Dissemination of multidrug resistant bacteria to the polar environment - Role of the longest migratory bird Arctic tern (*Sterna paradisaea*)

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Abstract

The ever-increasing prevalence of antibiotic-resistant bacteria (ARB), primarily due to the frequent use and misuse of antibiotics, is an issue of serious global concern. Migratory birds have a significant role in dissemination of ARB, as they acquire resistant bacteria from reservoirs and transport them to other environments which are relatively less influenced by anthropogenically. We have investigated the prevalence of ARB in a long-distance migratory bird, the Arctic tern (*Sterna paradisaea*) captured from the Svalbard Archipelago. The birds were tagged with geolocators to track their extraordinary long migration, and the cloacal samples were collected before the migration and after the migration by recapturing the same birds. The tracking of 12 birds revealed that during the annual cycle they underwent a total of 166 stopovers (11-18, mean=3.8) and recovery points along the Atlantic coast. Twelve major bacterial genera were identified from Arctic tern cloacal samples, which are dominated by *Staphylococcus* spp. and *Aerococcus* spp. The bacterial isolates showed resistance against 16 antibiotics (before migration) and 17 antibiotics (after migration) out of 17 antibiotics tested. Resistance to β-lactam and quinolone class of antibiotics were frequent among the bacteria. The study highlights the potential role of Arctic tern in the dissemination of multidrug resistant bacteria across far and wide destinations, especially to the polar environments.

Keywords: Antibiotic resistance, Bacteria, Arctic tern, Arctic, bird migration, wildlife

1. Introduction

The increasing prevalence of antibiotic resistance is a global problem that increases the pathogenicity of microorganisms, thus affecting conservation, biodiversity and economic loss (Berkner et al., 2014; Walsh, 2018; Ahmad and Khan, 2019). The World Health Organization listed this issue as one of the ten major threats to global health (Sprenger, 2019). The primary reason is attributed to the overuse and misuse of antibiotics in human medicine as well as in the control of diseases in animal production systems and aquaculture (Allen et al., 2010; Davies and Davies, 2010; Bush et al., 2011). The prevalence of antibiotic-resistant bacteria (ARB) is increasing in the environment since the beginning of the increasing usage of antibiotics, and the phenomenon is fuelled by frequent mutations and horizontal gene transfer mechanisms.

Polar environments are considered as relatively unpolluted due to its extreme weather and geographical conditions. However, these environments are also considered to be highly sensitive to perturbations (Boelter et al., 2016). The increases in anthropogenic activity and presence of migratory animals have favored introducing multidrug-resistant bacteria into the environment (Sjölund et al., 2008; Rabbia et al., 2016). Several studies have confirmed the prevalence of antimicrobial-resistant bacteria from polar environments (Yuan et al., 2014; McCann et al., 2019). The presence of ARB has been reported from birds such as gulls (Bonnedahl et al., 2014), *Branta leucopsis* (Hatha et al., 2013), *Calidris mauri, Branta bernicla* and *Larus hyperboreus* (Sjölund et al., 2008). Another relevant issue is the presence of 'historic' antibiotic-resistant genes in polar environments preserved for millions of years (Singh et al.,2017; Van Goethem et al., 2018), and the warming trend in the polar region leads to the release of this ARB to the environment.

Migratory birds can disperse ARB's across geographical boundaries because of their flying abilities (Stpień-Pyśniak et al., 2019; Zhao et al., 2020). In short periods of time, they can migrate longer distances (Bonnedahl and Järhult, 2014) and connect multiple sites across large geographical areas (Stillman et al., 2015). Previous studies reported that anthropogenic activity and bird's migration could mediate the dissemination of ARB (Allen et al., 2010; Wu et al., 2018). There are also reports showing the wild birds such as gulls can act as environmental reservoirs of ARB (Thomson, 2007). Recent studies reported the prevalence of colistin-resistant bacteria and resistance genes such as mcr-1 and mcr-2 in migratory birds (Lin et al., 2020). Colistin is considered as the "last resort" of defense against multidrug-resistant bacterial infections (Ahmed et al., 2020).

Each year, millions of birds migrate to and from the Arctic circle (Wauchope et al., 2016). Birds can act as biological and mechanical carriers of the microorganisms (Alcalá et al., 2016) and disseminate them to various geographical areas (Carter et al., 2018). Migration of birds to and from the six continents to the Arctic could lead to the dispersal of drug-resistant bacteria into the Arctic environment (Middleton and Ambrose, 2005; Sjölund et al., 2008; Ljubojević et al., 2016). Arctic terns (*Sterna paradisaea*) are known for their longest migration in the world (Egevang et al., 2010; Hromádková et al., 2020). They annually migrate back and forth over ~80,000 kms between the breeding areas in the Arctic and the non-breeding areas in the Antarctic (Volkov et al., 2017; Hromádková et al., 2020). Previous studies have identified several highly productive stopovers for energy refueling along their migratory route (Egevang et al., 2010; Mcknight et al., 2013). In the stopovers, migratory birds may intermix with other migratory and local birds. Aggression between the birds, self preeing and allopreening may lead to an exchange of pathogenic bacteria (Kulkarni and Heeb, 2007, Novcic, 2018). Consequently,

Arctic terns may become long-distance vectors and potential reservoirs for a wide range of pathogenic and antibiotic-resistant microorganisms that can be transmissible to the Arctic Circle.

In this study, we explored the role of Arctic terns in disseminating ARB's to the Arctic environment and identified the connection with their migratory route. The long-distance migration of the Arctic tern in short duration of time with many stopovers is likely to facilitate acquire and dissemination of ARB. To the best of our knowledge, this is the first study of the dissemination of ARB by Arctic tern into the Arctic environment.

2. Materials and Methods

2.1 Sampling

Twenty-two Arctic terns (3 or more years old) were live captured between 8 and 14th July 2017 using tent spring traps placed on their nests in breeding colonies in Longyearbyen town, Svalbard Archipelago (Coordinates 78.22537 N, 15.64622 E and 78.25001 N, 15.49682 E). The samples were collected within one week of arrival of the birds to minimize the contamination from the Arctic. Cloacal samples were collected directly from the cloacae of the bird using sterile swabs. The swabs were introduced into the bird's cloacae and inserted into a sterile tube containing transport media (Lobato et al., 2017). A small amount of blood (5–25 μl) was also collected and stored in 100 μl of 96% ethanol to examine the sex of birds. Body mass of birds were measured using Medio-Line Spring Scale, 300g/2g (Pesola, Switzerland). Sixteen birds were equipped with a lightweight tracking device called geolocator and released. The weight of the geolocator is 1.06 ± 0.05 g (SD) and the average weight of the bird is (106.2 ± 7.6 g). The cloacal samples were refrigerated at 4 °C in the Czech Arctic Research Station and subsequently transported to the Indian Arctic research station (Himadri, Ny-Ålesund). The samples were transported to Kingsbay Marine laboratory in ice cooled boxes kept in an insulated box. Bacterial

isolation is completed within a week in Kings Bay Marine Laboratory, Ny-Ålesund. Recapturing of the birds was carried out from 27 June and 12 July 2018. Using the method described above, 12 birds were recaptured from the same area (breeding grounds) in Longyearbyen, cloacal samples were taken and geolocators were removed for analysis of migration route.

2.2 Geolocator tracking

The birds were tagged with multi-sensor archival data loggers (geolocators; model Intigeo- W65A9-SEA, Migrate Technology) to track their migration path (Hromádková et al., 2020). We estimated the geographic locations and identify stopover regions of the tracked individuals throughout the annual cycle (Rakhimberdiev et al., 2017). Details of tracking and migratory behavior have been described in Hromádková et al. (2020).

2.3 Isolation of bacteria

Bacterial isolation was carried out in the MacConkey Agar (Himedia, India) and 1/4 strength nutrient agar media (Himedia, India) using spread plate method. The spread plates were incubated for 24 hrs at 37 °C. Single colonies of bacteria were aseptically picked from the agar plates, restreaked to ensure purity and transferred to full strength nutrient agar media. Pure cultures of the bacterial isolates on nutrient agar slants were transported to the laboratory (Cochin University of Science and Technology, Kerala, India) for further analysis.

2.4 Antimicrobial susceptibility testing

Antibiotic susceptibility of the isolates was carried out using the disc diffusion method following the protocols laid down by European Committee on Antimicrobial Susceptibility Testing (EUCAST). Mueller-Hinton agar (HiMedia, India) was used for the antimicrobial susceptibility test and the plates were incubated at 37 °C for 24 hrs. All the isolates (222) were tested against 17 different antibiotics (HiMedia, India) such as amikacin (Ak, 30 mcg),

ampicillin (Amp, 10 mcg), chloramphenicol (C, 30 mcg), ceftazidime (Caz, 30 mcg), cephalothin (Cep, 30 mcg), colistin (Cl, 10 mcg), co-trimoxazole (Cot, 25 mcg), cefpodoxime (Cpd, 10 mcg), erythromycin (E, 15 mcg), gentamicin (Gen, 10 mcg), nalidixic acid (Na, 30 mcg), penicillin G (P,10 unit), streptomycin (S, 10 mcg), sulphamethizole (Sm, 300 mcg), tetracycline (Te, 30 mcg), trimethoprim (Tr, 5 mcg) and vancomycin (Va, 30 mcg).

A multiple antibiotic resistance (MAR) index was determined (Krumperman, 1983) for each isolate. The MAR index, when applied to a single isolate is defined as a/b, where 'a' represents the number of antibiotics to which the isolate is resistant, and 'b' represents the number of antibiotics to which the isolate was exposed. The agar dilution method (Wu et al., 2015) was used to find out the lowest colistin concentration required to inhibit the visible growth of bacteria after overnight incubation. For MIC determination, different dilutions of colistin were prepared such as 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 µg/mL along with controls. Isolates were inoculated into the plates and incubated overnight at 37 °C. Bar charts, superimposed bar charts and chord diagrams were created using R version 4.1.0. T-tests were used to assess differences in total antibiotic resistance among the bacteria from female and male birds before and after migration.

2.5 Molecular sequencing of bacterial isolates

Two hundred and twenty isolates were obtained from the 22 swabs samples collected during 2017 (before migration)-(GenBank Accession No. OL851533 - OL851653) and 147 isolates during 2018 (after migration). Rep-PCR amplification-based fingerprinting technique was used with the BOX A1R primer (5'- CTACGGCAAGGCGACGCTGACG-3') to eliminate bacterial species subtypes and to select unique bacterial isolates for further studies (Rademaker et al., 1998). Finally, 222 isolates (129 from pre-migration in 2017 and 93 isolates after migration in 2018) were selected for genomic DNA extraction using Pure link genomic DNA

extraction kit (Invitrogen, USA; Catalog Numbers K1820-01), following the manufacturer's protocol. Amplification of the 16S region was carried out by using universal primers 27F 5'-AGAGTTTGATCM TGGCTCAG-3' and 1492R 5'-CGGTTACCT TGT TAC GAC TT-3'. The PCR product was purified using Invitrogen ChargeSwitch®-Pro PCR Clean-Up Kit (Catalogue #CS32250). Sequencing PCR was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The products were purified (BigDyeXTerminator™ Purification Kit) and sequenced on Genetic Analyzer 3500 (Applied Biosystems, Carlsbad, CA, USA) at National Center for Polar and Ocean Research, Goa, India.

2.6 Molecular determination of sex of Arctic terns

Molecular determination of sex was done based on the methodology of Ležalová-Piálková (2011). Genomic DNA was isolated from 10 µl of blood samples using the Genomic DNA Mini Kit (Geneaid Biotech Ltd, New Taipei, Taiwan) following the manufacturer's protocol. The avian sex primers 2550 F and 2718 R (Fridolfsson and Ellegren, 1999) were used in 10 µl PCR reactions following the protocol of Ležalová-Piálková (2011). The PCR products were separated by electrophoresis for 45–60 min at 7–10 V/cm using 3% agarose gels stained with SYBR® Safe (Life Technologies, Invitrogen, Carlsbad, CA). Heterogametic females were characterized by a two-band profile (~600 and ~450 bp), while homogametic males by only a single band (~450 bp).

2.7 Phylogenetic Analysis

Phylogenetic analysis of all the bacterial isolates were carried out using BioEdit 7.2.6.1 and MEGA X software programmes. The sequences were assembled using BioEdit sequence alignment editor version 7.0.5.3 and the consensus contig was checked for chimera using DECIPHER and Chimeric sequences were removed. The 16S rRNA gene nucleotide sequences

were used to identify their phylogenetic affiliation against already known sequences from published databases (NCBI, EzBioCloud). Phylogenetic analysis of the sequences was carried out in an R package 'ggtree' (Yu et al., 2016), 'ggtreeExtra' (Xu et al., 2021), 'ggplot2' (Wickham, 2016) and 'ggnewscale' (Campitelli, 2021) in R version 4.1.0. programme.

3. Results and Discussion

3.1 Migration route and stopovers

Although we have placed geolocators on 16 of the 22 Arctic terns captured in 2017, we could recapture only 12 of them after migration. Accordingly, we have analyzed the data from the geolocators of these 12 birds to study the migratory route of Arctic terns. Analysis of geolocator data from the 12 birds revealed that they had a total of 166 stopovers (minimum three days long) including 21 stopovers in the Antarctic during their interpolar migration. The migration route and stopovers are presented in Fig. 1. The terns started southbound migration by late August-early September from Svalbard. Most of the birds moved to their first stopover in the North Atlantic Ocean. Bird Nos BH011 and BH024 showed a looping behavior of the route by returning to Svalbard after the start of the journey in early October and then restarted their southbound migration. We have observed that five of the birds (BG997, BH001, BH004, BH005 and BH021 - 3 males and 2 females) traveled along the east coast of South America and the seven birds (BH002, BH011, BH013, BH015, BH017, BH023 and BH024 - 2 males and 5 females) migrated through the west coast of Africa. Results revealed that the two birds (BG997 and BH005) reached last stopovers in the South Atlantic Ocean by 5th November 2017. BH013 reached last by 25th November. All of them entered the Antarctic circle by the end of November and early December. The birds spend approximately four months in Antarctica; however, they do not breed within the Antarctic circle. From February to March, all the birds were located at the stopovers in the Weddell Sea. After spending approximately four months in the non-breeding

sites, they started the return journey to the breeding destinations in the Arctic. During their northward migration, the number of stopovers was limited, or they spent less than 3 days at each recovery point. BH011 was the first to reach the last stopover of northward movement in the North Atlantic Ocean on 3rd April 2018. BH005 was the last to reach the last stopover by 24th May and departed after three days. In late May−early June, the birds returned to the breeding colonies in Longyearbyen. BH004 spent 146 days in 17 stopovers. BH021 has high numbers of stopovers (18) and spent 128 days in the stopovers. BH004 spent 32 days in a single stopover in the Weddell Sea. The number of stopovers used by each of the birds were; BG997 (11), BH005 (11), BH001 (12), BH002 (12), BH011 (13), BH015 (13), BH013 (14), BH017 (14), BH024 (14), BH004 (17), BH023 (17) and BH021 (18). Similarly, BH015 spent 23 days at a single stopover in the Weddell Sea. Two stopovers of birds BH015 and BH011 were found at inland sites in Angola, Africa and Northern Ireland, except the stopovers in the Antarctic. But numbers of stopovers were different with birds on their southward and northward migration. BH015 spent 94 days at 13 stopovers and BH017 spent 92 days in stopovers. These twelve birds have together spent a total of 1428 days in all stopovers, averaging 119 days by each bird. They covered an average round trip distance of ~58500 km. The tracking and migratory route of these Arctic terns were well explained in the Hromádková et al. (2020).

In the present study, we observed that the first stopover of terns was in the North Atlantic Ocean. Further, the birds were chosen two distinct routes; four of them moved to later stopovers along the west coast of Africa and five birds followed the east coast of South America. A similar observation was made by previous studies on Arctic tern from Greenland, Iceland and Netherlands (Guilford et al., 2009; Egevang et al., 2010; Volkov et al., 2017). It was indicated that the birds chose both the west coast of Africa and east coast of North and South America for the southbound migration (Egevang et al., 2010). However, it is reported that the Arctic tern

population from Alaska chose the east pacific coast for their winter migration (Mcknight et al., 2013). During the migration, the birds inter-mixed with other birds, including terns from different locations. Migratory birds like Manx Shearwater (*Puffinus puffinus*) and Cory's Shearwater (*Calonectris diomedea*) used the same route for southbound and northbound migration, and they wintered in the South Atlantic (González-Solís et al., 2007; Guilford et al., 2009). Recent reports show that Antarctic seabirds and penguins carry ARB (Cerdà-Cuéllar et al., 2019).

The Arctic tern spent nearly 137 days (127 to 153 days) in Antarctica and started northbound migration by late March-early April. During this feeding behaviour they are unlikely to have significant direct contact with antibiotics, and were unlikely to pick up any significant load of ARB, except perhaps through food. Gudmundsson et al. (1992) had reported that during migration, the first stopover was observed in the Weddell Sea, during which very high numbers of Arctic terns were observed. This area is already known as a prime non-breeding area for Arctic terns and many seabirds (Egevang et al., 2010; Mcknight et al., 2013; Volkov et al., 2017). High productivity and the presence of Antarctic krill (*Euphausia superba*) in the Weddell Sea make it an excellent foraging ground for many seabirds (Atkinson et al., 2004). Further, it was observed that they follow a 'S' shaped pathway during their travel to the north. The birds from Greenland, Iceland and Longyearbyen chose two routes (West African Coast and East North American Coast) to reach the Arctic circle (Egevang et al., 2010; Mcknight et al., 2013; Hromádková et al., 2020). Our findings suggested that the terns were traveling with several stopovers along the polluted coasts of American (Hernandes et al., 2013; Leite et al., 2019), African (Faleye et al., 2018) and European (Håkonsholm et al., 2020) continents from where the birds could acquire ARB and antibiotic- resistance genes, which could transfer and alter the native resistome of the Arctic.

Tracking of Arctic terns by geolocators revealed that they migrate from Longyearbyen, Svalbard, the Arctic to Antarctica. Gudmundsson et al. (1992) observed that the Arctic terns migrate in small flocks of eleven individuals. The Arctic terns are opportunistic plunge-divers just above the surface of the water that mainly feed on small fishes, zooplankton and insects (Hatch, 2002; Newton, 2007). During this feeding behaviour, they did not have any direct contact with antibiotics and were unlikely to pick up any significant load of ARB. However, the birds also undergo several stopovers along the Atlantic Ocean for refueling, which are highly productive upwelling areas (Mcknight et al., 2013) and are also used by local and other migratory birds for feeding and resting. These areas could be potential locations of significant pickup of ARB from contact with other birds.

3.2 Genetic diversity of the isolates

From the 220 bacterial isolates obtained from the 22 Arctic tern cloacal samples before migration, we selected 129 bacterial isolates after bacterial subtyping through Rep-PCR. Major phyla of bacteria were Firmicutes (80%), Actinobacteria (10%) and Proteobacteria (10%). The most dominant genera were *Staphylococcus* (41.9%), *Aerococcus* (11.62%), *Micrococcus* (11.62%), *Alcaligenes* (10.8%), *Bacillus* (7%), *Enterococcus* (7%), *Flavobacterium* (3.1%), *Idiomarina* (2.3%), *Macrococcus* (2.3%), *Brevibacillus* (0.77%), *Kocuria* (0.77%) and *Lysobacter* (0.77%) (Fig. 2a & b). Ninety bacterial isolates were obtained from female birds and thirty-nine isolates from male birds. The swab samples collected in 2017 before the start of migration yielded twenty-five species of bacteria. Ten different genera were observed from the female birds, dominated by *Staphylococcus* (48%) followed by *Aerococcus* (12%) and Alcaligenes (10%) (Fig. 2a). Nine different genera were encountered in cloacal samples from male birds. Major genera of bacteria encountered in male birds were *Staphylococcus* (28%), *Micrococcus* (20.5%), *Alcaligenes* (13%), *Aerococcus* (10%) and *Enterococcus* (10%). Species

like *Idiomarina piscisalsi, Kocuria indica* and *Macrococcus goetzii* were only found in female birds. *Brevibacillus agri* and *Lysobacter concretionis* were found in male birds. Nine species of *Staphylococcus* were isolated in this study, in which *Staphylococcus arlette* (37%) and *Staphylococcus warmeri* (24%) were the dominant species. Various species seen under the genus Bacillus were *Bacillus cabrialesii, B. licheniformis, B. tequilensis* and *B. xiamenensis.* The detailed diversity of bacterial isolates from each bird and isolated species were presented in the supplementary table 1.

A total of 93 isolates were obtained from 12 birds after migration. The major bacterial genera were *Staphylococcus* (21.5%), *Aerococcus* (19%), Alcaligenes (19%), Bacillus (14%), *Micrococcus* (13%), *Enterococcus* (6%) and *Lysobacter* (6%) (Fig 2c & d.). In female birds the predominant genera were *Aerococcus* (22%), *Alcaligenes* (19%), *Bacillus* (18%), *Staphylococcus* (14.5%) and *Micrococcus* (13%) (Fig. 2c). *Staphylococcus* (35%), *Alcaligenes* (19%), *Aerococcus* (13%) and *Micrococcus* (13%) were the major isolates from male cloacal samples. Among the 14 different species that were obtained, species under the genus *Staphylococcus* was dominated. This included *S. caprae* (30%), *S. epidermidis* (20%), *S. warneri* (20%), *S. capitis* (15%), and *S. edaphicus* (15%) (Fig. 4d). The species such as *Aerococcus*, *Alcaligenes*, *Bacillus*, *Micrococcus* were also predominant in the samples; and higher occurrences of these genera were observed after migration.

The dominant species *Staphylococcus* spp. previously reported from the wild birds (Matias et al., 2018; Santos et al., 2012). *Aerococcus* spp.*, Micrococcus* spp. previously identified from the microbiome of European herring gulls (*Larus argentatus*) (Merkeviciene et al., 2017) and cloacal samples from long distance migratory birds (Kreisinger et al., 2015). *Alcaligenes* spp. were previously reported from the Australian wild bird species (Poiani and Gwozdz, 2002). These bacteria previously reported as a contaminant in the male birds

transmitted to female during copulation (Westneat and Birch Rambo., 2000). *Enterococcus* spp. is previously identified in cloacal samples from coraciiform birds (Splichalova et al., 2015) and psittacine birds (Devriese et al., 1995). *Bacillus* spp. were earlier identified from shoreline migratory birds (Santos et al., 2012) and reported as sexual behavior aid transmission of bacteria (Subhash, and Heeb, 2007).

3.3 Prevalence of ARB in the Arctic tern

The details of swab samples collected and analyzed before (2017) and after the migration (2018) is given in Table 1. The bacteria were isolated from the cloacal samples, identified and tested for resistance against nine classes of antibiotics before and after the migration (Fig. 3a & b). The results revealed that the overall prevalence of ARB was high among the bacteria from Arctic terns, with 95% of birds carrying bacteria resistant to various antibiotics tested (Fig. 3a). Nearly 94% (121 out of 129 isolates) of the bacterial isolates from the birds showed resistance to one or more different antibiotics; 56.5% (73 isolates) of the isolates showed multidrug resistance to three or more antimicrobial categories (Supplementary Table 1).

Most of the bacterial isolates were resistant to the antibiotic ceftazidime (71%), followed by penicillin G (67%), ampicillin (61%) and nalidixic acid (45%). With respect to the various classes of antibiotics tested, resistance was most frequently encountered against β-lactam and quinolones, followed by polypeptides. Interestingly, all the isolates were sensitive to tetracyclines (Fig. 4a). After migration (2018) 93 isolates were obtained from 12 birds which showed resistance against 17 antibiotics out of the 17 antibiotics tested. Among the isolates, 97% (90 isolates) showed resistance against one or more antibiotics. The highest resistance was observed against ceftazidime (59%), ampicillin (36.5%), nalidixic acid (32%) and penicillin G (31%) (Fig. 4b). All recaptured birds (12 birds, 100%) showed antibiotic-resistant carried isolates.

Multiple antibiotic-resistance (MAR) index and the resistance pattern of each isolated was worked out (Supplementary Table 1) to find the similarity and diversity of resistance pattern. MAR index varied from 0.06 to 0.65 (Table 2). A total of 92 different resistance patterns were observed. The most frequently observed resistance pattern was Caz, Na (5.43%) followed by Caz (3.87%) and Amp, Caz, Na, P (3.1%). The majority of the resistance patterns were expressed only once. The MAR index of isolates obtained from birds after the migration ranged from 0.06 to 0.53. Forty-five different patterns were found among the isolates from these birds. The most observed resistance patterns were Te, Va (7.07%) Caz, P (6.45%) and Sm, Tr (6.07%) (Table 3). The variation in the MAR index and the resistance patterns of the isolates from the samples collected before and after migration reveals great diversity and indicates the diverse origin of the isolates. The isolates with varying resistance patterns are likely to have originated from different sources. Forty-five isolates that showed colistin resistance in disk diffusion assay were further reconfirmed by MIC determination. Results revealed that 28 of them were confirmed as resistant to colistin and showed MIC of ≥4 µg/mL. One isolate (*Enterococcus faecalis*) showed high resistance (256 µg/mL) against colistin.

Previous studies have reported the existence of ARB in Arctic birds. Reasons such as de novo development through spontaneous mutations (Martinez and Baquero, 2000), horizontal gene transfer among natural reservoirs and bird microbiota (Maiden, 1998) and import of ARB into the region either by migratory birds or through human refuse (Sjölund et al., 2008) were suggested. Our results revealed that the resistance to β-lactam antibiotics was frequent among the bacteria from Arctic tern. This is one of the most widely consumed antibiotics in the world (Eley, 2018), which is also one of the antimicrobial drugs widely used in food animals. The selection pressure for the mutants resistant to β-lactam seems to be widespread, as reflected in the results of this study. Most of the bacterial isolates were resistant to β-lactam antibiotics such as

ceftazidime, penicillin and ampicillin. While penicillin is of natural origin, ceftazidime and ampicillin are semisynthetic or derivatives of natural origin antibiotics.

Our results also revealed fairly high antibiotic resistance to nalidixic acid, the first synthetic quinolone antibiotics. Quinolones (including Nalidixic acid) are also widely consumed 12% worldwide (Eley, 2018). The migratory birds mirroring human activity and its effect on the environment because of their diverse ecological niches, from where they can easily pick up ARB (Bonnedahl and Järhult, 2014). Our observations on prevalence of β-lactam resistance is supported by previous studies in the Arctic birds. Sjölund et al. (2008) revealed ARB in Arctic birds with frequent resistance to β-lactam, sulfamethoxazole, trimethoprim, chloramphenicol and tetracycline. Another study in Barnacle goose from Svalbard (Hatha et al., 2013) revealed modest levels of resistance against β-lactam antibiotics that include ampicillin and amoxicillin. Antibiotic resistance among the bacteria from diverse bird species has been reported previously. Migratory birds always have the opportunity to mix with several of them during their long journey, and possible pickup of diverse microflora/ exchange of genetic material is a real possibility. Foti et al. (2017) had reported relatively high resistance to amoxicillin, ampicillin, rifampicin and amoxicillin–clavulanic acid among the birds of the order Passeriformes. Further, Giacopello et al. (2016) had reported that bacterial isolates from 55 European wild birds most frequently exhibited resistance against trimethoprim/sulfamethoxazole, streptomycin, amoxicillin/clavulanic acid ampicillin and tetracycline. Ahlstrom et al. (2018) reported the cephalosporin-resistant bacteria from bald eagles and gulls that inhabit the landfills in Alaska. A study from Tagus estuary in Portugal, one of the stopover habitats of many migrating seabirds, have shown that the birds harbour potentially pathogenic bacteria such as Firmicutes, Actinobacteria, Helicobacter and Staphylococcus (Santos et al., 2012). Ampicillin resistant *Vibrio parahaemolyticus* isolated from Manx Shearwater (Cardoso et al., 2018). Another study

from Qinghai Lake in China, which is along the Central Asian Flyway, showed that migratory birds were carrying more ARGs than the surrounding environments (Lin et al., 2020).

Diverse antibiotic resistance patterns were observed in *Staphylococcus isolates* (16 patterns)*, Bacillus* spp. (14 patterns) *Micrococcus* spp. and *Aerococcus* spp. (13 each). The isolate *S. arlettae* showed resistance to 11 antibiotics. The details of resistance pattern of isolates to various antibiotics have been presented in the Supplementary Table 1. None of the isolates were resistant to high-level gentamicin or high-level streptomycin antibiotics. The antibiotic-resistant genes are often associated with plasmids (Bennett, 2008) and these genes can be horizontally transferred to other bacteria within the gut of the bird. In a global health perspective, the expansion of antibiotic resistance alters the microbiome at local and global levels (Huddleston, 2014; Hernando-Amado et al., 2019). In Arctic environments, multidrug resistant bacteria are reported widely in wild animals (Glad et al., 2010a, Glad et al., 2010b; Mogrovejo et al., 2020). This study reports a fairly high degree of resistance against colistin, which belongs to the antibiotic class polymyxins, one of the last-resort antibiotics for treating bacterial infections. Currently, colistin is widely used in veterinary medicine but restricted use in human health care due to its neuro and nephrotoxicity. Similar observations on colistin resistance were observed earlier in Barnacle goose, which has a comparatively shorter migratory route (Hatha et al., 2013), breeds in the Arctic tundra and winters in the Netherlands and the North of Germany (Kear and Hulme, 2005). European herring gulls were reported to carry colistin resistant ARB (Ruzauskas and Vaskeviciute, 2016).

Climate change and the increasing human population in the Arctic may contribute to selective pressure for increasing antibiotic resistant mutants of bacteria. Climate change is also changing the migration pattern of migratory birds because both the phenology and distributions of migratory birds respond to temperature. Therefore, the sites which migratory birds visit are

changing over time in response to climate change. This is important because climate change may therefore lead to birds dispersing ARB to new sites that are not currently used. These changes in migratory behavior caused by climate change are quite common and will affect many bird species (Nuijten et al., 2020).

3.4 Horizontal and sexual transmission of bacteria

Our study found that the bacterial isolates from female birds had a higher prevalence of ARB than male birds, before the migration ($t = 2.9183$, $df = 21.969$, p-value = 0.007) and after the migration ($t = 2.2149$, $df = 25.353$, p-value = 0.03). The high prevalence of ARB in females may be due to bacterial presence in the male ejaculate, which was deposited in the female cloaca (Brown, 2017). Sexual contact between birds also leads to the transmission of pathogens. This has resulted in more numbers of bacterial isolates from female Arctic terns and ARB than from male birds. However, there was no significant difference between the body weight of the birds and antibiotic resistance among the bacteria from them. Self and allopreening of the bird possibly increase the chances of horizontal bacterial infections. Bacteria from one's own plumage or from the plumage of another cohabited individual contaminate the cloaca of the bird and hence the gut (Kulkarni and Heeb, 2007).

4. Conclusion

Our results based on the direct collection of samples from the cloaca of Arctic tern, which have undertaken interpolar migration (as revealed by the geolocator data) may indicate the potential role of dissemination of the ARB to the Arctic. The cloacal microbiota has acquired antibiotic resistance, even to the last-resort antibiotics such as colistin, and they can act as reservoirs and potential vectors of ARB. The long migration route of Arctic terns with several number of stopovers, and mingling with the local and other migratory birds in such stopovers,

may make them a potential vehicle for harboring and dissemination of ARB into polar environments such as Arctic and Antarctic. Climate change and the increasing human presence in the Arctic may also contribute to selective pressure for the emergence of antibiotic resistant mutants.

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Figure Legends

Fig. 1. Arctic tern's migratory pattern includes stopovers along the Atlantic Ocean; a) shows the southbound migration and b) shows northbound migration stopovers. Maps coloured according to the usage of antibiotic defined daily doses (DDD) per 1,000 individuals from light red (low DDD/1000) to dark red (high DDD/1000). Source of antibiotic usage data: https://resistancemap.cddep.org/AntibioticUse.php.

Fig. 2. a) Percentage diversity of bacterial isolates (at genus level) observed in among the male and female Arctic tern before migration. b) Showed percentage diversity of bacteria (at genus level) isolated from 22 Arctic tern birds before migration. c) Percentage diversity of bacterial isolates (at genus level) observed in among the male and female Arctic tern after migration. d) Showed percentage diversity of bacteria (at genus level) isolated from 12 Arctic tern birds after migration.

Fig. 3. Chord diagram showing prevalence of antibiotic resistance in each bird. a) before migration and b) after migration.

Fig. 4. Total percentage of antibiotic susceptibility such as sensitive, intermediate, and resistance against 19 antibiotics, a) before migration and b) after migration (Ak-amikacin, Amp-ampicillin, C-chloramphenicol, Caz-ceftazidime, Cep-cephalothin, Cl-colistin, Cot-co-trimoxazole, Cpd-cefpodoxime, E-erythromycin, Gen-gentamicin, Na-nalidixic acid, P-penicillin G, S-streptomycin, Sm-sulphamethizole, Te-tetracycline, Tr-trimethoprim and Va-vancomycin).

Supplementary Figure 1. Collection of cloacal samples from Arctic tern using sterile swabs.

Supplementary Figure 2. Phylogenetic tree of bacteria isolated from *Sterna paradisaea*. In bird's sex, Red denotes the male birds and green denotes the female birds. a) The heatmap represents the prevalence of 129 isolates (before migration) to 9 antibiotic classes. b) The heatmap represents the prevalence of 93 isolates (after migration) to 9 antibiotic classes.

Table 1. Bird capturing locations in the Longyearbyen, bird's sex and weight of the birds.

Table 2. MAR index and Resistance pattern of bacterial isolates from the Arctic tern (before migration)

Table 3. MAR index and Resistance pattern of bacterial isolates from the Arctic tern (after migration)

Supplementary Table 1. Prevalence of Arctic tern carrying ARB to the Arctic. Data represents susceptibility of antibiotic of the isolates-Sensitive (-), Intermediate (+) and Resistance (++)
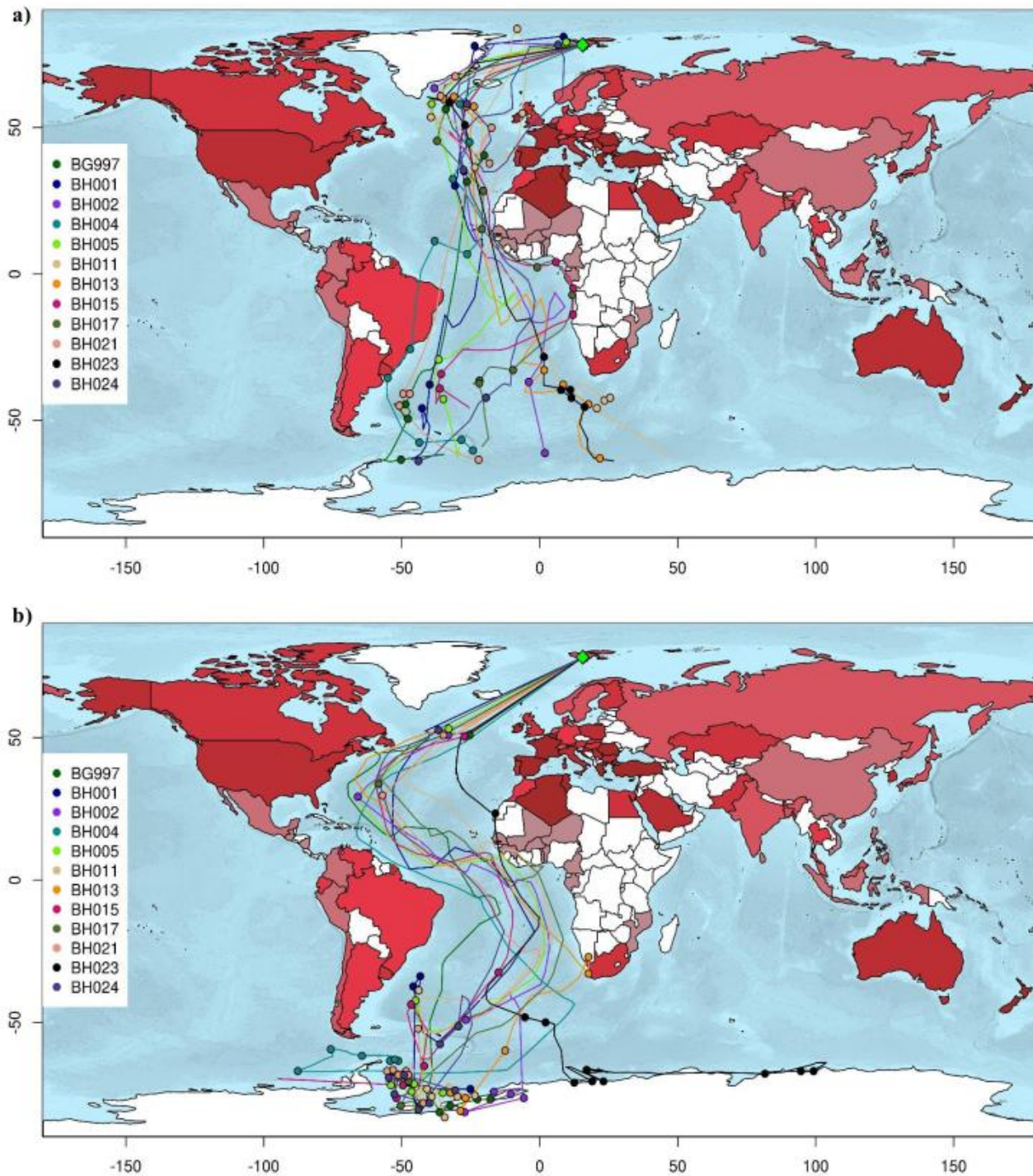
Fig. 1. Arctic tern's migratory pattern of 12 birds includes stopovers -longer than 3 d are marked with dots- along the Atlantic Ocean; a) shows the southbound migration and b) shows northbound migration. Breeding site in Longyearbyen, Svalbard, is marked with a green diamond. Choropleth maps indicates the world antibiotic usage, colored according to the usage of antibiotic defined daily doses (DDD) per 1,000 individuals, from light red (low DDD/1000) to dark red (high DDD/1000). Source of antibiotic usage data: https://resistancemap.cddep.org/AntibioticUse.php

Fig. 2. a) Percentage diversity of bacterial isolates (at genus level) observed in among the male and female Arctic tern before migration. b) Showed percentage diversity of bacteria (at genus level) isolated from 22 Arctic tern birds before migration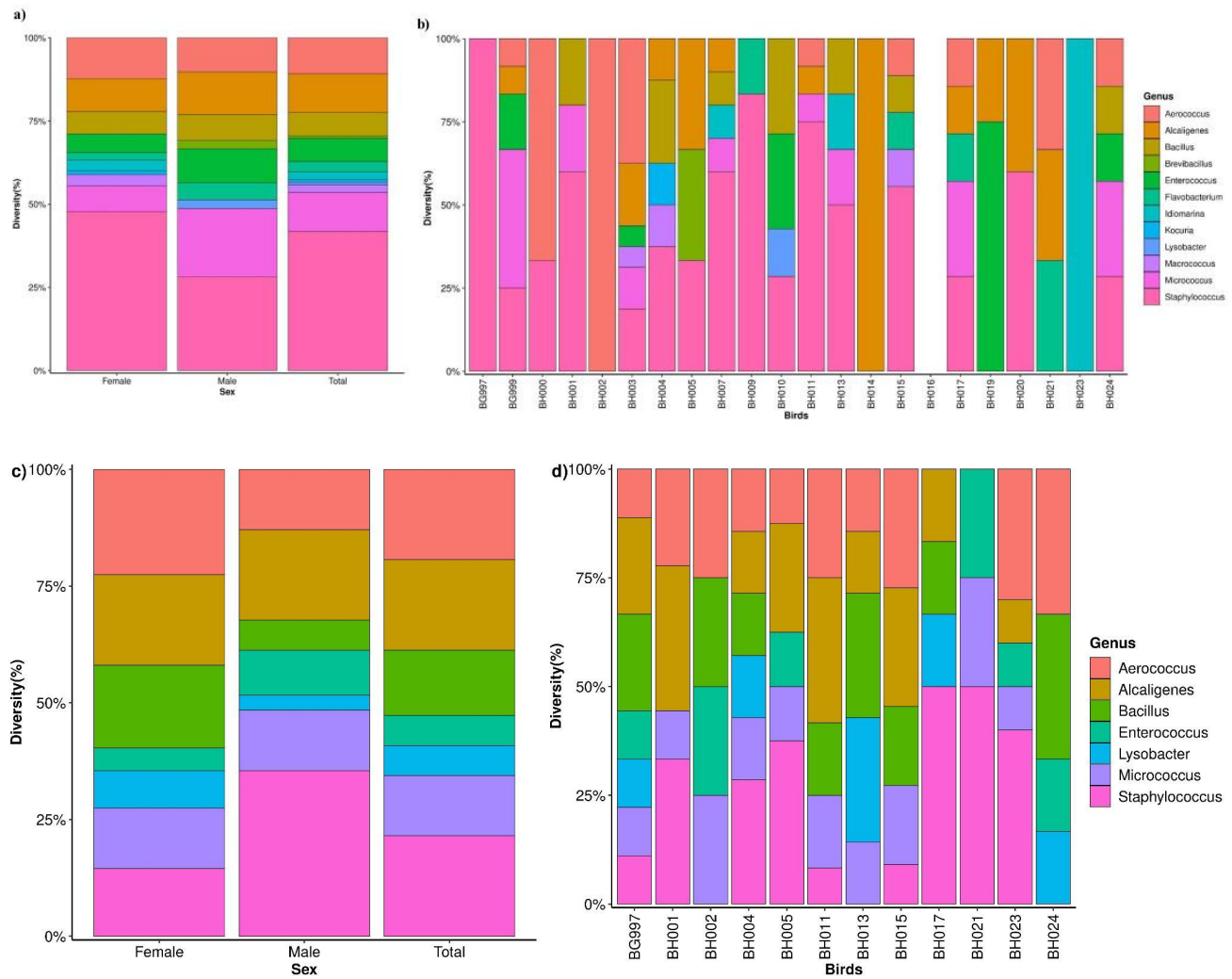. c) Percentage diversity of bacterial isolates (at genus level) observed in among the male and female Arctic tern after migration. d) Showed percentage diversity of bacteria (at genus level) isolated from 12 Arctic tern birds after migration.

Fig. 3. Chord diagram showing prevalence antibiotic resistance in each bird a) before migration and b) after migration.
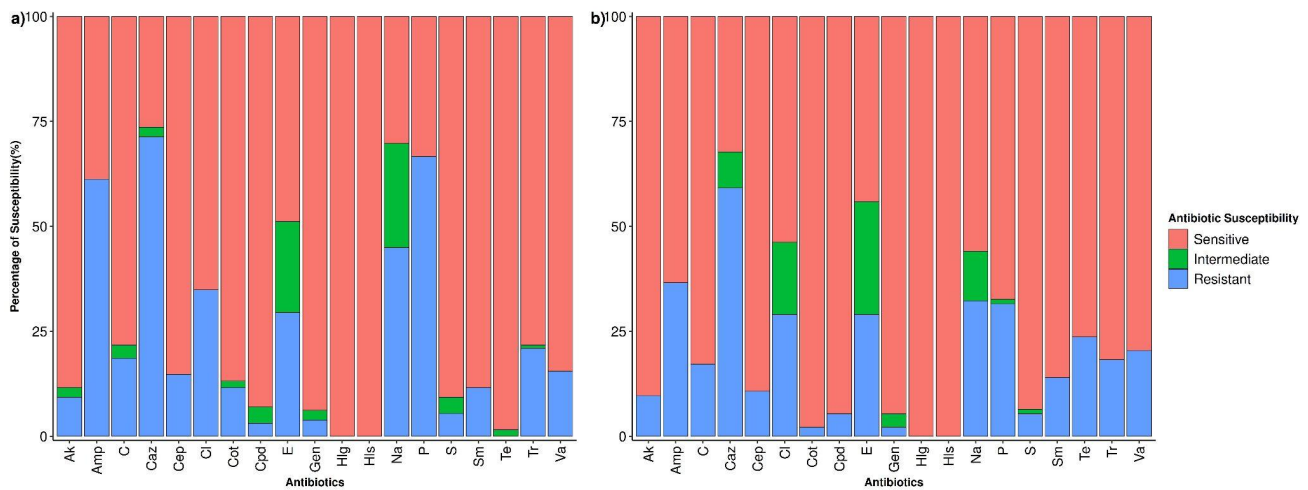
Fig. 4. Total percentage of antibiotic susceptibility such as sensitive, intermediate, and resistance against 19 antibiotics, a) before migration and b) after migration (Ak-amikacin, Amp-ampicillin, C-chloramphenicol, Caz-ceftazidime, Cep-cephalothin, Cl-colistin, Cot-co-trimoxazole, Cpd-cefpodoxime, E-erythromycin, Gen-gentamicin, Hlg-high-level gentamicin, Hls-high-level streptomycin, Na-nalidixic acid, P-penicillin G, S-streptomycin, Sm-sulphamethizole, Te-tetracycline, Tr-trimethoprim and Va-vancomycin).

Supplementary Figure 1. Collection of cloacal samples from Arctic tern using sterile swabs.

a)
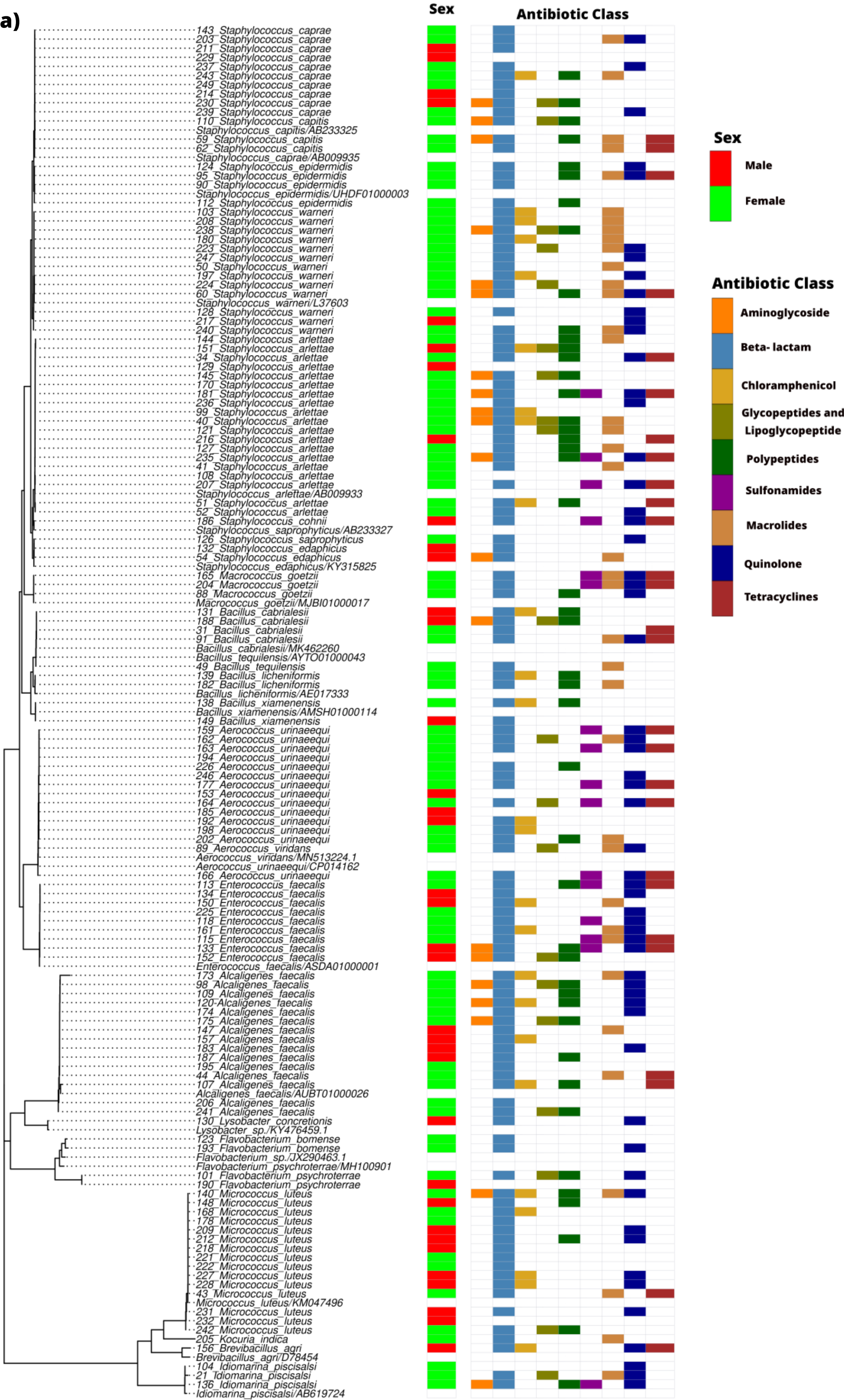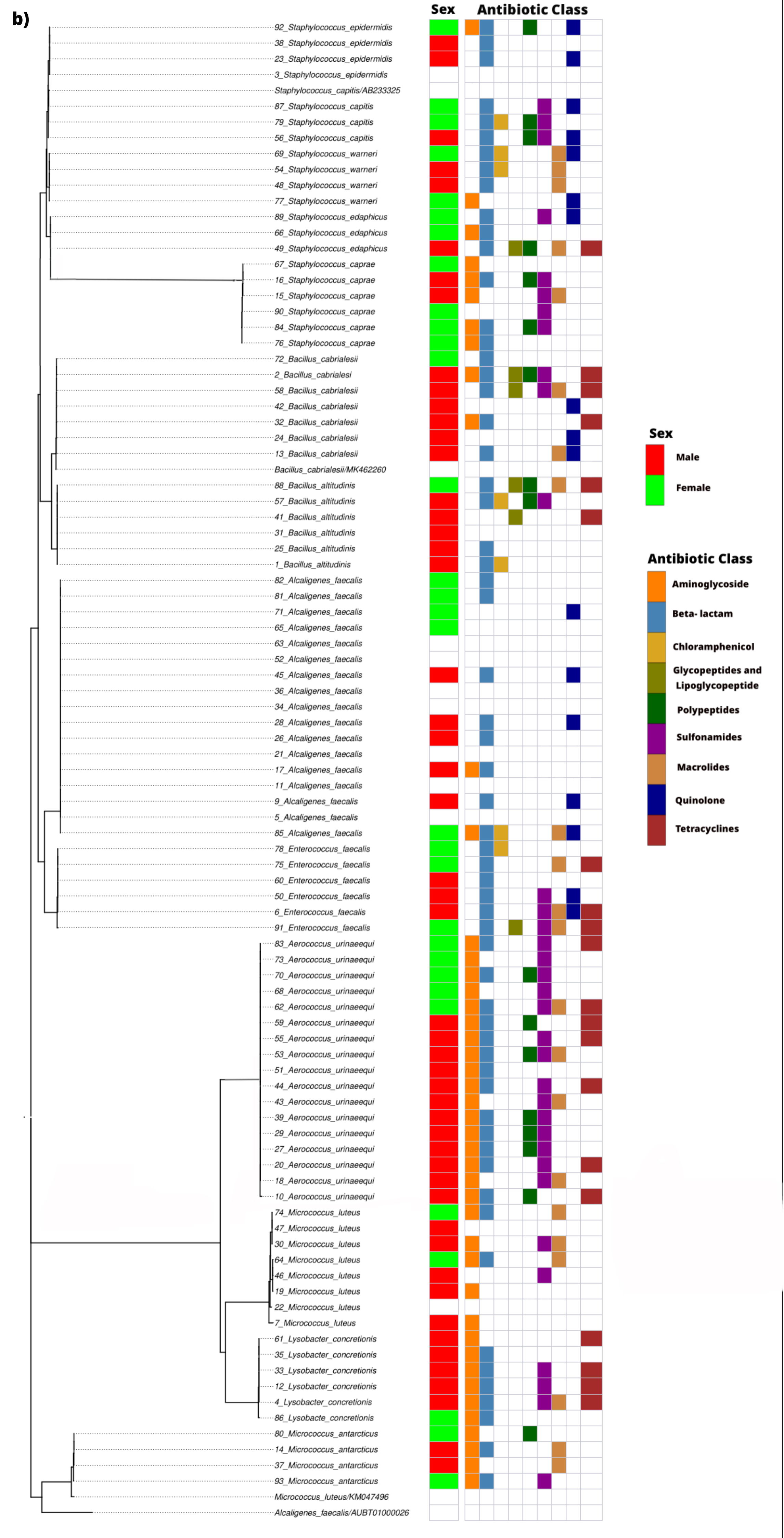
Table 1. Details of Arctic terns captured before (2017) and after migration (2018).

Sl No	Bird Id	Bird capture location (2017)	Bird (Male/Female)	Bird weight before migration (g)	Recapture location (2018)	Bird weight after migration (g)
1	BG997*	Sjøområdet, Longyearbyen	F	109	Před přepadem, Longyearbyen	107
2	BG999	Sjøområdet, Longyearbyen	M	110		
3	BH000	Hotellneset, Longyearbyen	F	101		
4	BH001*	Sjøområdet, Longyearbyen	M	101	Kosa, Longyearbyen	106
5	BH002*	Sjøområdet, Longyearbyen	M	100	Ostrov, Longyearbyen	--
6	BH003	Sjøområdet, Longyearbyen	F	98		
7	BH004*	Sjøområdet, Longyearbyen	F	105	Longyearelva, Longyearbyen	111
8	BH005*	Sjøområdet, Longyearbyen	M	102	Longyearelva, Longyearbyen	105
9	BH007	Hotellneset, Longyearbyen	F	99		
10	BH009	Sjøområdet, Longyearbyen	F	112		
11	BH010	Sjøområdet, Longyearbyen	M	114		
12	BH011*	Sjøområdet, Longyearbyen	F	113	Longyearelva, Longyearbyen	130
13	BH013*	Hotellneset, Longyearbyen	F	119	Longyearelva, Longyearbyen	117
14	BH014	Hotellneset, Longyearbyen	M	111		
15	BH015*	Sjøområdet, Longyearbyen	F	91	Před přepadem, Longyearbyen	101

16	BH016	Sjøområdet, Longyearbyen	M	112		
17	BH017*	Sjøområdet, Longyearbyen	M	103	Před přepadem, Longyearbyen	110
18	BH019	Sjøområdet, Longyearbyen	F	105		
19	BH020	Sjøområdet, Longyearbyen	F	114		
20	BH021*	Sjøområdet, Longyearbyen	M	120	Silnice, Longyearbyen	113
21	BH023*	Sjøområdet, Longyearbyen	F	108	Ostrov, Longyearbyen	111
22	BH024*	Sjøområdet, Longyearbyen	F	98	Longyearelva, Longyearbyen	102

*Recaptured birds after migration on 2018

Table 2. MAR index and resistance pattern of bacterial isolates from the Arctic tern before migration

MAR Index	Resistance Pattern	Occurrence (%)
0.06	Amp	1.55
	Caz	3.87
	Na	1.55
	P	2.32
0.11	Amp, P	1.55
	C, P	0.77
	Caz, Cl	0.77
	Caz, E	0.77
	Caz, Na	5.43
	Caz, P	0.77
	Na, P	0.77
0.18	Amp, Caz, Na	0.77
	Amp, Caz, P	1.55
	Amp, Cl, P	1.55
	Amp, E, P	0.77
	Amp, Na, P	0.77
	C, Caz, Cl	0.77
	C, Caz, Na	0.77
	Caz, Cl, Na	0.77
	Caz, Na, P	2.32

	Cep, Cl, Va	0.77
	Cl, E, P	0.77
0.23	Amp, C, Caz, P	1.55
	Amp, Caz, Cl, P	0.77
	Amp, Caz, Cl, Tr	0.77
	Amp, Caz, E, P	2.32
	Amp, Caz, E, Tr	0.77
	Amp, Caz, Na, P	3.1
	Amp, Caz, P, Tr	0.77
	Amp, Cl, Na, P	1.55
	Amp, E, Na, P	0.77
	C, Caz, E, Na	0.77
	C, Caz, E, P	0.77
	Caz, Cl, E, P	0.77
	Caz, Cl, P, Va	0.77
	Caz, E, P, Tr	0.77
0.29	Ak, Amp, Caz, Cl, Va	0.77
	Ak, Amp, Cl, P, Va	0.77
	Ak, Caz, Cl, E, Va	0.77
	Ak, Caz, Cl, S, Va	0.77
	Amp, C, Caz, Cep, P	0.77
	Amp, C, Caz, Cl, P	0.77
	Amp, C, Caz, Cl, Tr	0.77
	Amp, C, Caz, E, P	2.32
	Amp, Caz, Cl, E, P	0.77
	Amp, Caz, Cl, Na, P	0.77
	Amp, Caz, E, Na, Tr	0.77
	Amp, Caz, E, P, Tr	0.77
	Amp, Cep, Cl, E, P	0.77
	Amp, Cl, E, P, Va	0.77
	Amp, Cl, Gen, P, Va	0.77
	Amp, Cl, Na, P Va	0.77
	Amp, E, Gen, P, Va	0.77
	Amp, E, Na, P, Va	0.77
	C, Caz, Cep, Cl, Tr	0.77
	C, Caz, Cl, Na, S	0.77
	Caz, E, Na, P, Va	0.77
0.35	Ak, Amp, C, Caz, Cep, P	0.77
	Ak, Amp, Caz, Cep, E, P	0.77
	Ak, Amp, Cl, Gen, P, Va	0.77
	Amp, C, Caz, Cep, Cl, P	0.77
	Amp, C, Caz, Cep, Na, P	0.77
	Amp, C, Caz, Cl, E, P	0.77
	Amp, C, Caz, E, Na, P	0.77

	Amp, C, Caz, Na, P, Tr	0.77
	Amp, Caz, Cl, E, Na, P	0.77
	Amp, Caz, Cl, Na, P, Tr	0.77
	Amp, Caz, Cot, Na, P, Tr	0.77
	Amp, Caz, Cot, Na, Sm, Tr	0.77
	Amp, Caz, Cot, Na, Sm, Tr	0.77
	Amp, Caz, E, Na, P, Va	0.77
	Amp, Cep, Cl, Gen, P, Va	0.77
0.37	Ak, Amp, Caz, Cl, E, P, Tr	0.77
	Amp, C, Caz, Cep, Cl, P, Va	0.77
	Amp, Caz, Cep, Cot, Na, P, Sm	0.77
	Amp, Caz, Cep, E, Na, P, Va	0.77
	Amp, Caz, Cl, E, Na, P, Tr	0.77
	Amp, Caz, Cot, Na, P, Sm, Tr	0.77
	Amp, Caz, Cpd, Na, P, Sm, Tr	0.77
	Caz, Cep, Cot, Na, P, Sm, Tr	0.77
	Caz, Cot, E, Na, P, Sm, Tr	0.77
0.47	Ak, Amp, C, Caz, Cl, E, Na, P	0.77
	Ak, Amp, Caz, Cl, E, Na, P, Tr	0.77
	Amp, Caz, Cot, E, Na, P, Sm, Tr	0.77
	Amp, Caz, Cot, Na, P, Sm, Tr, Va	0.77
0.53	Ak, Amp, C, Caz, Cep, Cl, E, P, Va	0.77
	Ak, Amp, Caz, Cl, Gen, Na, P, S, Va	0.77
	Amp, Caz, Cep, Cl, Cot, Na, P, Sm, Tr	0.77
	Amp, Caz, Cep, Cot, E, Na, P, Sm, Tr	0.77
	Amp, Caz, Cl, Cot, Na, P, S, Sm, Tr	0.77
0.59	Amp, Caz, Cep, Cl, Cot, Cpd, Na, P, S, Sm	0.77
0.65	Amp, Caz, Cep, Cl, Cot, Cpd, Na, P, S, Sm, Tr	1.55

Table 3. MAR index and resistance pattern of bacterial isolates from the Arctic tern after migration

MAR Index	Resistance Pattern	Occurrence (%)
0.06	Ak	1.07
	Sm	1.07
	Na	3.22
	P	4.3
	Caz	5.37
0.11	Amp,C	1.07
	Ak,Caz	1.07
	Ak,Na	2.07
	Amp,Gen	3.07
	Amp,Va	4.07
	C,Te	5.07
	Sm,Tr	6.07
	Te,Va	7.07
	C,Caz	2.15
	Caz,Cl	4.3
	Caz,Na	5.37
	Caz,P	6.45
0.18	Ak,Caz,S	1.07
	Ak,Sm,Te	1.07
	Caz,E,Na	2.15
	Amp,Na,Tr	4.3
0.23	Ak,Caz,Cl,Na	1.07
	Amp,Caz,E,P	1.07
	C,Caz,Cl,Na	1.07
	C,Caz,E,Na	1.07
	Caz,Cl,Na,S	1.07

	Caz,E,P,Te	3.22
	Amp,Na,Sm,Tr	4.3
0.29	Ak,Caz,Cl,S,Va	1.07
	Ak,Caz,E,P,Te	1.07
	Ak,Caz,E,Sm,Te,Va	1.07
	Amp,C,Caz,E,P	1.07
	Amp,Caz,P,S,Te	1.07
	C,Caz,E,Na,S	1.07
	Amp,Cl,E,P,Va	3.22
	Caz,E,Sm,Te,Va	4.3
	Amp,Cl,E,Te,Va	5.37
0.35	Amp,C,Caz,E,Na,P	1.07
	Amp,Caz,Cep,Cl,P,Tr	1.07
0.411	Amp,C,Caz,Cep,Cl,Cpd,Tr	1.07
	Caz,Cep,Cl,Cot,Gen,Te,Va	1.07
	Amp,C,Cl,E,Na,Te,Va	2.15
	Amp,Caz,Cep,Cl,Na,P,Tr	2.15
0.47	Amp,C,Caz,Cep,Cl,Cpd,P,Tr	4.3
0.53	Amp,Caz,Cep,Cot,E,Na,P,Sm,Te	1.07