

1 A smelly business: microbiology of Adélie penguin guano (Point Thomas  
2 rookery, Antarctica)

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9 enzyme activity

10 **Abstract**

11 Adélie penguins (*Pygoscelis adeliae*) are the most numerous flightless bird group  
12 breeding in coastal areas of Maritime and Continental Antarctica. Their activity leaves a mark  
13 on the land in the form of large guano deposits. This guano is an important nutrient source for  
14 terrestrial habitats of ice-free Antarctic areas, most notably by being the source of ammonia  
15 vapors which feed the surrounding grass, lichen and algae communities. Although  
16 investigated by researchers, the fate of the guano-associated microbial community and its role  
17 in decomposition processes remain vague. Therefore, by employing several direct community  
18 assessment methods combined with a broad culture-based approach we provide data on  
19 bacterial numbers, their activity and taxonomic affiliation in recently deposited and decayed  
20 Adélie penguin guano sampled at the Point Thomas rookery in Maritime Antarctica (King  
21 George Island). Our research indicates that recently deposited guano harbored mostly bacteria  
22 of penguin gut origin, presumably inactive in cold rookery settings. This material was rich in  
23 mesophilic enzymes active also at low temperatures, likely mediating early stage  
24 decomposition. Fresh guano colonization by environmental bacteria was minor, accomplished  
25 mostly by ammonia scavenging *Jeotgalibaca* sp. cells. Decayed guano contained 10-fold  
26 higher bacterial numbers with cold-active enzymes dominating the samples. Guano was

27 colonized by uric-acid degrading and lipolytic *Psychrobacter* spp. and proteolytic  
28 *Chryseobacterium* sp. among others. Several spore-forming bacteria of penguin gut origin  
29 persisted in highly decomposed material, most notably uric-acid fermenting members of the  
30 *Gottschalkiaceae* family.

31

## 32 **1. Introduction**

33 Pygoscelis penguins are represented by three species of flightless birds and are found  
34 almost exclusively in Antarctica (Forcada et al. 2006). The most numerous among them are  
35 the Adélie penguins (*Pygoscelis adeliae*) with estimated population numbers around 14-16  
36 million individuals (Southwell et al. 2017). Most of their lives Adélie penguins spend in cold  
37 waters of the Southern Ocean, mostly in those covered or adjacent to sea pack ice. They feed  
38 primarily on Antarctic krill (*Euphausia superba*), foraging through its dense swarms. The  
39 penguins are seen on land mainly during the breeding season, which starts as early as late  
40 September and typically ends in February (Ainley 2002). Within that time frame groups of up  
41 to 1.5 mil individuals are seen in rookeries on land in coastal areas of Maritime and  
42 Continental Antarctica (Borowicz et. al. 2018).

43 Their activity leaves a mark on the land in the form of large guano deposits. This mass  
44 is highly heterogeneous and consists of three phases (fractions). The white fraction contains  
45 mainly urates (major end-product of nitrogen metabolism in birds), oxalates and phosphates.  
46 The red phase constitutes of carotenoid stained chitin from undigested krill shells. A green  
47 fraction which consists of proteins, lipids, cholic acids and undigested algae cells (presumably  
48 krill stomach content) is also present but rarely seen, most often when adult penguins have a  
49 prolonged period of fasting during egg incubation (Myrcha and Tatur 1991; Korczak-Abshire  
50 – personal communication).

51           One of the best studied places where the Adélie penguins breed is the Point Thomas  
52 rookery, located on the Western shore of Admiralty Bay, King George Island, Antarctica  
53 (Trivelpiece and Trivelpiece, 1990; Chwedorzewska and Korczak 2010; Korczak-Abshire et  
54 al. 2016; Sierakowski et al. 2017; Hinke et al. 2018). Breeding phenology at this site differs  
55 slightly from season to season but is roughly realized as follows: egg laying –  
56 October/November, chicks hatching – November/December, chicks gathering in crèches  
57 (nurseries) – December/January, adult and young penguins leave the rookery – February  
58 (Trivelpiece and Trivelpiece, 1990; Ciaputa and Sierakowski 1999; Korczak-Abshire –  
59 personal communication). Adélie penguin numbers increase in the colony when the chicks  
60 hatch, consequently so does the amount of guano being deposited. The height of this  
61 deposition occurs in late January, when chicks reach their target weight but are still fed by  
62 parent birds. The rookery at this point gives off a strong odor, mostly due to ammonia vapors.  
63 This phenomenon called “ammonia shadow” is responsible for the unique flora of the rookery  
64 characterized by lush *Deschampsia antarctica* (Antarctic grass) communities, extensive  
65 *Prasiola crispa* (alga) growth and colorful lichen assemblages comprising endemic *Caloplaca*  
66 and *Xanthoria* species (Tatur et al. 1997). This peculiar ammonia cloud is presumably derived  
67 from the ammonification of uric acid, present in the guano deposit (Lindeboom 1984).  
68 Furthermore, a very chemically aggressive liquid leaching from the decomposing guano  
69 reacts with the underneath loam and gravel, creating ornithogenic soils, providing plants with  
70 one of the few suitable habitats in those mostly barren areas (Myrcha et al. 1985). However,  
71 the mechanism behind this decomposition process or major players involved in it are still  
72 debated (Zhu et al. 2011).

73           The aim of this research was to investigate the bacterial community associated with  
74 deposited Adélie penguin guano at the height of the breeding season (maximal fresh guano  
75 deposition rate) but also after penguin departure from the rookery (advanced guano decay) to

76 elucidate the nature of processes involved in guano decay and its potential as a microbial  
77 habitat. Our hypothesis states that the decomposition of guano starts immediately after its  
78 deposition and is mainly driven by psychrophilic saprotrophs. We thus conducted a thorough  
79 microbiological survey of guano samples using direct assessment methods such as 16S  
80 targeted metagenomics and a culture based approach.

## 81 **2. Materials and Methods**

### 82 *2.1 Sites, sampling, pH and water content analysis*

83 Guano samples were collected during the Austral summer of 2016/2017 in December,  
84 January and March, once a month from three randomly picked sites within the Point Thomas  
85 penguin rookery (Western Shore of Admiralty Bay, King George Island, South Shetland  
86 Archipelago, Antarctica) (Table1). Guano was collected aseptically into sterile plastic bags  
87 and kept at -20°C until laboratory analysis. A total of nine bags (three per month) were filled  
88 with guano, 0.5kg each (4.5kg in total). Temperature during sampling was measured at 1 cm  
89 above guano but also of the guano itself with an Elmetron 410 multifunction meter  
90 (ELMETRON, Poland). Geographical coordinates were gathered with the use of a Garmin  
91 GPSMap 64s device. pH measurements were done according to guidelines provided by  
92 Minasny et al. 2011. Briefly: 1 g of guano was carefully dispersed by vortexing in 1 mL of  
93 ddH<sub>2</sub>O or 0.01 M CaCl<sub>2</sub> solution (flocculent). The guano suspensions were then centrifuged at  
94 5000 rpm for 5 min and pH was measured at 22°C (room temperature) with a Hanna HI 9025  
95 microcomputer pH meter. Measurements were done in triplicate. Dry weight was determined  
96 after 24 h at 65°C in a dry box with circulating air (Dowgiałło 1975).

### 97 *2.2 Bacterial cell extraction*

98 Approx. 1 g of fresh guano was weighted and placed in a 50 mL conical tube  
99 containing 20 mL of sterile and cool (4°C) dilution liquid composed of 1% (w/v) glycerol,  
100 Tween 80 (10 ppm) and 0.1% tetrasodium pyrophosphate (further termed GTP). The

101 suspension was then shaken for 30 min in a Tornado™ Vortexer at 2000 rpm at 4°C. The  
102 tubes were then placed in a VWR Ultrasonic Cleaner USC-TH filled with chilled water and  
103 sonicated for 60 sec. The tubes were vortexed afterwards for 30 sec to suspend detached cells.  
104 After a brief centrifugation (1 min.; 1000 rpm; 4°C) the suspension was submitted to further  
105 analysis. This procedure was adapted from Lunau et al. 2005 and Elliot and des Jardin 1999.  
106 This procedure was done in triplicate from each sampling site resulting in 27 suspensions for  
107 analysis.

### 108 *2.3 Cell count*

109 Microbial cell abundance was assessed as in Grzesiak et al. 2015. TCs (total counts)  
110 were determined by epifluorescence microscopy using 40,6-diamidino-2-phenylindole (DAPI)  
111 on black Nuclepore polycarbonate 0.2-µm-pore-size filters (Porter and Feig 1980), under a  
112 Nikon E-200 microscope with a 100 W Hg lamp and 100x CFI 60 oil immersion objective,  
113 with a digital DS Cooled Camera Head DS-5Mc-U1, and a filter block of wavelengths: EX  
114 330-380, DM 400, BA 420. Images of fields were analyzed in Nikon NIS Elements BR 3.1  
115 and MultiScan v. 14.02 (computer scanning systems). A minimum of 400 cells in 20 fields per  
116 sample were counted automatically in the image analysis system. Percentage contribution of  
117 active bacteria with intact membrane (MEM+) was assessed by the Live/Dead BacLight  
118 Bacterial Viability Kit (Molecular Probes-Invitrogen, UK). Samples of guano were used in  
119 triplicate for enumeration of alive bacteria (Schumann et al. 2003). For 1 mL of the  
120 aforementioned suspension a mixture of two BacLight Kit stains – SYTO 9 and propidium  
121 iodide was added (1:1 ratio, both dyes final concentration: 0.15%) then incubated for 20 min  
122 at room temperature in the dark, filtered through a 0.2 µm pore-size, black polycarbonate  
123 membrane filter (Millipore GTBP) and enumerated by epifluorescence microscopy (Dunalska  
124 et al. 2012). The percentage contribution of MEM+ bacteria was calculated as a ratio of  
125 MEM+ to the sum of MEM+ and MEM– bacterial cells.

126 *2.4 DNA extraction*

127 DNA was directly extracted from guano using the PowerSoil® DNA isolation kit  
128 (QIAGEN, Germany) according to manufacturer protocol. An approx. 0.2 g of guano per  
129 sampling bag was used in triplicate and pooled. DNA solutions were kept at 4°C until further  
130 analysis. Template was quality checked by DNA electrophoresis on 0.7% agarose gel. DNA  
131 from pure bacterial strains was extracted from a single colony. The procedure involved  
132 suspending the colony with sterile toothpick in 100 µL sterile MiliQ Water containing 5% of  
133 Chelex® 200 resin (Sigma) and 5% of garnet sand Lysing Matrix A (MP Biomedicals). The  
134 1.5 mL tube with the bacterial suspension was shaken in a Qiagen Retsch TissueLyserII for 5  
135 min at 39Hz – 1800 oscillation per min. The tubes were then placed in a thermoblock and  
136 heated for 5 min at 99°C. Then the tubes were centrifuged for 3 min at 12000 rpm. Fifty  
137 microliters of the clear supernatant was taken and used for PCR amplifications.

138 *2.5 Sequencing*

139 For Illumina 16S targeted amplicon sequencing pooled environmental samples from  
140 each month were examined. Phylogenetic study was performed by sequencing and analysis of  
141 prokaryotic 16S ribosomal RNA gene. A fragment of the 16S rRNA gene containing the V3  
142 and V4 variable regions was amplified using gene-specific primers: 16S\_V3-F and 16S\_V4-R  
143 positions 341-357F and 785-805R, respectively, according to *Escherichia coli* 16S rRNA  
144 gene reference sequence (Klindworth et al. 2013). Illumina Nextera XT overhang adapter  
145 nucleotide sequences were included in addition to the 16S rRNA gene-specific sequences,  
146 which allowed sample indexing and pooling. Each PCR amplification was done in triplicate  
147 using KAPA HiFi PCR kit (Roche) in a final volume of 20 µL per reaction according to the  
148 manufacturer's instructions. Obtained PCR products were pooled in equimolar ratio and  
149 indexed using Nextera XT barcodes (Illumina, San Diego, USA). Amplicon libraries were  
150 pooled and sequenced on Illumina MiSeq instrument (Illumina, San Diego, USA) in the DNA

151 Sequencing and Oligonucleotide Synthesis Laboratory (Institute of Biochemistry and  
152 Biophysics, Polish Academy of Sciences). Sequencing was done in paired-end mode ( $2 \times 300$   
153 bp) with the use of a v3 (600 cycles) chemistry cartridge which allowed generation of long  
154 paired reads fully covering 16S V3–V4 amplicons.

155         Amplification of 16S rRNA gene fragment from pure strains was performed using  
156 universal primers 27F and 1492R (Lane 1991). PCR amplification reaction conditions were as  
157 follows: 1 min of 95°C initial denaturation followed by 30 cycles of 95°C for 15 sec, 55°C  
158 annealing for 15 sec and elongation 72°C for 1min and 30 sec, using DreamTaq polymerase  
159 (Thermo Scientific-Fermentas). Obtained PCR products (~1500 bp for 16S rRNA gene  
160 fragment) were checked on 0.8% agarose gel and purified using Exonuclease I/Alkaline  
161 phosphatase mix (Thermo Scientific—Fermentas). 16S rRNA gene amplicons were  
162 sequenced using internal 16S rRNA gene primers: 341F, 518R and 928F (Weidner et al.  
163 1996) with the use of BigDye Terminator v.3.1 chemistry and ABI3730xl genetic analyzer at  
164 the DNA Sequencing Laboratory (Institute of Biochemistry and Biophysics, Polish Academy  
165 of Sciences).

## 166 *2.6 Activity assessment*

167         Fluorescein diacetate hydrolysis assay (FDA) was adapted from Jiang et al. 2016.  
168 Fluorescein diacetate hydrolysis is mediated by a variety of enzymes (proteases, lipases,  
169 esterases) present in environmental samples and is therefore considered mostly as indicator of  
170 microbial activity in soils (Green et. al. 2006). 15 mL of each guano suspension was amended  
171 with 0.3 mL of FDA stock solution (0.2 g powder FDA in 200 mL acetone). One set of  
172 suspensions (n=27) was incubated at 4°C (rookery temp.), the other (n=27) at 40°C (penguin  
173 gut temperature - Ropert-Coudert et al. 2000). After the addition of the FDA stock solution  
174 the guano suspensions were mixed thoroughly and immediately 1.5 mL was removed, mixed  
175 with 0.2 mL of acetone to stop the hydrolysis, centrifuged at 5000 x g for 5 min and the

176 supernatant absorbance was read at 490 nm in a Varioscan plate reader (Thermofisher  
177 Scientific) in 8 wells per sample containing 100  $\mu$ L each. Measurements were done every  
178 hour for 6 h. Fluorescein concentrations released by the hydrolysis was calculated based on a  
179 standard curve prepared according to Jiang et al. 2016.

180         Respiration activity by tetrazolium dye reduction was performed in Biolog EcoPlates.  
181 Guano suspensions were centrifuged at 6000 rpm for 5 min at 4°C, suspended in sterile, cool  
182 0.9% saline and were adjusted with sterile 0.9% saline to optical transmittance of 0.9. 100  $\mu$ L  
183 aliquots of each suspension were added to each well of Ecoplate microplates (Biolog Inc.,  
184 Hayward, CA, USA). The plates were incubated in darkness at 4°C, the color development  
185 was measured at 590 nm with a microplate reader (OmniLog) and cellular respiration was  
186 measured kinetically by determining the colorimetric reduction of tetrazolium dye. Data were  
187 collected approximately twice a week over a >65 day period. The Ecoplate Biolog assays  
188 assess the ability of a mixed microbial community to utilize any of 31 carbon compounds as  
189 the sole carbon source (+ one control well with no-carbon). Microbial communities were  
190 characterized for their ability to catabolize 10 different carbohydrates, 9 carboxylic and acetic  
191 acids, 4 polymers, 6 amino acids and 2 amines (Weber and Legge, 2009). Absorbance data  
192 from the different reading times were first blanked against the time “zero” reading and then  
193 OD 590 values, from a defined plate and reading time, were blanked against the respective  
194 control well containing no-carbon source

### 195 *2.7 Culture-based analysis*

196         Colony forming unit (CFU) enumeration was done using the drop plate method  
197 optimized by Herigstad et al. 2001. Each agar plate was divided into quadrants. One quadrant  
198 was dedicated for one dilution. Fifty  $\mu$ liters of a decimally diluted guano suspension was  
199 dispersed in five evenly spread 10  $\mu$ L drops onto the same quadrant. The plates were carefully  
200 transferred and stored face up for approx. 30 min at 10°C to let the agar absorb the liquid.



201 CFU numbers in a range of temperatures was examined in aerobic conditions on R2A agar  
202 (Biocorp). Plate replicas were incubated at 4, 10, 18, 22, 28 and 37°C in Liebher thermostat  
203 cabinets for 6 weeks. CFU numbers of copiotrophic microbes (tolerating high nutrient  
204 concentrations) were assessed on Rich Medium (Atlas, 2010) at 4°C in aerobic atmosphere.  
205 Culturable anaerobe abundance was examined on Thioglycollate Agar (Conda). Plates were  
206 incubated at 4°C in anaerobic conditions (bioMerieux Genbag Anaer System). Cultivable  
207 penguin gut bacteria were enumerated on BHI agar (Difco) at 40°C in microaerobic  
208 conditions (bioMerieux Genbag Microaer System).

209         Strains for morpho-physiological analysis were cultivated using the traditional spread  
210 plate method. 100 µL aliquots of the decimal dilutions of the aforementioned suspensions  
211 were plated on pre-cooled R2A agar plates. The plates were incubated at 10° C for 6 weeks to  
212 assure complete colony development. The strains were picked randomly, 100 per sampling  
213 month and purified by re-streaking onto fresh agar plates. All strains were sub-cultured on  
214 R3A broth (Atlas, 2010). Pure bacterial strains were tested for catalase and oxidase activity  
215 using 3% H<sub>2</sub>O<sub>2</sub> and Biomerieux Oxidase Reagent, Gram reaction by 3% KOH lysis and cell  
216 shape morphology (crystal violet staining). Further characteristics were assessed on agar  
217 plates by placing a 5µL drop of strain cell suspension onto an appropriate medium followed  
218 by a 2 week incubation at 10° C in aerobic conditions. Proteolytic activity of guano derived  
219 strains was examined on Skim Milk Agar (Atlas, 2010). Clearing around bacterial growth was  
220 scored as positive for protein degradation. Chitin agar was prepared according to Atlas 2010.  
221 5 g of chitin powder from crab shells (Sigma) was dissolved in 200 mL of cooled 37% HCl.  
222 The resulting liquid was poured into 1.5 L of cooled deionized water, mixed thoroughly and  
223 let sit overnight to precipitate colloidal chitin. The chitin was washed 5 times with deionized  
224 water by repeated centrifugation and resuspension in fresh dH<sub>2</sub>O and finally suspended in 1 L  
225 of deionized water. pH was adjusted to 7.2 using 3% NaOH solution. Then mineral salts were

226 added to make a mineral salts medium containing per L: Na<sub>2</sub>HPO<sub>4</sub> 1.1 g, KH<sub>2</sub>PO<sub>4</sub> 1 g, NH<sub>4</sub>Cl  
227 1 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g, CaCl<sub>2</sub> 0.05 g, Yeast extract 0.05 g, Fe-citrate 0.01 g, NaMoO<sub>4</sub>·2H<sub>2</sub>O  
228 0.005 g and agar 15 g, autoclaved and poured into Petri dishes. Clear zone formation around  
229 bacterial growth was scored as positive for chitinolysis. Strains' lipolytic abilities were tested  
230 with a modified lipoidal emulsion agar method. The medium consisted of two components:  
231 base agar (in g/L: Tryptone - 5, Yeast extract – 2.5, Agar – 15) and lipoidal emulsion (in g/L:  
232 Tween 80 – 2, Rapeseed oil – 200). After being separately autoclaved 970 mL of the base  
233 agar was amended with 30 mL of the lipoidal emulsion and poured onto petri dishes. After  
234 inoculation and a 2 week incubation at 10°C, the agar plates were flooded with a 5% CuSO<sub>4</sub>  
235 solution. The resulting bright blue halo (fatty acid copper salts) around bacterial growth was  
236 scored as positive for lipid degradation. Uric acid agar for uric acid utilization assay was  
237 prepared using the double layer technique mentioned by Bravo et al. 2015. A bottom layer of  
238 base mineral agar identical to the mineral salts medium described earlier was poured first.  
239 After it solidified a second, thinner layer of the same agar was poured but containing 10 g/L  
240 of suspended uric acid. After inoculation and a 2 week incubation at 10°C a clearing around  
241 bacterial growth was scored as positive for uric acid utilization. Calcium oxalate agar for  
242 oxalate degradation assay was prepared using the double layer technique mentioned by Bravo  
243 et al. 2015. After inoculation and incubation a clearing around bacterial growth was scored as  
244 positive for oxalate utilization. Pure bacterial strains were tested in terms of phosphate  
245 solubilizing abilities on the NBRIP medium (Nautiyal 1999). Acetate utilization was assessed  
246 on a Mineral Base Medium with Acetate (Atlas, 2010). Sodium acetate utilization by bacteria  
247 was scored as positive when the medium turned blue due to rise in alkalinity.

248 *Jeotgalibaca* sp. growth was tested on mineral salts liquid medium and R3A broth  
249 supplemented with nitrogen sources that are produced during uric acid degradation  
250 (Ramazzina et al. 2006): allantoin (0.73 g/L), urea (0.56 g/L) and ammonia (NH<sub>4</sub>Cl -1 g/L);

251 nitrification: nitrites ( $\text{KNO}_2$  – 1.58 g/L) and nitrates ( $\text{NaNO}_3$  – 1.58 g/L) and also with an  
252 amino acid mix (Casamino acids – 1 g/L). Nitrogen content was calculated to be equal as in 1  
253 g of  $\text{NH}_4\text{Cl}$  for each nitrogen source. Growth was measured as absorbance at 600 nm on a  
254 Varioscan device in a microtiter plate. *Jeotgalibaca* sp. growth enhancement experiments  
255 involved drop plating a suspension of *Jeotgalibaca* sp. cells on R3A agar in increasing  
256 distances from a colony of uric acid degrading *Psychrobacter* sp. growing on either a plug  
257 with uric acid agar or on R3A agar itself. Appropriate control were done with no  
258 *Psychrobacter* sp. inoculation. Additionally *Jeotgalibaca* sp. was grown the same way in  
259 vicinity of an agar cut well containing an  $\text{NH}_4\text{Cl}$  solution (3 g/l).

## 260 2.8. Data analysis

261 Obtained Illumina reads were quality checked using FastQC software  
262 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) (Andrews 2010). Raw  
263 sequencing data were cleaned, aligned and classified automatically by the EzBioCloud  
264 platform using the PKSSU4.0 database (Yoon et al. 2017). Illumina reads were deposited in  
265 the NCBI Sequence Read Archive (SRA) as BioProject PRJNA566128.

266 Strain sequence chromatogram files were analyzed using FinchTV ver. 1.4.0  
267 (Geospiza, Akron, USA). Consensus sequences were obtained with Seqman Pro ver. 9.1  
268 software (DNASTar, Madison, USA). 16S rRNA gene fragments sequences were aligned  
269 against 16S reference sequence database GenBank using BLAST (Altschul et al. 1990).  
270 Multiple sequence alignments were performed using ClustalW program. Phylogenetic trees  
271 were constructed using MEGAX software. Sequences were deposited in GenBank under  
272 accession numbers MN480548-MN480558.

273 Data compilation, basic statistical analysis and graphs were done using Excel (MS Office  
274 2016 for Windows).

## 275 3. Results

276 *3.1 Sampling site description and physicochemical factors.*

277 Table 1 contains a brief description of conditions at the Point Thomas penguin rookery  
278 with temperature data for air and guano *in situ*. Guano gathered in December had the highest  
279 temperature measured during the sampling period (av. 11.8°C) with average temperature 1cm  
280 above guano deposits equating 6.7°C, while air temperature measured by the meteorological  
281 station was 2.8°C. Guano temperatures in January were slightly lower than the ones registered  
282 in December (av. 8.4°C) with comparable temperature 1cm above guano (av. 6.1°C) but with  
283 higher air temperature (4.6°C). Guano sampled in March was the coldest, averaging 3.8°C,  
284 with temperatures 1 cm above guano 5.6°C on average and temperature registered by the  
285 meteo-station was 2.1 °C.

286 Guano pH in December was slightly acidic (6.7-6.9 in ddH<sub>2</sub>O and 6.6-6.8 in 0.01M  
287 CaCl<sub>2</sub>) while guano in January had a slight alkaline pH (7.1-9.0 in ddH<sub>2</sub>O and 7.1-8.8 in  
288 0.01M CaCl<sub>2</sub>) with pronounced variations between samples. Guano sampled in March had a  
289 uniformly alkaline pH (8.1-8.6 in ddH<sub>2</sub>O and 7.7-8.0 in 0.01M CaCl<sub>2</sub>).

290 *3.2 Direct cell count*

291 Bacterial total counts (TC) were similar in December and January. December-sampled  
292 guano counts ranged from 1.15 to 8.06x10<sup>10</sup> cells per gram dry weight (av. 3.42x10<sup>10</sup>), while  
293 January counts were in the range of 1.7 to 8.14x10<sup>10</sup> cells per g d.w. (av. 3.26x10<sup>10</sup>). A 10-  
294 fold increase in bacterial cell abundance was noted in March with 2.93x10<sup>11</sup> cells per g d.w.  
295 (1.75-3.54x10<sup>11</sup> cells/g d.w.) (Fig. 1).

296 Percentage of cells with intact membranes (“alive” MEM+) was the highest in March-  
297 sampled guano reaching 99% (98.1% on av.) with minor discrepancies between sampling  
298 sites. More than half of cells in guano sampled in January were “dead” (MEM-) although  
299 major differences between sites were noted (40-65%). The least “alive” cell contribution was

300 found in December-sampled guano (27.5%) also with high variation between sites (18-45%)  
301 (Fig. 2).

### 302 3.3 Phylogenetic affiliation

303 Valid reads numbers obtained from the samples were as follows: December - 73336,  
304 January – 71552, March – 73237. Phylum rank bacterial community composition is presented  
305 in Fig. 3 and family rank sequence contribution in Table 2. Sequences belonging to the  
306 Firmicutes phylum were the most numerous in all investigated samples (December – 70%,  
307 January – 44%, March - 53%). Within this phylum the family *Gottschalkiaceae* displayed a  
308 steady presence in all samples in the range of 18-19%. A fairly high percentage of sequences  
309 was maintained also by sequences belonging to the *Bacillaceae* (mean 4.8%),  
310 *Carnobacteriaceae* (10.23%), *Eurysipelotrichaceaea* (4.17%) and *Tissierellaceae* (6.27%).  
311 Bacteroidetes sequences were found to be the second numerous group (on av.) in guano  
312 samples (December – 10.6%, January – 37.6%, March – 25.4%). Two families that displayed  
313 high abundances (based on sequence percentage) were the *Flavobacteriaceae* (av. 11.97%)  
314 and *Porphyromonadaceae* (10.35%). The *Flavobacteriaceae* sequences were most abundant  
315 in January-sampled guano (26.27%) whereas the *Porphyromonadaceae* in March-sampled  
316 material (15.56% of all sequences). Proteobacterial sequences contribution (December –  
317 13.38%, January – 9.93%, March – 18.62 %) were mainly due to the presence of sequences  
318 belonging to two families: the *Moraxellaceae* (av. 7.12) and *Pseudomonadaceae* (av. 3.63).  
319 The *Moraxellaceae* contribution was the highest in December sampled guano whereas the  
320 members of *Pseudomonadaceae* family were the most numerous in March sampled material.  
321 Lastly, the phylum Actinobacteria (December – 4.9%, January – 6.9%, March – 2%) were  
322 represented mainly by the families *Intrasporangiaceae* (av. 1.08%) and *Nocardiaceae* (av.  
323 1.45%). Unaffiliated bacterial sequences and belonging to other phyla comprised less than 1%  
324 in each sample.

325           Eleven strains were chosen as representatives of groups that emerged in clustering by  
326 morpho-physiological features (data not shown) and subjected to identification based on 16S  
327 rRNA sequence similarity. Seven strains were affiliated to genus *Psychrobacter*, two to  
328 *Jeotgalibaca* and two to *Chryseobacterium*. *Psychrobacter* strains isolated from December  
329 (D1, D4) and January-sampled guano (J3) had the highest similarity based on 16S rRNA gene  
330 sequence coverage to *Psychrobacter adeliensis* (type strain) DSM15333 (98-100%) while  
331 *Psychrobacter* strains derived from March-sampled material displayed greater similarity to  
332 *Psychrobacter faecalis* (type strain) Iso-46. Discovered only on plates inoculated with guano  
333 sampled in December and January were members of the genus *Jeotgalibaca*, which displayed  
334 enough low similarity (96-97%) to the sequence of its closest described species *J.*  
335 *dankookensis* to be considered a new species. *Chryseobacterium* strains were isolated only  
336 from March-sampled guano and displayed closest similarity (by 16S rRNA gene sequence) to  
337 *Ch. antarcticum* type strain AT1013 (Fig. 4).

### 338 *3.4 Enzyme and respiration activity in P. adeliae guano*

339           Fluorescein release in December and January samples displayed considerable  
340 discrepancies between incubation temperatures. A 6.5-fold increase of fluorescein release (in  
341 mg/kg guano d. w. h<sup>-1</sup>) in December samples was noted at 40°C compared to 4°C. January  
342 material exhibited a similar response with an almost 4-fold increase. March-sampled guano  
343 releases were fairly similar in 4 and 40°C (32.7 and 47.6 mg/kg guano d. w. h<sup>-1</sup> respectively)  
344 (Fig. 5).

345           Substrate-dependent microbial community respiration measured as colorimetric  
346 responses in Biolog Ecoplates and displayed in Omnilog Arbitrary Units were (on average)  
347 lower in December and January samples (16.19 and 32.9 respectively) compared to March  
348 samples (163). Carbon sources that triggered greatest responses were: L-Asparagine (291),  $\alpha$ -

349 D-Lactose (266), D-Cellobiose (257), L-Arginine (244), L-Phenylalanine (232), L-Serine  
350 (221), Tween 40 (214),  $\alpha$ -Cyclodextrin (199) and Putrescine (197) (Fig. 6).

### 351 3.5. Bacterial cultivation

352 Colony forming unit (CFU) enumeration in different temperatures revealed major  
353 discrepancies between samples from different months. December samples displayed highest  
354 CFU counts ( $8.5 \times 10^6$ ) in 22°C (moderate mesophile range) while the lowest values were  
355 recorded at 18°C (psychrotolerant range). January-sampled guano CFU's ranged from  
356  $1.75 \times 10^5$  to  $2.36 \times 10^6$ , displaying highest and lowest values at 37°C and 10°C respectively.  
357 March sampled guano CFU's showed considerable variations between incubation  
358 temperatures (from  $1.32 \times 10^5$  in 37°C to  $3.79 \times 10^9$  in 18°C) (Fig. 7).

359 Psychrophilic (4°C) oligotrophic (R2A medium) CFU count was the highest in March-  
360 sampled guano reaching  $8.00 \times 10^8$  CFU/g d.w. followed by December ( $6.57 \times 10^6$ ) and January  
361 counts ( $1.05 \times 10^6$ ). Psychrophilic copiotrophic (RM medium) counts in March-sampled guano  
362 exhibited the highest CFU per g. d. w. of all conditions and media examined ( $1.89 \times 10^{10}$ ).  
363 December and January sampled guano also displayed high values ( $4.9 \times 10^7$  and  $1.5 \times 10^7$   
364 CFU/g d.w. respectively). Psychrophilic anaerobic count in December and March samples  
365 was comparable ( $1.91 \times 10^7$  and  $2.88 \times 10^7$  CFU/g d.w.), lower in January –  $7.7 \times 10^5$  g d.w.  
366 Simulated penguin gut conditions (BHI agar, microaerobic atmosphere) yielded uniform  
367 results in all samples (December –  $7.02 \times 10^5$ , January –  $5.95 \times 10^5$ , March –  $6.02 \times 10^5$  CFU/g  
368 d.w.) (Fig. 8). Cultivability (CFU/TC%) of guano-derived bacteria was extremely low,  
369 especially in January (0.000-0.039%) and December samples (0.001-0.145%). Relatively high  
370 cultivability was achieved in March samples, most notably on Rich Medium (RM) reaching  
371 6% (Table 3).

372 Bacterial strains derived from December-sampled guano overall had the widest range of  
373 properties involved in guano decomposition albeit only a small number of strains displayed

374 those features (uric acid degradation – 27%, calcium phosphate solubilization – 7%, chitin  
375 degradation – 2%, proteolysis –6%, calcium oxalate decomposition – 1%, acetate utilization –  
376 11%). 15% of December strains displayed 2 traits, 20% - one trait and 65% displayed no traits  
377 considered as useful in guano utilization. Strains derived from January samples displayed  
378 even less properties (uric acid degradation – 10%, calcium phosphate solubilization - 4%,  
379 proteolysis – 2%. Only 4% of January strains displayed more than 1 property, 85% showed no  
380 potential involvement in guano decomposition. A majority of strains isolated from March-  
381 sampled material displayed guano-decomposing properties. Uric acid degradation and  
382 lipolysis was exhibited by 42 and 44% of strains respectively. Proteolysis and acetate  
383 utilization was displayed by 31% and 23% of strains respectively. 16% of those strains  
384 displayed more than three guano-degrading features, 24% displayed two, 44% - one and only  
385 16% had none of the examined traits (Fig.9).

386 Growth of *Jeotgalibaca* sp. J13 was visibly enhanced in vicinity of uric acid degrading  
387 *Psychrobacter* sp. J3 growing on an uric acid agar plug (Fig. 1S A). Similar enhancement  
388 was observed in vicinity of an agar-cut well containing a concentrated  $\text{NH}_4\text{Cl}$  solution (Fig.  
389 1S B). There was no enhancement of *Jeotgalibaca* sp. S13 growth around uninoculated uric  
390 acid agar plug (Fig. 1S C) and only slight enhancement around *Psychrobacter* sp. S3 growing  
391 directly on R3A agar (Fig. 1S D).

392 On the premise that *Jeotgalibaca* sp. J13 growth was enabled due to catabolism  
393 products of uric acid degradation, a variety of compounds were tested as nitrogen sources.  
394 Organic uric acid degradation products (allantoin and urea) were negative in promoting  
395 growth on mineral (minimal) and full nutrient medium (R3A). So were nitrates and nitrites  
396 and surprisingly also Casamino acids. Only ammonia (final nitrogen-bearing product of uric  
397 acid degradation) in the form of  $\text{NH}_4\text{Cl}$  was able to support growth on R3A but not on  
398 minimal mineral medium with glucose (Table 1S).



#### 400 4. Discussion

401 Presented data highlight microbial community characteristics of recently deposited  
402 and decayed Adélie penguin guano at the Point Thomas rookery in Maritime Antarctica.  
403 Diversity, activity and abundance of resident bacterial cells have been assessed by direct and  
404 culturable approaches to examine the fate of deposited guano *in situ*.

##### 405 4.1 Early stages of decomposition

406 Recently deposited guano (December and January samples) was characterized by  
407 relatively high bacterial numbers, although the percentage of injured cells (MEM-) was  
408 substantial. 16S rRNA gene amplicon sequencing revealed that those samples were dominated  
409 by members of the phylum Firmicutes, including mesophilic, anaerobic bacteria belonging to  
410 the *Gottschalkiaceae*, *Erysipelotrichaceae*, *Peptostreptocaccaceae* and *Tissierellaceae*  
411 families (Poehlein et al. 2017, Verbarq et al. 2014, Slobodkin 2014, Alauzet et al. 2014).  
412 These bacteria, presumably of Adélie penguin intestine origin, may have been prone to  
413 damage by oxidative stress and freeze thaw cycles occurring at the rookery site (Plenzler et al.  
414 2019). Noteworthy here is the family *Gottschalkiaceae*, which consists currently of only three  
415 species: *Gottschalkia acidurici*, *Gottschalkia purinilytica* and *Andreessenia angusta*, all of  
416 which are strictly anaerobic, spore-forming, uric acid fermenters (Poehlein et al. 2017).  
417 Sequences clustering within this family have been recovered from bird-associated materials,  
418 most notably from penguin nesting sites (Aislabie et al. 2009a, Kim et al. 2012, Banks et al.  
419 2009), suggesting their major involvement in guano decomposition. However, their optimum  
420 growth temperature is noted to be 30-37°C, which is considerably higher than the measured  
421 guano temperature *in situ* in this study, suggesting negligible activity after deposition  
422 (Poehlein et al. 2017). Therefore, ammonia released during active breeding period measured  
423 by several researchers (Mizutani et al. 1985, Theobalt et al. 2013, Riddick et al. 2016) did

424 presumably originate from uric acid degradation occurring before rather than after defecation  
425 with ammonia being released from the deposited guano largely by physical or chemical  
426 processes (Zhu et al. 2011).

427         Microbial respiration on different organic substrates serving as electron donors was  
428 minor at low temperatures (4°C) as measured by tetrazolium salt reduction in Biolog  
429 Ecoplates. Moreover, cultivability of bacterial cells was also very low, despite using a range  
430 of different media, temperatures and oxygen concentrations. Minor cultivability of bacteria in  
431 fresh Adélie penguin guano has also been observed by Zdanowski et al. 2005. Culture  
432 techniques recover mostly opportunistic microbes (Siegler and Zeyer, 2004; Zdanowski et al.  
433 2013), those that are known to start decomposition processes of a variety of organic  
434 compounds (Mooshammer et al. 2014; Naeem et al. 2000). Low respiration rates and  
435 cultivability (especially at low temperatures) suggests negligible colonization by  
436 psychrophilic bacteria at this stage of decomposition. High deposition rates might be one of  
437 the causes of this phenomenon, where psychrophilic decomposer bacteria derived in moderate  
438 quantities by various means are being constantly buried and/or diluted by ongoing guano  
439 supply. This is apparent when comparing strain activities derived from December and January  
440 sampled guano. Percentage of psychrophilic guano decomposers in December is considerably  
441 higher, probably due to lower fecal deposition rates (smaller chicks are fed less frequently)  
442 and therefore higher chance of cold-adapted saprotroph population establishment. However,  
443 CFU numbers are comparable, suggesting some other factors might also exert influence, like  
444 high concentration of ammonia, amine compounds or even phytoplankton-derived acrylic acid  
445 (Thouzeau et al. 2003; Sieburth 1959, Sieburth 1960). pH does not seem to have the potential  
446 to influence bacterial colonization greatly, as it falls within a “safe” physiological range.

447         Interestingly, the FDA assay confirmed the mesophilic nature of enzymes present in  
448 December and January sampled guano which displayed considerable activity in 40°C

449 (penguin gut temperature) compared to 4°C (Struvay and Feller 2012; Ropert-Coudert Y et al.  
450 2000). This suggest they may be of penguin and/or penguin gut bacteria origin. Surprisingly,  
451 their activity at 4°C was comparable to enzyme activity in March sampled material (advanced  
452 stage of decomposition), where psychrophilic enzymes were present (similar activity at 4 and  
453 40°C), which points towards them still being active in recently deposited guano *in situ*,  
454 implying the possibility of guano components degradation without active microbial  
455 participation (Myrcha et al. 1985). This would explain low percentage of strains bearing  
456 guano decomposing traits isolated from December and January sampled material. Rather than  
457 degrading complex molecules, primal colonizers scavenge the already digested nutrients  
458 (Ghosh et al. 2017).

459         One such scavenger has been reveal in this study. The recovered *Jeotgalibaca* sp.  
460 represents an interesting case of an ammonia scavenger. Its growth was very poor when no  
461 ammonia was present. Growth was greatly enhanced when ammonia salts were added or  
462 ammonia producing bacteria grew next to it. *Jeotgalibaca* sp. also required complex media to  
463 grow, further confirming its auxotrophic and scavenging life style. Colonization by  
464 *Jeotgalibaca* sp. presumably occurs after deposition as the isolated strains were psychrophilic  
465 with their growth being hampered in media above room temperature (data not shown). And  
466 there are no reports of it forming heat resistant endospores (Lee et al. 2014; Zamora et al.  
467 2017). Reports of its sister genus *Trichococcus* say this group is associated with penguin  
468 guano deposits, however there is no mentioning of it requiring NH<sub>3</sub> for growth (Pikuta et al.  
469 2006).

470         Despite having some similar traits, the bacterial community composition of December  
471 and January samples differed even at the phylum level. January sampled guano had a  
472 considerable amount of Bacteroidetes sequences. This might be connected to the age of young  
473 penguins and the state of their intestine flora. Barbosa et al. (2016) observed such differences

474 in fecal samples from chicks and adults of chinstrap penguins (*Pygoscelis antarctica*), where  
475 adult birds tended to have more bacteria of the phylum Bacteroidetes, whereas chicks  
476 harbored more Firmicutes.

#### 477 *4.2 Late decomposition stage*

478 March samples represent guano in an advanced decomposition stage as there has been  
479 no guano deposition for at least a month at this point. In those samples colonization by  
480 psychrophilic saprotrophs was apparent. FDA assay shows similar enzyme activity at 4°C and  
481 40°C pointing towards psychrophilic enzyme presence (Struvay and Feller 2012). Elevated  
482 microbial numbers compared to December and January samples indicate bacterial  
483 proliferation, implying also new niche establishment, similarly as observed by Zdanowski et  
484 al. (2005). Furthermore, a great majority of cells had intact membranes, suggesting their  
485 adaptation to prevalent conditions. Microbial respiration rates at 4°C were considerably  
486 higher than in early decomposition stages confirming microbial activity *in situ*. However,  
487 penguin gut derived bacteria like the *Gottschalkiaceae* remained at similar abundance level as  
488 in recently deposited guano. This suggests adaptation to harsh Antarctic conditions or what is  
489 more plausible presence of inactive endospores as these bacteria (formerly in genus  
490 *Clostridium*) have been proven to possess this ability (Olguín-Araneda et al. 2015; Poehlein et  
491 al. 2017). This scenario could also be applied to several other penguin gut related spore  
492 formers persisting in decayed guano (Verbarg et al. 2014, Slobodkin 2014, Alauzet et al.  
493 2014). Not so, for the *Porphyromonadaceae* family members, all of which are strict non-spore  
494 forming anaerobes (Sakamoto 2014). Their contribution increases in later stages of  
495 decomposition, which suggests their proliferation on site therefore implying anaerobic  
496 microhabitat establishment. The *Porphyromonadaceae* in Antarctic settings have been  
497 enriched in media at anaerobic conditions from wind dispersed cryoconite material  
498 (Zdanowski et. al. 2017), hinting toward their ability to survive oxic atmosphere conditions.

499 Frequently associated with penguins are bacteria belonging to the *Carnobacteriaceae*  
500 (Firmicutes) and *Moraxellaceae* (Proteobacteria) families (Yew et al. 2017; Kim et al. 2012,  
501 Dewar et al. 2013). Several genera within *Carnobacteriaceae* family have been found in  
502 association with penguin-on-land activity, including *Carnobacterium*, *Atopostipes*,  
503 *Trichococcus* and discovered in this study *Jeotgalibaca* (Shekh et al. 2009; Aislabie et al.  
504 2009a, Pikuta et al. 2006). Besides the latter, their role in guano and guano derived materials  
505 (ornithogenic soils) have not been elucidated. However, their source seems to be  
506 environmental rather than intestinal due to their psychrophilic/psychrotrophic nature and no  
507 ability to produce heat-resistant spores (Lawson and Caldwell 2014). Belonging to the  
508 *Moraxellaceae* family *Psychrobacter* spp. have been found as an integral part of penguin and  
509 other birds' excrements (Romaniuk et al. 2018; Dewar et al. 2017). It's main role is that of an  
510 aerobic uric acid degrader as seen in December and January samples. However,  
511 *Psychrobacter* strains isolated from March-sampled guano also had the ability to degrade  
512 triglycerides (lipolytic activity). This implies depletion of the aforementioned digested  
513 nutritional compounds and colonization by active saprotrophic degraders rather than  
514 scavengers. This is further confirmed by the cultivation of proteolytic strains of the genus  
515 *Chryseobacterium*, found only in decayed guano. Very little chitinolytic strains have been  
516 recovered from guano which is consistent with some of the previous observations (Aislabie et  
517 al. 2009b; Zdanowski et al. 2004, Pietr et al. 1983). Zdanowski et al. 2005 found that 40% of  
518 chitin present in guano after deposition remains even after 42 days of decay in rookery  
519 conditions, while CFU numbers of aerobic chitin degraders were low ( $10^5$  g d.w.). Presumably  
520 chitin decomposition is mediated by gut derived chitinases in recently deposited guano and if  
521 in later stages chitin degradation still occurs unculturable and/or anaerobic microbes may be  
522 responsible. Aerobic, cultivable chitinolytic bacteria may occur in detectable quantities in  
523 even later stages of guano decay, as they have been found to live in ornithogenic soils

524 (Aislabie et al. 2009a). Several analyzes suggest that in these stages scavengers also occur.  
525 Acetate and putrescine, common waste products of bacterial metabolism (Wolfe 2005; Pircher  
526 et al. 2007), were catabolized by bacteria in March gathered samples, which implies that a  
527 different group of scavenging microbes thrived on the end products of saprotrophic guano  
528 degradation. Despite that penguin guano is said to be rich in mostly insoluble phosphates  
529 (Tatur and Barczuk 1985), a minor number of phosphate solubilizing bacteria have been  
530 recovered. This could imply either that the guano has enough labile phosphorus sources  
531 (dissolved phosphates, organic P) or that the solubilization of phosphate minerals might be  
532 releasing toxic substances like fluorine or heavy metals and is therefore unfavorable  
533 (Zdanowski et al. 2005; Romaniuk et al. 2018). Cellulose and lactose consumers were also  
534 present, as hinted by the EcoPlates. Lactose metabolism might be explained by the presence  
535 of lactic acid bacteria of the aforementioned family *Carnobacteriaceae* (Lawson and Caldwell  
536 2014). Cellulose as catabolic substrate may occur due to colonization of the guano by the  
537 ornithocoprophyllic alga *Prasiola crista* (Smykla et al. 2007) and due to remnants of  
538 microscopic algae deposited with the guano as krill stomach content (Myrcha and Tatur  
539 1991).

## 540 **5. Conclusions**

541 Psychrophilic saprotrophs rather slowly colonized recently deposited guano, while the  
542 bulk of bacteria at that time was of penguin gut origin. Enzymes present in fresh guano were  
543 also mostly of gut origin but had the potential to contribute to guano decomposition *in situ*, at  
544 least for some period of time. This set the stage for simple compound scavenger colonization.  
545 One of the first psychrophiles to colonize the guano were ammonia scavengers of the  
546 *Jeotgalibaca* genus, which fed on dissolved ammonia produced by anaerobic bacteria in  
547 penguin guts that was being released after deposition. Psychrophilic, aerobic uric acid  
548 decomposers like *Psychrobacter* sp. colonization was apparent but negligible. Their presence

549 was more noticeable in later stages while a specific succession occurred within this genus  
550 with a broadening of utilized substrate spectrum. Decayed guano also harbored proteolytic  
551 *Chryseobacterium* sp., acetate and putrescine scavengers and also cellulose and lactose  
552 consumers. Several spore forming intestinal bacteria persisted throughout the season in guano  
553 material but their activity *in situ* was questionable.

554 This is the first study that showcases the severe changes that naturally occur within the  
555 Adélie penguin guano microbial community throughout the summer season. Due to the  
556 variety of methods used it was possible to conclude, that large bacterial groups that show up  
557 in high throughput amplicon sequencing data might be dead or inactive, especially in cold  
558 polar region settings. The cultivable approach provided information on how to increase the  
559 cultivability of guano associated bacteria as well as pinpointed the timeframe in which to  
560 prospect for bacteria bearing certain traits that are of value for biotechnological application.  
561 Nonetheless, a more detailed investigation is needed, preferably spanning a whole year of  
562 sampling to gain a more precise insight into the microbiological interactions within this  
563 unique habitat.

564

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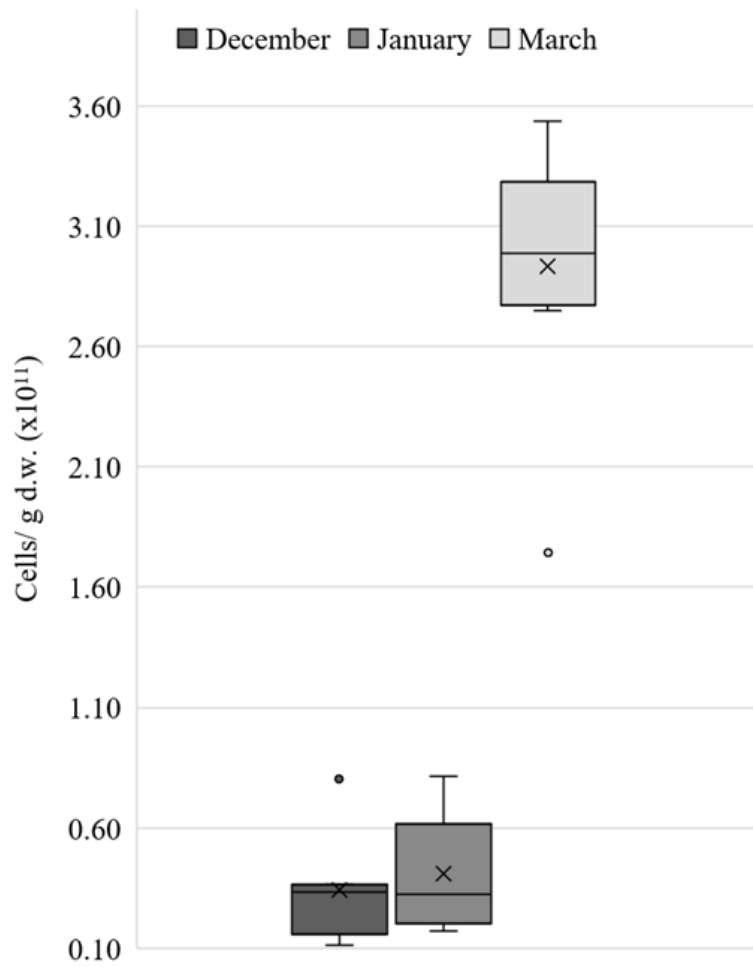
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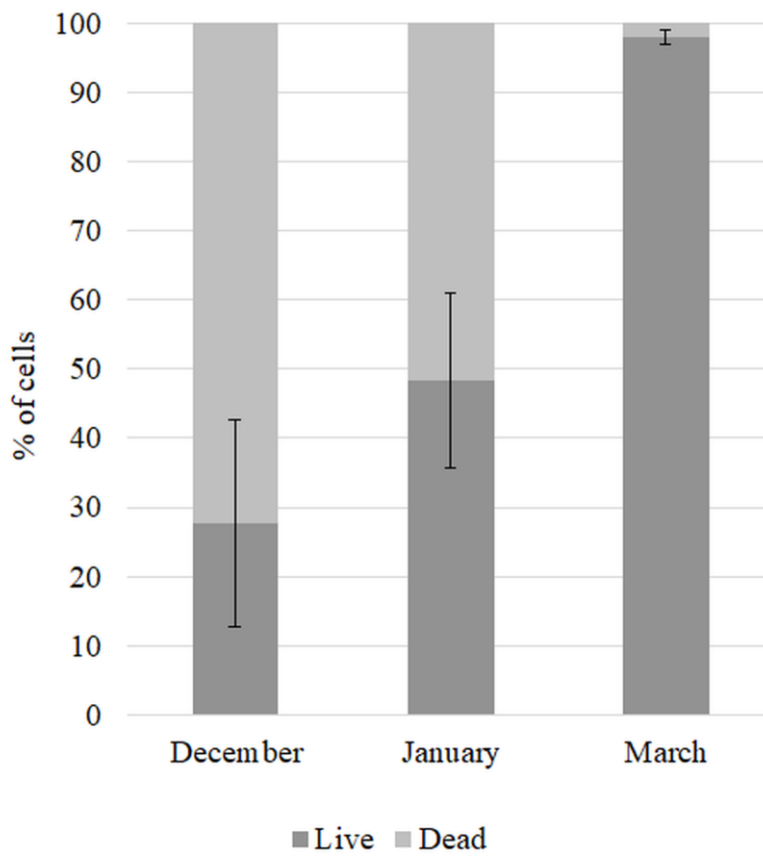
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864 **Fig.1. Bacterial total count in penguin guano. Each boxplot represents the distribution**  
 865 **of cell numbers per gram dry weight (g. d. w.) of guano in each sampling month. X-**  
 866 **mean value.**

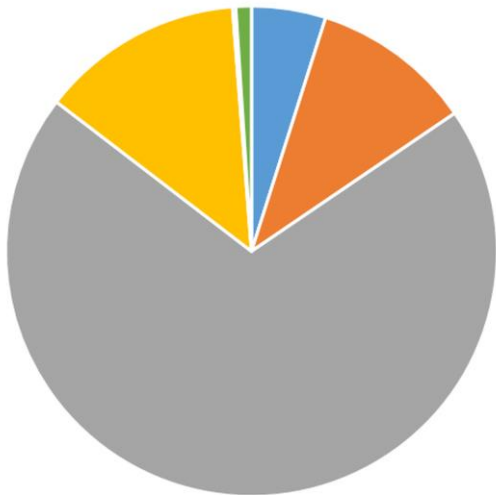


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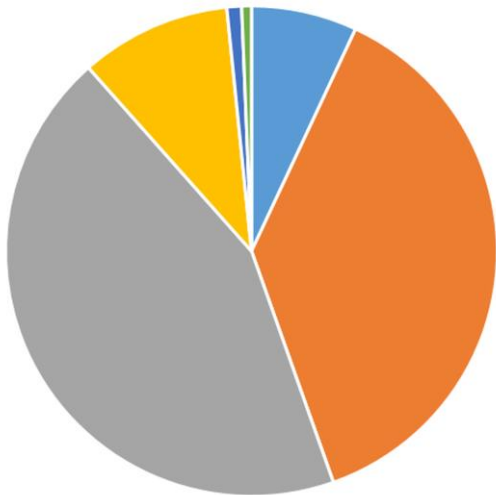
868 **Fig.2. Percentage of bacterial cells with intact (Live) and disrupted membranes (Dead)**

869 **within guano samples from each month. Mean values with standard error.**

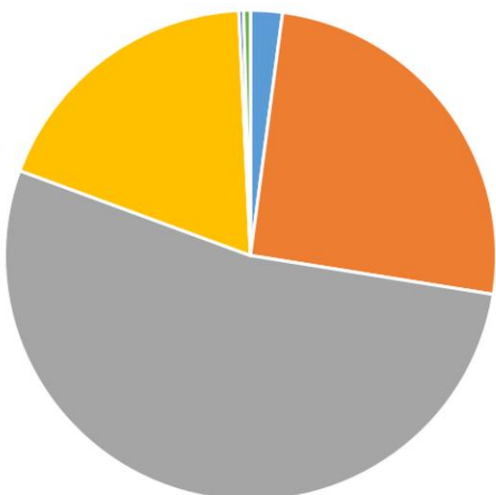
**December**



**January**



**March**



- Actinobacteria
- Bacteroidetes
- Firmicutes
- Proteobacteria
- Saccharibacteria\_TM7
- Others

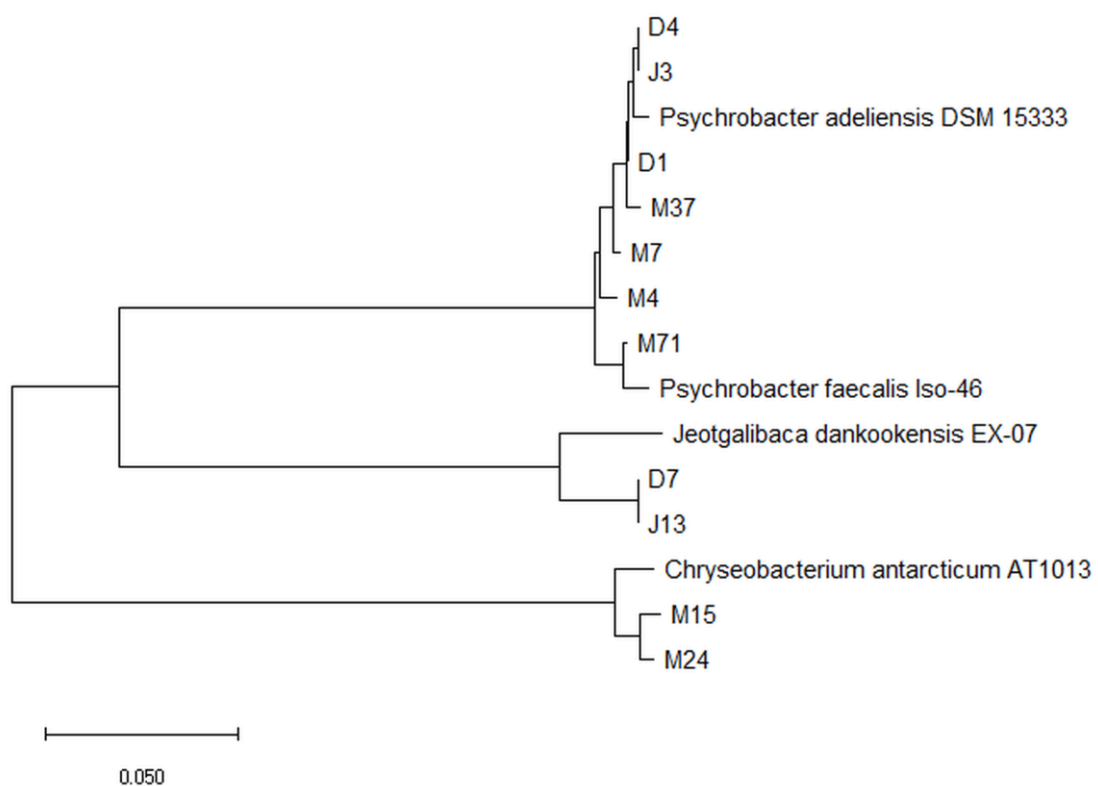
871 **Fig.3. Pie charts with sequence percentage contribution identified at phylum level for**  
872 **pooled samples from each month obtained by 16S rRNA gene fragment amplicon**  
873 **sequencing.**

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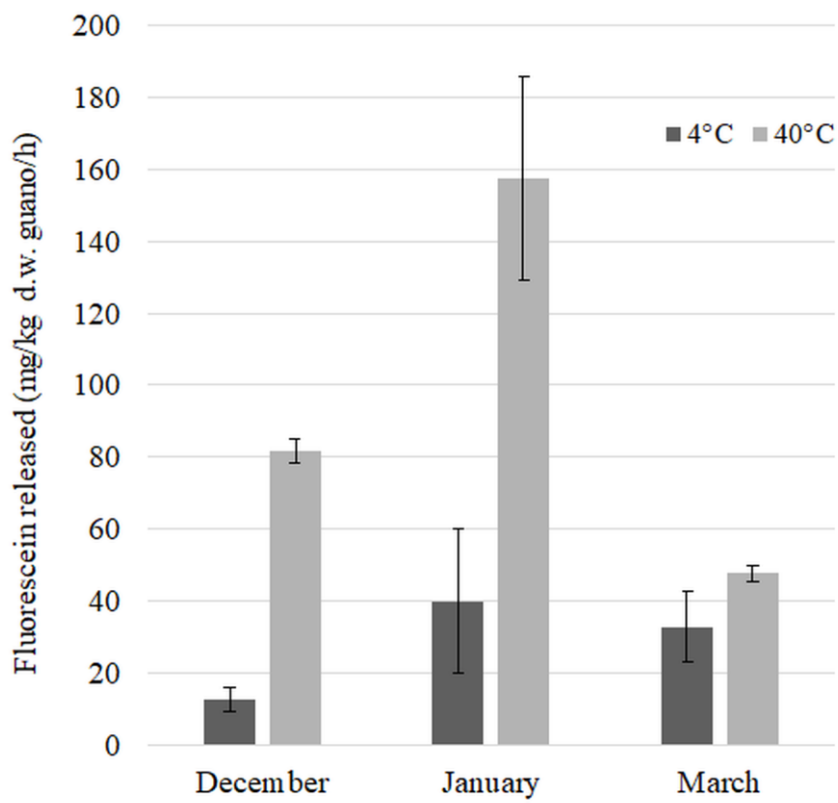
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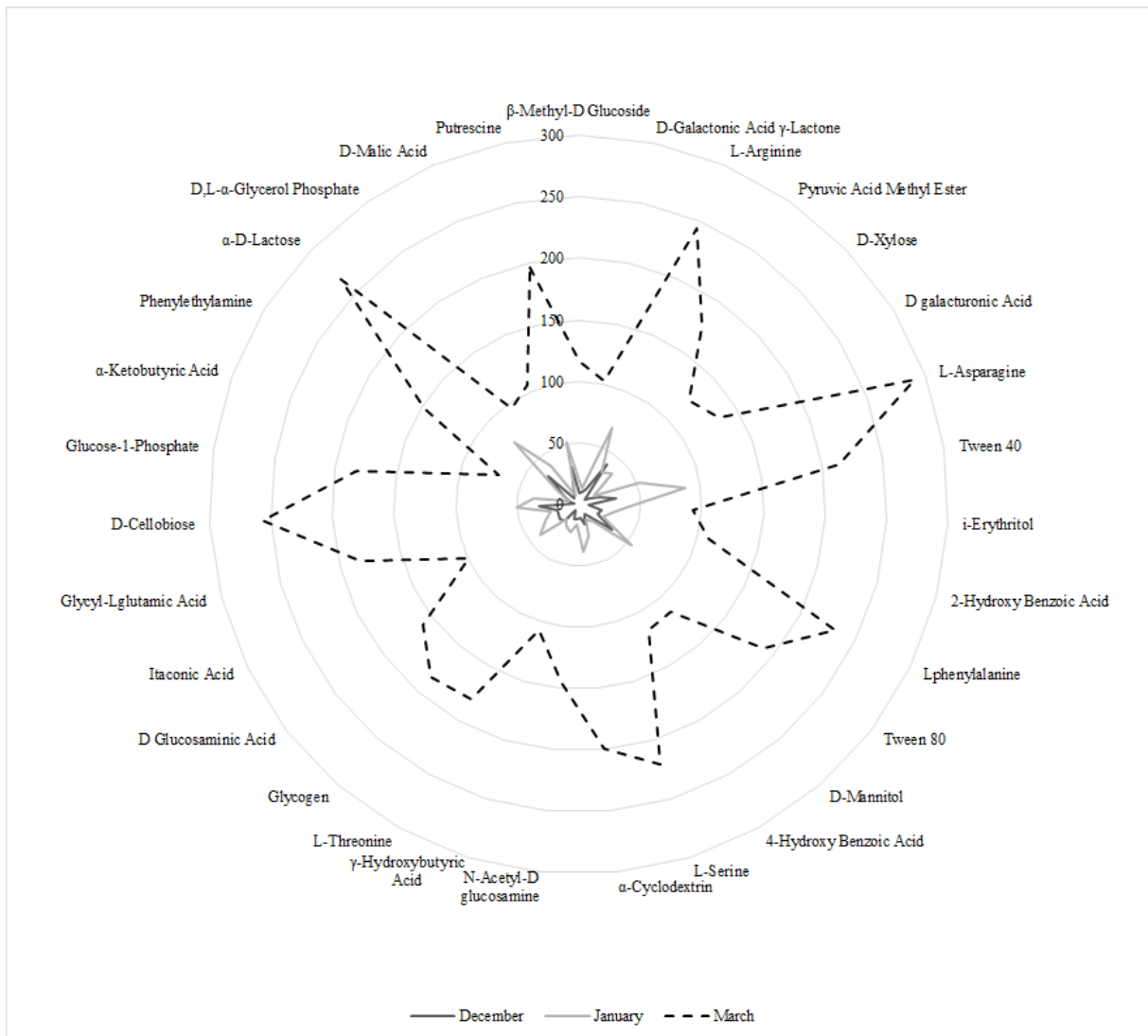
879 **Fig.4. Phylogenetic tree constructed using partial 16S rRNA gene sequences of guano-**  
880 **derived bacterial isolates. Designations indicate sampling month: D-December, J-**  
881 **January, M-March. The tree was built using the neighbor-joining method.**



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883 **Fig. 5. Enzymatic activity in guano samples as fluorescein release rate in mg of**  
 884 **fluorescein per kg of guano dry weight per hour. Mean values with standard error bars.**

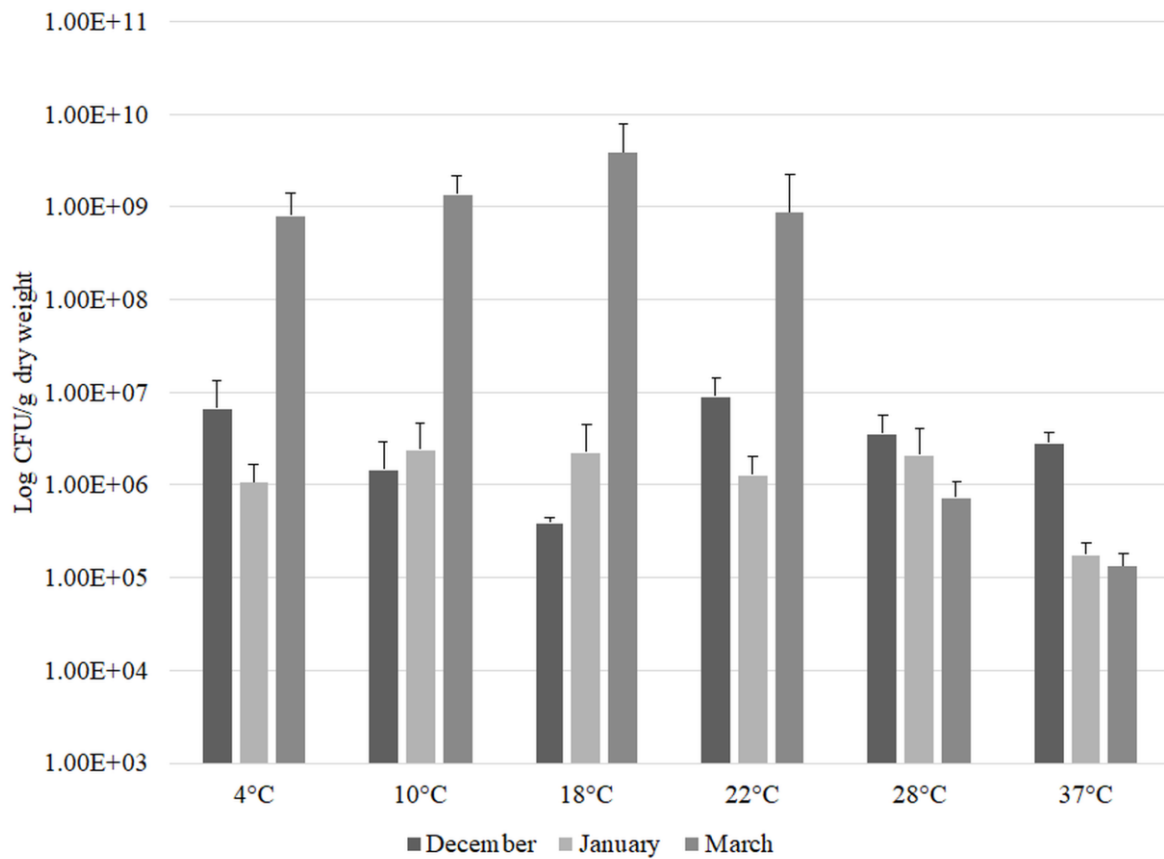




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886 **Fig. 6. Radar chart displaying microbial community responses in Biolog Ecoplates.**

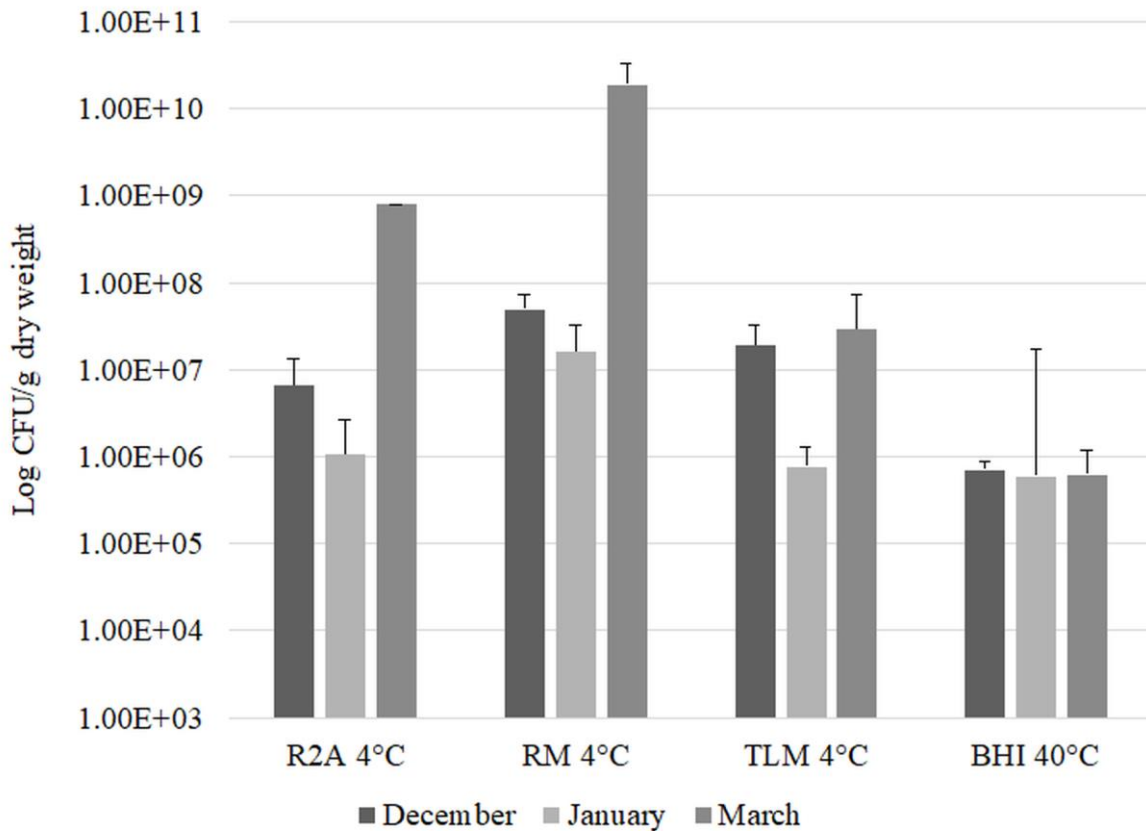
887 **Mean values are given in Omnilog Arbitrary Units, n=9.**



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889 **Fig.7. Aerobic colony forming unit (CFU) count on R2A agar after incubation in given**  
 890 **temperature expressed as log CFU per gram dry weight (g.d.w.). Bars represent mean**  
 891 **values with standard error, n=9.**

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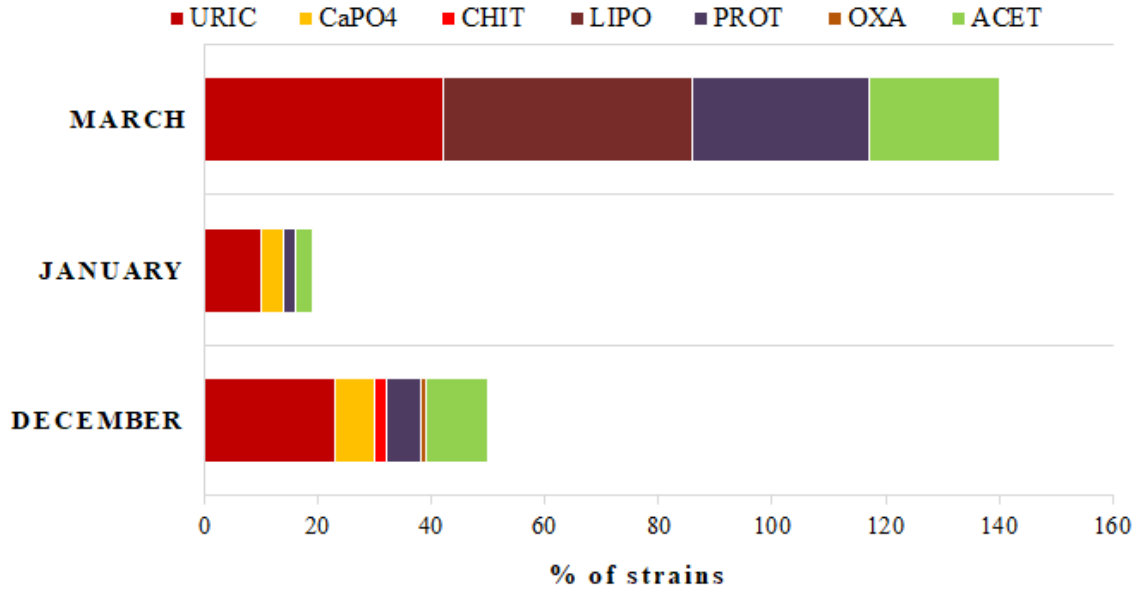
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894 **Fig.8. Colony forming unit (CFU) count on different media and conditions expressed as**  
 895 **logCFU per gram dry weight of guano. Bars represent mean values with standard error,**  
 896 **n=9. R2A 4°C – R2A agar medium at 4°C in aerobic conditions; RM4°C – RM agar**  
 897 **medium at 4°C in aerobic conditions; TLM 4°C – Thioglycollate agar medium at 4°C in**  
 898 **anaerobic conditions; BHI 40°C – Brain Heart Infusion agar medium at 40°C in**  
 899 **microaerophilic conditions.**

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905 **Fig. 9. Percentage of strains expressing guano decomposing abilities isolated from**  
 906 **material sampled in each month. URIC-uric acid decomposition; CaPO4-calcium**  
 907 **phosphate solubilization; CHIT-chitin degradation; LIPO-lipolytic activity; PROT-**  
 908 **proteolytic activity; OXA-oxalic acid utilization; ACET-acetate consumption.**

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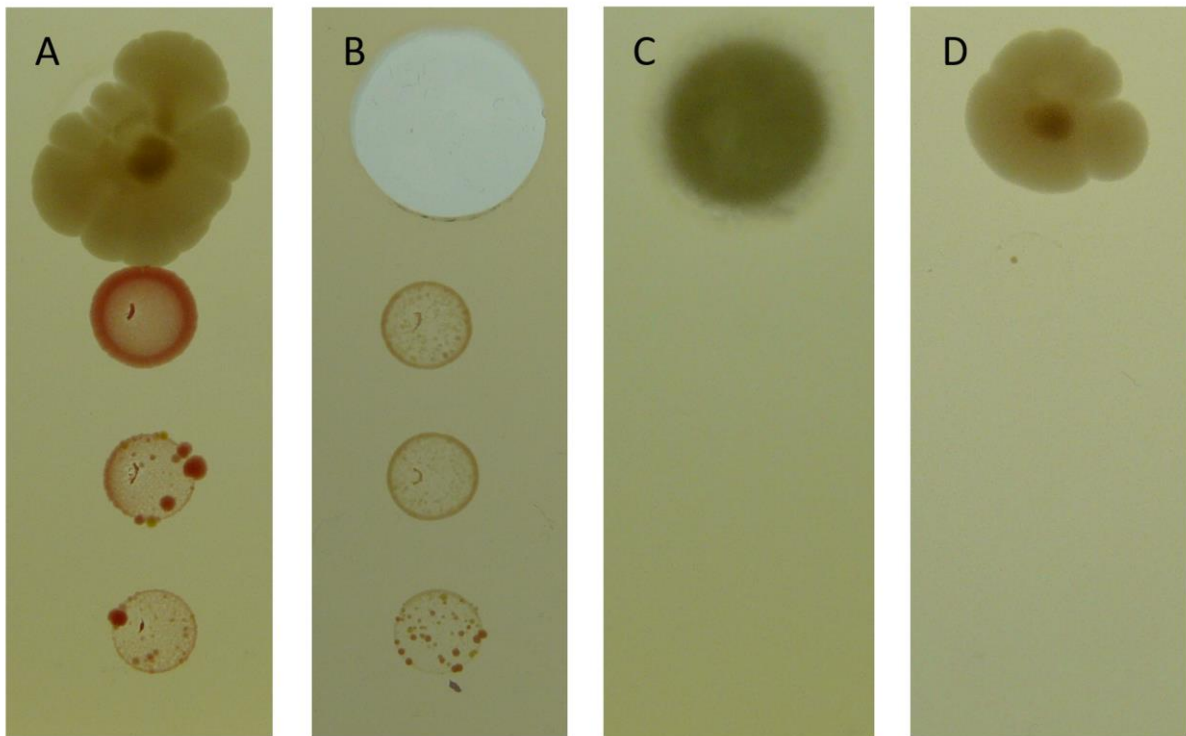
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923 **Fig. 1S. Growth of Jeotgalibaca sp. S13 inoculated as 5 $\mu$ l liquid culture to the surface of**  
924 **R3A agar medium. A – growth stimulation of Jeotgalibaca sp. S13 near an irregular**  
925 **colony of uricolytic Psychrobacter sp. growing on uric-acid agar plug. B - growth**  
926 **stimulation of Jeotgalibaca sp. S13 near ammonium chloride solution filled agar-cut**  
927 **well. C – control without Psychrobacter sp. inoculation. D – control without uric-acid**  
928 **agar plug underneath Psychrobacter sp. growth.**

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Sample ID	Coordinates	Guano temperature	Air temperature (1cm above sampling site)	Atmospheric conditions during sampling*	Breeding site description
D1	62 09.811 S, 58 27.762 W	10.8°C	6.0°C	Temperature: 2.8°C; Humidity: 63%; Wind: 3.6 m/s; Air pressure: 999 hPa	P. adeliae chicks gathered in nurseries, begin to shed feathers.
D2	62 09.816 S, 58 27.698 W	13.5°C	7.1°C		
D3	62 09.821 S, 58 27.688 W	11.2°C	6.9°C		
J1	62 09.811 S, 58 27.762 W	9.2°C	7.0°C	Temperature: 4.6°C; Humidity: 85%; Wind: 0.2 m/s; Air pressure: 992 hPa	Most chicks with new feather coat, begin to leave the colony for sea shore; colony begins to deplete in adult penguins.
J2	62 09.816 S, 58 27.698 W	8.2°C	5.7°C		
J3	62 09.821 S, 58 27.688 W	8.0°C	5.7°C		
M1	62 09.811 S, 58 27.762 W	3.8°C	5.7°C	Temperature: 2.1°C; Humidity: 73%; Wind: 7m/s; Air pressure: 993 hPa	Rookery devoid of penguins since early February.
M2	62 09.816 S, 58 27.698 W	4.5°C	6.3°C		
M3	62 09.821 S, 58 27.688 W	3.3°C	4.8°C		

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937 Tab. 1. Site description, guano temperature and atmospheric conditions during sampling. Sample  
938 abbrev.: D – December samples, J – January samples, M – March samples. \*Data obtained from the  
939 meteorological station at Arctowski Polish Polar Station.

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	December	January	March
<i>Bacillaceae</i>	6.35	3.18	5.00
<i>Carnobacteriaceae</i>	14.93	6.70	9.05
<i>Clostridiaceae</i>	2.99	1.47	2.44
<i>Crocinitomicaceae</i>	0.01	0.81	1.19
<i>Erysipelotrichaceae</i>	5.55	3.12	3.84
<i>Flavobacteriaceae</i>	2.09	26.27	7.55
<i>Gottschalkiaceae</i>	18.57	18.40	19.28
<i>Intrasporangiaceae</i>	0.95	1.99	0.28
<i>Lachnospiraceae</i>	2.12	0.94	4.79
<i>Moraxellaceae</i>	10.90	5.25	5.21
<i>Moritellaceae</i>	0.00	0.74	1.66
<i>Neisseriaceae</i>	0.00	0.90	0.00
<i>Nocardiaceae</i>	1.89	1.51	0.94
<i>Peptostreptococcaceae</i>	11.27	1.21	0.67
<i>Planococcaceae</i>	1.41	2.57	0.60
<i>Porphyromonadaceae</i>	8.00	7.51	15.56
<i>Pseudomonadaceae</i>	0.39	0.30	10.20
<i>Sphingobacteriaceae</i>	0.07	1.37	0.26
<i>Tissierellaceae</i>	6.03	5.85	6.94
<i>Xanthomonadaceae</i>	1.01	0.87	0.28

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956 Tab.2. Heatmap displaying percentage contribution of family-rank affiliated sequences  
 957 derived from pooled guano samples within each sampling month obtained by 16S rRNA gene  
 958 fragment amplicon sequencing.

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Culture conditions	December	January	March
R2A 4°C	0.019%	0.003%	0.273%
R2A 10°C	0.004%	0.006%	0.463%
R2A 18°C	0.001%	0.005%	1.293%
R2A 22°C	0.026%	0.003%	0.297%
R2A 28°C	0.010%	0.005%	0.000%
R2A 37°C	0.008%	0.000%	0.000%
RM 4°C	0.145%	0.039%	6.443%
TLM 4°C	0.056%	0.002%	0.010%
BHI 40°C	0.002%	0.001%	0.000%

969

970 Table 3. Cultivability of guano-derived bacteria expressed as contribution percentage of  
 971 average colony forming units for particular culture conditions in each month bacterial total  
 972 count for that month. Color intensity indicates highest values within sampling month for  
 973 temperature range and within culture condition for the remaining data.

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<b>Medium</b>	<b><i>Jeotgalibaca</i> sp. D7</b>	<b><i>Jeotgalibaca</i> sp. J12</b>
MM	-	-
MM+NH <sub>4</sub> Cl	-	-
MM+Urea	-	-
MM+KNO <sub>2</sub>	-	-
MM+KNO <sub>3</sub>	-	-
MM+Allantoin	-	-
MM+Casamino acids	-	-
R3A	-	-
R3A+NH <sub>4</sub> Cl	+	+
R3A+Urea	-	-
R3A+KNO <sub>2</sub>	-	-
R3A+KNO <sub>3</sub>	-	-
R3A+Allantoin	-	-
R3A+Casamino acids	-	-

990

991 Table 1S. Growth characteristics of *Jeotgalibaca* sp. Isolated from *P. adeliae* guano on  
992 different media supplemented with various nitrogen sources. MM- mineral medium devoid of  
993 a nitrogen source, R3A – R3A medium. + growth, - no growth.

994