

Nitrate reduction by fungi in marine oxygen-depleted laboratory microcosms.

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Running title: Fungal activity in marine oxygen-depleted laboratory microcosms.

Abstract

Fungi are major contributors to the process of denitrification in terrestrial environments, but their role as denitrifiers in the ocean has not been addressed thus far. We measured the nitrate reducing capacity of fungi based on the respiratory electron transport system (ETS) activity and nitrite accumulation in oxygen-depleted laboratory microcosms. Specific fungal and bacterial activity in these microcosms was studied by fortifying the sediments with antibiotics and anti-fungal compounds, respectively. The higher ETS activity in the microcosm with antibiotics indicated higher fungal than bacterial activity. Activity of two fungi, # An-2 (MTCC Accession number 9376) and # MT-811 (JMC Accession number 11502) in pure cultures under aerobic and anaerobic conditions, and in the microcosms was also studied. ETS activity in the fungal cultures grown under anaerobic conditions was half the activity measured under aerobic growth conditions. Simulated laboratory microcosm data show that fungi participate in the denitrification activity in sediments from marine hypoxic environment.

Key words: Arabian Sea, denitrification, microcosms, fungi.

Introduction

Fungi are generally regarded as aerobic heterotrophs that require oxygen for growth and metabolic activity. The only exceptions are the members of a specialized group called the “rumen-fungi” that are fermentative forms inhabiting the anaerobic gut of herbivores (Orpin 1977). Colonization of fungi on wood in the marine environment is slower or almost zero in oxygen minimum zones (Kohlmeyer and Kohlmeyer 1979). Diversity and the ecological role of fungi in natural oxygen-depleted environments are considered to be insignificant (Mansfield and Barlocher 1993, Dighton 2003). However, recent molecular studies on total micro-eukaryotic diversity have shown vast and novel diversity of many groups such as alveolates, straminopiles and fungi in oxygen-depleted marine environments (Takishita *et al.* 2005, 2007a, b, Stoeck *et al.* 2006).

Several terrestrial forms such as *Fusarium oxysporum* Schldl., *Aspergillus oryzae* (Ahlb.) E. Cohn and yeasts *Trichosporon cutaneum* (Beurm., Gougerot and Vaucher bis) M. Ota, *Cylindrocarpon tonkinense* Bugnic. are all capable of denitrification (Shoun and Tanimoto 1991, Shoun *et al.* 1992). The well-known denitrification process in bacteria involves reduction of nitrate and nitrite to nitric oxide (NO), which is reduced to nitrous oxide (N₂O) by nitric oxide reductase, which is further reduced to the final product nitrogen gas (N₂). But, fungi possess an incomplete denitrification process, where the pathway largely stops with the reduction of NO to N₂O. Fungal denitrification accounts for nearly 80% of the microbial nitrous oxide production in grassland ecosystems (Laughlin *et al.* 2009). Fungi are also involved in co-denitrification and chemo-denitrification to produce N₂ gas (Laughlin and Stevens 2002).

Microbes involved in the denitrification pathway are studied by screening culturable bacteria for their ability to utilize nitrate or nitrite in the absence of oxygen (Simbert and Kreig 1981). Mahne and Tiedje (1995) have suggested that the characteristic feature of respiratory denitrification is that the reduction of nitrogenous oxide to nitrogen gases is coupled with an active electron transport system. Therefore, the two criteria that must be met for an organism to be called a respiratory denitrifier are (a) the production of nitrogen gases, principally N₂ and N₂O as products of nitrite or nitrate reduction, and (b) an increase in growth yield. This method is the most straightforward and simplest way to establish the denitrification activity.

The objective of the present study was to quantify the denitrifying activity of bacteria and fungi in oxygen-depleted marine sediments of the Arabian Sea by setting up various simulated laboratory microcosms. The Arabian Sea is influenced largely by the south-west and northeast monsoons, which

lead to upwelling of bottom water nutrients and high productivity (Warren 1994, Naqvi 1994). This has resulted in the formation of one of the world's largest perennially oxygen-depleted environment in the open ocean and a seasonal hypoxic zone along the western coast of India. The coastal hypoxic condition develops along the continental shelf down to 200 m depth, leading to intense sedimentary denitrification activity (Naqvi *et al.* 2000). The hypoxic condition develops annually from June to October and reaches a maximum intensity in September and October, and the conditions are sometimes so intense that all the nitrates are depleted and sulphate reduction begins. Dissolved oxygen (DO) levels during anoxic condition drop down to $<20 \mu\text{M}$ (the oxic DO level is $\sim 180 \mu\text{M}$) (Naqvi *et al.* 2006). We recently surveyed oxygen-depleted environments of the Arabian Sea and carried out studies to definitively demonstrate fungal abundance and diversity from this region (Jebaraj and Raghukumar 2009). Fungal colony forming units (CFUs) were isolated from hypoxic marine sediments, and some of the isolates screened showed denitrification activity under laboratory conditions (Jebaraj and Raghukumar 2009). Culture-independent fungal diversity in this region demonstrated a substantial number of sequences that were closely related to environmental sequences from a range of other oxygen-depleted marine habitats, but distantly related to known sequences of described fungi (Jebaraj *et al.* 2010). Here, we report electron transport system (ETS) activity and nitrate reduction in laboratory microcosm experiments to compare the relative role of fungi and bacteria in the denitrification process.

Materials and methods

Fresh sediment and near-bottom water were collected from station CSt-26, $15^{\circ}29.951'\text{N}$, $73^{\circ}40.827'\text{E}$ located within the coastal hypoxic zone off Goa, India during the pre-monsoon season in April 2009. Sediment samples were collected with a Van-Veen grab, and a Niskin water sampler (5 l) was used to collect water from 26 m depth. The samples were brought to the laboratory on ice and five different microcosms were set-up (Table 1) under (a) natural nutrient levels (Mic-1a to Mic-5a) and (b) with 10 mM nitrate (Shoun *et al.* 1998, Jebaraj and Raghukumar 2009) supplementation (Mic-1b to Mic-5b).

For all microcosm sets, sediment slurry was prepared by mixing 15–20 g of sediment and ~ 100 ml of near-bottom water. The slurry was added to 100 ml serum bottles and flushed with N_2 gas to remove the dissolved oxygen present. These bottles were closed with butyl rubber stoppers and sealed immediately with aluminum crimps.

In the first microcosm (Mic-1a), plain sediment was incubated under anaerobic conditions without any supplementation. To study the activity of fungi, bacterial growth was inhibited by addition

of streptomycin (0.1 g in 100 ml medium) and penicillin (40,000 Units in 100 ml medium) in microcosm-2 (Mic-2a). Fungal activity was specifically suppressed with an anti-fungal compound Bavistin (commercial grade) to a final concentration of 0.5% in microcosm-3 (Mic-3a). Bavistin is a commonly used benzimidazole fungicide containing methyl benzimidazol-2-yl carbamate. The concentrations of anti-bacterial and anti-fungal agents used were standardized by growing three isolates of bacteria and fungi in varying concentrations of both inhibitors. The concentrations that did not show cross-inhibition (ie. fungicide not inhibiting bacteria and vice versa) were used in the study. In microcosm-4 and 5 (Mic-4a and Mic-5a) culture suspensions containing approximately 30 mg (dry weight) of fungal cultures *Fusarium* sp. # An-2 and # MT-811 were added to the respective sediment slurries (Table 1). # An-2 is a marine isolate from the Arabian Sea coastal hypoxic zone and deposited in the Microbial Type Culture Collection at Chandigarh, India under the accession number MTCC 9376 (Jebaraj and Raghukumar 2009). # MT-811 is a much-studied fungus because of its denitrification activity (Shoun *et al.* 1998); it is deposited in the Japanese collection of Microorganisms under accession number JCM 11502 and was included as a positive control (donated by Dr H Shoun, Tokyo University, Japan)

As the initial nutrient concentration was very low in the fresh sediment, a set of experiments with nitrate (sodium nitrate) supplementation to a final concentration of 10 mM was run simultaneously i.e., Mic-1b to Mic-5b. The experimental bottles were incubated for 7 days under controlled conditions at 30°C with mild agitation to prevent settling of the slurry to the bottom.

Concentrations of nitrate, nitrite, DO levels and the redox potential (Eh) of the sediment in the slurry were determined at the start and at the end of the experiment. The DO levels were determined by spectrophotometric methods (Pai *et al.* 1993). Eh is the electrical measurement of reducing or oxidizing capacity of a system; it was recorded with a pH meter (PHAN, LabIndia, Thane, India). Nitrate in seawater used in the experimental set-up was measured following Strickland and Parsons (1968). All chemicals used were analytical grade.

At the end of the experiment incubated sediment was freeze-dried in sterile plastic containers. Electron transport system (ETS) activity in the freeze-dried sediment was determined by homogenizing the sediment (approximately 600 mg) in 6 ml of 0.05 M phosphate buffer (pH 7.7) containing 18 mg of polyvinyl pyrrolidone and 1 mg of dithiothreitol. Precautions were taken to minimize bacterial contamination throughout the experimental procedure by preparing the buffers in filter sterilized water

and using sterilized glassware. The crude homogenized mixture was centrifuged at 6000×g for 5 min at 4°C. The supernatant was immediately transferred to clean tubes and the volume obtained was recorded. One ml of the crude enzyme extract was incubated in darkness for 60 minutes at room temperature with 1 ml of 4 mM 2(p-Iodophenyl)-3(p-nitrophenyl)-5 phenyl tetrazolium chloride (INT) and 3 ml of substrate solution containing 0.6 g NADH, 0.2 g NADPH, 36 g sodium succinate hexahydrate and 2 ml Triton X 100 per liter of 0.05 M phosphate buffer (pH 8). During the incubation process, INT is reduced to its formazan giving a pink coloration to the reaction mixture. The reaction was terminated by addition of 1 ml of 1:1 (v/v) solution of 1 M H₃PO₄ and concentrated formalin. The quenched reaction mixture was centrifuged at 6000×g for 5 min at 4°C. The absorbance was recorded at 490 nm and 760 nm within 3 h at room temperature in a UV–Vis 2450 spectrophotometer (Shimadzu, Kyoto, Japan). The peak at 490 nm is proportional to INT-formazan produced and the 760 nm reading serves as turbidity blank. A pigment blank consisting of an assay run without pyridine nucleotides or succinate on the clarified homogenate and a reagent blank was run with each assay (Packard *et al.* 1983).

ETS activity was similarly measured in the fungal biomass grown under aerobic and anaerobic conditions, which were maintained as described by Jebaraj and Raghukumar (2009). For this, the cultures were grown in a mineral medium containing 30 g glucose, 1.3 g KH₂PO₄, 0.2 g MgSO₄, and 2 g peptone prepared in artificial sea water (24 g NaCl, 0.7 g KCl, 7 g MgCl₂ in 1000 ml deionised water) and supplemented with 10 mM sodium nitrate. The cultures were maintained under aerobic conditions in 100 ml conical flasks (plugged with cotton) containing 20 ml of medium under anaerobic conditions in 100 ml serum bottles sealed air tight with butyl rubber stoppers and steel crimps after flushing with nitrogen gas through the medium for 2 min. Dissolved oxygen (DO) was determined spectrophotometrically (Pai *et al.* 1993) at the start and end of the experiment on day 10. Cultures were harvested every alternate day up to 10 days, and ca. 100 mg of freeze dried mycelia were used for the assay.

ETS activity was calculated from the following equation and expressed as nano equivalents (neq h⁻¹ μg⁻¹ protein).

$$\text{ETS} = 60 \times S \times (\text{COD} - \text{RB}) / 1.42 \times f \times t \times 5.6$$

where S = volume of quenched reaction mixture, COD = corrected absorbance, RB = reagent blank, f = concentration of protein in reaction mixture (μg), t = incubation time, the constant 60 converts the time dimension to hours, and the 1.42 conversion factor is based on the molar extinction coefficient of the

INT formazan, the stoichiometry of INT reduction and the stoichiometry of oxygen consumption by the respiratory electron transport system. The constant 5.6 is used to convert ETS activity obtained in $\mu\text{l O}_2 \text{ h}^{-1} \mu\text{g}^{-1} \text{ protein}$ to $\text{neq h}^{-1} \mu\text{g}^{-1} \text{ protein}$. Total protein concentration in the crude homogenate was determined by the Bradford method using the commercially available Bradford reagent (Sigma-Aldrich, Bangalore, India).

Results and Discussion

Sediment samples were collected during the pre-monsoon season under suboxic conditions; the dissolved oxygen (DO) level in near bottom water was $112 \mu\text{M}$, Eh was -9 mV , and nitrate and nitrite were undetectable ($<1 \text{ nM}$). Sediment was incubated in anaerobic microcosms as described (Table 1) for 7 days. At the end of the incubation period, DO level and Eh values were reduced to $3.81 \mu\text{M}$ and -19.9 mV , respectively in Mic-1a. In the microcosms supplemented with antibiotics and anti-fungal compound (Mic-2a and 3a), Eh values were -3.5 mV and -25.8 mV , respectively. In the microcosm with addition of fungal inocula (Mic-4a and 5a), Eh values were further reduced to -45 and -34.4 , respectively (Fig. 1a).

ETS activity in the sediment before incubation on day zero was about $90 \text{ neq h}^{-1} \mu\text{g}^{-1} \text{ protein}$ (Fig. 1a). The activity decreased to $15 \text{ neq h}^{-1} \mu\text{g}^{-1} \text{ protein}$ by the end of the incubation period under anaerobic conditions (Mic-1a). ETS activities in the microcosms resulting from specific bacterial and fungal activities were 21.43 and $38.88 \text{ neq h}^{-1} \mu\text{g}^{-1} \text{ protein}$, respectively. Thus, specific activity of fungi was high when bacterial growth was preferentially suppressed using antibiotics in the microcosms under natural nutrient conditions (Mic-2a). ETS activities in the microcosms show that microbial respiratory metabolism was active under anaerobic conditions. However, nitrite accumulation unmeasurable, perhaps because nitrate was unavailable under natural conditions since initial nitrate and nitrite levels were below detection values on day zero.

Denitrification activity was observed as nitrite accumulation in all microcosms with 10 mM nitrate supplementation (Mic-1b to 5b). In Mic-1b, nitrite accumulation due to the total microbial community was about 12.6 nM . Nitrite accumulation in Mic-2b (bacterial communities suppressed) was about 5.5 nM ; in Mic-3b (fungal activity suppressed) it was about 16 nM . Nitrite accumulation after additions of external fungal inocula # An-2 and # MT-811 increased to 53 nM and 358 nM , respectively.

Nitrite accumulated under micro-aerobic and anaerobic conditions when fungal cultures # An-2 and # MT-811 were present. Cultures maintained in flasks had initial DO values of 115.2 μM ; as the cultures grew, DO was utilized and micro-aerobic conditions developed (18 - 97 μM) on days 4–6 depending on the biomass increase. As conditions became suboxic, cultures started to utilize nitrate for respiration, and nitrite accumulated in the culture flasks. In anaerobic cultures (following flushing with N_2 gas), initial DO was 9.5 μM , and at the end of the experiment DO was undetectable. Fungal cultures #An-2 and # MT-811 accumulated maxima of 434.5 nM and 199.4 nM, respectively, over 10 days when grown in inorganic media containing 10mM sodium nitrate (Jebaraj and Raghukumar 2009). ETS activity in fungal cultures grown under anaerobic conditions were half those under aerobic growth conditions (Fig 2a, 2b). Culture # An-2 had higher ETS activity than control culture # MT-811 under both conditions.

Nitrite formation and ETS activity increased proportionally with nitrate supplementation in the microcosm (Fig 1b). Fungal-specific ETS activity was higher (43 $\text{neq h}^{-1} \mu\text{g}^{-1}$ protein in Mic-2b) than bacterial specific activity (30.98 $\text{neq h}^{-1} \mu\text{g}^{-1}$ protein in Mic-3b), showing that the fungal contribution to denitrification activity in the microcosm exceeded that from bacterial activity. ETS activities with additional fungal inoculations were 70.92 and 29.87 in the presence of # An-2 and # MT-811, respectively. Denitrification activity was detected by nitrite accumulation in all microcosms with nitrate supplementation, but there was no significant correlation between ETS activity and nitrite accumulation. The absence of a clear relationship may be due to the fact that intermediary compound nitrite may have already been reduced to nitric oxide, nitrous oxide or to nitrogen gas, which were not measured in this study. There is also a possibility that reduction of nitrate was through an ammonia fermentation process. This is another fungal dissimilatory pathway under completely anaerobic conditions; it is not associated with the respiratory chain (Zhou et al., 2002). Detailed investigation of all reduction products of nitrate under denitrification conditions and a study of the ammonia fermentation procedure will help detailed interpretation of the role of fungi under reduced oxygen conditions.

In the present study, nitrate supplementation had a profound impact on fungal activity in the anaerobic microcosm. The requirement for nitrate clearly shows the need for an alternate electron acceptor in the absence of oxygen. Addition of nitrate to the microcosms resulted in increased biomass and subsequent reduction of Eh values which ranged between – 15.9 to – 51.9 in comparison with microcosms without nitrate (Fig.2a and 2b). Fungal- and bacterial-specific activities in the microcosms show that (in addition to bacteria) fungi participate in the denitrification process. Hence, the viable and active fungi that survive in anaerobic conditions; could be playing an important role in marine systems,

as they do in terrestrial soils (Laughlin *et al.* 2009). Studies on denitrification in wetlands indicate that both bacteria and fungi contribute significantly to denitrification under reducing conditions. However, fungal denitrification might well be of greater ecological significance under aerobic or moderately reducing conditions as they contribute to greenhouse gas emission and can denitrify efficiently under widespread micro-aerobic conditions (Seo and DeLaune 2010). Fungi act as denitrifiers in terrestrial ecosystems such as grassland soil (Laughlin and Stevens 2002), fumigated soil (Spokas *et al.* 2006) and woodland (Castaldi and Smith 1998). Mixed cultures of the fungus *Fusarium oxysporum* and the bacterium *Pseudomonas stutzeri* (Lehmann and Neumann) Sijderius under anaerobic conditions was able to remove nitrate and nitrite faster than individual cultures (Liu *et al.* 2006). Studies of fungal denitrification under anaerobic conditions will contribute to better understanding of natural biogeochemical cycling of nitrogen. They will also contribute to biotechnological developments in sewage treatment plants, as filamentous fungi can perform both nitrifying and denitrifying process even under low oxygen conditions (Guest and Smith 2002, Greben *et al.* 2007).

Acknowledgements

We are grateful to the Director, National Institute of Oceanography for the financial support and encouragement. We thank Dr. Shoun H, Tokyo University, Japan for culture MT-811. We acknowledge the help rendered by Dr. S.Raghukumar for scientific discussion while preparing this manuscript. Authors would like to thank both the anonymous reviewers for their critical and constructive review and greatly acknowledge the scientific editing done by Dr. Anthony R.O. Chapman Editor-in-Chief, Botanica Marina that helped us improve this manuscript. This is NIO's contribution No.

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Figure Legends

Fig. 1. ETS activity and nitrite formation in laboratory microcosms measured on day zero and after seven days (a) without nitrate supplement and (b) with nitrate supplement (see Table 1 for the detailed description of the experimental set ups). * Nitrite concentration was 10-fold higher than shown in the graph. Eh (mV) values are negative. Values are mean of 6 replicate samples and the error bar is the \pm standard deviation to the calculated mean value (n = 6).

Fig. 2. ETS activities of two fungal cultures (An-2, MT-811) grown for ten days under (a) aerobic and (b) anaerobic conditions. Values are mean of 6 replicate samples and the Standard error bar is the \pm standard deviation to the calculated mean value (n = 6).

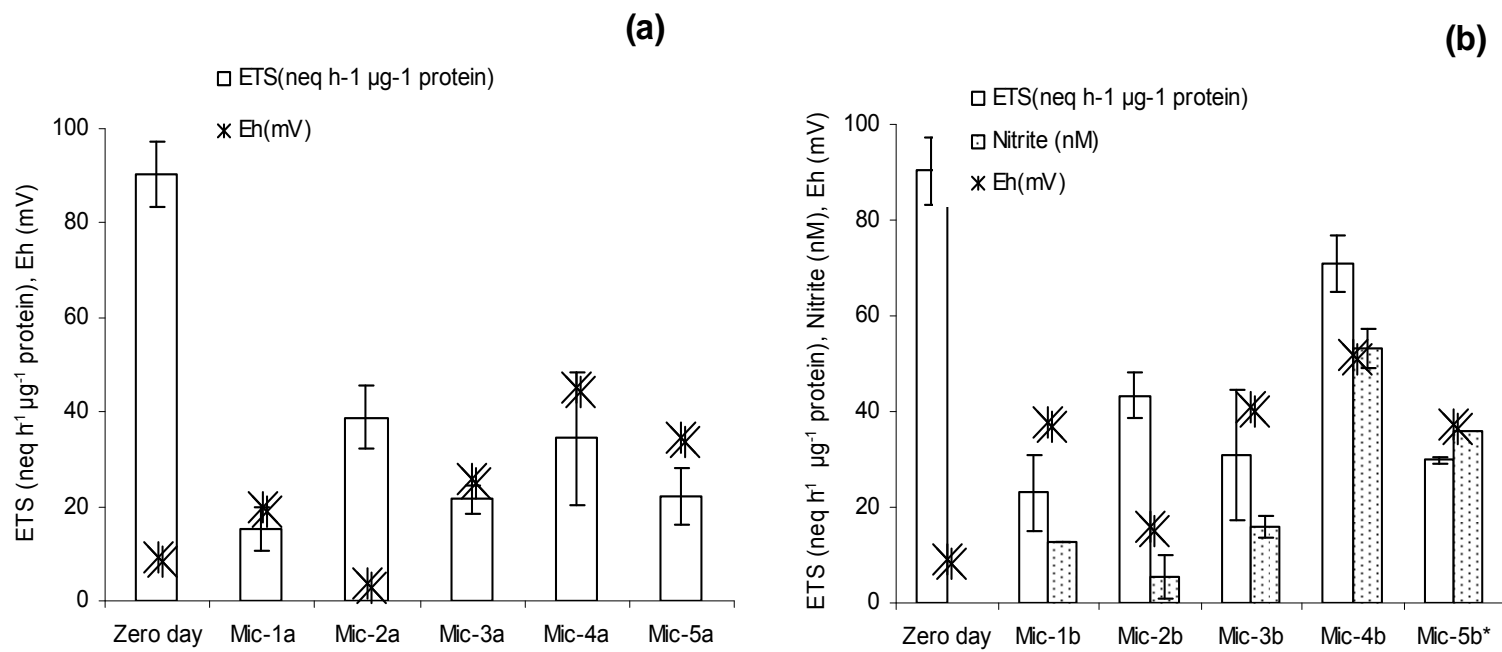


Fig. 1

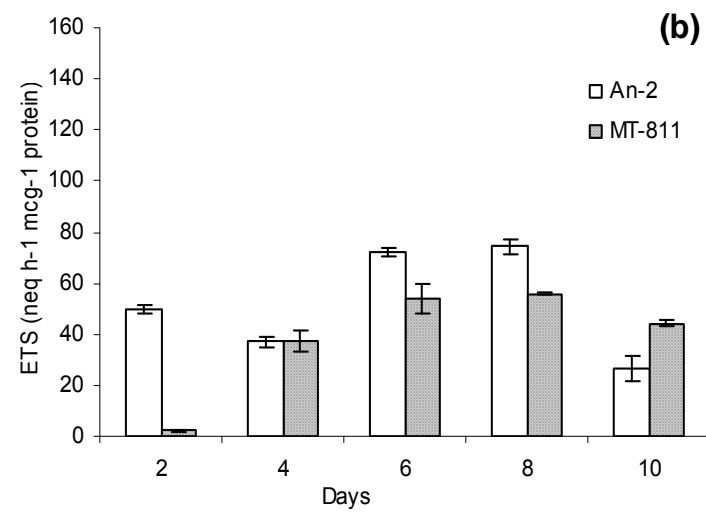
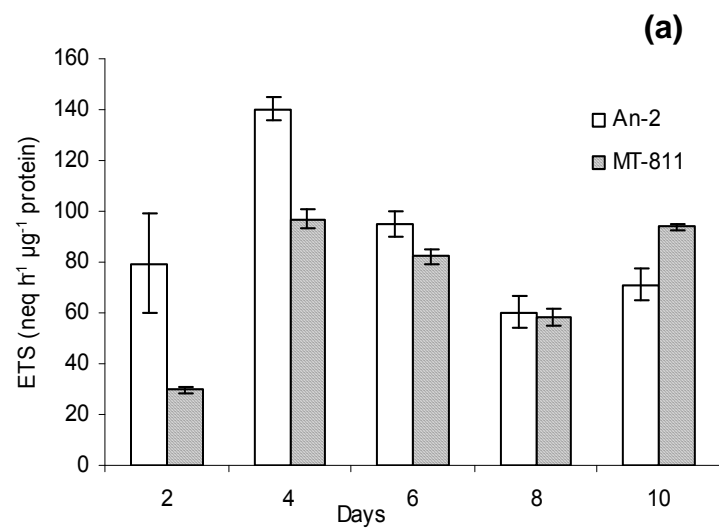


Fig. 2

Table 1. List of laboratory microcosms

Microcosm	Components	Objective
Mic-1a	Plain sediment slurry	To study total microbial activity
Mic-2a	Sediment slurry with anti-bacterial agents	To study fungal activity by suppressing bacterial growth
Mic-3a	Sediment slurry with anti-fungal agents	To study bacterial activity by suppressing fungal growth
Mic-4a	Sediment slurry with culture suspension of # An-2	To study activity of fungal culture # An-2 under <i>in-situ</i> conditions
Mic-5a	Sediment slurry with culture suspension of # MT-811	To study activity of fungal culture # MT-811 under <i>in-situ</i> conditions

Note: Similar microcosms (Mic-1b through Mic-5b) were set up with a 10 mM nitrate supplementation to the sediment slurry.