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N_2-Fixation in Cyanobacterial Mats from Ponds on the McMurdo Ice Shelf, Antarctica

E. Fernández-Valiente,[1] A. Quesada,[1] C. Howard-Williams,[2] I. Hawes[2]

[1] Dpto. Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco 28049-Madrid, Spain
[2] National Institute of Water and Atmospheric Research, P.O. Box 8602, Christchurch, New Zealand

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ABSTRACT

We have investigated the ecological importance of N_2-fixation in cyanobacterial mats, dominated by oscillatorean species, in ponds of the Bratina Island area of the McMurdo Ice Shelf, Antarctica (78°S, 166°E). Nitrogenase activity, estimated as acetylene reducing activity (ARA), was found in all the mats investigated (n = 16). The average ARA was 75.9 μmol ethylene m^{-2} h^{-1}, ranging from 6 to 201 μmol ethylene m^{-2} h^{-1}. Nitrogenase activity was positively correlated with dissolved reactive phosphorus concentration in pondwater and the C/N ratio of the mat, and was negatively correlated with pondwater NH_4^+–N concentrations and natural abundance of ^{15}N in the mats. ARA was restricted to the upper, oxic layer of the mats. Experiments conducted to ascribe ARA to different groups of prokaryotes suggested that ARA was mainly conducted by heterocystous cyanobacteria, since no activity was found in the dark and the activity was inhibited by the photosystem II inhibitor DCMU (3-[3,4-dichlorophenyl]-1,1-dimethyl urea). In spite of 24 h of daylight, nitrogenase activity showed a diel cycle with maximum activity at midday (10–18 h) and minimal activity at early morning (6–10 h) when pond temperatures were at their minima. Light dependency of nitrogenase activity for three cyanobacterial communities showed that the irradiance required for saturating ARA was low, in every case lower than 100 μmol photon $m^{-2}s^{-1}$. Irradiance rarely fell below 100 μmol photon $m^{-2}s^{-1}$ during Antarctic summer days and ARA was likely to be light saturated for much of the time. We estimate that N_2 fixation represented on average a N input into the ponds of over 1 g $m^{-2}y^{-1}$. This value appears to be the highest N input to this Antarctic ecosystem.

Introduction

Microbial communities dominated by cyanobacteria are a major component of terrestrial and freshwater polar ecosystems. These benthic microbial mats have been reported from many aquatic habitats in both, maritime and continental Antarctica [4, 12, 21, 32]. The richest accumulations of cyanobacteria in the polar regions occur at the bottom of lakes, ponds, and streams where cyanobacteria form benthic mats up to several centimeters thick [43]. The benthic microbial

Correspondence to: E. Fernández-Valiente; Fax: 34-913978344; E-mail: eduardo.fernandez@uam.es

mats typically consist of a matrix of filamentous cyanobacteria, especially oscillatorians, together with inorganic material and other organisms including diatoms [22] and meiofauna such as nematodes, rotifers, and tardigrades [42]. Heterocystous N_2-fixing cyanobacteria particularly *Nostoc* and *Nodularia* species, are also present in most of the microbial mats, but usually do not represent a major proportion of the mat biomass.

One of the most extensive developments of cyanobacteria anywhere in the polar regions occurs over a 1500 km^2 area of the McMurdo Ice Shelf (78°S, 166°E) [22, 43]. This area contains a variety of small lakes and ponds of different chemical and biological characteristics, ranging from low to high conductivity [21, 22]. Most of these ponds are lined with cyanobacterial mats. Despite the variability of the chemical characteristics in the lakes and ponds of the McMurdo Ice Shelf ablation zone, most of the water bodies are considered to be N limited [16]. In fact, the ponds of the McMurdo Ice Shelf at Bratina Island have very low concentrations of dissolved inorganic nitrogen when compared with other waters across Southern Victoria Land [44]. Under these circumstances any N input in the ecosystem will be of significance for the biota.

Biological N_2-fixation is known to play an important role in the nitrogen budgets of some ecosystems in the polar regions, in both the Arctic [25, 26] and the Antarctic [6, 27, 29]. Most of the studies have been conducted either on the macroscopic colonies of the cyanobacterium *Nostoc* [7] or on cyanobacterial communities dominated by heterocystous N_2-fixing species and associated with other organisms such as mosses [6, 31, 39], lichens [27], and angiosperms [27]. N_2-fixation was found to be a small component of the nitrogen budget of the nutrient enriched ponds in the maritime Antarctic [8] and to provide 10% of nitrogen required to support benthic cyanobacterial mats in Antarctic glacial melt streams [20].

Within the microbial mats of the McMurdo Ice Shelf a variety of microenvironmental conditions have been recorded, from oxygen-supersaturated microzones to anoxic layers [46]. Under such conditions there are a number of prokaryotes adapted to each microenvironment that can potentially carry out N_2-fixation. The relative contribution of each group to whole mat N_2-fixation probably varies during diel and seasonal cycles [3]. Among cyanobacteria, all heterocystous species are capable of fixing dinitrogen under fully oxic conditions in the light. In addition, a large number of filamentous and unicellular nonheterocystous cyanobacteria are also capable of fixing dinitrogen under oxic conditions [40]. Most of these nonheterocystous cyanobacteria, including oscillatorians, confine their nitrogenase activity to periods of darkness. Thus, N_2-fixation in natural communities of cyanobacteria shows distinct spatial and temporal patterns, depending on the type of organism [40].

The aim of this work was to study the role of N_2-fixation in cyanobacterial mat communities of the McMurdo Ice Shelf. We first evaluated the range of nitrogenase activity found in microbial mats and *Nostoc* colonies from waterbodies with different chemical and physical characteristics. We then investigated the spatial distribution of N_2-fixation within the mats and the metabolic origin of energy and reductants for N_2-fixation to determine which groups of microorganisms were carrying out this activity. Finally, we measured diel cycles of nitrogenase activity and the effect of naturally varying irradiance on N_2-fixation.

Materials and Methods

The Study Site

All the experiments were conducted in January 1998 and in January 2000 on the McMurdo Ice Shelf ablation zone close to Bratina Island (78°00′S, 165°35′E). Lenses of mirabilite ($Na_2SO_4 \cdot 10H_2O$), seawater intrusions, and different pond ages provide a widely varied series of water chemistries in the ponds. We studied 16 ponds within a few hundred meters of each other of similar morphometry but widely differing water chemistry and microbial mat biomass. All ponds were colonized by dense mats of cyanobacteria with some diatoms and coccoid chlorophytes. Oscillatoriaceae accounted for 70% or more of the total cell counts in most ponds [22]. The pond names given in this paper are unofficial, but correspond to the names found elsewhere in the literature and are recorded on New Zealand Department of Survey and Land Information Map NHS:37/165 of 1991.

Air temperature and irradiance were recorded automatically every 10 min at a meteorological station on the Ice Shelf, situated no more than 2 km from any sampling site. The station was based on a Campbell CR-10 data logger system and used a Campbell Scientific 107 thermistor for air temperature measurements and a LiCor Li190 quantum sensor for irradiance. Additional Campbell 107 thermistors were located in waters at 30 cm depth in two ponds close to the station. During experiments on these and other ponds, water and sediment temperatures were recorded at the beginning and the end of each experiment with a thin (<2 mm) digital thermometer probe.

Water Analysis

Water samples for nutrient analysis were collected in acid-washed polyethylene bottles. All samples were filtered using Whatman

glass-fiber filters (grade GF/C) and the filtrate stored frozen prior to analysis. Filtrates were analyzed using a Technicon II autoanalyzer system for NO_3–N, NH_4^+–N, total dissolved N (TDN), dissolved reactive P (DRP), and total dissolved P (TDP). Methods are fully described by Downes [11].

Community Structure, Chlorophylls, Carbon, and Nitrogen Analysis

Samples from mats were taken with a metal corer (11 mm inner diameter) from homogeneous areas of the microbial mats. Live mat material was teased out and examined at the site by light and epifluorescence microscopy. The classification system of Anagnostidis and Komarek [2, 24] was used throughout. Chlorophyll *a* (Chl *a*) concentration in the microbial mats was measured following overnight extractions using 90% aqueous methanol. The extract was then filtered through a GF-F Whatman filter. Absorbance of the extract at 665 nm was measured in a JASCO spectrophotometer. Extraction was repeated on the same pellet using fresh solvent until no more pigment was extracted; usually between 3 and 5 extractions were necessary. Chl *a* concentration in the extracts was determined using the extinction coefficient of Marker et al. [28] and total concentration (μg cm^{-2}) by summing the concentration of all the extractions. Total C and N contents of subsamples of dried, and weighed cores were determined using an elemental analyzer (Perkin-Elmer 2400CHN) with a thermal conductivity detector. Natural abundance of ^{15}N was analyzed with an IRMS Micromass-Isochrom mass spectrometer.

N_2 Fixation

N_2-fixation of microbial mats was estimated *in situ* using the acetylene reducing activity (ARA) technique [41]. Cores from mats were incubated in triplicates either in 11 ml sterile plastic tubes (Vacuette) (one 11 mm diameter core per tube) or in 250 ml glass Erlenmeyer flasks (between 2 and 4 cores per flask) with 3 ml or 50 ml of lake water added to the plastic tubes or flasks, respectively. Controls comprised tubes or flasks with water but no mats. Special care was taken to place the cores with the surface layer facing up and to avoid overlapping. Erlenmeyer flasks were capped with reversible rubber stoppers that were gas-tight for at least 24 h, and 10% of the air was replaced by the same volume of chemically pure acetylene. Incubations were conducted in the surface waters of the ponds where cores were taken, for 4 h. When ARA was measured in plastic tubes, the incubation was terminated by injecting 1 ml formaldehyde (4% final concentration) and the samples maintained in cold and darkness until the ethylene could be determined. When the incubation was undertaken in Erlenmeyer flasks samples of the gas mixture at initial and final incubation times were taken in preevacuated Vacuette tubes using a double needle. The gases typically found in the samples remained unchanged in those tubes for at least 40 days. Ethylene resulting from the reducing activity was measured, in triplicate for each sample, using gas chromatography (Shimadzu model GC-8A), with flame ionization detection and a Porapak N column. Small amounts of ethylene generation were measured in some experimental controls without acetylene, but this always was lower than the experimental detection limit (about 2 μmol ethylene m^{-2} h^{-1}).

ARA in Nostoc *Colony Types*

Nitrogen fixation associated with three different morphologies of macroscopic *Nostoc* colonies was studied: spherical brown colonies growing on the microbial mat lining the ponds, foliaceous deep blue-green colonies growing vertically attached to the lake bottom and some times floating, and thick-walled, cylindrical hollow colonies. Thick, cylindrical hollow colonies were made up of a mixture of *Nostoc* sp. and *Phormidium* sp., whereas the spherical and thin flattened colonies comprised almost exclusively *Nostoc. Nostoc* colonies could not be studied in the same way as other mats. Instead, colonies of the three morphologies were collected and each type was divided into three aliquots. As the size of the spherical colonies ranged between 2 and 12 mm, all colonies were sorted out by size, ensuring that each replicate had the same colony size spectrum. Every replicate contained between 26 and 32 colonies. Several thin flattened colonies 7–8 cm long were detached from the bottom of ponds and were cut in 2 cm lengths, all mixed in a tray, and distributed in three aliquots at random. Finally, a single thick flattened cylindrical colony of around 4 cm diameter, 8 cm long, was detached from the sediment and sliced into three similar-sized sections. ARA was measured in Erlenmeyer flasks and incubated in situ for 4 h. The biomass used in the experiment was measured as fresh weight and separated into two identical aliquots. One aliquot was used for pigment extraction and the other to measure the dry weight by drying to stable weight at 60°C and ash-free dry weight by combustion at 475°C for 12 h and subtracting ash weight from the previous one.

Vertical Distribution of ARA

The vertical distribution of nitrogen fixation within the mats was studied on communities from two different ponds, JA and P-70. Cores were cut horizontally with a scalpel blade to section them in two layers that differed in color and coincided with the upper oxic and lower anoxic zones. The upper layer (ca. 3 mm thick) was defined by an orange-green color and the layer below this was defined by a black color in both mats. Sectioned samples were incubated for ARA in Erlenmeyer flasks in triplicates.

In situ profiles of oxygen were measured in these mats with a Clark-type microelectrode attached to a micromanipulator.

Metabolic Requirements of ARA

Metabolic requirements of the mat ARA were studied *in situ* in Erlenmeyer flasks in three different pond communities (JA Pond, P-70 Pond, and Casten Pond). Before the acetylene was added to the flasks, the samples were kept in darkness at ambient water temperature for 1 h, to reduce the energy storage of cells. Autotrophic metabolism was measured under full surface light, and

heterotrophic metabolism was investigated under dark conditions, by observing the stimulation of ARA from fructose and glucose additions (5 mM of each, final concentration). Mixotrophic metabolism was also investigated by adding the sugars in the light. In order to distinguish whether heterocystous or nonheterocystous cyanobacteria were the main contributors to nitrogen fixation, 5 μM (final concentration) DCMU (3-[3,4-dichlorophenyl]-1,1-dimethyl urea), an inhibitor of photosystem II, was added. In the presence of DCMU, oxygen concentration within cells will decrease. Under these conditions, if ARA depends mostly on nonheterocystous cyanobacteria, the value will be maintained or increased; if heterocystous cyanobacteria are involved, a decrease in ARA is expected [23]. It should be noted that the results obtained using these procedures are directly related to cellular metabolic requirements at the time of the experiment and not to the storage capabilities of the communities.

Light Dependence of ARA

Experiments to study the light dependence of ARA were conducted in the same way as the metabolic experiments for nitrogenase activity. A light gradient was obtained with a log series of plastic neutral density screens, with transmissions of 100, 50.5, 29.1, 16, 5.8, 3, and 0% of the surface irradiance. During the experimental period the surface irradiance ranged between 530 and 570 μmol photon m^{-2} s^{-1} for Casten Pond, 500 and 700 μmol photon m^{-2} s^{-1} for JA Pond, and between 725 and 1210 μmol photon m^{-2} s^{-1} for P-70 Pond. The data from each community were fitted to a quadratic hyperbola [18], defining ARA_{max} as the maximum activity, α as the initial slope of the nonsaturated part of the curve, and E_k as the irradiance from which ARA is saturated ($E_k = ARA_{max}/\alpha$).

Diel Cycles in ARA

Daily variation of nitrogen fixation was studied in January 2000 with a semi-*in situ* methodology in 300 ml tissue culture flasks. Three cores each of 1.33 cm^2 from different microbial communities were placed in each flask with 50 ml of pond water. Four replicates were allocated to each lake. To avoid acetylene inhibition, assays were conducted in successive incubation periods of 4–6 h, since longer incubations have been found to be potentially toxic for cyanobacteria [9]. Different cores were used for each incubation.

Statistics

Where appropriate, ANOVA with Tukey post-hoc testing was used to compare replicated measurements. Where data did not conform to assumptions required for parametric testing, nonparametric tests were used. Statistica for Windows 2.0 (Statsoft Inc., 1999) or SygmaStat 2.0 (SSPS, Inc., 1997) were used for all statistical procedures.

Results

Screening of Ponds

The 13 study ponds all showed significant ARA (Table 1). The 13 ponds represented the widest possible range of pond types on the McMurdo Ice Shelf in terms of two orders of magnitude variation in conductivity, dissolved organic nitrogen, and dissolved phosphorus (Table 1). Dissolved inorganic nitrogen was less variable than the other parameters. Ammonium showed higher concentrations than nitrate in all ponds, though dissolved organic nitrogen always exceeded both. The highest NH_4^+–N and NO_3^-–N concentrations were found in the most saline ponds, Salt Pond and Brack Pond. The dissolved inorganic N:P ratios were extremely low in all ponds.

Particulate C in cyanobacterial mats ranged over an order of magnitude from 19.7 to 159.5 mg g^{-1}, with an average value of 66 mg g^{-1} (Table 1). Particulate N ranged from 1.7 to 22.5 mg g^{-1}, with an average value of 7.7 mg g^{-1}. The highest C and N concentrations were recorded in Russell Pond. The C:N ratio varied by a factor of 2 from 6.6 to 15.7 among ponds, with an average value of 9.9. Natural abundance of ^{15}N showed two extreme values of 8.4 in Orange Pond and −0.8 in Russell Pond; in the other ponds $\delta\ ^{15}N$ ranged from 1.0 to 3.8. Benthic mat Chl *a* concentrations ranged from 3.5 to 52.5 μg cm^{-2}, with an average value of 25.5 μg cm^{-2}.

The main constituents of the microbial mats were nonheterocystous filamentous cyanobacteria, tentatively identified as species of *Phormidium, Oscillatoria, Lyngbya,* and *Leptolyngbya.* The heterocystous cyananobacterial genera *Anabaena, Nodularia,* and *Nostoc* were also present in most of the microbial mats investigated and ranged from rare to subdominant, but never represented a major proportion of the biomass.

ARA per unit of surface area ranged from 6 to 201 μmol ethylene m^{-2} h^{-1} across the ponds (Table 1). ARA exceeded the 2 μmol m^{-2} h^{-1} detection limit in microbial mat cores from all the water bodies investigated, except for a single core sample from Orange Pond. Standard deviations ranged from 22 to 135% of the mean. A one-way analysis of variance showed that variance in ARA between ponds was significantly ($P = 0.002$) greater than within-pond variability. The mean ARA over all the mats was 75.92 μmol ethylene m^{-2} h^{-1} (standard deviation 88.9). ARA values expressed on a Chl *a* basis ranged from 52 nmol ethylene mg Chl a^{-1} h^{-1} in Orange Pond to 1389 nmol ethylene mg Chl a^{-1} h^{-1} in Casten Pond. The mean of the activity was 445 nmol ethylene mg Chl a^{-1} h^{-1}.

Determination of the chemical characteristics that explain the most variability in ARA was carried out by multiple regression analysis (Table 2). When ARA was normalized to chlorophyll *a,* the regression explained 56% of total variance.

Table 1. Acetylene reducing activity (ARA), and some chemical characteristics from waters and from benthic mats in the studied ponds

Pond	Water characteristics							Mat				ARA/chl (nmol C_2H_4 mg Chl^{-1} h^{-1})	ARA/surface (μmol C_2H_4 m^{-2} h^{-1})
	Conductivity (μS cm^{-2})	DRP (μg L^{-1})	DOP (μgL^{-1})	NH_4^+–N (μg L^{-1})	NO_3^-–N (μg L^{-1})	DON (μg L^{-1})	DIN/DRP	C (mg g^{-1})	N (mg g^{-1})	C/N	$\delta^{15}N$ (‰)	Chl (μg cm^{-2}	
Brack	15,380	85.8	288.0	28.5	3.3	4,984	0.37	96.8	10.3	9.4	1.2	52.6	154	81
Casten	565	145.0	20.5	9.1	1.5	618	0.07	65.8	9.5	6.9	2.7	7.8	1,389	108
Conophyton	1,649	3.7	5.4	9.1	ND	540	2.46	70.2	10.1	7.02	3.0	45.7	90	41
Fresh	301	67.1	1.3	6.8	2.0	254	0.13	68.8	6.8	10.1	3.5	3.5	280	10
JA	1,197	2.6	2.4	7.7	ND	722	2.96	43.2	4.3	10.2	1.9	15.5	439	68
Login	1,456	—	—	—	—	—	—	43.9	4.4	10.0	1.3	34.7	256	89
Nostoc	1,693	24.6	9.5	8.8	ND	875	0.36	19.7	1.7	11.6	3.1	10.3	329	34
Pancreas	—	37.9	<1	13.9	ND	99	0.36	23.2	1.9	13.4	2.4	13.9	1,108	154
Orange	1,802	42.7	89.2	15.5	7.4	2042	0.54	74.3	7.1	10.5	8.4	11.5	52	6
P70	3,700	17.9	49.2	6.2	3.4	2,734	0.54	62.0	4.0	15.7	2.0	38.4	523	201
Russell	—	50.4	72.5	8.8	7.3	1,647	0.32	159.5	22.5	7.3	–0.8	44.5	165	71
Salt	56,200	120.8	729.6	26.2	8.4	14,500	0.29	—	—	—	1.0	42.7	91	39
Skua	407	131.7	19.0	8.2	1.9	553	0.08	63.8	9.7	6.6	3.8	11.1	749	83

ND = not detected

Table 2. Multiple linear regression between ARA and the chemical characteristics from water and from mats[a]

Variable	Acetylene activity (ARA) reducing	
	Chl basis	Surface basis
Conductivity	—	—
DRP	0.00577	—
DOP	—	—
NH_4^+–N	−0.0335	—
NO_3–N	—	—
DON	—	—
DIN/DRP	—	—
C	—	—
N	—	—
C/N	—	8.810
$\delta^{15}N$	—	−12.803
Constant	0.435	27.996
R^2adj	0.559	0.367
P	0.016	0.041

[a] The coefficient for each variable, constant, adjusted R^2 (R^2 adj), and *P* values are shown. The models were chosen following stepwise linear regressions.

Dissolved reactive phosphorus and ammonium dominated the multiple linear regression equation. ARA was positively correlated with DRP and negatively correlated with NH_4^+–N. When ARA was normalized to surface area, the regression model explained only 37% of total variance with C/N ratio and $\delta^{15}N$ as individually significant positive and negative parameters respectively.

ARA Associated with Nostoc *Colonies*

The three different morphologies of *Nostoc* had different structural composition and ARA activities. Thick cylindrical colonies had the highest percentage of dry weight (Table 3). Most of this dry weight was inorganic sedimentary material, as indicated by the low percentage of ash-free dry weight and the low Chl *a,* C, and N contents. In contrast, spherical colonies showed the lowest dry weight fraction, the highest ash-free dry weight fraction, and the highest content of Chl *a,* C, and N. The thin flattened colonies showed intermediate values. When values of Chl *a,* C, and N were normalized to ash-free dry weight, there were no differences in C and N contents among the three morphologies (39–49 mg C g $AFDW^{-1}$ and 4.5–4.9 mg N g $AFDW^{-1}$). However, the concentration of chlorophyll *a* was significantly higher in spherical colonies than in the other two morphologies (1.99 versus 0.86 and 0.74 μg Chl mg $AFDW^{-1}$) ($P < 0.001$).

Table 3. Dry weight (DW), ash free dry weight (AFDW), chlorophyll *a*, C and N contents, and natural abundance of ^{15}N in three different morphologies of *Nostoc* colonies[a]

Colony morphology	DW (% of fresh wt)	AFDW (% of dry wt)	Chl *a* (μg mg dry wt^{-1})	C (mg g dry wt^{-1})	N (mg. g dry wt^{-1})	$\delta^{15}N$
Thick cylindrical	11.7 ± 2.3	26.5 ± 6.0	0.23 ± 0.02	13.0	1.2	−0.5
Thin flattened	6.5 ± 1.3	63.4 ± 6.5	0.47 ± 0.2	25.0	3.0	−1.2
Spherical	1.2 ± 0.04	92.0 ± 1.8	1.83 ± 0.1	40.5	4.5	−1.3

[a] Data are the mean of three replicates ± SD.

Thick cylindrical colonies had the lowest nitrogenase activity (Table 4), expressed on both a Chl *a* and a dry weight basis. ARA expressed per dry weight was higher in the spherical colonies than in the thin flattened morphology, although the contrary was found when expressed on a Chl *a* basis because of the higher Chl *a* content of the spherical colonies.

Vertical Distribution of ARA within the Mats

Distribution of ARA was examined in two ponds, JA Pond and P-70 Pond. The mat community found in JA Pond formed a thicker (about 10 mm thick), but looser mat than in P-70 Pond. The matrix in JA Pond was formed by a narrow (1–2 μm diameter) band of Oscillatoriaceae species accompanied by *Anabaena* and *Nodularia.* Small (up to 1–2 mm diameter) *Nostoc* colonies were unevenly distributed within the microbial mat. The community in Pond P-70 was dominated by 2–3 μm diameter oscillatoriacean cyanobacteria, although a 4–5 μm thick oscillatoriacean was also abundant, and *Anabaena. Nodularia,* and *Nostoc* colonies were also present. The P-70 Pond community formed conical structures similar in appearance to the Precambrian "Conophyton," and the mat structure was 5–6 mm thick.

Oxygen profiles through the two pond mats were different (Fig. 1). In JA Pond mat the oxygen concentration during the experiments was below saturation even near the surface, and declined progressively with increasing depth until it became anoxic at about 9 mm depth. In Pond P-70 the oxygen profile showed a layer of supersaturation (110% of air-equilibrium values) at ca. 2 mm depth. The concentration then fell sharply with increasing depth, becoming almost anoxic at about 5–6 mm depth.

ARA was mainly distributed in the upper 3 mm layer of both mat communities (Table 5). In JA Pond, ARA in the upper layer represented 94.2% of the total activity. In P-70 Pond ARA was confined exclusively to the surface layer. Chl *a* was also mostly found in the upper layer in both communities, reaching the 82.8% and 60.71% of the total Chl *a* in the mat profile in JA Pond and P-70 Pond, respectively. These results suggested that nitrogenase activity was closely associated with the photosynthetic layer of both mats.

Metabolic Requirements of ARA

These experiments were undertaken in three ponds, JA and P-70 Ponds described above and Casten Pond. The microbial mat in Casten Pond contrasted slightly from those in JA Pond and P-70 Pond. It consisted of a thin (2–3 mm thick) mat with a matrix of narrow (1 μm diameter) cyanobacteria tentatively classified as *Lyngbya,* accompanied by filaments of *Nodularia, Anabaena,* and thicker Oscillatoriaceae such as *Phormidium* and *Oscillatoria* [36].

In dark conditions ARA was found to be very low (Table 6), and in most cases values remained below the detection limit. Fructose and glucose addition did not stimulate dark ARA in any of the communities. The addition of DCMU in assays performed in the light triggered significant reductions in ARA of between 32% in Casten Pond and 45% in P-70 Pond, both with or without sugars (ANOVA $p < 0.001$ in JA Pond and P-70 Pond and $p < 0.011$ in Casten Pond). No significant ARA activity was found that could be associated with mixotrophic metabolism.

Light Dependence of ARA

With the above results confirming that ARA was a photodependent process in the mats, the relationship between ir-

Table 4. Acetylene-reducing activity (ARA) in three different morphologies of *Nostoc* colonies[a]

Colony	ARA/DW (nmol C_2H_4 g dry wt^{-1} h^{-1})	ARA/AFDW (nmol C_2H_4 g ash-free dry wt^{-1} h^{-1})	ARA (nmol C_2H_4 μg Chl^{-1} h^{-1})
Thick cylindrical	60.2 ± 60.8	209.6 ± 189	0.26 ± 0.2
Thin flattened	1637.8 ± 332	2593.2 ± 475	3.48 ± 1.9
Spherical	2006.9 ± 120	2178.4 ± 239	1.11 ± 0.1

[a] Data are the means of three experiments ± SD.

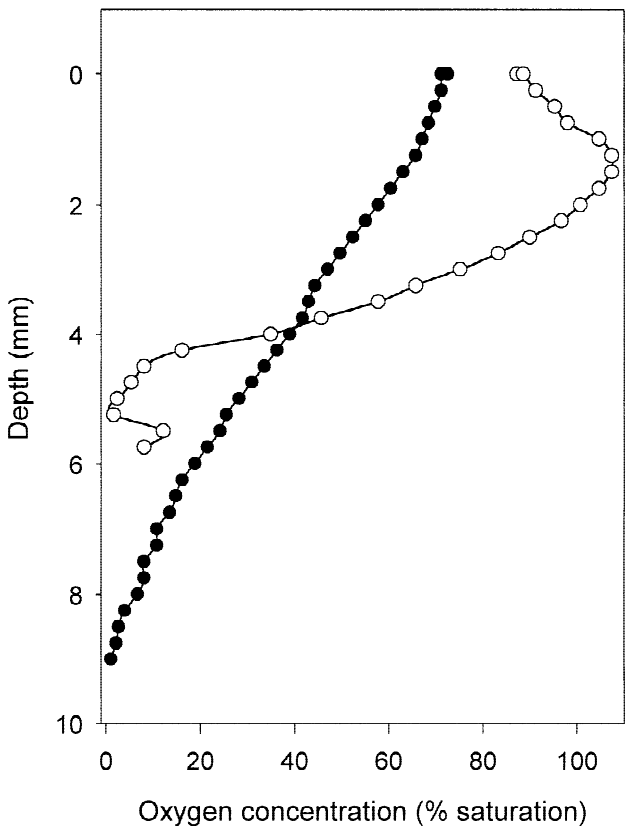

Fig. 1. Vertical profiles of oxygen concentration (expressed as % air equilibrium at 5°C) in two cyanobacterial mats, under clear sky conditions on 22 January 1998. ●JA Pond, ○P-70 Pond.

radiance and ARA was investigated in the same three mat communities. Data shown in Fig. 2 demonstrate that ARA was saturated under relatively low environmental irradiance for a shallow water benthic mat community. The community from Casten Pond showed a fivefold steeper slope of saturation [α = 1.95 μmol ethylene m^{-2} h^{-1} (μmol photon m^{-2} $s^{-1})^{-1}$] than the community from JA Pond [α = 0.39 μmol ethylene m^{-2} h^{-1} (μmol photon m^{-2} $s^{-1})^{-1}$]. In both cases irradiance at saturation (E_k) was below 100 μmol photon m^{-2} s^{-1}, being 28 μmol photon m^{-2} s^{-1} in Casten Pond and 97 μmol photon m^{-2} s^{-1} in JA Pond. The models utilized for the two previous communities significantly described the ARA versus irradiance curves ($p < 0.001$). Data from the Pond P-70 community showed high variability (standard deviation ca. 75% of the mean) and ARA was saturated at the first experimental irradiance utilized (36 μmol photon m^{-2} s^{-1}) (data not shown).

Table 5. Distribution of acetylene reducing activity (ARA) within the mat structure[a]

Layer	P-70 Pond	JA Pond
Whole mat	27.9 ± 16	37.6 ± 26
Surface layer	25.3 ± 7	42.6 ± 19
Bottom layer	0.0 ± 0	2.6 ± 2

[a] Data are expressed as μmol ethylene m^{-2} h^{-1}. Mean values of three experiments ± SD.

Table 6. Effect of DCMU and sugars on acetylene reducing activity (ARA) assayed under light and dark conditions

Incubation	Additions	JA Pond	P-70 Pond	Casten Pond
Dark	None	0	0	2.4 ± 3^{a}
Dark	Sugars*	0	0	3.7 ± 3^{a}
Light	None	71.8 ± 22^{a}	29.3 ± 1^{a}	82.7 ± 15^{b}
Light	DCMU†	43.5 ± 7^{b}	16.0 ± 2^{b}	56.5 ± 8^{c}
Light	Sugars*	83.8 ± 16^{a}	28.0 ± 7^{a}	64.2 ± 11^{bc}
Light	Sugars* + DCMU†	39.1 ± 9^{b}	14.5 ± 3^{b}	48.0 ± 24^{c}

Data are the means of three replicates ± SD. Units in μmol ethylene m^{-2} h^{-1}. Values in the same column followed by the same letter are not significantly different at 0.05% by Duncan's mean range test.
* 5 mM glucose + 5 mM fructose; †5 μM

Diel Cycles of N_2-Fixation

During the experimental period (January 1–25, 2000), solar irradiance at the automatic meteorological station remained above 100 μmol photon $m^{-2}s^{-1}$ for 93% of the total time and

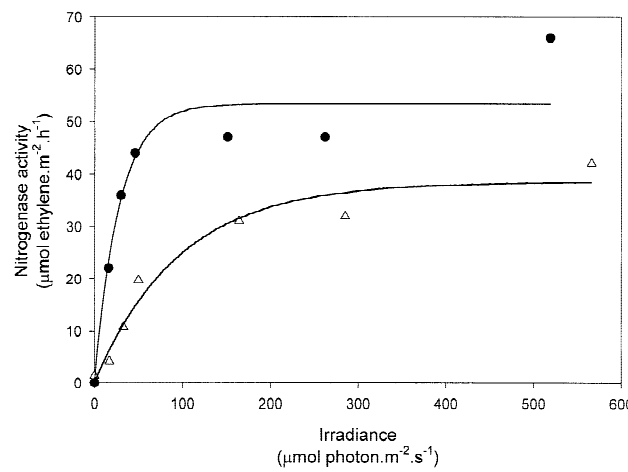

Fig. 2. Nitrogenase versus irradiance curves for cores of two cyanobacterial mats incubated under ambient light with neutral filters on 9–10 January 1998. ●Casten Pond; Δ JA Pond. Data are the means of three replicates. Standard deviation was less than 20% of the mean.

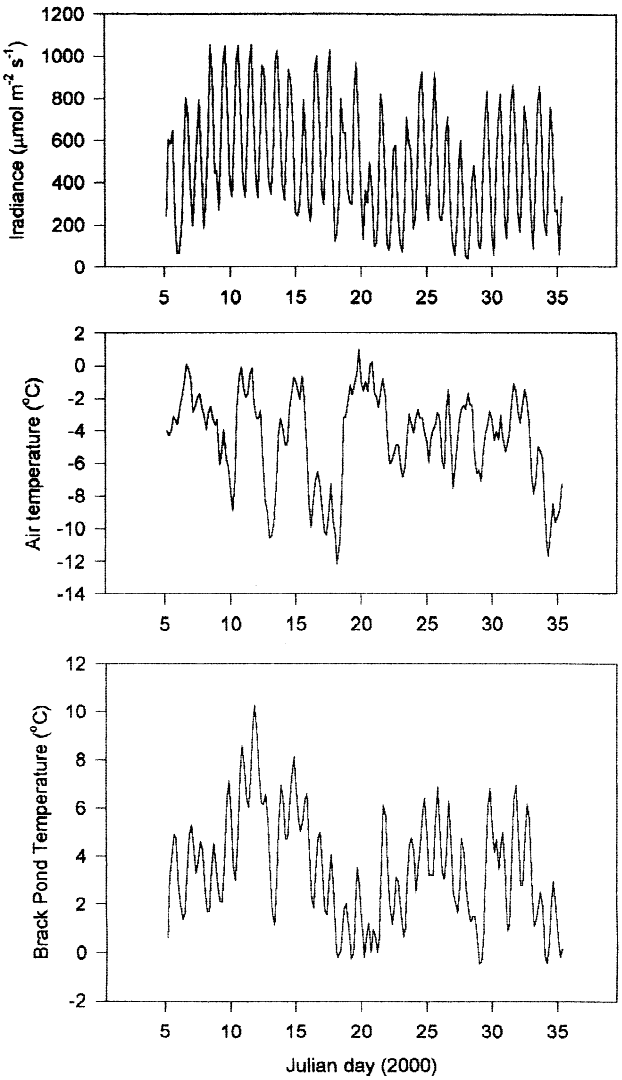

Fig. 3. Solar radiation, air temperature, and water temperature for Brack Pond over the study period.

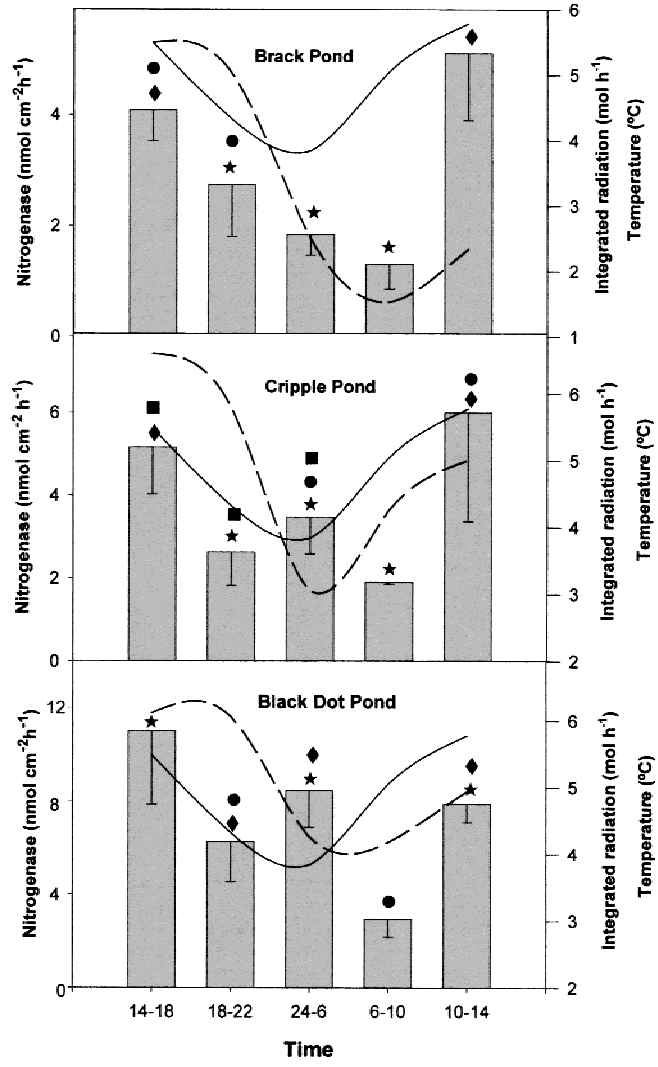

Fig. 4. Daily variation of nitrogenase activity (bars) in three cyanobacterial mats on 22–23 January 2000. The solid lines represent light variation during the day and the broken lines represent water temperature. Data are the means of four replicates. Bars with the same symbol are not significantly different at 0.05% by Duncan's mean range test.

was lower than 50 µmol photon m^{-2} s^{-1} for only 50 min (Fig. 3). During this time air temperature fluctuated between −12°C and 1°C, while pond surface temperatures showed a diel pattern overlain on a longer period oscillation. Brack Pond temperatures ranged from 0 to 10.5°C with typical daily fluctuations of 4°C. Minimum daily pond temperatures were typically at 6:00 AM.

Daily variation of nitrogenase activity in three different communities (Brack Pond, Cripple Pond, and Black Dot Pond) (Fig. 4) indicated that the activity was higher at the hours of maximal light intensity. The highest activity was reached between 10 and 14 h in Brack Pond and Cripple Pond, and between 14 and 18 h in Black Dot Pond. Nitrogenase activity fell during the period of lowest solar radiation (18–6 h), and reached its minimum values at early morning (6–10 h) when pond temperature was minimal and solar radiation was rising. Statistical analysis (ANOVA and *t*-test) indicated that the differences between the day time intervals (10–14 h and 14–18 h) were not significant. Similarly, differences between the two periods of lowest solar radiation (18–22 and 24–6 h) were not significant. However, there were significant differences among the daytime intervals (10–18 h), the 18–22 h interval, and the early morning (6–10 h) interval ($P < 0.001$).

Discussion

N_2-fixation has been described in both arctic [25] and antarctic [27] polar ecosystems and has been found to be a significant input of N. In most cases N_2-fixation has been described in cyanobacterial communities dominated by *Nostoc* (e.g., [5]) or associated with other organisms such as mosses (e.g., [6, 29]) or lichens, although in Victoria Land N_2-fixation has been described from microbial mats [1, 20]. Our results indicate that N_2-fixation is a common process in microbial mats in antarctic ponds, appearing in all the communities assayed from ponds with a wide range of chemical characteristics.

Water bodies in Bratina Island have been described as N limited [16, 21] with low concentrations of dissolved inorganic N (DIN) and high concentrations of dissolved reactive phosphorus (DRP), leading to very low DIN/DRP ratios. N_2-fixation in glacial melt stream communities in the nearby Dry Valleys was positively correlated with DRP. The results shown in Table 1 for the 1998 sampling season agree with these previous observations. Under these conditions of low DIN and high DRP, N_2-fixation would be an advantage [38]. Multiple regression (Table 2) with ARA normalized to chlorophyll *a* showed a positive correlation between nitrogen fixation and DRP, and a negative correlation between nitrogen fixation and NH_4^+–N, which is the main component of DIN (Table 1) in the ice shelf ponds studied. Similar correlations were reported in cyanobacterial Arctic communities [5] and are well known in temperate systems [13]

The average biomass of benthic mats, expressed as chlorophyll per unit of area, was in the range of that previously reported from the same ponds [21]. Biomass was highest in the two most saline ponds, a fact that agrees with previous observations in several temperate water bodies that showed a positive correlation between conductivity and the number of cyanobacteria (as colony forming units) [34] up to conduct of working of up to 1500 μScm^{-1}. Multiple regression analysis (Table 2) showed a positive correlation between nitrogen fixation per unit area and the C/N ratio. A similar correlation was reported in free-living cyanobacteria from the high Arctic [26], suggesting N-limiting conditions in communities with a high C/N ratio.

Natural abundance of ^{15}N has been used to identify N_2-fixing organisms and to estimate the fractional contribution of atmospheric N_2 to N_2-fixing organisms in terrestrial and aquatic systems [14, 19]. These differences in $\delta^{15}N$ between N_2-fixing plants and non fixing plants are due to the higher $\delta^{15}N$ of soil nitrogen (terrestrial systems) or DIN (aquatic systems) as compared to atmospheric N_2 and to the isotope fractionation that occurs during N_2-fixation [10]. In arctic and subarctic lakes, the $\delta^{15}N$ of N_2-fixing cyanobacteria was shown to be significantly lower than the $\delta^{15}N$ of nonfixing green algae and that 58–75% of the total nitrogen of a bloom of *Anabaena flos-aquae* was obtained by N_2 fixation [14]. The negative correlation between nitrogen fixation and $\delta^{15}N$ in the ice-shelf ponds (Table 2) is consistent with the literature and indicates a different fractional contribution of atmospheric-derived nitrogen to nitrogen in the different mats. The observation thus supports the view that N_2-fixation is a significant source of nitrogen within the mats of the McMurdo Ice Shelf.

The presence of *Nostoc* colonies with different morphologies has been reported both in arctic [25] and antarctic [7] aquatic ecosystems. In the ponds of the McMurdo Ice Shelf we found three different morphologies of *Nostoc* colonies that differed in ash and chlorophyll contents and nitrogenase activity. As has been reported in the Arctic, thin flattened colonies showed the highest rates of nitrogen fixation when this activity was normalized to chlorophyll or to ash-free dry weight, although the differences with spherical colonies were not significant. Values of nitrogenase activity in these *Nostoc* colonies were higher than those reported for other locations in Antarctica [6, 7] and are in the range of those reported in the Arctic [25]

Benthic cyanobacterial mat communities are laminated systems of vertically stratified phototrophic, chemotrophic, and heterotrophic microorganisms that support most of the major biogeochemical cycles within a vertical scale of a few millimeters [30]. Cyanobacterial mats in the McMurdo ice shelf ponds have a similar layered structural composition to mats from more temperate zones [17, 45]. Usually cyanobacterial mats are dominated by filamentous nonheterocystous strains and, in these cases, nitrogen fixation is limited to the deeper anoxic layers of the mat, and at night, when O_2 concentrations are lowered by aerobic respiration and chemical oxygen demand [30, 40]. However, the 24-h light conditions and low temperatures experienced by antarctic mats during the summer provide a very different metabolic environment to temperate ponds and raised the question as to what organisms were responsible for the observed N_2 fixation rates (6–201 μmol ethylene $m^{-2}h^{-1}$), similar in magnitude to the average range found in temperate ecosystems (e.g., 12.9–108.7 μmol ethylene $m^{-2}h^{-1}$ in temperate rice fields [35]).

This study has shown that N_2-fixation was essentially restricted to the upper oxic layer of the ice-shelf pond mats

(Figure 1, Table 5), where most chlorophyll was found, suggesting a major role for autotrophic cyanobacteria. Furthermore, the experiments conducted to determine the metabolic pathways related to N_2-fixation (Table 6) clearly indicated that photoautotrophic organisms were mainly responsible for N_2-fixation, since no activity was observed under dark conditions. The photosystem II inhibitor, DCMU, results in a decrease in the oxygen concentration in cells and helps in distinguishing N_2-fixers with oxygen protective structures (e.g., heterocysts) from those lacking such structures [23]. DCMU inhibits nitrogen fixation in heterocystous cyanobacteria [33] and results in increased nitrogen fixation in nonheterocystous cyanobacteria [3, 40]. The DCMU-induced reduction of N_2-fixation (Table 6) indicates that this activity was relatively independent of O_2 concentration since O_2 concentrations within the mats decline rapidly in the absence of photosynthesis [17]. Thus, most of the ARA was apparently due to heterocystous cyanobacteria. However, in these mats Oscillatoreaceae accounted for 70% or more of the total cell counts and were associated with diatoms and coccoid chlorophytes. Heterocystous strains of cyanobacteria were found to comprise ca. 10% or less of total cell counts [21, 46]. This suggests a different behavior in oscillatorean mats from Antarctica relative to those from other latitudes.

ARA versus irradiance curves showed saturation irradiances for autotrophic N_2-fixation of less than 100 μmol photon m^{-2} s^{-1}, similar to those for N_2-fixation in glacial meltwater in stream habitats in Antarctica [20] and in the Arctic [5]. Similar E_k values for photosynthesis are characteristic of Antarctic cyanobacterial mats [15]. An E_k of <100 μmol photon is lower than the daily minimum irradiance typically found in the Antarctic summer. The absolute minimum irradiance during the first 25 days in January 2000 was 38 μmol photon m^{-2} s^{-1}, which only occurred for 10 min, and during the 25-day experimental period only 42 h were recorded where irradiance was lower than 100 μmol photon m^{-2} s^{-1} (Fig. 3). These data suggest that during the summer period light is not limiting for photodependent N_2-fixation on the McMurdo Ice shelf. However, despite this, we recorded a diel variation of nitrogen fixation (Fig. 4) with three marked periods. The highest rate was observed at midday (1000–1800 h), with a decline in activity between 1800 h and 0600 h, to the lowest values in early morning between 0600 and 1000 h. This diel variation at light-saturating conditions implies that N_2-fixation is influenced by factors other than light. Temperature has been shown to influence N_2-fixation in antarctic communities of cyanobacteria [7], and in our study temperature each day were at their minimum at 6:00 A.M.

Assuming an ethylene: N_2 conversion factor of 4 [37], the average ARA is equivalent to 12.75 mg N m^{-2} d^{-1} in the McMurdo Ice Shelf ponds. With 85 days of 24 h light at the same rate, this converts to 1083 mg N m^{-2} y^{-1}. If we consider that irradiance is higher than 100 μmol photon m^{-2} s^{-1} for at least 12 h per day during at least 73 more days [17], the input of N would increase to 1500 mg N m^{-2} y^{-1}. This value represents an important input of N into the pond ecosystem, considering that the standing stock in these communities ranges between 1 and 10 g N m^{-2}. Comparisons with other polar ecosystems are difficult since in most cases N_2-fixation estimates in the literature refer to discrete communities of *Nostoc* unevenly distributed over the surface, and not to cyanobacterial communities in widespread microbial mats. Comparisons with similar communities from other latitudes [40] show that the average daily values from Bratina Island ponds are in the same order of magnitude. However, hourly rates were lower in Antarctic microbial mats than maximum rates found in cyanobacterial communities from temperate climates. This lower maximum activity is almost certainly related to the lower temperatures of polar habitats, although some type of nutrient limitation can not be discarded. Howard-Williams et al. [20] recorded a Q_{10} for N_2-fixation in Antarctic melt-stream cyanobacterial mats of 2.4, and the corresponding Q_{10} for photosynthesis was 1.6 [15]. However, N availability could be similar during the active growing period because of the 24 h of activity of photodependent N_2-fixation during polar summers.

Estimates of the N requirement of pond benthic communities are approximately 3 g m^{-2} y^{-1} [16]. N input from N_2-fixation estimated in our study could satisfy one-third of this demand and is the most important source of new N to this ecosystem. Nitrogen from precipitation has been estimated to be much lower (ca. 36 mg N m^{-2} y^{-1} [16]). Animal depositions are an important N input in some polar ecosystems, but they are unlikely to play an important role in the microbial communities of the McMurdo Ice Shelf since vertebrates are rarely seen in the area. The remainder of the N demand of the microbial communities in the McMurdo Ice Shelf ponds is likely to be satisfied from internal recycling.

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