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Bacterial role in the decomposition of marine-derived material (penguin guano) in the terrestrial maritime Antarctic

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Abstract

Bacterial decomposition of Adélie Penguin (*Pygoscelis adeliae*) guano was followed during 42 days exposure in a rookery in Admiralty Bay, King George Island, South Shetland Islands, Antarctica. Bacterial abundance, both total counts (TC) determined by epifluorescence microscopy, and colony forming units (CFU) determined on nutrient media, was enhanced by an air temperature of between 7 and 11 °C, while temperatures above and below this negatively affected abundance. Optimal temperatures for guano decomposition ranged from 3 to 11 °C. Increasing wind velocity positively influenced photoautotrophic bacteria (TAC: total autotrophic bacteria count); photoautotrophs were enumerated during epifluorescence microscopy by their autofluorescence. Guano dry weight attained the lowest value of 74% of initial dry weight after 20 days in situ exposure. Changes in guano resulted from decomposition of the component fats, proteins, chitin, nitrogen and carbon, as well as of mineral recycling (Ca, P, Mg, and others). All transformations accompanied bacterial growth, with TC and CFU both attaining 10^{11} cells g^{-1} d wt of guano. Total bacterial biomass (TBB) increased from 594 μg C g^{-1} dry weight in fresh guano, to 9101 μg C g^{-1} dry weight after 42 days exposure in situ. Mean cell volume (MCV) also increased from 0.236 to 0.343 μm^3. Photoautotrophic bacteria were numerically the smallest TC fraction throughout the incubation, with TAC to TC ratio ranging from 0.01 to 0.22%. Culturable bacteria abundance in guano increased dramatically from 0.28% of TC (in fresh guano) to 26% (3-h day), and 90% (42nd day) of TC. Within the total CFU population, copiotrophic bacteria were ca. two orders of magnitude more abundant than oligotrophic bacteria. Chitinolytic bacteria in guano were detected only late in the incubation; by 42 days, >40% of the initial chitin content remained. This material may comprise a significant fraction of the soil in the penguin rookery. Bacteria cultivated from the penguin guano displayed high morphophysiological diversity.

Keywords: Antarctica; Bacterial diversity; Penguin guano; Decomposition

1. Introduction

Significant amounts of organic and inorganic materials are exchanged between the land and sea in Admiralty Bay (Rakusa-Suszczewski, 1995). Much of the organic material deposited on land is in the form of penguin guano and the faeces of pinnipeds. The most abundant penguins in the bay are Adélies (*Pygoscelis adeliae*; Trievelpiece et al., 1987). The Point Thomas rookery where this experiment was conducted, hosts 7000 to 11,000 breeding pairs of Adélie penguins annually (Ciaputa and Sierakowski, 1999). Tatur and Myrcha (1984) considered that over the breeding season, some 10 kg m^{-2} of penguin guano (dry weight) are deposited in rookeries. Intensive fertilization with penguin guano initiates the formation of ornithogenic soils (Ugolini, 1972; Tatur, 2002). Guano from rookeries located a few hundred meters from the shore is distributed across a larger area by local streams, trampling by penguins, other birds and seals, and by groundwater (Myrcha et al., 1985; Juchnowicz-Bierbasz, 1999). Penguins thus influence the biological and chemical nature of the soil (Zdanowski and Weglenski, 2001). Conversely, a portion of this matter, including microorganisms, flows into the water of Admiralty Bay itself (Zdanowski, 1995).

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The Adélie diet is composed almost exclusively (95.4%) of krill (*Euphausia superba*; Trievelpiece et al., 1987, 1990). As a result, the microbial flora of the penguin guano is largely of marine origin, such as that associated with the krill (Zdanowski, 1995; Donachie and Zdanowski, 1998). Once deposited on land, however, penguin guano is transformed into material that ultimately becomes ornithogenic soil (Tatur and Myrcha, 1984). Complex biological and physical factors are involved in the long-term decomposition of guano.

The Antarctic climate varies from severe continental to less severe in the maritime Antarctic (Rakusa-Suszczewski, 2002). The latter is characterized by an average annual air temperature in Admiralty Bay of ca. −1.8 °C (from −32.3 to +16.7 °C; Rakusa-Suszczewski et al., 1993), and wind velocity that may attain 70 m s^{-1} (Kowalski and Wielbińska, 1989). In spite of these conditions, bacterial processes in the maritime Antarctic terrestrial environment continue, reflecting the adaptive potential of autochthonous and allochthonous bacterial populations.

Soil is one of the warmest exposed environments in Maritime Antarctic regions. At *Henryk Arctowski* Station, the mean annual soil temperature between 5 and 100 cm depth was positive between 1979 and 1998 (Zwolska and Rakusa-Suszczewski, 2002). The highest temperature recorded in loose soil (Gelic Regosols) on a south-facing slope on sunny and windless days was 35.2 °C at a depth of 1 cm (Bölter et al., 1995). Conversely, the lowest temperature (−14.0 °C) was recorded in winter at 5 cm depth (Kratke and Wielbinska, 1981). During the breeding season, the soil temperature in rookeries is significantly higher than in the immediate vicinity because of the soil's physical properties and its modification by nesting penguins (Moczydłowski, 1986).

Understanding the role of bacteria in guano degradation and relationships between bacterial abundance and environmental factors is a prerequisite for describing the functioning of the Antarctic terrestrial ecosystem (Pietr et al., 1983; Pietr, 1993; Zwolska and Rakusa-Suszczewski, 2002).

The main objectives of this paper are: (1) to evaluate environmental factors that regulate bacterial abundance during guano decomposition; (2) to estimate the rate of decomposition of guano with an emphasis on percentage changes in fats, protein, chitin, ash, TOC (total organic carbon), nitrogen, phosphorus, and some other elements; (3) to examine the quantitative and qualitative transformations in the bacterial population during penguin guano decomposition; (4) to isolate and identify the dominant culturable bacteria as a first step in evaluating their diversity, and to preselect strains for further phylogenetic analysis.

2. Materials and methods

2.1. Field experiments—sites and sampling

Adélie penguin (*P. adeliae*) guano was collected from the Point Thomas rookery close to Rakusa Point, Admiralty Bay, King George Island, Antarctica (62°09′50″S, 58°28′W), between 29 December 1999 and 9 January 2000. The Pt Thomas rookery is at an elevation of ~50, and ~250 m from sea. According to Trievelpiece et al. (1987), Adelie penguin parents return to the rookery to feed their chicks after ~24 h foraging at sea, so fresh guano is deposited daily in and around the rookery.

We collected freshly deposited penguin guano with no underlying soil into sterile 50 ml polypropylene tubes using a sterile plastic spatula. Hundreds of deposits of 1–3 g each totaling >0.5 kg were collected within a few hour period from rookery edge. Samples were returned immediately to the laboratory at the Henryk Arctowski station, whereupon 60 subsamples of 5–7 g each were wrapped in sterile bolting-cloth (pore size 0.3 mm) bags. All transfers were conducted aseptically at an air temperature below 10 °C. Each wrapped sample was returned to the collection site, placed directly on the soil surface, and surrounded by a steel mesh enclosure to protect against trampling. Samples were undisturbed for up to 42 days.

On days 0, 2, 6, 10 and then every 5 days up to 30 days (with the last collection on day 42), 6–7 bags were randomly selected and removed from the enclosure and returned to the laboratory for biochemical and microbiological analyses.

Using mercury maximal and minimal thermometers placed at the sampling site, diurnal extreme air temperatures 1 cm above the ground were recorded daily. Some meteorological data such as air temperature at 2 m above ground, including precipitation, humidity, cloud cover and wind velocity were obtained from the meteorological station at Henryk Arctowski Station. Means were calculated from data obtained during the three days preceding sampling, plus those on the actual sampling day.

2.2. Biochemical analyses

The pH in 1 g wet weight of sample suspended in 100 ml distilled water was determined in a Hanna Instruments HI 9025 pH meter, in three replicates randomly selected from 6 to 7 bags. Wet weight was determined by weighing directly after sampling. Dry weight was determined after 24 h at 65 °C in a dry-box with circulating air (Zdanowski, 1988). The mean and standard error in weights were determined from 6 to 7 bags. Subsamples of dried guano were powdered, pooled and used in 2–3 replicates for elemental CHNS analysis (Carlo Erba CHNS-O-meter, EA 1108). Phosphorus was determined in pooled material by colorimetric meta-vanadate method, and other elements by atomic absorbtion spectroscopy (Tatur et al., 1997). Fluorine was indirectly estimated from Ca^{2+} content, based on the assumption that most of the F^{-} is in fluoroapatite ($Ca_5(PO_4)_3F$; Rai et al., 2000), where the molar ratio of fluorine to calcium is exactly 0.2 (Tatur, 1987). Note that molar ratios of P to Ca and F in guano fit exactly to stochiometric formula of fluorapatite (Tatur and Keck, 1990). Fats were measured gravimetrically from

the difference in dry weight before and after threefold extraction with ethyl ether (Dowgiałło, 1975). Protein was determined spectrophotometrically (absorbance at 562 nm) using the PIERCE BCA Protein Assay (Brenner and Harris, 1995) in an alkaline extract of defatted samples. Chitin was determined gravimetrically in the defatted and deproteinized material from difference in dry weight before and after combustion at 450 °C (Dowgiałło, 1975). Ash content was obtained by weighing the residue after sample combustion at 450 °C. The mean and standard error in fats, protein, chitin and ash content were determined in powdered and pooled material, from three measurments.

2.3. Microbiological analyses

Bacterial counts (total bacterial counts: TC, total autotrophic counts: TAC, colony forming units: CFU), bacterial cell morphometry, and biomass) were determined in 1 g (wet weight) of material comprising sub-samples of ~0.2 g from each bag pooled in a glass homogenizer. Each 1 g pooled sample was homogenized in 10 ml phosphate-buffered saline (PBS, 0.15 M potassium phosphate, 0.85% NaCl, pH 7.0; McDermott, 1997) centrifuged for 7 min at $250\times g$, and the supernatant decanted to a sterile flask. The pellet was resuspended and washed twice by centrifugation in sterile PBS. All fractions were pooled a in sterile flask and serially diluted (10^0–10^7) in PBS. A decimal series to 10^{-7} of the homogenate was prepared. For direct counts, homogenates were stabilized with buffered formalin to a final concentration of 1% and sealed in glass ampoules until cells were counted by epifluorescence microscopy. Direct counts by epifluorescence microscopy were performed using 4′6-diamidino-2-phenylindole (DAPI) on black Nuclepore polycarbonate 0.2 μm pore size filters (Porter and Feig, 1980) under a Nikon E-200 microscope equipped with a 100 W Hg lamp and a 100× CFI 60 oil immersion objective, with a COHU 4910 video camera, Mutech IV-410 card, and a LUCIA 4.60 image processing and analysis system (Laboratory Imaging, Prague, CZ). This system allowed high quality images to be captured and single and aggregated bacterial cells to be distinguished. Total bacteria were counted using the UV-2A standard filter block of wavelengths EX 330–380, DM 400, BA 420. Photoautotrophs, including cyanobacteria and photoautotrophic eukaryotes, were counted under blue light excitation of 450–490 nm (B-2A) and green EX 510–560 nm (G-2A; Putland and Rivkin, 1999), and distinguished from heterotrophs by gold-yellow or red autofluorescence (Rassoulzadegan and Sheldon, 1986). A minimum of 400 bacterial cells per sample were counted automatically in the image analysis system. Standard error (SE) was estimated for average values from three measurements using three independently prepared filters.

Images of the microscope fields were evaluated for total bacterial count, cell volume, total bacterial biomass, cell carbon and protein content (Sieracki et al., 1985; Święcki, 1997) through image analysis. Cell volume (V) was converted to bacterial biomass on the basis of cell carbon (C) using C (fg) $=120\times V^{0.72}$ (Simon and Azam, 1989). A diversity analysis was conducted with the Shannon index based on specific morphological forms (cocci, rods, curved forms) evaluated in five classes of volumes <0.1; 0.1–0.2; 0.2–0.5; 0.5–1.0, and >1.0 mm^3 (Gurienowich, 1995; Nübel et al., 1999).

Culturable bacteria (colony forming units, CFU) were enumerated by the spread plate method on: (a) full strength nutrient Soil Extract Agar (SEA) for the isolation of copiotrophic bacteria, and 10× diluted NA for the bacteria more oligotrophic (Ogram and Feng, 1997). Agar plates were inoculated with 100 μl from each dilution in a decimal series of the homogenate. One liter of NA includes bacto beef extract (3 g), bacto-tryptone (5 g), bacto-agar 15 g (Fenchel and Hemmingsen, 1974) in 1 l of soil extract prepared according to Klement et al. (1990). CFU growth was examined every 2–3 days through 25–30 days incubation at 10 °C. This was done to compare the kinetics of bacterial colony increment among bacterial populations, isolated in different stages of guano decomposition. At the end of incubation, CFU counts were calculated. The standard error (SE) in CFU counts was determined from four replicates. For each experimental day (0, 2, 6, 10, 15, 20, 25, 30, 42), an average of 15 colonies were randomly selected, and subcultured for purification through repeated transfers on the same medium, and physiological investigations. In total, 136 colonies were so isolated.

Chitinolytic bacteria were enumerated on a solid medium containing soluble dye-labeled chitin (CM-Chitin-RBV solution; LOEWE Biochemica GmbH) prepared according to Klement et al. (1990).

Morphological, physiological and biochemical features of the isolated bacteria were determined on the basis of 49 tests, including growth at 4, 22 and 32 °C, Gram stain, morphology (rods or cocci), motility (determined in hanging drop preparations), colony pigmentation (examined macroscopically), growth in 4% NaCl, and responses in API 20NE and API ZYM systems (API bioMérieux; Tearle and Richard, 1987; Zdanowski and Donachie, 1993; Zdanowski and Weglenski, 2001). A dendrogram showing the hierarchical classification of isolates based on the tests described was constructed by the simple matching coefficient of Sokal and Michener (1958) in association with the weighted pair-group-average algorithm (Sneath and Sokal, 1974).

3. Results

3.1. Environmental factors

During the austral summer of 1999–2000, air temperature near the soil surface was considerably higher than at 2 m above the ground (Fig. 1). Surface temperature ranged

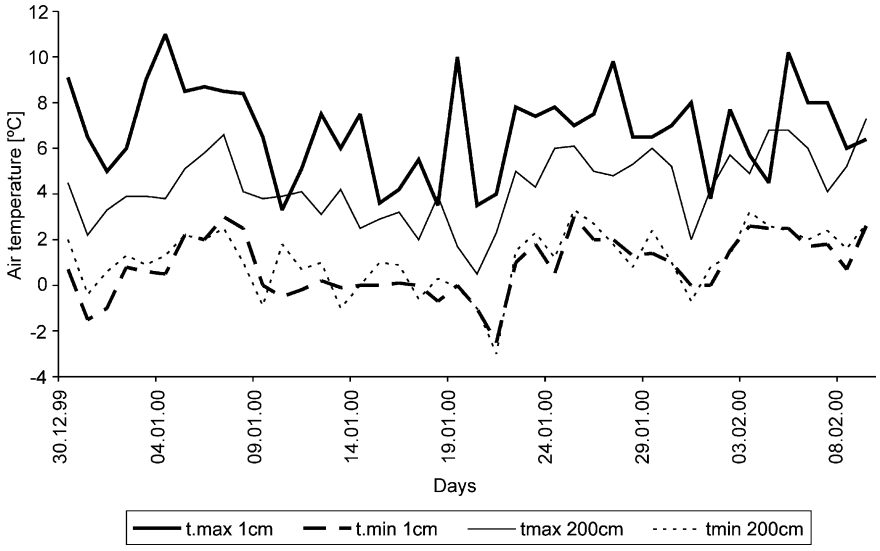


Fig. 1. Maximal and minimal air temperature fluctuations, at 1 and 200 cm above ground level, during penguin guano exposure at the edge of Pt Thomas penguin rookery.

from 2.5 to 11.0 °C; in the same period, standard air temperature was 3.8 °C, with a minimum of 3.0 °C. The soil surface on sunny days was especially warm; soil moisture decreased and temperature exceeded air temperature. A maximum temperature of between +7.1 and +9.0 °C was recorded at midday on over one-third of days. Daily temperature fluctuations on cloudy days were relatively small, and differences between soil and air temperature insignificant. Minimum temperatures of between –0.9 and 1.0 °C were recorded on 50% of days, and between +1.1 and 3.0 °C were observed on 40% days.

Using multiple stepwise linear regression analysis, we aimed to determine what might account for changes in numbers of TC and CFU bacteria, and in the rate of guano decomposition rate. Temperatures close to the highest recorded at the surface (11 °C) negatively impacted abundance in all bacterial fractions, whereas a surface temperature above the lowest (−2.5 to +3 °C), or even higher than that 2 m above ground (1–7 °C) caused an increase in bacterial abundance. Wind also positively influenced bacterial abundance, particularly that of the autotrophic bacteria.

3.2. Loss of guano wet and dry weight

The wet weight of penguin guano varied considerably during the experiment (Table 1). Correlations between wet and dry weight losses were positive yet insignificant, and were mainly affected by moisture levels that in turn depended upon the amount of precipitation. Indeed, over 66% of the variance could be explained through precipitation. This phenomenon was supported by multiple stepwise regression between wet weight and physical data (Table 2). Among the biological factors, only TAC was significantly correlated with the loss of wet weight (at $p<0.05$). Conversely, dry weight losses and fluctuations were much smaller. With the aid of multiple stepwise regression we determined that bacterial abundance positively correlated with surface air temperature (Table 2).

3.3. Guano properties upon exposure time

Quantitative changes in the composition of guano are shown in Fig. 2. Initially relatively high contents of ethyl ether-extractable fats and alkaline extracted protein in fresh penguin guano decreased to ~20 and 15% by dry weight within ca. 10 days, and until the end of exposure were relatively stable. The delay in commencement of chitin degradation was remarkable, beginning only with the appearance of bacterial chitin degraders. The ash share in guano increased in the first 10 days, and coincided with the loss of fat and protein.

Some elements (Ca, F, Mg, P, Fe, Mn, Zn) were concentrated in the ash (Table 1) as time progressed and organics decomposed. Among macroelements, Ca was the most abundant in ash during the experiment, while phosphorus was the most abundant after 42 days. Among microelements, the Zn concentration showed the greatest increase during guano decomposition. Fe was the most abundant but showed less of an increase. Other minerals (C, N, Na) were washed out, or oxidized during combustion. Note negative correlations between these and the concentrated elements. Potassium did not correlate with the ash content nor with other elements during the experiment.

The content of C, H, N, S as well as C:N ratio varied with exposure time (Table 1). High N and relatively low C contents (C:N=3) in fresh guano coincided with high protein and chitin contents (Fig. 2). An increase in the C:N ratio to 5 after the first eight days might indicate organic nitrogen mineralization and NH_3 release; this increase was

Table 1
Changes in wet weight and dry weight, pH, diversity index (Shannon I), organic carbon, hydrogen, nitrogen, C/N, sulphur (all above ± SE), total organic matter (OM), phosphorus content, C/P ratios, and mineral compounds content in the course of penguin guano decomposition

	Day								
Compound	0	2	6	10	15	20	25	30	42
Wet weight (%)	100 ± 2.6	127,1 ± 5.7	70.1 ± 3.0	32.4 ± 1.8	45.1 ± 3.0	30.7 ± 2.1	36.8 ± 1.7	73.0 ± 3.4	45.8 ± 2.2
Dry weight (%)	100 ± 2.4	92.9 ± 3.7	89.5 ± 1.7	88.8 ± 6.2	89.6 ± 8.7	73.7 ± 5.4	75.1 ± 5.5	78.5 ± 5.1	74.2 ± 4.6
PH	6.33 ± 0.09	7.20 ± 0.06	7.42 ± 0.02	8.82 ± 0.02	9.38 ± 0.02	8.66 ± 0.05	9.00 ± 0.05	8.94 ± 0.01	8.94 ± 0.02
Shannon I	0.72 ± 0.017	0.79 ± 0.012	0.84 ± 0.015	0.92 ± 0.015	0.92 ± 0.025	0.94 ± 0.021	0.94 ± 0.012	0.92 ± 0.015	0.9 ± 0.026
C (%)	32.08 ± 0.14	34.41 ± 0.25	30.02 ± 1.61	28.28 ± 1.67	25.13 ± 1.38	29.31 ± 0.25	29.0 ± 0.09	28.53 ± 1.21	27.79 ± 1.47
H (%)	5.26 ± 0.01	5.16 ± 0.03	3.83 ± 0.07	3.85 ± 0.04	3.1 ± 0.04	3.84 ± 0.04	3.72 ± 0.05	3.36 ± 0.06	3.38 ± 0.02
N (%)	10.9 ± 0.46	7.5 ± 0.34	5.68 ± 0.13	6.15 ± 0.48	5.66 ± 0.12	6.43 ± 0.26	6.33 ± 0.47	6.17 ± 0.08	5.7 ± 0.13
C:N	2.97 ± 0.11	4.6 ± 0.24	5.3 ± 0.3	4.61 ± 0.09	4.45 ± 0.33	4.57 ± 0.22	4.61 ± 0.36	4.62 ± 0.14	4.89 ± 0.37
S (%)	1.36 ± 0.13	1.61 ± 0.15	1.27 ± 0.16	1.37 ± 0.12	1.44 ± 0.13	1.05 ± 0.12	1.28 ± 0.17	0.95 ± 0.01	0.8 ± 0.08
OM (%)	83.22	81.40	71.59	72.44	61.88	70.34	71.38	68.24	69.49
P (%)	1.58	1.27	2.43	2.42	3.28	3.82	3.52	3.80	4.05
C:P	20.31	27.09	12.33	11.69	7.66	7.67	8.24	7.59	6.86
Ca (%)	3.80	3.74	4.83	4.77	5.46	6.66	5.82	4.21	6.32
~~F (%)~~	~~0.36~~	0.35	0.46	0.45	0.52	0.63	0.55	0.40	0.60
Mg (%)	0.91	0.81	1.08	0.98	1.37	1.59	1.50	0.98	1.74
K (%)	0.59	0.76	0.72	0.68	0.78	0.64	0.72	0.60	0.57
Na (%)	0.94	1.01	0.78	0.69	0.78	0.56	0.70	0.51	0.48
Fe (μg g^{-1})	3436	3684	5616	4936	5040	4724	4652	6196	5552
Mn (μg g^{-1})	68	72	140	120	172	116	128	128	160
Zn (μg g^{-1})	144.4	173.3	178.8	222.2	202.9	338.1	341.1	415.6	646.1

Table 2
Multiple stepwise regression between total bacterial count (TC), total autotrophic bacteria count (TAC), colony forming units: copiotroph (CFU-C), oligotroph (CFU-O), chitynolitic (CFU-CH), wet weight (WET WT), dry weight (DRY WT) and physical variables

Variable	β	p-Level	Explained variance (%)
TC			$r^2=0.70$
TS MAX	−1.248	0.019	20.5
WIND	0.824	0.059	32.6
TS MIN	0.473	0.173	17.3
TAC			$r^2=0.73$
TS MAX	−1.383	0.001	64.7
WIND	0.463	0.017	7.9
CFU-C			$r^2=0.39$
TA MAX	1.476	0.028	26.1
TS MIN	−1.004	0.088	12.6
CFU-O			$r^2=0.55$
TS MAX	−1.143	0.037	29.5
TA MIN	0.781	0.118	25.1
CFU-CH			$r^2=0.82$
TS MAX	−1.609	0.006	18.4
TA MIN	1.202	0.009	56.8
WIND	0.371	0.215	7.1
WET WT			$r^2=0.98$
PRECIPIT	0.716	0.003	66.2
TS MAX	0.589	0.012	9.5
TS MIN	−0.69	0.008	19
WIND	−0.266	0.063	3.4
DRY WT			$r^2=0.65$
TS MAX	0.912	0.016	49.8
TS MIN	−0.437	0.165	14.8

β-standardized coefficient of regression; p: level of significance of the slopes in the regression equations. Results are presented for mean data (3–4 measurements) collected during the full period of this study ($n=9$). Physical variables: diurnal extreme air temperatures 1 cm above the surface (TS MAX, TS MIN); air temperatures 200 cm above the surface (TA MIN, TA MAX); precipitation (PRECIPIT); wind velocity (WIND).

accompanied by an increase in guano pH (Table 1) and the characteristic odor of ammonia. Loss of H coincided with the disappearance of carbohydrate materials. The sulphur content also decreased.

3.4. Total bacteria

Total bacterial counts (TC) increased from 1.01×10^{10} g^{-1} dry weight in fresh guano, to 3.83×10^{11} g^{-1} after 30 days of exposure, and then decreased slightly (Fig. 3). Photoautotrophic bacteria were detected throughout the experiment as the smallest TC fraction, with TAC to TC ratio ranging from 0.01 to 0.22% (Fig. 4). Total bacterial biomass, along with TC, increased markedly, reaching a peak between 25 and 30 days of exposure (Fig. 5). Autotrophic bacteria biomass reached a maximum after 15 days, peaking at over four orders of magnitude less than TCB. The mean bacterial cell volume in guano after 0–2 days exposure (0.215 ± 0.029 μm^3 cell^{-1}) was lower than that after 42 days (0.330 ± 0.045 μm^3 cell^{-1}; Fig. 5). Largest cell volumes were observed in both TC and TAC on the 15th and final days of exposure. The most numerous cells were <0.1 μm^3 (Fig. 6). Rods of this size constituted 36.4 ± 10% of the population, while curved forms were 16.9 ± 4.0%, and cocci 1.4 ± 0.8% of the total within each class. A significantly higher percentage of the smallest rods were determined in the first six days of exposure days (mean 47.3 ± 8.1) compared to the remaining 32 days (30.9 ± 5.1). The ratio between morphological forms of bacteria, i.e. rods to cocci to curved forms averaged 66:4:30, and did not vary significantly during the entire experiment. When frequency of forms in each size class (Fig. 6) was replaced by their biomass (Fig. 7), the smallest forms dominated, especially of the rods, and only in fresh guano. In the following phases of exposure, the biomass was dominated by the largest cells, especially the rods and curved forms. To describe changes in the total bacterial community in penguin guano with time, a modified Shannon index based on cell morphotypes was applied. A significant increase in the diversity index was observed (Table 1). On the basis of nonlinear regression we determined that morphotype diversity of bacterial communities and exposure time were significantly and positively correlated during up to 14 days of exposure ($p=0.02$).

3.5. Colony forming bacteria

The numbers of culturable bacteria (CFU) increased significantly with exposure time (Fig. 3). The CFU:TC ratio increased significantly, reaching a very high value after 42 days (Fig. 4). Of the CFU population, copiotrophic bacteria constituted about two orders of magnitude more cells than 'oligotrophic' bacteria growing on diluted media. Chitinolytic bacteria appeared in guano remnants with considerable delay (Fig. 3). Morphophysiological properties of these three bacterial groups varied significantly, according to 49 tests (Fig. 8). A general tendency for the number of positive responses to increase was observed with the transition from copiotrophic through oligotrophic to chitinolytic isolates. Such a tendency is demonstrated by the occurrence of glucose assimilation. Up to 76% of chitinolytic isolates assimilated glucose, whereas only 32 and 16%, respectively, of oligotrophic and copiotrophic isolates did so. A similar pattern was observed with carbohydrate assimilation as well as the glycoside hydrolases. As many as 100% of isolates grew at 4 °C, while not all grew at 22 °C. Just a handful of (mainly chitinolytic, noncopiotrophic) isolates grew at 32 °C. More than 80% of oligotrophs grew in 4% NaCl, compared to 60% of copiotrophs that did so.

Morphophysiological characteristics of the bacterial community changed as the guano degraded, particularly in the early (0–10 days) and later (15–42 days) phases. The early phase can be characterized by following examples; an increase in microbial diversity index and C/N in guano (Table 1); rapid decrease in lipid and protein content (Fig. 2), and lack of chitinotrophs and chitin decomposition, which appeared only after 10 days of exposure. Changes in the rate of colony formation also testify to the fact that

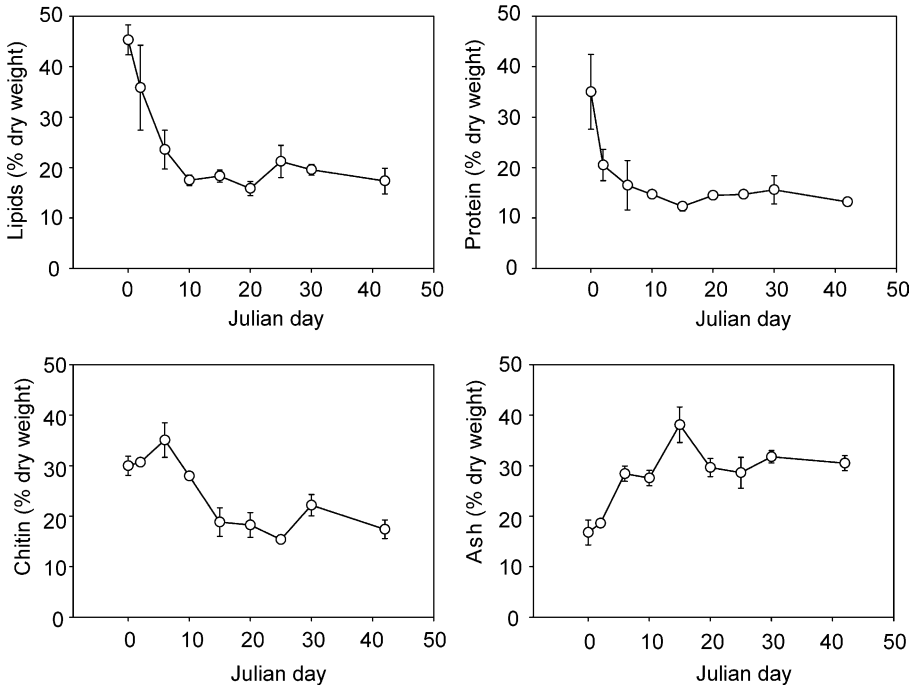


Fig. 2. Penguin guano composition during in situ exposure: fat, protein, chitin and ash content as a percentage of residual dry weight.

the population composition had changed. When guano homogenates were incubated on nutrient agar, colonies were first visible after 3–4 days incubation at 10 °C. The highest number of colonies developed in the next 5–6 days, and then slowed. The kinetics of colony formation changed during guano decomposition (Fig. 9). This was illustrated by regression equations calculated for consecutive sampling days. Note the increasing slope of the line, which is most distinct in the phase of rapid growth. Quantitatively, copiotrophs always outnumbered oligotrophs, but the trend in kinetics of CFU development was similar for both.

We determined phenotypic characteristics of 136 randomly selected and thence purified bacteria strains. Isolates were grouped on the basis of their responses in 49 morphophysiological tests (Fig. 10). Eleven groups (1–11) comprising 2–44 strains were distinguished on the basis of a similarity level of $\geq$60%, while nine strains formed of single isolate 'clusters'. On the basis of their numerical profiles in API 20NE, only 34 of 136 strains (25%) could be related to species in the bioMerieux database. Clusters 6 and 7 comprised 44 and 5 isolates, respectively, 26 of which were related to the *Pasteurellaceae* and *Moraxellaceae/Pseudomonadaceae* (API 20NE bioMéreiux database). The other two clusters (4 and 5) comprised isolates related to the Sphingobacteriaceae and Flavobacteriaceae. Among the selected strains, only eight shared a numerical profile that was identical to one in the database, while another 26 had profiles that had only minor mismatches. Several strains sharing a numerical profile in the database were subsequently found on the basis of other data not to be related to the initial match.

4. Discussion

The fertilizing capacity of seabird guano has been known for a long time. In the maritime Antarctic, continuous inputs of organic and inorganic-rich penguin guano (Tscherko et al., 2003) are nutrient sources for ice-free terrestrial areas. Microbial transformation of guano occurs on and below the soil surface, and ultimately leads to the formation of ornithogenic soils (Tatur, 2002). We investigated processes of guano degradation on the soil surface.

In contrast to other work in the field, our samples were incubated exclusively in the field and in a rookery occupied by several thousand penguins (Trievelpiece et al., 1987; Ciaputa and Sierakowski, 1999). This site, situated in the area of water run-off from the rookery, was fully exposed to both climate and rookery conditions. Climate is especially changeable and dynamic in the maritime Antarctic (Wynn-Williams, 1990; Zwolska and Rakusa-Suszczewski, 2002). Rookery conditions modify local climate, creating specific microclimates in the nesting area (Moczydłowski, 1986). Components of the environmental factors either positively or negatively influenced guano decomposition rate, bacterial growth, and bacterial community composition.

One of the most important physical factors influencing transformations during guano decomposition was temperature (cf. Fig. 1). With its influence on general thermal characteristics of the soil and heat exchange between the soil and atmosphere, temperature is one of the most important factors controlling microbial processes anywhere, but especially so in Antarctica. We believe that significantly higher surface temperature at the rookery is not only

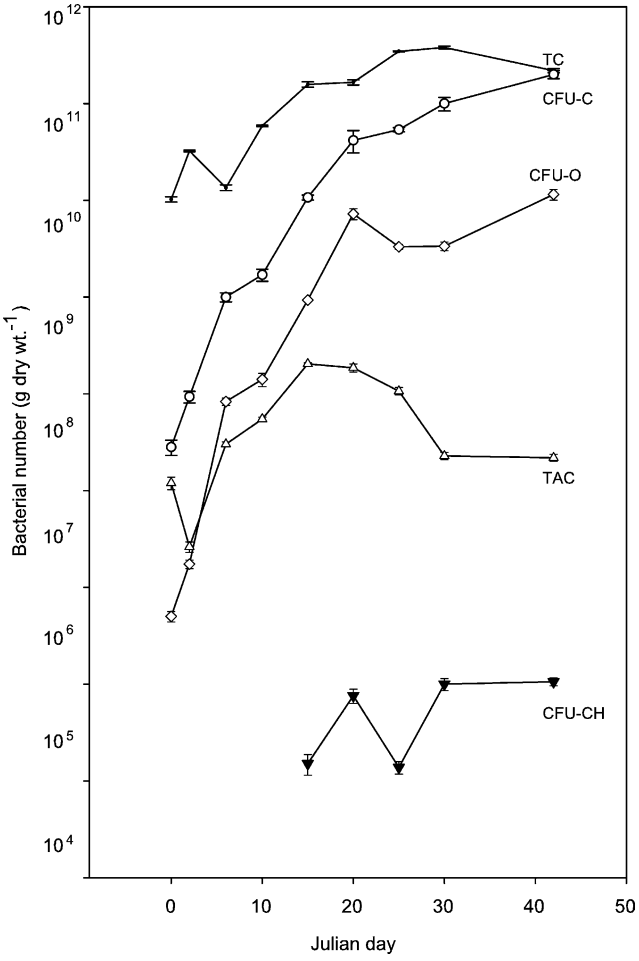


Fig. 3. Total bacterial count (TC), total autotrophic count (TAC), colony forming units of bacteria: copiotrophic (CFU-C), oligotrophic (CFU-O), chitinotrophic (CFU-CH) during penguin guano decomposition process.

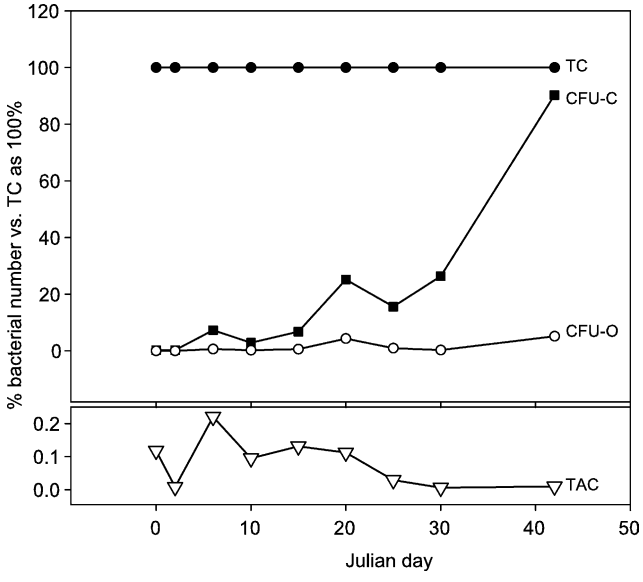


Fig. 4. Relationship between TAC, CFU-C and CFU-O to TC (%), in different phases of guano decomposition.

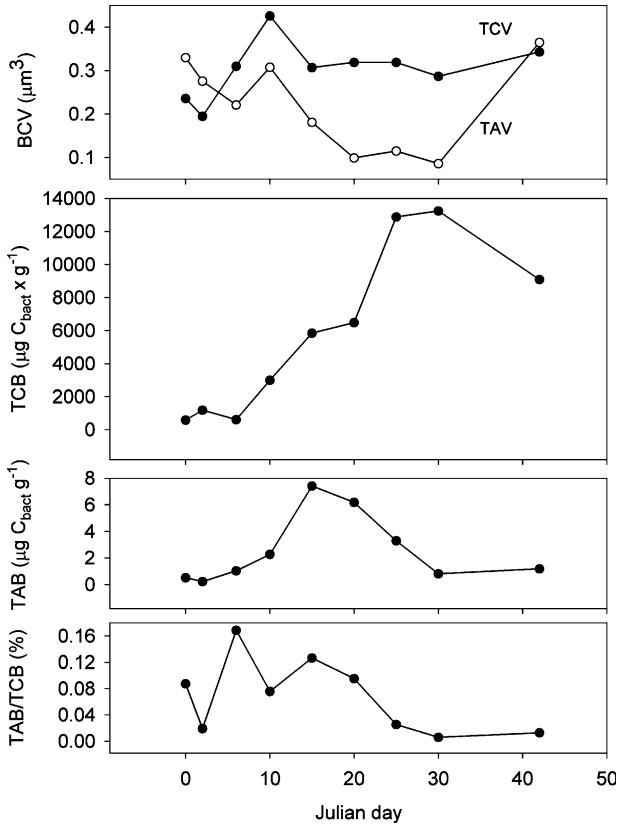


Fig. 5. Bacterial cell volumes (BCV), total bacterial biomass (TCB) and autotrophic bacteria biomass (TAB) during guano decomposition.

the result of the presence of large populations of birds (Moczydłowski, 1986), but may also be the result of microbial activity enhancing temperature in the ground. During the 2004 austral summer, highly significant differences in mean temperatures (measured 3 cm below the ground surface) occurred between the middle of rookery (3.96 ± 0.24 °C) and the area outside it (3.21 ± 0.26 °C; two samples t-test, $t_{23} = 8.473$, $p < 0.000$; Zdanowski, personal observation). Support for this thesis, however, needs further experimental studies.

The second important physical factor was precipitation. According to Fisher (1990), humidity and temperature in Arctic soil at +8 to +12 °C affect metabolism, O_2 consumption and CO_2 production as a function of water availability. The general opinion that water is not a limiting factor for biological processes in the maritime Antarctic (Wynn-Williams, 1990) is true, but water availability can control the rates of these processes. The rate of guano decomposition depends mainly on bacterial activity, which, as most biological processes, also depends on water availability. During the 36 days of measurement in our experiments, precipitation was above 10 mm m^{-2} only on four days, whereas 23 days were characterized by precipitation below 1 mm m^{-2} (6 days with 0 mm m^{-2}). In this time, the water content in guano samples fluctuated between 31.7 and 81.7% of wet weight.

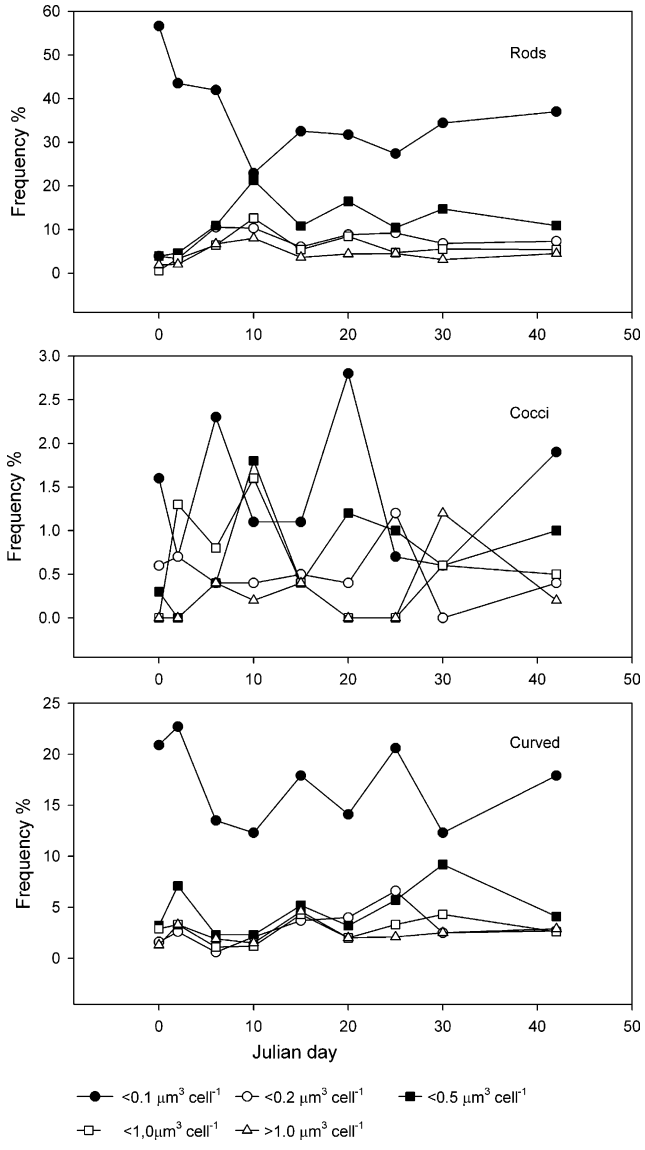


Fig. 6. Temporal changes in percentage of rods, cocci, and curved forms evaluated in five classes of cell volumes.

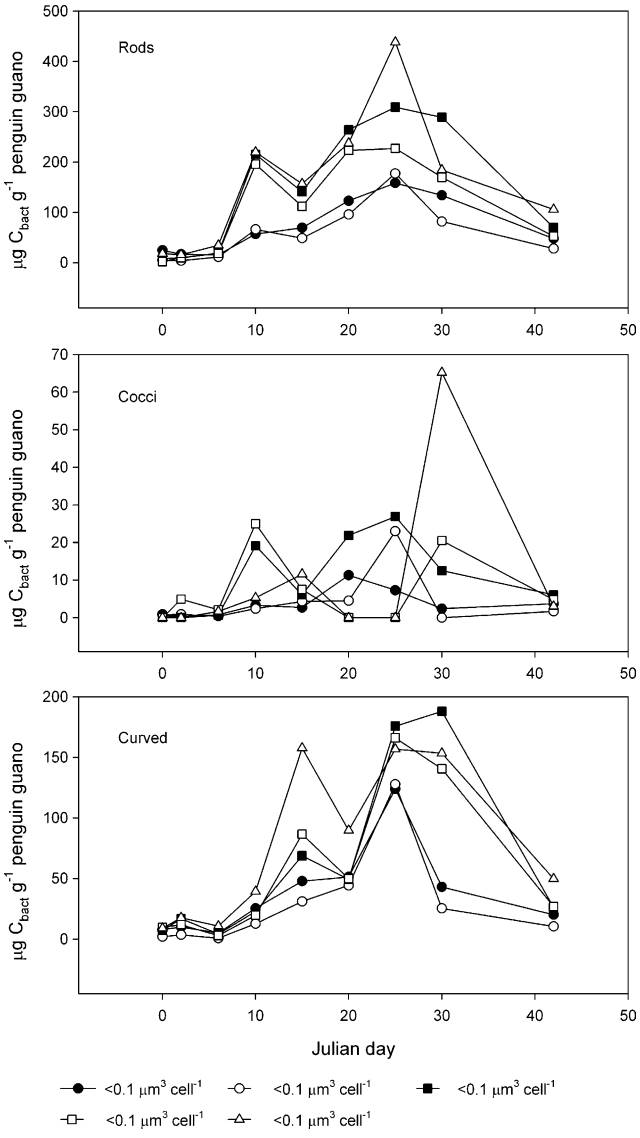


Fig. 7. Temporal changes in biomass (μg bacterial C g^{-1} penguin guano dry weight) of rods, cocci, and curved forms evaluated in five classes of cell volumes.

It should be noted that long-term changes in soil and air temperature (Rodriguez et al., 1996; Keina, 1999; Zwolska and Rakusa-Suszczewski, 2002), and cyclic changes in climatic conditions in Antarctica and the El Niño Southern Oscillation (Carleton, 1988; Smith et al., 1996), can modify bacterial decomposition processes in penguin guano. Such climatic effects and marked differences in experimental set-ups deployed by different authors complicate the comparison of respective data sets.

Significant differences were observed in efficiency of chitin decomposition between two experiments, namely ours during summer 1999/2000, and Pietr et al. (1983) in summer 1979/1980. Half the amount of chitin we determined after 42 days exposure (17.4%) was reported by Pietr et al. (1983) after 21 days laboratory-and-field exposure (8.5%). We calculated that 43.1% of the initial chitin content remained intact, and may thus be a major component of soil humus. If it is assumed after Tatur and Myrcha (1984), that the guano deposition on the rookery may reach 10 kg dry weight m^{-2} during the breeding season, and that 32.1% (3.21 kg) of fresh guano is chitin, we see that the chitin deposition is 1.38 kg dry weight m^{-2}. This relatively difficult to decompose material promotes a soil structure that is efficiently aerated, which in turn promotes bacterial activity and production.

No comparable data on total protein and fat decomposition in penguin guano is available. On the basis of our results, however, we conclude that neither protein nor fat were totally eliminated. After 42 days exposure, both still comprised ~28% of their initial contents. In part at least, the lipids and proteins we detected during the incubation were part of the bacterial standing crop (both live and dead).

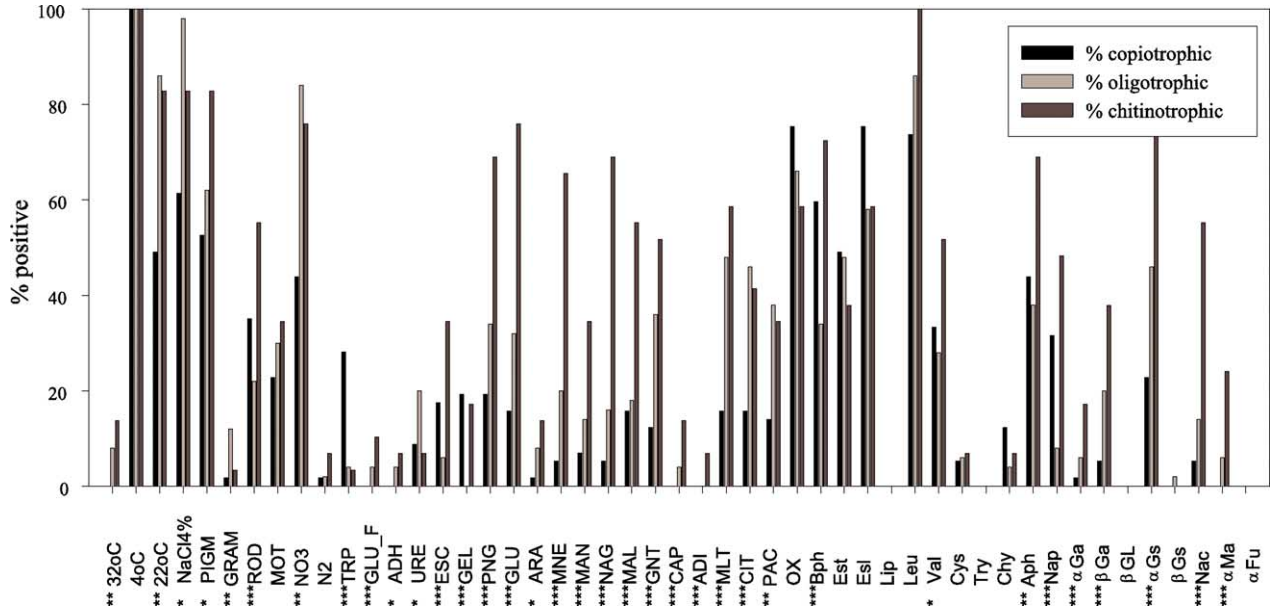


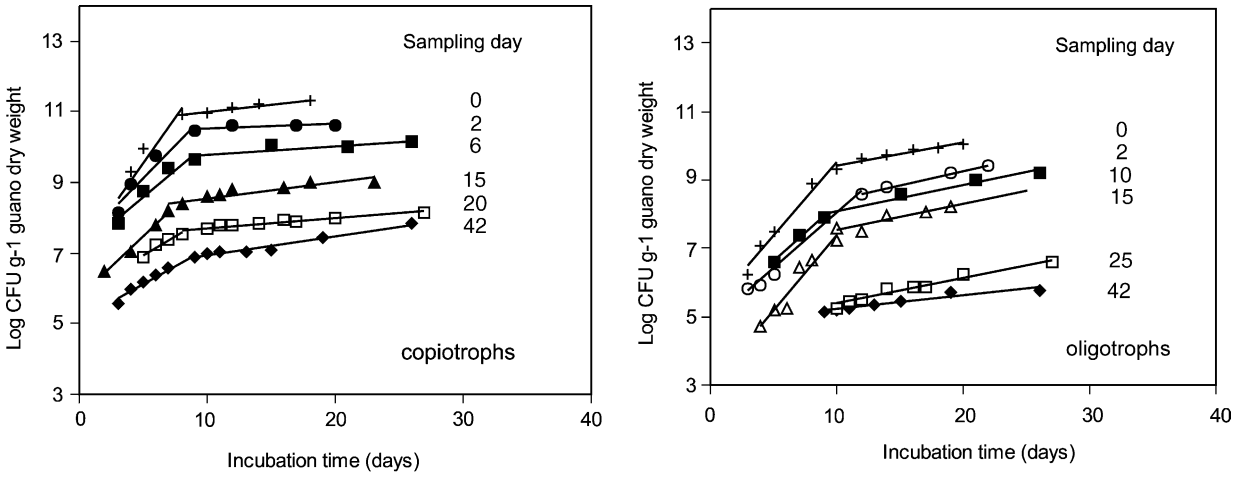
Fig. 8. Differences between morphophysiological characteristics of three CFU bacterial populations. Significance level ($*p<0.05$, $**p<0.01$, $***p<0.001$) between frequency of positive responses to tests shown by copiotrophic, oligotrophic and chitinolytic groups of isolates (χ^2_2). The following tests were conducted: Ability to grow at 32, 4, 22 °C, and to grow on nutrient agar containing 4%, w/v NaCl (NaCl 4%). Morphological analyses comprised counts of pigmented colonies (PIGM), three microscopic tests: gram reaction (GRAM), morphology (ROD), motility (MOT). The API 20NE system allows 10 biochemical tests: reduction of nitrate to nitrite (NO3), and to nitrogen (N2); indole production (TRP); fermentation of glucose (GLU_F); arginine dihydrolase (ADH); urease ((URE); β-glucosidase (ESC); gelatin hydrolysis (GEL); β-galactosidase (PNG); cytochrome oxidase (OX); and 12 tests for assimilation of carbohydrates as sole carbon sources; glucose (GLU); arabinose (ARA), mannose (MNE); mannitol (MAN); *N*-acetyl-glucosamine (NAG); maltose (MAL); gluconate (GNT); caprate (CAP); adipate (ADI); malate (MLT); citrate (CIT); phenyl-acetate (PAC). The API ZYM system tests for the presence of 19 constitutive enzymes: alkaline phosphatase (Bph); esterase-C_4 (Est); esterase lipase-C_8 (Esl); lipase-C_{14} (Lip); leucine arylamidase (Leu); valine arylamidase (Val); cystine arylamidase (Cys); trypsin (Try); chymotrypsin (Chy); acid phosphatase (Aph); naphthol-AS-BI-phosphohydrolase (Nap); α-galactosidase (αGa); β-galactosidase (βGa); β-glucuronidase (βGl), α-glucosidase (aGs); β-glucosidase (βGs); *N*-acetyl-β-glucosaminidase (Nac); α-mannosidase (αMa); α-fucosidase (αFu).

Using crude calculations (Bratbak and Dundas, 1984; Zdanowski, 1995, and unpublished) in which bacterial biovolumes were converted to bacterial carbon and protein, we determined bacterial standing stock protein content after 30 days as 1.54% of guano dry weight. The remainder might result from conversion of native lipids and protein into part of the recalcitrant humic fraction.

The percentages of lipids, proteins, chitin and ash in balance equations calculated during successive phases of guano degradation were over 100% up to the 6th day, then in the next period decreased to 78.5% by the last day (cf. Fig. 2). This shed light on two factors: (a) over estimation of equation components as a result of methodological error; (b) from the 10th to 42nd day, accumulation of products that could not be detected by our methods. Worth noting is the significant increase in the ash content in the remnants. This may show a relative increase in the ash fraction with respect to other components, and may shed light on the significant influence of transport by wind and runoff of materials from the surrounding area. Strong onshore winds bring volatile nutrients in the marine aerosol, and ornithogenic or terrestrially derived nutrients in the form of dust (Wynn-Williams, 1990).

Guano deposition by penguins is the major source of carbon, nitrogen, phosphorus and other inorganic macro and micronutrients in the terrestrial Antarctic environment. According to Pietr et al. (1983) about 50% of C and N were volatilized in the first three weeks of decomposition in laboratory experiments (Myrcha et al., 1985). In the same time, the P content almost doubled. Some general trends were apparent during our field experiment: After 30 and 42 days, respectively, only 30.2 and 35.7% of C, 52.0 and 58.7% of N, was lost, whereas a nearly 100% increase in P content was observed. It is noteworthy that nitrate reducers were not detected by Pietr et al. (1983), in contrast to our findings. Among our 136 randomly selected CFU isolates, up to 60% in the first phase (0–10) days of the experiment (N 52 isolates) were nitrate reducers, and about 70% in the second phase (15–42) days (N 84 isolates). A few isolates that reduced NO_3 to N_2 were observed (cf. Fig. 8). A significant pH increase in guano (cf. Table 1) might reflect ammonia release during mineralization of amino acids and uric acid (Pietr et al., 1983; Lindeboom, 1984; Lindeboom and Sandee, 1992). The increase of the C:N ratio (cf. Table 1) in the first few days may indicate volatilization of N/NH_3 during microbial mineralization of organic N.

Of the elements detected in guano (Table 1), fluorine may be a toxic component. Krill consumed by penguins contain ~0.11% fluorine (Buchholz, 1983), which is concentrated in the penguin's digestive tract. Soils fertilized



Regression equations

0a) y = 0.21x + 5.08;R2 = 0.969
0b) y = 0.06x + 6.37;R2 = 0.966
2a) y = 0.21x + 5.86;R2 = 0.959
2b)y = 0.03x + 7.40;R2 = 0.966
6a) y = 0.34x + 5.75;R2 = 0.993
6b) y = 0.05x + 8.05;R2 = 0.827
15a) y = 0.30x + 7.09;R2 = 0.950
15b) y = 0.37x + 9.51;R2 = 0.767
20a) y = 0.01x + 7.32;R2 = 0.934
20b) y = 0.01x + 10.42;R2 = 0.569
42a) y = 0.52x + 6.98;R2 = 0.888

Regression equations

0a) y = 0.21x + 5.08;R2 = 0.969
2a) y = 0.21x + 5.86;R2 = 0.959
10a) y = 0.03x + 7.40;R2 = 0.966
10b) y = 0.34x + 5.75;R2 = 0.993
15a) y = 0.30x + 7.09;R2 = 0.985
15b) y = 0.37x + 9.51;R2 = 0.972
25a) y = 0.01x + 7.32;R2 = 0.966
25b) y = 0.01x + 10.42;R2 = 0.993
42a) y = 0.52x + 6.98;R2 = 0.970
42b) y = 0.05x + 10.56;R2 = 0.918

Fig. 9. Kinetics of CFU counts increments for bacterial cells incubated on plates. Each plot represents CFU communities isolated from guano in different phases of guano decomposition. Regression equations were calculated for: (a) the phase of rapid growth, (b) the phase of slow growth.

by penguin guano contain high concentrations of calcium phosphate through its release, or formation during chitin mineralization (Tatur, 2002). The toxicity of fluorine is limited, however, perhaps through its precipitation as fluoroapatite (Tatur, 1987) which restricts F diffusion (Rai et al., 2000).

Considerable changes also occurred in guano bacterial populations. Among Antarctic environmental data, the highest bacterial abundances have been reported in bird-impacted soils of sub-Antarctic islands. In albatross nest sites on subAntarctic Marion Island, French and Smith (1986) and Grobler et al. (1987) counted 1.1×10^{11} and 1.78×10^{11} bacteria g^{-1} dry weight of soil, respectively. Similar numbers were reported for sites affected by penguins (French and Smith, 1986; Ramsay and Stannard, 1986). We recorded similar scale increases in TC after 30 days incubation. It is clear that at the transition from greatest bacterial abundance (days 30–42), TC decreased while culturable bacterial counts (CFU) significantly increased. Dramatic increases in CFU, however, were observed throughout our experiment. Very high CFU:TC ratios in the late phase of guano transformation contrast with reports that cultivated bacteria number five or six orders of magnitude less than the total soil microbial community. Conversely, extremely large fractions of the total bacterial community may be culturable in very fertile terrestrial environments (Balicka et al., 1982; Zdanowski and Weglenski, 2001).

Bölter (1995) considers that mean bacterial cell volume may be a useful descriptor of the bacterial community. We used this parameter to develop information on diversity within bacterial communities at different stages of guano decomposition. Complementary information on diversity was obtained after transforming these data to bacterial biomass in classes of cell volumes. Clearly, just a few large bacterial cells can significantly increase the amount of biomass in that class. Data discussed here were generated through procedures based on an image analysis system and can hardly be compared to earlier data obtained by direct observations. Our data show a prevalence of straight and curved rods over cocci, with dominance by the smallest cells. This finding contradicts earlier observations that show much larger percentages of cocci in soil samples from a penguin colony (Ramsay and Stannard, 1986). A possible explanation for this change may lie with that described by Amy and Morita (1983), through which rod-shaped bacteria in culture (cf. nutrient rich guano) may shrink to coccoid forms when starved (cf. oligotrophic soils). More than 40% of randomly isolated bacteria in our work were cocci, which is comparable to the 30% isolated by Sieburth (1963) from

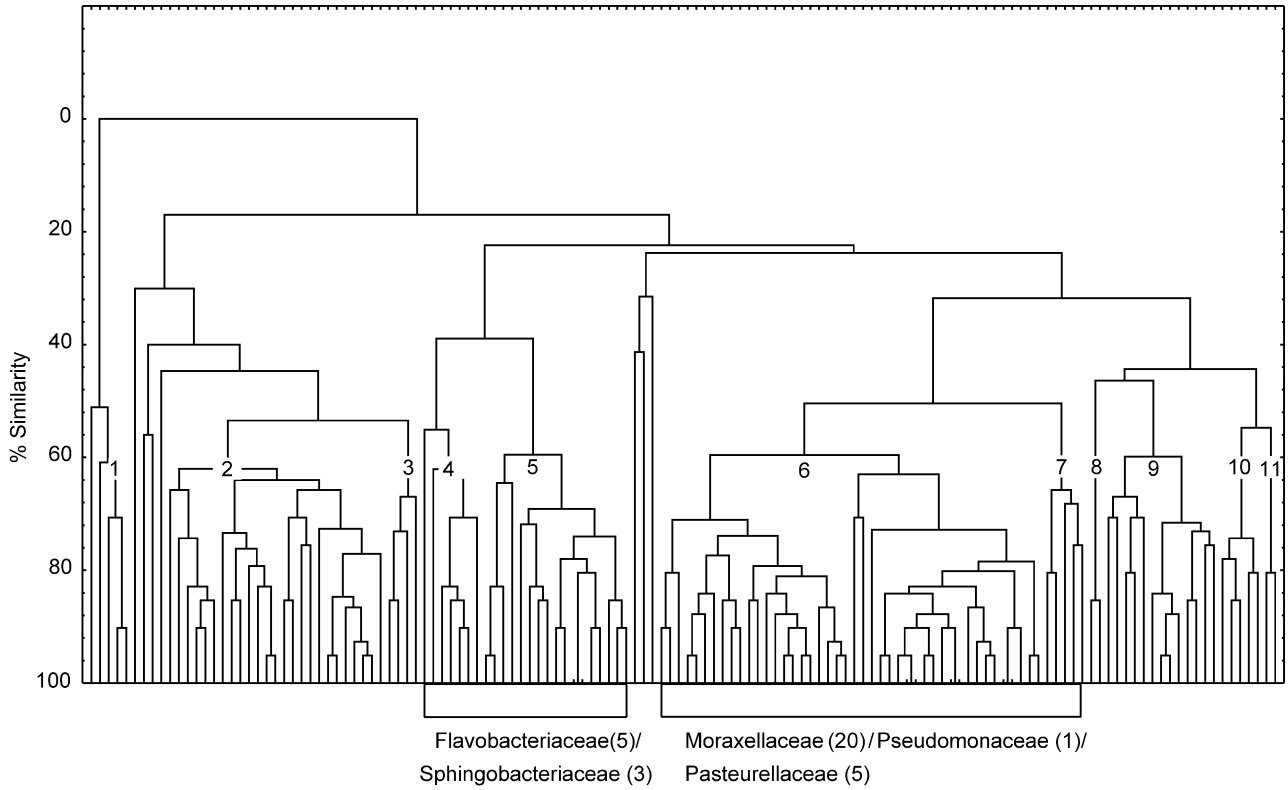


Fig. 10. Hierarchical classification of 136 bacterial isolates based on their responses in 49 morphophysiological tests (as described in Fig. 8). Eleven clusters are enumerated with ciphers 1–11. In brackets are numbers of isolates which show numerical profiles having matches in the bioMérieux database.

penguin nests. The increase in counts of cocci with the transition from copiotrophy to oligotrophy was significant (cf. Fig. 8).

Changes in soil bacterial populations have been investigated through different analyses of colony-forming curves. Zdanowski and Weglenski (2001) used the time course of colony formation of bacteria on agar plates to refine soil CFU diversity in each sampling site in the vicinity of Henryk Arctowski Station. Hashimoto and Hattori (1989) and Gorlach et al. (1994) were using the first order reaction (FOR) model to describe the formation process of visible colonies from paddy field soils on a plate as a function of incubation time. Mitsui et al. (1997) investigated the correlation between phylogeny and bacterial colony grouping by a colony-forming curve (CFC), based on the FOR model. In all these studies, the relationships between the number of colonies and incubation time was presented. We used another way to present this relationship and to demonstrate differences in CFU increment kinetics at different phases of guano decomposition. In this study the transition from <1 to 90% CFU of TC is a prominent feature, and suggests highly dynamic transformations are taking place in the guano. Time courses of the colony formation process of penguin guano bacteria on agar plates (cf. Fig. 9) can be considered a function of proliferating bacterial cells that show higher rate of colony formation during the course of guano decomposition.

Many data in the literature confirm the high phylogenetic diversity of bacteria in soil (e.g. Holmes, 1996). According to Curtis et al. (2002), one ton of suburban garden soil contains 4×10^6 different taxa, or twice that of the entire ocean. The high diversity in bacterial habitats and community composition reported here is consistent with previous reports for Antarctic soil (Ramsay, 1983; Pietr, 1986; Bölter et al., 1997; Zdanowski and Weglenski, 2001). The unique nature of Adelie penguin guano and its microflora depends primarily on the penguin diet. After deposition on land, the guano microflora may comprise microbes of diverse origin (Donachie and Zdanowski, 1998), and we believe, of the penguin's own enteric flora. Once deposited on land, however, the guano microflora develops under different environmental parameters. Morphophysiological analyses presented by hierarchical classification of isolates showed high diversity within our randomly selected bacterial collection.

In such diversified communities, one can expect high diversity in digestive and assimilatory potential of bacteria. Bölter (1993) studied the effect of carbohydrates and leucine on growth of bacteria from Antarctic soils, and showed that growth of CFU bacteria is stimulated by several sugars, among them glucose, maltose and mannitol, and to a lesser extent by the amino acid leucine. In our studies, we found varying capacity to assimilate these carbohydrates within different groups (copio-, oligo-, and chitinotrophs), and in the case of glucose, between groups of isolates from different

phases of guano degradation. Similarly, the digestive potential of bacteria revealed by the percentage of isolates producing enzymes varied markedly. Noteworthy was the fact that most isolates grew in media containing sodium chloride equivalent to that in seawater. This contrasts sharply with the numbers of halotolerant bacteria isolated from soil in the area of Arctowski station (Pietr, 1993) and a stream in the same area (Zdanowski, 1995). About one-fifth of isolates from the first phase of guano degradation expressed chymotrypsin, and a little more hydrolyzed gelatin. This also contrasts sharply with data on the percentage (up to 75%) of proteolytic bacteria in different ornithogenic soils (Pietr, 1986). It is not surprising that most of isolates from penguin guano were cytochrome *c* positive, especially in the second phase of guano degradation (Zdanowski, unpublished). The rather porous structure of guano, with its high content of fibrous chitin debris, ensures it can be aerated just as an Antarctic soil (Vishniak, 1993).

Our data for molecular and morphophysiological approaches (Zdanowski et al., 2004) suggest these approaches agree in terms of the composition of the respective groups. They further demonstrate that a select range of morphophysiological tests can rapidly group isolates to the same standard as that based on a fragment of the 16S rRNA gene. This approach may allow affiliation of cultivated bacteria with a bacterial group previously defined by phylogenetic and phenotypic methods.

5. Conclusions

Quantitative and qualitative changes in guano composition and in bacterial populations in guano were followed under in situ conditions. Temperatures between ca. 7 and $<11\,^{\circ}C$ positively affected bacterial numbers, while temperatures outside this range had a negative impact. Optimal temperatures for guano decomposition ranged between 3 and 11 °C. Among other (edaphic) factors, increasing wind velocity positively influenced autotrophic bacterial abundance. Wet weight changes correlated significantly with precipitation. Changes in the guano resulted from decomposition and transformation of organic components: fats, proteins, chitin, and other forms of nitrogen and carbon, as well as of minerals. These transformations stimulated bacterial growth to levels characteristic only for materials of the highest nutritional values for bacteria (Zdanowski, 1995). Morphometric and morphophysiological tests demonstrated the diverse nature of bacteria in guano.

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