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Released coral mucus does not enhance planktonic N₂ fixation rates

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ABSTRACT: Dinitrogen (N₂) fixation by prokaryotic microorganisms provides bioavailable nitrogen in oligotrophic marine ecosystems such as coral reefs. The widespread though largely unknown heterotrophic diazotrophs require dissolved organic matter (DOM) for their nutrition. In coral reef ecosystems, DOM-rich mucus released by corals potentially sustains heterotrophic diazotrophic growth and activity. In this study, we investigated the effect of coral mucus on planktonic N₂ fixation in *in situ* experiments over a seasonal cycle within a New Caledonian lagoon, as well as in *in vitro* experiments in which the effect of mucus was monitored for 72 h. During the field experiment, N₂ fixation rates ranged between 0.12 and 7.90 nmol N l⁻¹ d⁻¹. Despite the highest N₂ fixation being found after mucus release, no significant difference was measured between the seawater surrounding the coral before and after mucus release. Similarly, the addition of mucus during the *in vitro* experiment enhanced N₂ fixation rates 1.5-fold, but this increase was not significantly different from the control. The abundance of 2 dominant populations of diazotrophs associated with corals and their surrounding seawater environments (unicellular cyanobacteria and rhizobia) found within pure mucus samples was on average 18-fold higher than in the surrounding seawater in the summer period and 400-fold higher in the winter. Our results suggest that although coral mucus does not influence planktonic N₂ fixation, the release of large numbers of diazotrophic cells associated with the mucus likely influences the abundance and diversity of diazotroph populations within the lagoon waters.

KEY WORDS: Diazotrophs · Coral mucus · Dissolved organic matter · Pelagic–benthic interactions

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INTRODUCTION

Coral reefs are among the most productive and diverse ecosystems on the planet (Sorokin 1993), despite their location in generally oligotrophic waters with low concentrations of nutrients such as bioavailable nitrogen (Furnas 1992). Nitrogen limitation in coral reefs is notably counteracted by dinitrogen (N₂) fixation (Wiebe et al. 1975), which takes place in a variety of reef compartments, including the substrata

(Wilkinson et al. 1984), algal turfs (Den Haan et al. 2014, Rix et al. 2015), microbial mats and seagrass meadows (Charpy et al. 2012, Cardini et al. 2014), coral skeletons (Larkum 1988, Davey et al. 2008), endosymbionts of hard and soft corals (Shashar et al. 1994, Lesser et al. 2004, 2007, Lema et al. 2014, Bednarz et al. 2015, Cardini et al. 2015, Rådecker et al. 2015), as well as in pelagic plankton communities (Biegala & Raimbault 2008, Riemann et al. 2010, Turk-Kubo et al. 2015). Among these sources, primary

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production in coral reef lagoons is likely sustained by benthic diazotrophs and particularly by cyanobacterial mats (Casareto et al. 2008). However planktonic diazotrophs also have a role, with previous work suggesting that their N_2 fixation activity could sustain high levels of planktonic primary production in a New Caledonian lagoon (Garcia et al. 2007).

Autotrophic diazotrophs such as filamentous and unicellular cyanobacteria (UCYN) were long thought to be the sole contributors to pelagic N_2 fixation (Capone et al. 1997, Moisander et al. 2010). However, recent studies have demonstrated that heterotrophic diazotrophs such as the non-photosynthetic UCYN-A, Archaea and other bacterial diazotrophs are more widespread and abundant on a global basis (Farnelid et al. 2011, Luo et al. 2012), and especially active in the South Pacific (Halm et al. 2012, Moisander et al. 2014). Yet, their contribution to global fixed N_2 inputs to the oceans and their ecology remain elusive. Non-autotrophic diazotrophs rely on external dissolved organic matter (DOM) inputs for their nutrition and growth, and therefore to understand their ecology their interactions with *in situ* organic matter pools needs to be investigated (Riemann et al. 2010).

The presence and activity of heterotrophic (i.e. DOM-dependent) diazotrophs in the pelagic compartment of coral reef ecosystems could be sustained by organic substances produced and released by the coral as mucus. Coral mucus is a carbohydrate complex rich in polysaccharides, lipids, triglycerides, proteins and other compounds all of which provide an adequate substrate for bacterial growth (Nakajima et al. 2009, Tanaka et al. 2011). While it is well known that the activity and abundance of heterotrophic planktonic bacteria respond to coral mucus release (e.g. Huettel et al. 2006, Allers et al. 2008), the relationship between coral mucus and planktonic diazotrophs remains unknown. Because heterotrophic diazotrophs are more abundant than autotrophic ones in the world's oceans (Halm et al. 2012, Turk-Kubo et al. 2015), their dependence on DOM and effect on nitrogen dynamics within coral reef ecosystems needs to be elucidated.

The objective of this study was to investigate the relationship between the activity of planktonic diazotrophs and the DOM released by corals into seawater in the form of mucus. With this aim we measured N_2 fixation activity in the waters above and around coral colonies, before and after mucus release within the New Caledonian lagoon and experimentally tested the effect of mucus additions on N_2 fixation *in vitro*. In addition, we quantified 2 groups of diazotrophs present in the seawater column and in pure mucus samples.

MATERIALS AND METHODS

Study area and sample collection

Seawater and coral mucus samples were collected within 2 contrasted reefs in New Caledonia (south-western tropical Pacific): an offshore station (L2) located 17 km off the coast on the barrier reef 'Recif Aboré' (22° 33.87' S, 166° 28.2' E), which is an environmentally protected area (Kulbicki et al. 1996); and a coastal station (D39) located near the mouth of the Dumbéa River at Pointe Bovis (22° 18.9' S, 166° 21.9' E), which presents less oligotrophic character due to river runoff (Jacquet et al. 2006). The sampling was performed on a monthly basis between February (austral summer) and July (winter) 2014 (see Table 1 below). The depth of the water column during sampling was 5 to 6 m.

At each station seawater was collected into twelve 4.3 l transparent polycarbonate bottles to measure planktonic N_2 fixation and diazotroph abundance. Seawater was collected using an air-compressed Teflon pump (Astii) connected to PVC tubing (1) from the surface (0.5 m depth), (2) from ~6 m above the coral colonies before stressing the corals, and (3) after the corals had been stressed and started releasing mucus. Mucus release was induced by applying mechanical stress with sand as described in Mitchell & Chet (1975). After the sand had settled onto the coral colony (~3 min), predominantly composed of *Acropora* sp. and once corals started releasing mucus, seawater containing mucus strands were collected by scuba divers using polycarbonate bottles as above. An additional set of samples included pure mucus samples, obtained by cutting 5 or 6 *Acropora* sp. branches (10 to 15 cm long), which were gently washed with sterile 0.2 μ m-filtered seawater and placed upside down to release the mucus. The mucus released over the first 30 s was discarded to avoid any contamination with coral surface bacterial communities (Naumann et al. 2009). We always used *Acropora* sp. for our experiments, as it is one of the 3 dominant hermatypic coral species in the New Caledonian lagoon (Adjeroud et al. 2010).

Hydrography and nutrient analysis

All seawater samples were collected in acid-washed 20 ml polyethylene vials and preserved with $HgCl_2$ (1% final concentration) for the analysis of nitrate plus nitrite (NO_3^-) and phosphate (PO_4^{3-}). All samples were kept in the dark at 4°C until analysis.

Table 1. Measured values of core parameters (temperature, salinity) and inorganic nutrient concentrations NO_x (nitrate plus nitrite) and PO₄³⁻ at 1 coastal station (Stn D39) and 1 offshore station (Stn L2) on coral reefs in the New Caledonian lagoon. Seawater samples were measured in surface water (0.5 m depth; Surface), in water above coral colonies (~6 m; Above coral), and water above the colonies after the corals had been stressed and started releasing mucus (~6 m; After stressing). n/a: data not available

Date (in 2014)	Temp. (°C)	Salinity	Sample	NO _x (μmol l ⁻¹)	PO ₄ ³⁻ (μmol l ⁻¹)
Stn D39					
17 Feb	27.03	34.54	Surface	0.60	0.04
			Above coral	0.43	0.09
			After stressing	0.57	0.03
03 Apr	26.07	34.85	Surface	0.70	0.04
			Above coral	0.37	0.03
			After stressing	0.40	0.19
30 Apr	25.97	35.06	Surface	0.34	0.02
			Above coral	0.58	0.04
			After stressing	0.49	0.51
21 May	23.34	n/a	Surface	0.51	0.04
			Above coral	0.50	0.04
			After stressing	0.40	0.11
16 Jul	22.15	n/a	Surface	0.07	0.01
			Above coral	0.14	0.00
			After stressing	0.19	0.02
Stn L2					
17 Feb	26.16	35.3	Surface	0.41	0.00
			Above coral	0.62	0.06
			After stressing	0.67	0.07
03 Apr	25.60	35.18	Surface	1.30	0.06
			Above coral	1.36	0.11
			After stressing	1.10	0.09
30 Apr	25.83	35.24	Surface	0.58	0.04
			Above coral	0.60	0.04
			After stressing	0.51	0.69
21 May	22.93	35.66	Surface	0.87	0.14
			Above coral	0.89	0.14
			After stressing	0.93	0.26
16 Jul	22.50	35.62	Surface	0.15	0.04
			Above coral	0.14	0.03
			After stressing	0.13	0.03

Nutrient concentrations were determined according to K  rouel & Aminot (1997) using a Bran+Luebbe III nutrient autoanalyzer. Temperature and salinity were measured using a SBE56 conductivity-temperature-depth (CTD) probe.

N₂ fixation rates

To measure planktonic N₂ fixation rates, seawater sampled before and after mucus release was collected in triplicate (n = 3) and amended with ¹⁵N₂-enriched seawater as described in Gro  kopf et al. (2012). To prepare the ¹⁵N₂-enriched seawater (from the same site where the corresponding sample was collected),

water was filtered through 0.2 μm and degassed for 1 h at a flow rate of 450 ml min⁻¹ using a degassing membrane (Membrana, Minimodule) connected to a vacuum pump (<200 mbar). The degassed seawater was transferred into a 4.5 l tedlar bag and 45 ml of ¹⁵N₂ (98.3 atom% ¹⁵N₂, Cambridge Isotope Laboratories) were injected. The bag was then struck with a wooden stick until the ¹⁵N₂ bubble was completely dissolved. ¹⁵N₂-enriched seawater was added to the incubation bottles at 5 % vol:vol (i.e. 225 ml), sealed without headspace using silicon septum screw caps. The bottles were incubated for 24 h under *in situ* simulated conditions in an incubator covered with neutral density screening and cooled with circulating water to reproduce irradiance and temperature conditions corresponding to the original sampling depth. Two extra 4.3 l bottles were filled with surface seawater and with the water closer to the corals, spiked with ¹⁵N₂ and immediately filtered to determine the natural ¹⁵N₂ enrichment of the particulate organic N (PON) at time zero (T₀), which is required for calculations of N₂ fixation rates (Montoya et al. 1996).

At the end of the incubation period, the bottles were filtered through pre-combusted (450°C, 5 h) 25 mm GF/F filters (Whatman), stored at -20°C and dried at 60°C for 24 h before analysis. The PON content and its ¹⁵N enrichment were measured on an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS, SERCON Integra CN). N₂ fixation rates were calculated as described in Montoya et al. (1996). Rates were considered significant when the ¹⁵N₂ enrichment of the PON was higher than 3 times the standard deviation obtained from T₀ samples.

Quantification of diazotrophs

UCYN and rhizobia were chosen to quantify diazotroph abundance in our seawater and mucus samples as these 2 groups are abundant in Southwest Pacific coral reef ecosystems (Biegala & Raimbault 2008, Lema et al. 2014). *Trichodesmium* and diatom-diazotroph associations are also abundant in the New Caledonian lagoon (Turk-Kubo et al. 2015) but were not targeted by our TSA-FISH (tyramide signal amplification fluorescent *in situ* hybridization) analyses as our objective was to study the effect of mucus on heterotrophic planktonic diazotrophs, and cyanobacterial

diazotrophs associated to diatoms are autotrophic. To quantify UCYN and rhizobia, the 4.3 l seawater samples were collected in April (summer) and July (winter) at each station under the 3 conditions of sampling described above. The samples were sequentially size-fractionated through 47 mm polycarbonate filters of 10, 3 and 0.2 μm (Millipore). For the 10 and 3 μm size fractions, 3.5 to 4 l and 2 to 2.5 l were filtered by gravity, respectively, while 200 to 500 ml were filtered under low vacuum pressure (200 mm Hg) for the 0.2 μm fraction. To quantify the abundance of UCYN and rhizobia within the mucus, 5 ml of pure mucus were diluted in 100 ml of 0.2 μm -filtered sterile seawater, and then size-fractionated as previously detailed. All filters were fixed with 1 % paraformaldehyde at room temperature for 15 min. Subsequently, the cells were dehydrated with 100 % ethanol for 10 min and stored at -80°C until analysis.

UCYN and rhizobia cells were quantified using the TSA-FISH method according to Biegala & Raimbault (2008), Bonnet et al. (2009), Ramm et al. (2012) and Agawin et al. (2014) with slight modifications. The cells were permeabilized with lysozyme (5 mg ml^{-1}) at 37°C for 1 h and achromopeptidase (60 U ml^{-1}) at 35°C for 30 min. To reduce the autofluorescence of cells and coral debris, a bath of H_2O_2 at 3 % (10 min) was added to the protocol. UCYN and rhizobia were respectively hybridized with probes Nitro821 (5'-CAA GCC ACA CCT AGT TTC-3') and Rhiz1244 (5'-TCG CTG CCC ACT GTC ACC-3'), both labeled with horseradish peroxidase. The general bacteria probe EU338mix (5'-GCW GCC WCC CGT AGG WGT-3') was also used as a positive control. All samples were also counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vectashield). The cells were counted under an epifluorescence microscope (Axioskop 2) equipped with a mercury lamp (HBO 100 W) and dichroic filters of 360 ± 20 excitation, 410 ± 5 emission for DAPI (blue fluorescence) and 480 ± 20 excitation, 535 ± 40 emission for fluorescein (green fluorescence). Due to the low abundance of diazotrophs, the entire surface of each filter portion was counted.

Mucus addition experiment

In addition to the measured N_2 fixation rates in the seawater surrounding coral colonies after being stressed (i.e. mucus released), we also tested the effect of coral mucus on N_2 fixation rates and planktonic community structure by adding 2 ml of pure mucus to 4 sets of triplicate 2.3 l bottles ($n = 12$) containing seawater from Stn L2. Four further sets ($n = 12$)

of triplicate 2.3 l bottles were also sampled and served as untreated controls. Two sets of triplicates were used as T_0 samples (initial conditions; $n = 6$), with 1 triplicate used as the control (no mucus addition; $n = 3$), and 1 triplicate containing mucus ($n = 3$). Control and treated bottles were incubated for 24, 48 and 72 h ($n = 6$ each). At T_0 and at each time point, triplicate bottles were subsampled in order to measure the evolution of inorganic nutrient concentrations (NO_x and PO_4^{3-}), N_2 fixation rates and picoplankton abundance (including bacteria, *Prochlorococcus*, *Synechococcus* and picoeukaryotes).

Picoplankton was quantified on 1.8 ml samples fixed with 2 % paraformaldehyde (final concentration) using a FACSCalibur flow cytometer (BD Biosciences). For the quantification of bacteria, the samples were stained with SYBR Green I (Invitrogen) at room temperature in the dark for 15 min. Fluorescent microspheres (Trucount and 2 μm beads, BD Biosciences) were added as an internal standard. The samples were run in medium mode for 1 min. Red versus green fluorescence, and green versus side scatter cytograms were used to gate the bacteria using the FlowJo software.

Statistical tests

The effect of the release of mucus on the N_2 fixation rate on the water column and the effect of sampling station were performed with an unpaired t -test (N_2 fixation rates were previously log transformed to make them normally distributed). For the mucus addition experiment, an unpaired t -test was also used to compare the variation of N_2 fixation rate in time, as well as the picoplankton abundance, between control and treated samples. These comparisons were done for each time point (0 h, 24 h, 48 h and 72 h) of the experimentation. All statistical analyses were performed with the GraphPad Prism 5 (GraphPad Software). Statistical significance was accepted at $p < 0.05$. All mean values measured between replicate sampling were expressed with their standard deviation.

RESULTS

Environmental parameters

Between the sampling period of February and July 2014, seawater temperatures decreased from ~ 27 to $\sim 22^{\circ}\text{C}$ at the coastal station D39, and from ~ 26 to $\sim 23^{\circ}\text{C}$ at the offshore station L2 (Table 1). Salinity

increased slightly from 34.5 to 35 at Stn D39 between February and April 2014 (data from May and July are not available), while it was stable throughout the whole sampling period at Stn L2 (~35.2 to 35.5) (Table 1).

NO_x concentrations were <1.5 µM and were, in most cases, lower at Stn D39 compared to Stn L2. Vertical differences throughout the water column were not observed (even when seawater included coral mucus), suggesting a high degree of mixing in these shallow waters as previously reported in the New Caledonian lagoon (Ouillon et al. 2010). Conversely, PO₄³⁻ concentrations showed contrasting values between different samples collected throughout the water column and from coral mucus. In most cases, they were higher at Stn D39 than at Stn L2. The PO₄³⁻ content in the seawater containing mucus doubled or tripled in samples from March, April and May, but such differences were not observed in February and July (Table 1).

N₂ fixation rates in field samples

Overall, the lowest rates of N₂ fixation were in surface waters and they were similar at both Stns D39 and L2 (ranging between 0.1 and 0.7 nmol N l⁻¹ d⁻¹, Fig. 1) except in early April when a peak of activity was observed at Stn D39 (>2.0 nmol N l⁻¹ d⁻¹), being 3.4-fold higher than that measured at Stn L2 (*t*-test,

p < 0.01). Although N₂ fixation rates found in the waters above coral (i.e. seawater without mucus) were up to 2.7-fold higher than those measured at the surface, no significant differences were observed between them (*p* > 0.05), except in April at Stn D39 (*p* < 0.001), when N₂ rates reached a maximum of 4.6 nmol N l⁻¹ d⁻¹.

The highest N₂ fixation rates were measured in seawater samples above the corals after mucus release (ranging between 0.3 and 1.5 nmol N l⁻¹ d⁻¹), being similar between the 2 stations. Nevertheless, again in early April, a peak of activity was observed, when N₂ fixation rates were 5.8-fold higher at Stn D39 than at Stn L2 (*t*-test, *p* < 0.01). However, only the N₂ fixation rates measured in February, at the beginning of April and in May (*p* < 0.05) were significantly higher after stressing the corals than those measured at the surface for the coastal station D39 (Fig. 1a). At the offshore station L2, only the rates measured in February for the water after stressing the corals were significantly higher (*p* < 0.01, Fig. 1b) than those measured in the surface water.

N₂ fixation rates found after stressing the corals were not significantly different (*t*-test, *p* > 0.05) than those measured in seawater above the corals not containing mucus except in February (*p* < 0.01) and then only at Stn D39 (Fig. 1a), where the N₂ fixation rates measured after stressing coral (i.e. mucus-affected) were 5.0-fold higher than those not containing mucus.

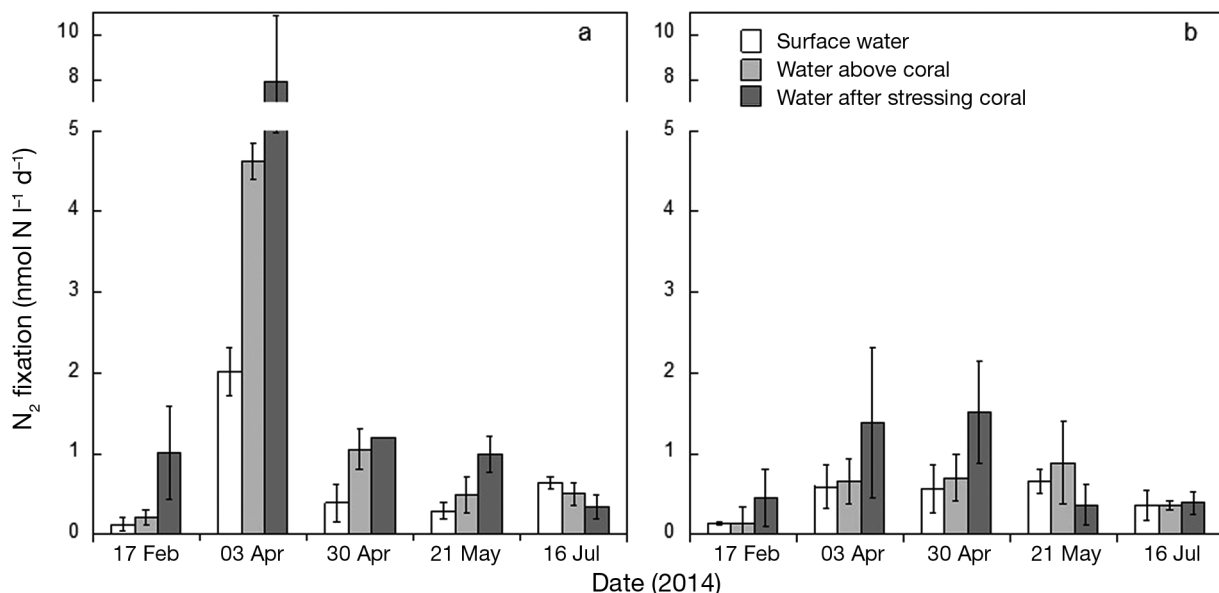


Fig. 1. Coral N₂ fixation rates at stations in the New Caledonian lagoon, measured in surface water (0.5 m depth), water above coral (~6 m), and water above the coral after stressing the corals: (a) coastal station D39; (b) offshore station L2. Error bars represent the standard deviation of the mean

Quantification of diazotrophs

The abundance of UCYN in the water column before and after the release of mucus for the summer (April) and winter (July) periods ranged from 0.5 to 13.9 cells ml⁻¹ and was generally higher at Stn D39 than at Stn L2, and in summer as compared to winter (Fig. 2a–f). UCYN were less abundant in surface waters (Fig. 2a,b) compared to waters above corals (Fig. 2c,d), and to waters above corals after stressing the corals, i.e. containing mucus (Fig. 2e,f). In mucus samples, UCYN abundances were on average 286- and 616-fold higher than in the water column at Stns D39 and L2, respectively (Fig. 2g,h), reaching a maximum of 719.8 cells ml⁻¹ (Fig. 2g,h). Cells from the picoplanktonic fraction (retained on 0.2 µm filters) dominated over nano- and microplanktonic cells (collected on 3 and 10 µm filters) in all cases (Fig. 2), averaging ~60% of the total UCYN community in summer and >90% in winter.

The abundances of rhizobia in the water column ranged from 1.5 to 125.8 cells ml⁻¹ and were ~0.5- to 12-fold higher than those of UCYN. Similarly to UCYN, the rhizobia were more abundant in the summer than in the winter (Fig. 3a–f), but the differences between Stns D39 and L2 were less pronounced than those observed for UCYN. The abundances of rhizobia were similar throughout the water column and were on average 3.5-fold higher after stressing the corals (Fig. 3e,f) than in waters not containing mucus (surface waters and waters above corals collected before mucus release), with greater abundance detected at Stn L2 than at Stn D39. As observed for UCYN, the highest abundance of rhizobia was detected in pure mucus samples at both stations, reaching 526.2 cells ml⁻¹. For both stations the abundance of rhizobia found in the pure mucus was 1.5-fold higher in winter compared to summer.

Mucus addition experiment

Mucus additions to natural seawater during a 72 h incubation period demonstrated varying degrees of enhanced N₂ fixation rates, which ranged from 0.14 ± 0.01 to 4.94 ± 0.93 nmol N l⁻¹ d⁻¹ for the water amended with coral mucus compared to 0.29 ± 0.05 to 2.76 ± 0.84 nmol N l⁻¹ d⁻¹ for the controls without mucus addition. Despite the addition of mucus leading to a peak of activity observed at 24 h, it did not result in a significant change with respect to the control (*t*-test, *p* > 0.05) over the 72 h of the experiment. The highest N₂ fixation rates were measured after

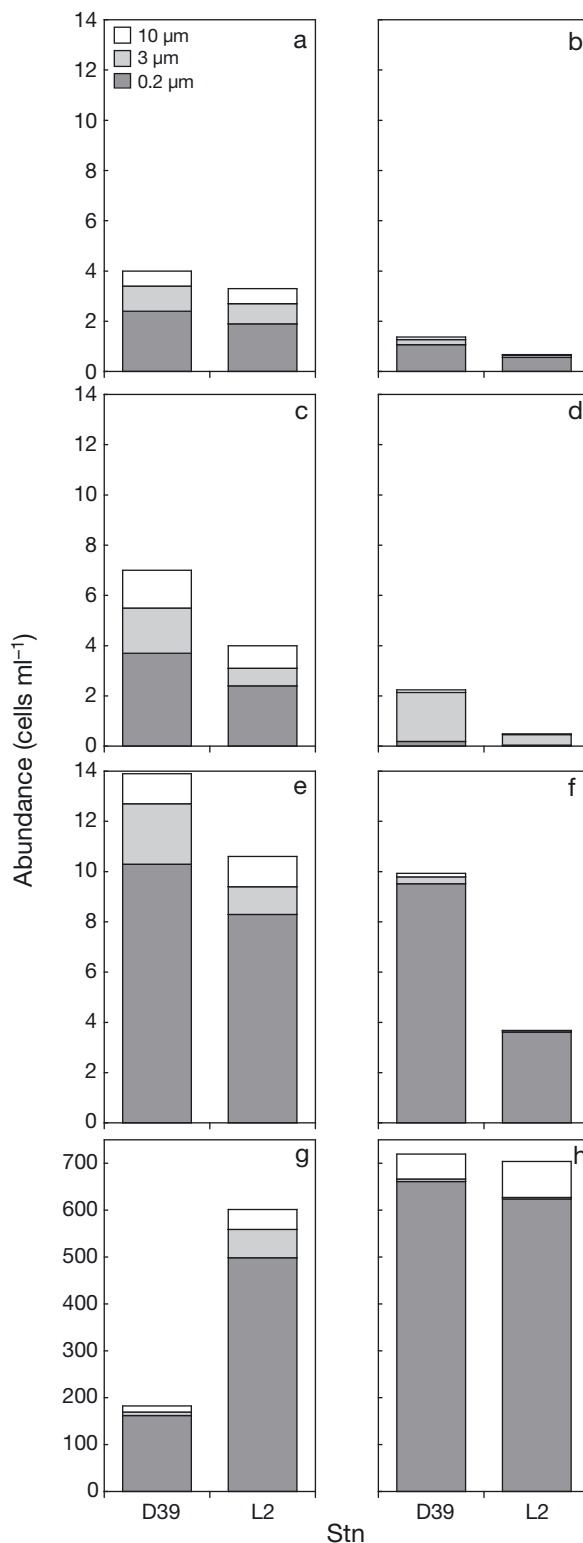


Fig. 2. Relative contribution of the different size fractions (0.2, 3 and 10 µm) to overall Nitro821-positive cells counted with the TSA-FISH technique for Stns D39 and L2 in summer (left panels) and winter (right panels) in (a,b) surface water, (c,d) water above the coral colonies, (e,f) water above the colonies after stressing the corals and (g,h) coral mucus

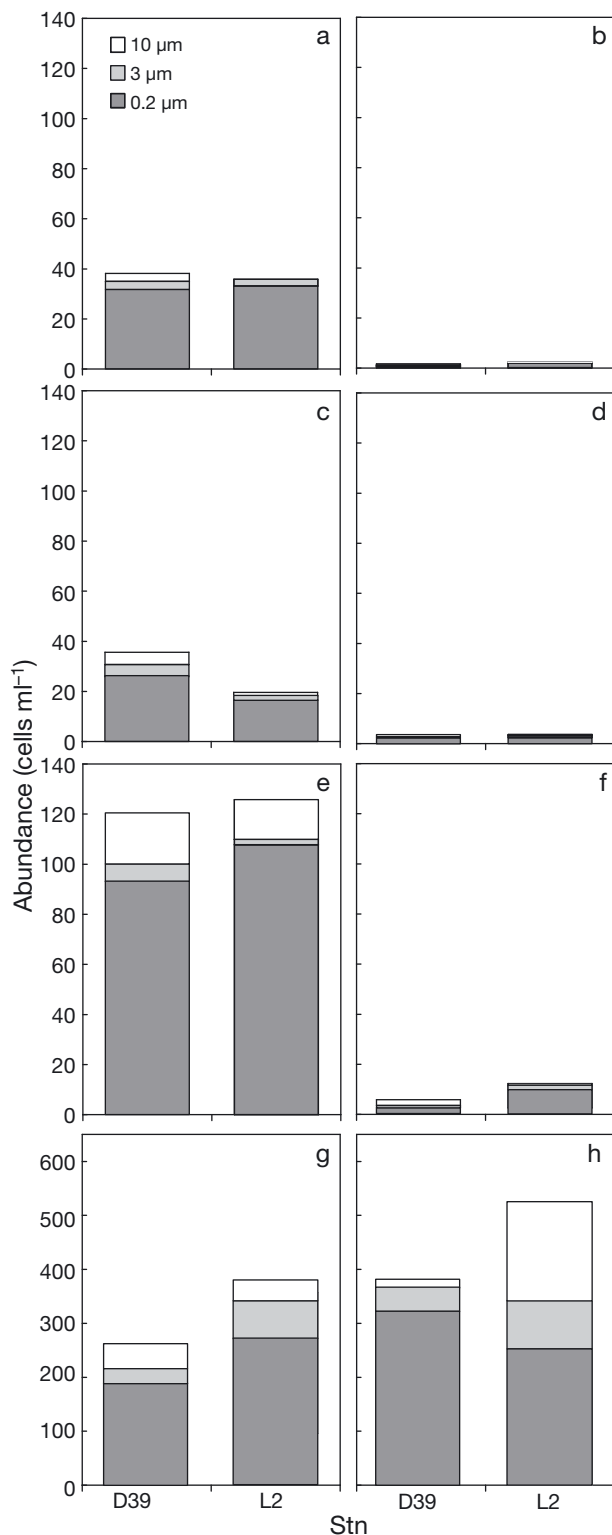


Fig. 3. Relative contribution of the different size fractions (0.2, 3 and 10 μm) to overall Rhizobia-positive cells counted with the TSA-FISH technique for Stns D39 and L2 in summer (left panels) and winter (right panels) in (a,b) surface water, (c,d) water above the coral colonies, (e,f) water above the colonies after stressing the corals and (g,h) coral mucus

24 h (~ 3 and $\sim 5 \text{ nmol N l}^{-1} \text{ d}^{-1}$ for control and mucus-amended samples, respectively; Fig. 4a); rates then decreased to values close to 1.2 and $1.5 \text{ nmol l}^{-1} \text{ d}^{-1}$ for the control and mucus-amended samples at the 48 and 72 h time points, respectively (Fig. 4a).

NO_x and PO_4^{3-} concentrations decreased over the incubation period, ranging from ~ 0.6 to $0.3 \mu\text{M}$ and from ~ 0.1 to $0.01 \mu\text{M}$ for NO_x and PO_4^{3-} (Fig. 4b,c). NO_x concentrations measured at 24 h were significantly higher (t -test, $p < 0.05$) in the mucus-amended treatments compared to the control, and PO_4^{3-} concentrations were significantly ($p < 0.05$) higher in the mucus-amended treatments at T_0 and after 48 h of incubation. NO_x and PO_4^{3-} concentrations decreased from T_0 to 72 h both for the control and for the mucus-amended treatments.

Bacterial and *Prochlorococcus* abundance increased by 2- to 3-fold at 24 h with respect to T_0 and then decreased towards the end of the incubation (Fig. 4d,e), while the abundance of *Synechococcus* and picoeukaryotes increased progressively over time for the mucus-amended treatments and control (Fig. 4f,g). Bacteria were significantly more abundant in the mucus-amended treatments than in the control samples at T_0 , 24 and 48 h ($p < 0.05$). These differences were not significant (t -test, $p > 0.05$) for *Prochlorococcus*, *Synechococcus* and picoeukaryotes.

DISCUSSION

Coral mucus does not enhance planktonic N₂ fixation

Studies have previously shown that bacterial abundance and production in the pelagic compartment is enhanced when coral mucus is released into the seawater or isolated samples are amended with mucus (e.g. Allers et al. 2008, Nakajima et al. 2009, Shnit-Orland & Kushmaro 2009). In this study, we hypothesized that the activity and/or abundance of planktonic heterotrophic diazotrophs could be enhanced by organic matter-rich coral mucus released into the water column. To the best of our knowledge this study is the first to directly test the effect of mucus on pelagic diazotrophy.

Our results indicate that mucus derived from corals can increase the abundance of bacteria within the seawater adjacent to the corals (Fig. 4d) and that it contains a high abundance of diazotrophs (e.g. up to 790-fold higher in winter; Figs. 2 & 3). Although planktonic N₂ fixation rates were higher after mucus was released into the surrounding seawater at the

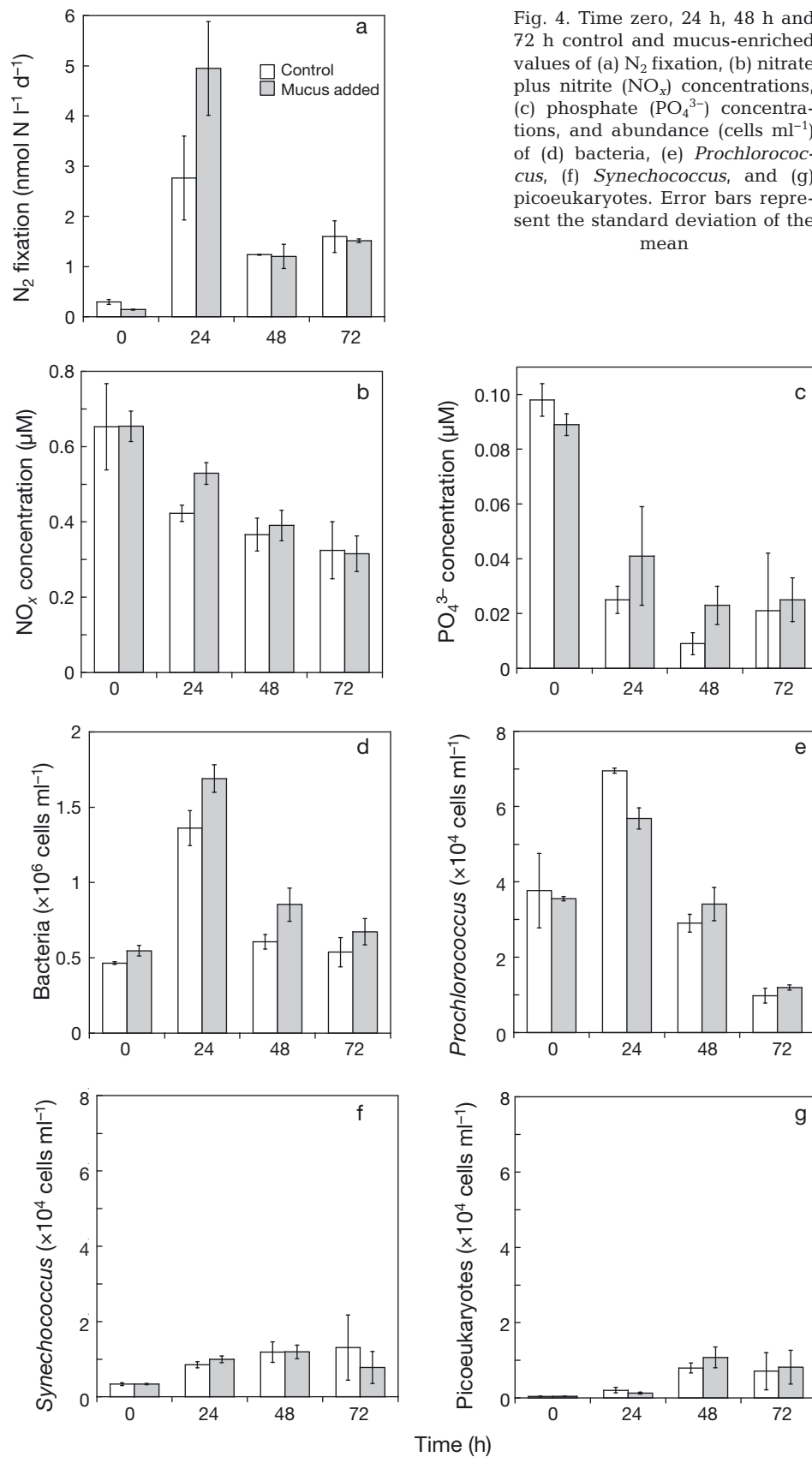


Fig. 4. Time zero, 24 h, 48 h and 72 h control and mucus-enriched values of (a) N_2 fixation, (b) nitrate plus nitrite (NO_x) concentrations, (c) phosphate (PO_4^{3-}) concentrations, and abundance (cells ml^{-1}) of (d) bacteria, (e) *Prochlorococcus*, (f) *Synechococcus*, and (g) picoeukaryotes. Error bars represent the standard deviation of the mean

coastal station (Stn D39) during our field experiments, this increase was not always significant. No increase was observed at the offshore station (Stn L2), except in February. Given the high organic matter content of mucus, it was expected that a significant increase of heterotrophic N₂ fixation activity would be observed as it was observed after addition of carbohydrates to open ocean seawater samples (e.g. Bonnet et al. 2013, Benavides et al. 2015). The high PO₄³⁻ content of mucus (see Table 1) is also known to enhance heterotrophic bacterial growth (Nakajima et al. 2015) and N₂ fixation in the New Caledonian lagoon (Berthelot et al. 2015). Our field-based results were consistent with the *in vitro* addition of mucus to seawater samples (mucus addition experiment), with N₂ fixation rates not significantly higher than those observed in the control treatments, i.e. where no mucus was added (Fig. 4a). This is despite the quantity of mucus added to clear seawater likely being much higher *in situ* after stressing corals as compared to the 2 ml added to 2.15 l samples during our *in vitro* experiment.

Our results contrast with previous studies that have observed active N₂ fixation in mucus particles released by corals in the Red Sea (Grover et al. 2014). There are at least 4 possible explanations for the lack of an enhancement of N₂ fixation activity. First, the chemical composition of the mucus during our experiments may have not been labile enough to trigger heterotrophic diazotrophic activity, as suggested by the high percentage of refractory carboxyl-rich alicyclic molecules (CRAM) present in these mucus samples (M. Benavides et al. unpubl. data). Moissander et al. (2012) found that the low N₂ fixation rates as well as growth rates of bacteria are due to a lack of energy given in the form of bioavailable carbon. The chemical composition of the mucus and its physical characteristics vary extensively among coral species and environmental conditions (Meikle et al. 1988, Rohwer et al. 2002, McKew et al. 2012), as well as depending on the collection strategy used, since artificially stressed corals release mucus which differs in composition to that released naturally (Ferrier-Pagès et al. 1998, Klaus et al. 2007, Tanaka et al. 2008), and as a result of its degradation over time following collection (Wild et al. 2004, Tanaka et al. 2011). While our experimental setup does not allow us to discern whether naturally released mucus would have caused a different diazotrophic response than mucus released after artificial stress, we believe that the degradation of mucus was minimal in our samples since <1 h passed between the time of collection and sample processing in the laboratory. Sec-

ondly, the dissolved organic carbon concentration of coral mucus ranges between ~300 and 4000 µM, with values as high as 7500 µM in some cases (Brown & Bythell 2005, Huettel et al. 2006, Tanaka et al. 2011); thus it is possible that the addition of 2 ml of mucus introduced to 2.15 l incubation bottles during our *in vitro* experiments may not have been enough to trigger heterotrophic N₂ fixation. The quantity of mucus released *in situ* after stressing natural coral colonies cannot be assessed with our data. Third, the low abundance of heterotrophic diazotrophs in the waters of the New Caledonian lagoon (Turk-Kubo et al. 2015) could prevent the observation of any significant effects upon DOM (coral mucus) additions. Moreover, the phylotype-targeted quantitative PCR quantifications of Turk-Kubo et al. (2015) do not necessarily cover the overall diversity of heterotrophic diazotrophs present in the lagoon's waters. Nevertheless, the heterotrophic diazotrophs dominate over autotrophic ones on a global basis (Farnelid et al. 2011). Finally, other sources of DOM to the Caledonian lagoon, such as riverine and atmospheric sources (Mari et al. 2007, 2014), may alleviate organic carbon limitation in heterotrophic plankton. Indeed, a recent study suggests that heterotrophic bacteria within the New Caledonian lagoon are more limited by nitrogen than by carbon (Van Wambeke et al. 2015). The effect of DOM on heterotrophic planktonic diazotrophs may vary seasonally according to DOM composition (organic carbon and nitrogen content) and its bioavailability.

Although mucus did not affect pelagic N₂ fixation rates in our study, it caused an increase in diazotrophic abundance in the column water whatever the season. This increase is due to high abundance of diazotrophs inside the mucus which are introduced in the column water upon its release. The activity of these diazotrophs seemed to be low, not affecting N₂ fixation rates in the surrounding waters. Moreover, the mucus did affect the abundance of heterotrophic bacteria in general as previously observed (Huettel et al. 2006). It is thus likely that non-diazotrophic heterotrophic bacteria grew faster than diazotrophic ones in our experiment, which is not surprising given that NO_x concentrations were not totally exhausted in lagoon waters at the time of the mucus addition experiment (Fig. 4b). As it requires ~25 % more energy to reduce N₂ (87 kcal) than nitrate (69 kcal) to ammonium (Falkowski 1983), it is thus not surprising that heterotrophic diazotrophs did not fix N₂, or were less competitive than non-diazotrophs given that nitrate was not depleted. Nevertheless it is possible that the proportion of heterotrophic diazotrophs present in sea-

water was not sufficient to observe a change in the N_2 fixation rates (Turk-Kubo et al. 2014).

Moreover, the variability in abundance and activity of diazotrophs was likely affected by temperature (Shiozaki et al. 2014, 2015). Except in February, N_2 fixation rates were highest at higher temperatures. The differences in temperature observed between the 2 stations sampled agreed with those of Ouillon et al. (2005) and were likely caused by the more efficient water renewal closer to the reef barrier as compared to the coastal station. These differences led to a water warming and to a greater development of diazotrophs at Stn D39 than at Stn L2. The abundance and community composition of diazotrophs in the tropical Southwest Pacific have been shown to be related to changes in temperature, with greater abundances at temperatures above 25°C (Garcia et al. 2007, Bonnet et al. 2015b) and it is likely that N_2 fixation is influenced by environmental conditions rather than by coral mucus in this region. Negative and positive linear relationships have been found between the abundance of UCYN-A and UCYN-B and temperature, respectively (Moisander et al. 2010, Bonnet et al. 2015a). Nevertheless, the abundance and activity of diazotrophs in the present study were generally lower than those observed in previous studies (e.g. Biegala & Raimbault 2008, Turk-Kubo et al. 2015). Biegala & Raimbault (2008) observed UCYN abundances ~3-fold higher than those observed in April in our study, and Bonnet et al. (2015b) measured N_2 fixation rates of ~10 nmol $N\ l^{-1}\ d^{-1}$ in February 2013 close to Stn L2 (i.e. 70-fold higher than the rates recorded in our study in the same season). This may be due to the specific location where our experiments were performed (in shallow waters, and very close to the coast for Stn D39), thus harboring NO_x concentrations known to inhibit N_2 fixation (Holl & Montoya 2005, Knapp 2012), whereas the stations sampled in the studies mentioned above were located in the middle of the lagoon where water flow is more constant (Ouillon et al. 2010) and NO_x concentrations lower (Fichez et al. 2010). Nevertheless, during our study NO_x concentrations at the coastal station D39 were 2-fold lower than at Stn L2, suggesting a diminished influence of the Dumbea River runoff during this season.

Coral mucus harbors important UCYN and rhizobia communities

Corals form mutualistic associations with diverse prokaryotic communities including diazotrophs (Ol-

son et al. 2009, Reis et al. 2009) which have been found in the skeleton (Shashar et al. 1994, Davey et al. 2008), tissue (Lesser et al. 2007, Lema et al. 2014) and mucus of corals (Lema et al. 2012). The high heterogeneity of diazotroph communities living within the mucus of corals such as *Acropora* sp. (Nithyanand et al. 2011, Lema et al. 2012) likely provides a significant source of fixed nitrogen for corals and the wider reef ecosystem (Cardini et al. 2014). Moreover, diazotrophs inhabiting coral mucus matrices may play a key role in coral health (Reis et al. 2009). Although we only quantified UCYN and rhizobia, these 2 groups provide a good representation of the diazotrophic communities associated to coral reefs as they have often been identified in several coral species of the Southwest Pacific such as *Acropora* sp., which were the dominant coral species of our sampling sites (Lema et al. 2012, 2014, Krediet et al. 2013), and are also abundant in the pelagic compartment of tropical waters (Biegala & Raimbault 2008, Le Moal et al. 2011). The abundance of diazotrophs within the mucus was always higher than in the water column whatever the season (Figs. 2g,h & 3g,h). This was especially notable in winter, when diazotrophs were scarce in surrounding waters. The permanent high abundance of diazotrophs within the coral mucus suggests that these microorganisms are important for the nitrogen nutrition of the coral holobiont and for the water column (Lema et al. 2012, Cardini et al. 2015).

Although our mucus samples contained abundant diazotroph populations when compared to seawater samples (Figs. 2 & 3), the lack of statistically significant differences between seawater and seawater containing mucus N_2 fixation rates (Fig. 1) suggests that these colonizing diazotrophs were not especially active, or at least not more active than planktonic diazotrophs. These results contrast with those of Grover et al. (2014), who found significant N_2 fixation rates in mucus flocs. The activity and community composition of coral-associated microbes are highly dynamic and we do not discount that N_2 fixation activity by mucus-colonizing diazotrophs provides an important source of nitrogen in coral reef ecosystems under environmental conditions different to those present during our study (Rädecker et al. 2015). Planktonic N_2 fixation influenced by coral mucus release may in turn influence benthic communities. Knapp et al. (2015) have shown that the largest fraction of primary production within the New Caledonian lagoon is sustained by N_2 fixation in the water column and exported to benthic communities, either directly through diazotroph micro-

organism sedimentation (Bonnet et al. 2015b) or indirectly through fixed N₂ transfer to non-diazotrophic microorganisms and consequent sedimentation. This is confirmed by a recent study showing that diazotrophs significantly contribute to the nitrogen nutrition of both corals and zooxanthellae (M. Benavides et al. unpubl. data).

CONCLUSIONS

The diversity of active N₂ fixation sites present in coral reef ecosystems (e.g. hard and soft corals, seagrasses, sediments and sponges) indicates that diazotrophic activity provides an important source of combined nitrogen to these ecosystems. While planktonic N₂ fixation is also important in coral lagoon waters, our results suggest that this activity is not strongly influenced by organic materials released by corals such as mucus. However, our results do show that coral mucus harbors abundant diazotrophic communities that are released into the water column and may provide an additional source of fixed nitrogen to the pelagic coral reef ecosystem. In contrast to the diazotrophic communities in the seawater column which varied greatly over time, the abundance of diazotrophs within the mucus remained stable over the study period, and ~20- to ~400-fold higher than seawater abundances, in summer and winter, respectively (Figs. 2 & 3). Together these results indicate a key role for diazotrophs associated with the coral holobiont. Diazotrophs also influence the pelagic and benthic compartments within the reef ecosystems as they are released into the seawater associated with mucus and subsequently undergo sedimentation.

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