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Rhizobacterial population density and nitrogen fixation in seagrass community of Gulf of Mannar, India

Author Details

S. Raja	Centre of Advanced Studies in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, India
T.Thangaradjou (Corresponding author)	Centre of Advanced Studies in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, India e-mail : umaradjou@gmail.com
K.Sivakumar	Centre of Advanced Studies in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, India
L. Kannan	Centre of Advanced Studies in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, India

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Abstract

Seagrass rhizosphere generally supports high bacterial population density which plays a major role in determining the nutrient cycles of the sea. Higher densities of total heterotrophic bacteria (26.3 ×10^6 CFU g^{-1}), nitrogen fixing (27.3 ×10^3 CFUg^{-1}), ammonifying (44.66 X 10^6 MPN g^{-1}) and nitrifying bacteria (42.33 X 10^6 MPN g^{-1}) have been registered in the seagrass areas than the non seagrass area. In particular, all these rhizosphere microbial population was higher in *Thalassia hemprichii*. The rates of nitrogen fixation was recorded in the different species of seagrasses such as *Enhalus acoroides* (1.166 n mol $g^{-1}d^{-1}$), *Halophila ovalis* (0.166 n mol $g^{-1}d^{-1}$), *Thalassia hemprichii* (18.5 n mol $g^{-1}d^{-1}$), *Cymodocea serrulata* (10.5 n mol $g^{-1}d^{-1}$), *Halodule uninervis* (5.375 n mol $g^{-1}d^{-1}$) and *Syringodium isoetifolium* (0.666 n mol $g^{-1}d^{-1}$) using gas chromatography. The average nitrogen fixation by the seagrasses of Gulf of Mannar alone was estimated to be 7640.58 n mol m^{-2} d^{-1} and the contributions from the rhizosphere microbes will increase the quantity to many fold.

Key words

Rhizosphere bacteria, Nitrogen fixation, Seagrasses, Gulf of Mannar

Introduction

Seagrasses are flowering plants growing submerged in shallow marine and estuarine environments of the world. The true importance of seagrass meadows to the coastal marine ecosystem is not fully understood and is generally underestimated (Marten and Carlos, 2000). Seagrass biomass is the prime factor influencing the organization of marine micro and macro faunal communities. Structure of seagrass beds modify the physical and chemical characteristics of the water column and sediments of the areas colonized. The structure of seagrass canopy, for instance, modifies water current velocity and enhancing sedimentation of suspended particles (Koch *et al.*, 2001) and they prevent sediment resuspension (Gacia and Duarte, 2001). Similarly, the network of rhizomes and roots effectively binds the sediments deposited in the seagrass rhizospheres and prevents coastal erosion.

Physical and chemical conditions of the seagrass colonized areas constrain benthic microbial communities thereby influencing the processes of mineralization of organic matter and regeneration of nutrients in the coastal areas. Several species of seagrass have obligate microbial populations inhabiting their roots, leaves and

rhizomes (Ravikumar et al., 2010). Seagrass beds, therefore, play an important engineering role in controlling coastal biogeochemistry (Jones *et al.*, 1997). The input of organic matter and the accumulation of seagrass detritus in the sediments increase the amount of microbial substrates in the sediments (Gacia and Duarte, 2001).

Generally, bacteria mediate various nutrient cycling processes biologically by using their extracellular enzymes and thus help in initiating the food-web process. Among the various nutrient cycling processes, nitrogen and phosphorus cycles are important and the organisms involved in these processes are essential as nitrogen and phosphorus are the two major nutrients that support the life system in the earth (Touchette and Burkholder, 2000).

Nitrogen fixation also contributes significantly both to the nitrogen cycling in the sediments of tropical seagrass beds and sediment nitrogen fixation has been estimated to provide with more than 50% of plant nitrogen demand, whereas it tends to be much lower in temperate sediments, usually less than 12% (Welsh, 2000) or even <5% (McGlathery *et al.*, 1998). About one third of the nitrogen fixation activity in the rhizosphere sediments is directly associated with the roots and rhizomes, suggesting that nitrogen fixing bacteria colonize the roots (Nielsen *et al.*, 2001).With this background, present investigation was carried out to estimate the total heterotrophic bacteria (THB), nitrogen fixing, ammonifying and nitrifying bacteria from the rhizosphere soil of individual seagrass species and to assesse the rate of nitrogen fixation of different species of seagrass meadows of the Gulf of Mannar, India.

Materials and Methods

Seagrass samples [*Enhalus acoroides* (L.F.) Royle, *Halophila ovalis* (R.Br.) Hook, *Thalassia hemprichii* (Ehrenb.) Asch, *Cymodocea serrulata* (R.Br) Asch and Magnus, *Halodule uninervis* (Forsk.) Asch and *Syringodium isoetifolium* (Asch.) Dandy)] were collected from the shallow coastal waters of Pamban (8º 35'-9º 25' N latitude; 78º 08'-79º 30' E longitude) in the Gulf of Mannar region of Tamil Nadu, India. Species were identified in the field itself with the help of the field keys of Kannan and Thangaradjou (2006) and confirmed by following Ramamurthy *et al.* (1992), in the laboratory. Physico-chemical characteristics of rhizosphere soil of the different seagrass species were analyzed during the time of sampling (July 2008). Temperature, pH and Redox potential (Eh) of the rhizosphere soil samples were measured with the appropriate electrodes (pH 315i/SET, Germany.) Salinity of sediment samples was measured using a hand refractometer (Atago hand Refractometer, Japan). Rhizosphere sediment samples were analyzed for nutrients such as nitrogen, phosphorous and potassium following Kjeldahl method (Subbiah and Asija, 1956) and colorimetric method (Olsen *et al.,* 1954) respectively and the values were expressed in kg ha^{-1}.

Total heterotrophic bacteria (THB) and free living nitrogen fixing bacteria (NFB) were studied using Zobell Marine Agar (2216) and nitrogen free mannitol agar medium respectively. Ammonifying bacteria and nitrifying bacteria in the rhizosphere sediments of different seagrass species were enumerated, following the MPN method described by Chandramohan (1988). Experiments were conducted in triplicate.

Biomass was estimated using the quadrate (0.5m^2) method. All the seagrass materials found in the quadrate were collected and washed thoroughly with the habitat water to remove the debris. The samples were then treated with 5-10 % v/v orthophosphoric acid to remove the epiphytes and calcareous substances. They were washed several times with water to remove the traces of acid. Moisture from the samples was removed using blotting papers and the samples were weighed to get the total biomass. The mean of four quadrate sampling was considered for computing the seagrass biomass per square meter. Samples were then separated species wise and the below ground biomass was estimated. Rhizome and roots were treated as below ground biomass (Ramamurthey *et al.,* 1992).

Nitrogen fixation: Rhizome and root were detached from the seagrasses, after removing the attached sediments by washing with seawater. Roots were measured for the fresh weight and then gently rinsed with filter sterilized seawater. To ensure nitrogen fixation by the seagrass roots, they were surface sterilized for 15 sec. using 0.05% of hypochlorite solution so as to remove all the microbes associated with the root surfaces. Assay for nitrogen fixation (acetylene reduction assay) was carried out following the modified method of Smith and Hayasaka (1982).

Seagrass roots were incubated for 24 hr in sterilized seawater (15 ml) in 25 ml syringe bottle and with acetylene gas. After incubation, ethylene production was analyzed in gas chromatograph (Hewlett- Packed, Modal No- 5840 A, Splitless injector, Poropak- R, Stainless steel 1/8" width and 1 m length of Column, Flame Ionization detector) temperature of the injector was 100 ˚C and detector and column temperature was maintained 60 ˚C. Nitrogen carrier gas and hydrogen gas flow rates was maintained at 30 ml min^{-1}. Ethylene peak heights were compared with known standard concentrations. Ethylene production was converted to nitrogen fixed by a 3:1 molar ratio. Triplicate measurements were made in both filed and laboratory testing so as to get the standard deviations in the results. Biomass values recorded in the present study were used to convert nitrogen fixation by 1 g of individual seagrass species to nitrogen fixation by individual seagrasses per m^2.

Statistical analysis: Field and laboratory testings were done in triplicate so as to get the standard deviations in the results. Physico-chemical parameters and microbial characteristics recorded in the different seagrass samples were subjected to ANOVA and correlation test (SPSS 11.5) to find out significant variations and relation among the parameters between different seagrass species and within the individual seagrass species.

Results and Discussion

Physico-chemical characteristics of seagrass rhizosphere and non-seagrass soils are given in Fig.1. Temperature in the rhizosphere soil ranged from 28.81 to 29.98^0 C. Minimum was noticed (28.81^0 C) in *E. acoroides* rhizosphere soil and maximum was (29.98^0 C) noticed in *C. serrulata* rhizosphere soil. pH ranged from 6.5 to 7.6 with the minimum (6.5) in the *E. acoroides* rhizosphere soil and the maximum (7.6) in the *C. serrulata* rhizosphere soil. Redox potential (Eh) ranged from -17.41 to 22.03 mV. The minimum (-17.41 mV) in the *E. acoroides* rhizosphere soil and maximum (22.03 mV) in the *T. hemprichii* rhizosphere soil. Soil salinity ranged from 30.7 to 37.7 ‰ with minimum (30.7 ‰) in non-seagrass samples and maximum (37.7 ‰) was recorded in the *S. isoetifolium* and *E. acoroides* rhizosphere soil.

Major sediment nutrient values recorded in seagrass rhizosphere and non-seagrass are shown in Fig. 2. Nitrogen content in the rhizosphere samples ranged from 34.22 to 68.4 kg ha^{-1} with minimum in the non-seagrass soil and maximum in *T. hemprichii* rhizosphere soil. Similarly, phosphorus concentration ranged from 25.41 to 52.26 kg ha^{-1} with the minimum in the non-seagrass soil and the maximum in the *T. hemprichii* rhizosphere soil. Potassium content ranged from 104.25 to 153.3 kg ha^{-1} with minimum in the non-seagrass area and maximum in the *T. hemprichii* rhizosphere soil.

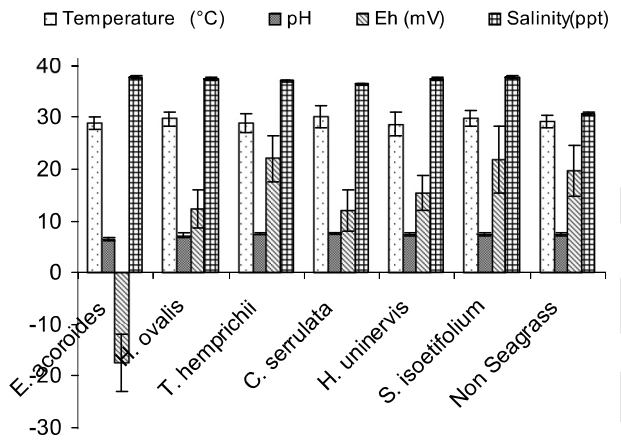

Fig.1: Characteristics of the seagrass rhizosphere and non-seagrass soil samples

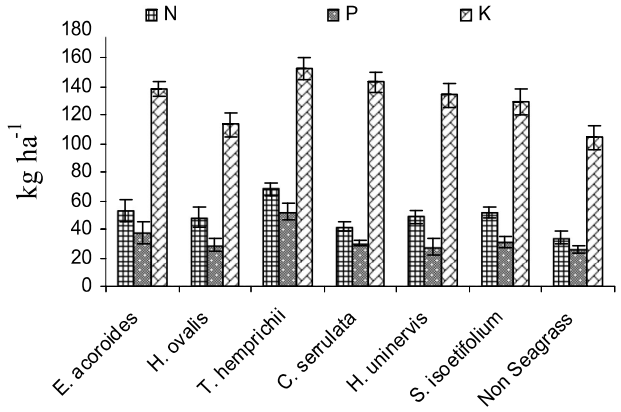

Fig.2: Nutrients recorded in seagrass rhizosphere and non-seagrass soil samples

The THB population in the rhizosphere soil samples ranged from 12.2 to 26.3 ×10^6 CFU g^{-1}. The minimum was recorded in non seagrass soil sample and maximum (26.3 ×10^6 CFU g^{-1}) in *T. hemprichii* rhizosphere soil. Population of nitrogen fixing bacteria ranged from 17.6 to 27.3 ×10^3 CFU g^{-1} with minimum (17.6 ×10^3 CFUg^{-1}) in the non-seagrass soil and maximum (27.3 ×10^3 CFU g^{-1}) in the *T. hemprichii* rhizosphere soil. Density of ammonifying bacteria ranged from 7.36 to 44.66 X 10^6 MPN g^{-1} with minimum in non-seagrass soil and the maximum in *T. hemprichii* rhizosphere soil. Nitrifying bacterial load was registered minimum (7.33 X 10^6 MPN g^{-1}) in the non-seagrass soil sample and maximum (42.33 X 10^6 MPN g^{-1}) in the *T. hemprichii* rhizosphere soil (Table 1).

Below ground biomass (BGB) ranged from 189.70 to 2603.49 g f.wt.m^{-2} with the minimum in *H. ovalis* and the maximum in *E. acoroides* (Table 2).

Nitrogen fixing rate in the roots of the seagrass species ranged from 0.166 to18.5 n mol g^{-1} d^{-1}. The minimum was (0.166 n mol g^{-1} d^{-1}) in *H. ovalis* and the maximum (18.5 n mol g^{-1} d^{-1}) in *T. hemprichii*. Nitrogen fixation rate ranged from 31.61 to 30529.07 n mol m^{-2} d^2, with minimum in *H. ovalis* and maximum in *T. hemprichii* (Table 2).

Dissolved organic compounds, volatile fatty acids, and dissolved carbohydrates are generally more in seagrass sediments compared to non-vegetated sites (Holmer and Nielsen, 1997; Burdige and Zimmerman, 2002) which indicates that there is more substrate available for the bacteria in the seagrass rhizosphere sediments for remineralizing processes. Major sediment nutrients such as nitrogen, phosphorus and potassium were found in higher concentrations, which may be mainly due to the discharge of domestic waste and solid wastes in the coastal area (Thangaradjou and Kannan, 2007).

Present investigation on THB, nitrogen fixing bacteria, ammonifying bacteria and nitrifying bacteria revealed that the various microbial populations showed significant relation in different seagrass rhizosphere soils. In general, the microbial load was higher in the seagrass vegetated region compared to non-seagrass region. Population density of THB recorded in the seagrass environment is higher when compared to the adjacent coral reef environment (Kannan *et al.,*1998) of the Gulf of Mannar. Abundance of seagrass biomass can promote the total heterotrophic population (Cotner *et al.,* 2000; Donnelly, 1999) thereby helping to decompose the biomass into particulate organic matter.

Though population density of THB showed significant variation (0.1% level) between different seagrass soil samples, the

Table- 1: Total heterotrophic bacteria (THB), Nitrogen fixing bacteria (NFB), Ammonifying and nitrifying bacteria recorded in seagrass rhizosphere and non-seagrass soil samples

Rhizosphere soil of seagrass	THB (CFU $g^{-1} \times 10^6$)	NFB (CFU $g^{-1} \times 10^3$)	Ammonifying bacteria (MPN g^{-1} $x10^6$)	Nitrate reducing bacteria (MPN g^{-1} x 10^6)
E. acoroides	18.63 ± 5.2	21.43 ± 3.86	16.1 ± 2.5	12.6 ± 2.18
H. ovalis	15.93 ± 3.5	20.34 ± 3.65	14.46 ± 2.05	14.03 ± 2.25
T. hemprichii	26.32 ± 4.6	27.34 ± 4.07	44.66 ± 3.53	42.33 ± 4.15
C. serrulata	21.35 ± 3.8	24.98 ± 3.63	32.76 ± 3.55	32.43 ± 3.8
H. uninervis	15.87 ± 3.4	21.52 ± 3.79	14.1 ± 1.86	15.1 ± 0.83
S. isoetifolium	18.82 ± 3.2	26.28 ± 3.77	15.93 ± 2.05	21.26 ± 2.48
Non Seagrass	12.21 ± 3.3	17.62 ± 3.64	7.36 ± 1.5	7.33 ± 1.76

Table- 2: Below ground biomass and Rate of nitrogen fixation by different seagrass

S.No	Seagrasses species	Below ground biomass (g f. wt. m^2)	Nitrogen fixation (n mol m^{-2} day^{-1})	Nitrogen fixation (n mol m^{-2} day^{-1})
1	*Enhalus acoroides*	2603.5 ± 21.8	3.5	9112.26
2	*Halophila ovalis*	189.7 ± 49.6	3.5	94.85
3	*Thalassia hemprichii*	1650.2 ± 84.9	55.5	91587.21
4	*Cymodocea serrulata*	923.3 ± 33.7	31.75	29312.56
5	*Halodule uninervis*	391.9 ± 36.6	16.125	6318.91
6	*Syringodium isoetifolium*	552.4 ± 45.5	2	1104.72

difference was found to be less which indicates that irrespective of species, presence of seagrass rhizosphere and supply of organic matter by the seagrass species promote bacterial growth in the seagrass vegetated areas than the non seagrass regions. This is mainly because of the seagrass production and organic carbon inputs. But, Lopez *et al*. (1995) reported that the bacteria and seagrass growth metabolism are inversely related, apparently due to the competition of microbes and seagrasses for inorganic nutrients.

Among the nitrogen fixing, ammonifying and nitrifying bacteria, there were significant variations between different seagrass species and non seagrass areas. ANOVA indicates that there is 5% level variation in nitrogen fixing bacteria while 0.1% level variation in ammonifying bacteria and 1% level variation in nitrifying bacteria. This indicates that depending upon the rate of nitrogen fixation, the presence of other groups of bacteria would vary. All these bacteria were recorded in lesser densities in the non seagrass areas than the seagrass vegetated areas. In turn, microbes can enhance the uptake of ions by plant roots and promote root growth by producing growth regulators (Dubey, 1998).

Nitrogen fixation is one of the significant phenonmena in nutrient cycling in any system including the seagrass ecosystem, which provides with substantial inputs of nitrogen in the root zones of a variety of marine macrophytic communities including tropical seagrass (Capone and Taylor, 1980), salt marsh and mangrove (Zuberer and Silver, 1978) systems. Present study recorded different rates of nitrogen fixation in different species of seagrasses. Nitrogen fixation rate by the rhizomes and roots of different seagrass species ranged from 0.166 to18.5 n mol $g^{-1}d^{-1}$. This finding of nitrogen fixation rates in the different seagrass species beds is in agreement with previous studies on several seagrass species from different locations of the world (Capone, 1982; Capone and Taylor, 1980).

Root system of *Z. marina,* which is generally found in anoxic sediments, are presumed to provide with a conduit for the transport of gases and release of organic compounds into the surrounding sediments (Wetzel and Penhale, 1979; Wood and Hayasaka, 1981). The plants thereby function to create a rhizosphere effect conducive for enhanced bacterial activities in the vicinity of the plant roots. It is worth mentioning that higher rates of N_2 fixation have been recorded in association with intact rhizosphere sediments of *Z. marina* communities rather than in the adjacent non-vegetated areas (Capone, 1982).

Present study showed that the roots and rhizomes of the individual seagrass species (without rhizosphere sediments) are fixed with different rates of nitrogen. At the sametime, the values recorded in present study were lower when compared to the previous studies conducted with the seagrass rhizosphere sediments (Patriquin and Knowles, 1972; Capone and Taylor, 1980; O'Donohue *et al.,* 1991). This indicates that the rhizosphere microbes play a major role in the nitrogen fixation of the seagrass meadows and the contribution from seagrasses is relatively very less.

Nitrogen fixation in the individual seagrass species was worked out per unit area (m^2) by taking the below ground biomass. This indicated that *H. ovalis* recorded the minimum of 31.62 n mol m^{-2}

d^{-1} followed by *S. isoetifolium* (368.24 n mol m^{-2} d^{-1}), *H. uninervis* (2106.3 n mol m^{-2} d^{-1}), *E. acoroides* (3037.4 n mol m^{-2} d^{-1}), *C. serrulata* (9770.85 n mol m^{-2} d^{-1}) and *T. hemprichii* (30529.07 n mol m^{-2} d^{-1}) in the increasing order. In an average, 7640.58 n mol m^{-2} d^{-1} of nitrogen is fixed per m^2 in this part of Bay of Bengal by the seagrasses alone. It has been estimated that 55.15 sq.km of seagrasses are found to be distributed in the Gulf of Mannar region alone (Susila, 2009).This means that 42,13,77,987 n mol d^{-1} (5.902 kg d^{-1}) of nitrogen is fixed by the seagrasses of the region. This excludes the microbial nitrogen fixation in this region, which could be much higher then the estimated seagrass fixation. However, in the case of seagrass nitrogen fixation, one has to confirm that whether the seagrasses are doing nitrogen fixation or the endophytic microbial communities present in the rhizome are involved in the processes.Present study has clearly shown that the seagrass rhizosphere is having higher densities of bacteria which could play a major role in determining the local nitrogen cycling process.

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