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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF URIC ACID IN SOIL

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SUMMARY

The large deposits of organic nitrogen in sea-bird rookeries may be responsible for an establishment of an ornithocoprophilous plant community. Uric acid, a major end-product of avian nitrogen metabolism, must play an important role in making this unique ecosystem. The acid was extracted from soil into potassium phosphate solution and separated from other components in the extract by high-performance liquid chromatography. Although the adsorption of the acid by soil prevents its quantitative recovery, the method is adequate to be applied to a study of nitrogen metabolism in rookeries. A large input of uric acid during the breeding season, its localization on the soil surface and its *in situ* decomposition were found to occur in the rookeries. The method is easily modified for preparative purposes, which makes it possible to perform isotopic analysis at a natural level and to elucidate further the nitrogen dynamics in the ecosystem.

INTRODUCTION

Uric acid is excreted by birds as a major end-product of their nitrogen metabolism and is often 70–90% of the total nitrogen excreted by avian species. During their breeding season, some birds form rookeries on islands that, for secure breeding, are often located several hundred or thousand kilometres from the nearest continent. A large amount of nitrogen is deposited there in the form of uric acid in the excreta and also of feathers, eggs and carcasses of chicks and adult birds.

Hence, the breeding activity of birds, which results in large deposits of nutrients essential for the development of the flora and fauna in these islands must be significant in determining the ecological features of the islands. Most of the nutrients on such bird rookeries are provided by sea-birds[1] and a heavy plant growth consequently resulted in contrast to the generally sparse vegetation outside the rookeries[2,3]. As most of the nitrogen in avian excreta is in the form of uric acid, which is not directly utilized by higher plants, its transformation by uric acid bacteria must occur at the beginning of the nitrogen cycle in rookery.

As previous investigators perceived a distinct smell of ammonia above, for

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instance, a penguin rookery in Marion Island[4–7], the degradation of uric acid to ammonia and its evaporation may play an important role in the nitrogen cycle in bird rookeries. However, the previous studies did not quantify the rates of faecal production or nitrogen transformation, and our knowledge of the quantitative significance of this phenomenon in natural ecosystems remains minimal[8].

In order to follow the fate of uric acid in a bird rookery, a method for the determination of the amount of uric acid present in rookery soil is necessary. However, we failed to find such a method in the literature, and decided to develop a high-performance liquid chromatographic (HPLC) method. As isotopic studies on the nitrogenous substances in an ecosystem often elucidate their biogeochemical nitrogen dynamics[9,10], we considered it desirable that the method can be applied with little modification to the isolation of uric acid from soil for determination of its isotopic composition.

Consideration of the future utility of the method for isotopic determinations imposes some restrictions on our search for the method, as is discussed elsewhere[11,12], and distinguishes it, in addition to the fact that it handles biogeochemical samples such as soils, from recent HPLC determinations of uric acid in plasma and urine[13–21], in animal feedstuffs containing added poultry waste[22], in grain and cereal products as a measure of insect infestation[23] and in rat tissues[24].

In this paper, we describe an HPLC method for the separation of uric acid from soil and present an example of its application to the study of rookery soils, which elucidates their unique biogeochemical characteristics. The isotopic application of this HPLC method is reported in an accompanying paper[11].

EXPERIMENTAL

Chemicals

Water was distilled from demineralized water in an all-glass Yamato Model WAG-24 Auto Still (Yamato Kagaku, Tokyo, Japan). Uric acid, ethanol, sodium phosphate salts and potassium phosphate salts were of guaranteed reagent grade from Wako (Osaka, Japan).

Standard soils

A standard clay mineral (Swy-1 Na^+-montmorillonite from Crook County, WY, U.S.A.) was obtained from the Source Clay Mineral Repository (Department of Geology, University of Missouri, Columbia, MO, U.S.A.) and was used without further treatment. The paddy field soil was from a ploughed layer (0–13 cm from the soil surface) of a long-term fertilization experimental paddy field at the Central Agricultural Experiment Station (Konosu, Saitama Prefecture, Japan). The field had been fertilized only with organic manure for more than 50 years. The soil was dried, passed through a stainless-steel sieve (2 mm) to eliminate large particles and then crushed to pass through a 0.5-mm stainless-steel sieve.

Preparation of uric acid-containing soils

Na^+-montmorillonite and paddy field soil that contained uric acid were obtained as follows. An appropriate amount of a 0.1 g/l aqueous solution of uric acid was added to the clay and to the soil. They were then vigorously shaken at 23°C for

2 h. After shaking, they were vacuum dried for 2 days, lightly crushed and passed through a 0.5-mm stainless-steel sieve. Bacterial and/or chemical decomposition of uric acid during the above procedure were unlikely[11,25]. Prior to the addition of the uric acid solution, the absence of indigenous uric acid was demonstrated by the method given in this paper for extracting and determining the content of the acid.

Soils from Black-tailed Gull (*Larus crassirostris*) rookeries were collected on July 24 and September 11, 1981, and January 21, 1982, at the Kabushima rookery (Hachinohe, Aomori Prefecture, Japan) (location: 40°32′12″N, 141°33′41″E) and on June 5, 1982, at the Tsubakishima rookery (Rikuzentakata, Iwate Prefecture, Japan) (location: 38°55′59″N, 141°43′14″E). For the study of the vertical profile of the uric acid level, the soil from the Kabushima rookery was cut to expose a vertical section, and four layers of thickness 5 cm each were sampled, starting with the lowest layer (15–20 cm depth) and ending at the top layer (0–5 cm depth) in a stainless-steel cylinder (height 50 mm, I.D. 50 mm) with as much care as possible not to disturb the original structure of the soil. Immediately after collection, 8.6 g of ethanol were added to the soil in the cylinders. For the soil from the Tsubakishima rookery, the same procedure was employed except that the top layer was further separated to yield two layers (0–1 and 2–5 cm depth).

For the study of the seasonal variation of the uric acid content in the soil, only surface soil (0–5 cm) was collected with a core sampler (core diameter 20.5 mm). The core sample was transferred into a plastic vial, which was filled with ethanol and sealed tightly. Small portions of the soil were kept separately in a vial without addition of ethanol for the measurement of its water content.

Both the cylinders and the vials were placed in a refrigerated container and moved to the laboratory. On arrrival, the vial was opened and the contents were vacuum dried using a Labconco FDC-8 freeze-dryer (Labconco, Kansas City, MO, U.S.A.). After drying, the soil was passed through a 2-mm stainless-steel sieve to eliminate large particles such as pebbles, plant debris and animal remains. The soil was then homogenized to pass a 0.5-mm sieve and subjected to extraction of uric acid.

Extraction of uric acid

Approximately 1 g of dried soil was weighed in a 100-ml beaker and 20 ml of 0.1 *M* KH_2PO_4 solution were added. The beaker was covered with a piece of aluminium foil and the soil suspension was stirred with gentle heating for 10 min. The suspension was then transferred into a 50-ml centrifuge tube and centrifuged at 3000 rpm for 10 min using a Kokusan H-103N table-top centrifuge (Kokusan Centrifuge, Tokyo, Japan). After centrifugation, the supernatant was pressure-filtered through a pre-heated (at 420°C for more than 5 h) Whatman glass-fibre filter (GF/C; diameter 4.7 cm; Whatman, Maidstone, U.K.) and a 0.45-μm microporous filter (Type HA; diameter 4.7 cm; Millipore, Bedford, MA, U.S.A.).

Chromatography

Potassium phosphate solution (0.1 *M* KH_2PO_4) for use as the HPLC mobile phase was pressure-filtered through a 0.4-μm microporous polycarbonate membrane (Nuclepore, Pleasanton, CA, U.S.A.). The flow-rate of the mobile phase was 1.5 ml/min unless indicated otherwise.

The HPLC apparatus consisted of an Erma ERC-3310 degasser (Erma Optical Works, Tokyo, Japan), a Waters Model 6000A dual-piston reciprocating pump (Waters Assoc., Milford, MA, U.S.A.), a Waters Model U6K universal injector equipped with a standard injection loop and a Waters Model 440 absorbance detector. The detector monitored at both 280 and 254 nm; the former was used to follow the elution of uric acid and the latter for detecting contaminants from soil (*cf.*, Table I). Chromatograms were recorded on a Pantos Unicorder U-225MS32 two-pen recorder (Nippon Denshi Kagaku, Kyoto, Japan). The entire apparatus was operated at 19–23°C.

The analytical column selected was a commercial 50 × 0.8 cm I.D. stainless-steel column (SUS 316) packed with polyhydroxyalkyl methacrylate gel (Shodex OHpak B-804; Showa Denko, Tokyo, Japan). A pre-column (Shodex OHpak B-800P) was used to protect the analytical column.

The chromatographic peak heights were read by a Kontron MOPAM 02 semi-automatic image analyser (Kontron Messgeräte, Munich, F.R.G.) and the data were transferred to a Hewlett-Packard HP-85 personal computer that handled the data and printed out the amount of uric acid present in each sample.

Chemical analysis

Organic nitrogen in a sample was converted into ammonia by Kjeldahl digestion for 4 h in the presence of a small amount of HgO–SeO–K_2SO_4 (3:1:4, w/w) as a catalyst[26]. The ammonia thus produced was steam-distilled and collected in a 0.125 *M* H_2SO_4 trap. The ammonium sulphate solution obtained was diluted to volume with water in a 100-ml volumetric flask. Total nitrogen was determined by using an aliquot of the diluted ammonium sulphate solution. The phenol–hypochlorite method[27] was employed for the determination, and the absorbance at 640 nm was measured with a Shimadzu UV-210A double-beam spectrophotometer (Shimadzu Seisakusho, Kyoto, Japan). Standard solutions of ammonium chloride were used for calibration.

In order to measure the total organic carbon in a sample, it was combusted to form carbon dioxide as described elsewhere[28], and the amount of the gas thus generated was measured manometrically. The volume was converted to give the organic carbon content of the sample.

RESULTS AND DISCUSSION

Examination of extractant and eluent

The cation-exchange capacity (CEC) of Na^+-montmorillonite was 1.01 mequiv./g dry clay[29] and that of the paddy field soil was 0.20 mequiv./g dry soil[30]. One gram of the dried paddy field soil contained 34.1 mg of organic carbon and 3.9 mg of organic nitrogen, whereas dried Na^+-montmorillonite contained 1.7 mg of organic carbon and 0.036 mg of organic nitrogen per gram.

Preliminary experiments with hot water as the extractant failed to extract uric acid effectively from the paddy field soil. Further, earlier experiments using Sephadex G-10 and 1 *M* sodium hydroxide solution as the extractant and as the mobile phase did not yield promising results.

Although the nature of the interaction between uric acid and soil is virtually

unknown and many possibilities exist concerning the mode of interaction[31], there are several observations that should be taken into account before setting up the extraction method. First, the two major types of soil of organic matter, humic and fulvic acids, are known to be extracted from soil efficiently with an alkaline solution. This may imply that an acidic solution is desirable as the extractant so that any interference of soil organic matter with the chromatographic procedure is minimized. However, most nucleotide bases are adsorbed to some extent on bentonite[32], and a study on the adsorption of several nucleotide bases on Na^+- and Ca^{2+}-montmorillonites showed that the adsorbed bases are released from the clays when the pH becomes high[33]. Further, the solubility of uric acid is dependent on pH, and more uric acid is dissolved under alkaline than acidic conditions.

Therefore, the following five aqueous solutions of different pH were examined as possible extractants: 0.1 *M* potassium hydroxide solution, 0.1 *M* potassium phosphate dibasic salt solution (pH 8.8), a mixed solution of sodium phosphate dibasic and monobasic salts with final concentrations of 0.02 and 0.01 *M*, respectively (pH 7.2), a 1:1 mixture of 0.1 *M* solutions of potassium phosphate dibasic and monobasic salts (pH 6.8) and a 0.1 *M* solution of potassium phosphate monobasic salt (pH 4.6).

After an extraction by the procedure given in "Extraction of uric acid", an aliquot of the pressure-filtered solution was injected into the analytical HPLC column using the extractant solution as the mobile phase, except with 0.1 *M* potassium hydroxide solution. Using the latter solution, an appropriate amount of a mixture of phosphoric acid and potassium hydroxide was added after the centrifugation to an aliquot of the supernatant in order to make a 0.1 *M* potassium phosphate solution that had the same concentration of the salt as that of the eluent used as the HPLC mobile phase. The addition made the solution cloudy, as a portion of humic acid that was extracted by the alkaline extractant was precipitated under such conditions. The cloudy solution was subjected to HPLC analysis after filtration through a Millipore 0.45-μm microporous filter (Type HA; diameter 2.4 cm).

The chromatograms of the extract from the paddy field soil had two major peaks, due to uric acid and soil organic matter. Mobile phases of higher pH resulted in an insufficient separation between the two peaks, whereas 0.1 *M* potassium phosphate monobasic salt solution (pH 4.6) gave a better separation. Further, extractants of high pH extracted more soil organic matter and consequently resulted in a larger peak of this material.

As far as the extraction of the acid is concerned, 0.1 *M* potassium hydroxide solution seems to be the most efficient. Lowering of the pH of this extractant, which was necessary for the HPLC analysis, however, appeared to reduce the final recovery when the acid was extracted from the paddy field soil. This was probably due to the precipitation of humic acid and consequent co-precipitation of uric acid. This is a great disadvantage of the use of potassium hydroxide solution as the extractant, as an isotopic fractionation was likely to occur during the occlusion reaction, which should be avoided if the method is intended to be modified for isotope analysis.

The separation between soil organic matter and uric acid on the chromatogram was less distinct with K_2HPO_4 as the mobile phase, which also is to be avoided as far as possible if the method is intended for isotopic determinations. In fact, our later experience in isolating uric acid from gull excreta showed that the excreta also contained substances whose elution was similar to that of soil organic matter and that

even the eluent of pH 7.2, which was between K_2HPO_4 (pH 8.8) and KH_2PO_4 (pH 4.6), still resulted in a poor separation when the excreta was subjected to the extraction and isolation procedures. The purity of the isolated uric acid was 94% with this eluent, whereas KH_2PO_4 gave 100% purity when the same procedures were used. Therefore, a 0.1 *M* solution of potassium phosphate monobasic salt (pH 4.6) was chosen as the eluent.

Fig. 1 shows the relationship between the initial amount of adsorbed uric acid and that recovered. As the KH_2PO_4 extraction of uric acid resulted in a higher recovery than the KOH extraction, 0.1 *M* potassium phosphate monobasic salt solution was chosen as the extracting solution.

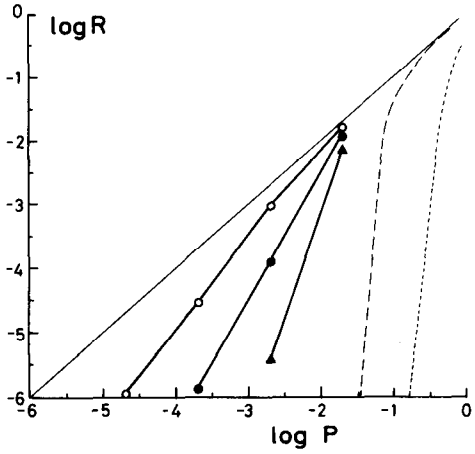

Fig. 1. Relationship between the amount of uric acid present in standard soils and that recovered by extraction. *P* is the concentration of uric acid (g/g dry sample) in the paddy field soil or the Na^+-montmorillonite, and *R* is that extracted from the soil or the clay. The solid line without symbols indicates complete extraction of the acid, the long broken line (——) represents no release of uric acid adsorbed to the full extent of CEC on the paddy field soil and the short broken line (---) is the same on Na^+-montmorillonite. ○, KH_2PO_4 extract from the paddy field soil; ●, KOH extract from the field soil; ▲, KOH extract from Na^+-montmorillonite. The mobile phase for analytical HPLC was 0.1 *M* KH_2PO_4 solution for all the experiments shown. All the results lie between complete recovery and complete adsorption, and the KH_2PO_4 extract gave the highest recovery from the field soil. No uric acid was recovered from Na^+-montmorillonite by extraction with KH_2PO_4 when the amount of the acid present in the clay was up to 12% of its CEC.

If the result obtained from the paddy field soil can be considered to be representative, the use of 0.1 *M* KH_2PO_4 solution as both the eluent and the extractant indicates that about half of the uric acid adsorbed on a soil would be extracted when the total amount of the adsorbed acid is about 6% of the CEC of the soil, and that nearly 90% of the acid would be extracted when the total acid equals to 60% of the CEC.

Separation of uric acid from other compounds in soil

Although the degradation pathway of uric acid by uric acid bacteria is not yet fully understood, the probable route under aerobic conditions involves sequentially

allantoin and carbon dioxide, allantoic acid, urea and glyoxylic acid, and oxalic acid, carbon dioxide and ammonia, in this order. Table I shows the chromatographic characteristics of these compounds and also those of uric acid and soil organic matter.

It is apparent that none of these metabolites of uric acid and the soil organic matter interfere with the detection of uric acid.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF URIC ACID, ITS METABOLITES AND SOIL ORGANIC MATTER

The flow-rate of the mobile phase (0.1 *M* KH_2PO_4) was 1.3 ml/min. mA_{254} is the peak-height absorbance (mAbs) at 254 nm and mA_{280} is that at 280 nm. Neither urea, ammonia nor ammonium can be detected by these wavelengths. The last column shows the ratio of the molar absorptivity at 280 nm to that at 254 nm. Only uric acid gives a ratio of more than unity. Note that the amount of uric acid used to produce a comparable peak height is far smaller than that necessary for other compounds. Although allantoic acid absorbs 254 nm and 280 nm light very weakly, it was not detected, under the conditions employed, during 60 min after its injection. Glyoxylic acid was used as the monohydrate and oxalic acid as the dihydrate. Soil organic matter was obtained from the paddy field soil. Three different extractants, 0.1 *M* KOH, 0.1 *M* K_2HPO_4 and 0.1 *M* KH_2PO_4, yielded soil organic matter that gave the same elution time on the chromatogram. Glyoxylic acid and soil organic matter gave more than one peak, and the elution time given is that of the largest peak. None of the other peaks had an elution time close to that of uric acid.

Compound	*Amount injected (μg)*	*Elution time (min)*	mA_{254}	mA_{280}	$\varepsilon_{280}/\varepsilon_{254}$
Uric acid	0.0017	22.7	0.035	0.10	2.9
Allantoin	4.0	17.4	0.59	0	0
Allantoic acid	4.0	N.D.*	–	–	–
Glyoxylic acid	35.2	14.8	3.5	0.61	0.17
Oxalic acid	14.8	14.0	17.0	5.2	0.30
Soil organic matter	Unknown	13.6	5.0	3.6	0.73

* No peak observed.

Reproducibility and linearity of uric acid determination

The reproducibility of the chromatography was evaluated by making a series of six injections of ten different 0.1 *M* KH_2PO_4 extracts (totaling 60 injections) and determining the relative standard deviation of the peak heights. An average coefficient of variation of 3.8% was obtained for these replicate injections.

In order to test the linearity, a series of nine standard uric acid solutions in 0.1 *M* KH_2PO_4 were analysed. The amount of uric acid injected ranged from 5 ng to 2 μg, and a linear response (correlation coefficient = 0.998) was obtained over this range.

Uric acid in extract and in soil

The uric acid content was calculated directly from that found in the extracts, and no effort was made to take into account uric acids tightly adsorbed to the soils. As rookery soils are often not fully weathered, and as even the paddy field soil yielded a considerable recovery when the uric acid content was more than a few tens of ppm, we believe that the uric acid content of the extract gives a reasonable indication of the true uric acid content in the soils.

In order to obtain the true content, a series of adsorption and extraction experiments must be carried out for each soil studied. Without a knowledge of the true content to begin with, however, a complete understanding of the adsorption and extraction characteristics of a soil would be impossible. Further, the bacterial decomposition of uric acid is very rapid in some rookery soils[25]. The impracticability of eliminating this bacterial activity without sacrificing other soil characteristics would make the understanding even more difficult. Even after the series of experiments, the contents obtained would still be approximate. Therefore, as a first-order approximation, the value calculated directly from that found in the extracts was adopted in this paper.

Uric acid in rookery soil

The Kabushima rookery soil sampled in July was examined in order to study the vertical distribution of uric acid, and the results are given in Table II. The topmost soil (0–5 cm from the surface) contained more than 1% dry weight of uric acid, whereas lower soils contained no uric acid. This indicates that the uric acid deposited on the soil in the form of bird excreta remains on the surface and little, if any, penetrates into the lower layers. This localization of the acid was also found in the Tsubakishima soil. The topmost layer (0–1 cm) contained more than 2.5% dry weight of uric acid, whereas the 2–5 cm and deeper layers contained no uric acid.

TABLE II

SOIL CHARACTERISTICS AND VERTICAL DISTRIBUTION OF URIC ACID

Dried solid matter that did not pass the 2 mm stainless-steel sieve was classified into two groups: pebbles and detrita. Detrita consisted of animal and plant remains and occasionally of anthropogenic material such as pieces of plastics and metals that could be derived from fishing and other human activities. There was no difficulty in distinguishing between pebbles and detrita.

Depth (cm)	*Composition of wet soil (%)*				*Uric acid content (ppm of dried soil)*
	Water	*Pebbles*	*Detrita*	*Soil (< 2 mm)*	
0–5	42.9	3.2	0.4	53.5	$1.0 \cdot 10^4$
5–10	48.1	0.2	0.6	51.1	0.00
10–15	48.3	0.8	0.3	50.6	0.00
15–20	43.7	21.3	0.0	35.0	0.00

Normally, Black-tailed Gulls arrive at Kabushima in February after an absence during autumn and winter, and the activity in the rookery lasts until August[34]. Table III shows the seasonal variation of the uric acid content in the surface soil. It is clear that, after the gulls have left the rookery, the uric acid content declines rapidly. The considerable amount of uric acid in winter soil must be due to the early visit of gulls during the day; at the time of the sampling, a large number of gulls were found to stay on the rookery during the daytime and to leave the rookery at night to feed on fish. The chromatogram for the winter soil that yielded $2.3 \cdot 10^2$ ppm uric acid is shown in Fig. 2 as an example of the analysis of the uric acid level in soil.

TABLE III

SEASONAL VARIATION OF URIC ACID IN ROOKERY SOILS

Surface soil (depth 0–5 cm) was collected for this study. The approximate phase of breeding activity is given according to ref. 35.

Date	*Approximate phase of breeding activity*	*Uric acid content (ppm of dried soil)*
July 24	In the midst of leaving nests	$1.0 \cdot 10^4$
September 11	1 month after the leaving rookery	1.4
January 21	3 weeks before return to the rookery	$2.3 \cdot 10^2$

As the uric acid does not penetrate into the soil, the rapid decrease in uric acid content of surface soil may be due (1) to flushing out by rain and sea water spray, (2) to losses to the air by wind or (3) to *in situ* aerobic decomposition by uric acid bacteria.

Aerobic decomposition of uric acid would lead to the production of ammonia, and the vertical profile of the soil ammonium content in the Kabushima soil in which

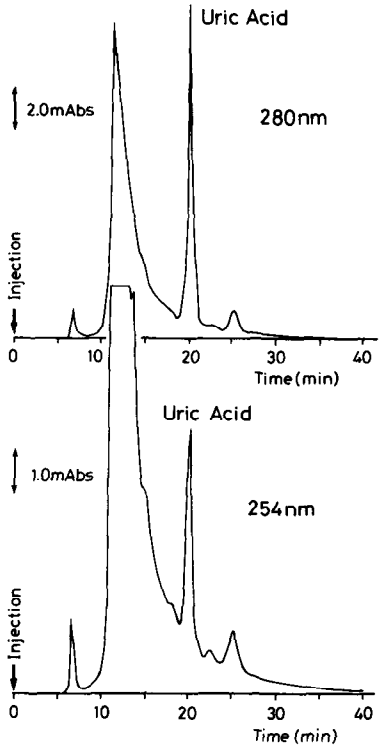

Fig. 2. High-performance liquid chromatograms of uric acid extracted from Kabushima rookery soil in winter. The concentration of uric acid in the soil was calculated to be $2.3 \cdot 10^2$ ppm. The top chromatogram shows the absorbance of the eluent at 280 nm and the bottom one that at 254 nm. The flow-rate was 1.5 ml/min. The large peak on the 254 nm chromatogram is soil organic matter.

uric acid was found only in the surface layer suggested that the decomposition was really taking place. The ammonium content measured according to the method given in the accompanying paper[11] was 2.3, 0.78, 0.44 and 0.21 mg N/g dry soil for the 0–5, 5–10, 10–15 and 15–20 cm layers, respectively. The same trend was found for the Tsubakishima soil, the ammonium contents being 3.3, 0.51, 0.24, 0.24 and 0.21 mg N/g dry soil for the 0–1, 2–5, 5–10, 10–15 and 15–20 cm layers, respectively. Further, field and laboratory experiments that eliminated the possible flushing out of uric acid and the effect of the wind did not reduce the loss of uric acid in the soil[36]. Therefore, the rapid decrease in uric acid content must reflect the microbial activity of the rookery soil and hence this activity would dominate the nutritional status of the rookery, which must have a great influence on the whole rookery ecosystem.

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