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STUDIES ON CERTAIN NITROGEN CYCLE BACTERIA IN THE PRAWN CULTURE FIELDS OF KERALA

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Introduction

In the Southwest coast of India, certain paddy fields found in the vicinity of Cochin Backwater, traversed by tides, are used for a traditional prawn culture. In the perennial fields, prawns alone are cultured throughout the year and in the seasonal fields known as "Pokkali fields", prawns and paddy are cultivated on a rotation basis depending upon the prevailing ecological conditions.

The productivity of these brackishwater fields or ponds depends to a great extent on the physicochemical properties of the water and sediment, particularly the availability of the nutrients such as nitrogen and phosphorus and the production of prawn food organisms. The salinity of the water in these ponds does not remain constant throughout the year, but fluctuates during the different seasons of the year, attaining maximum values during the summer and minimum during the monsoon. Such changes in the water salinity during the different seasons are likely to bring about profound influence on the bacteria associated with the biogeochemical transformation of the nutrients. A series of chemical and biochemical reactions continuously takes place within the bottom soil resulting in the release of nutrients to the overlying water which are mediated by specific groups of micro-organisms including bacteria. The dynamics of these processes influence greatly the growth and population of micro-organisms in these ponds.

Objectives

Despite the extensive studies on the Cochin Backwater detailed investigations on the microbial ecology of these prawn culture ponds are lacking. Recognising the importance of nitrogen in the productivity of aquatic ecosystems and the involvement of various groups of micro-organisms in the turnover of nitrogenous matter, the present study was carried out on the nitrogen cycle bacteria in selected perennial and seasonal prawn ponds.

Material and methods

The investigation was carried out during 1982 - 1985, at Narakkal (76°14'E - 10°03' N), a fishing hamlet in the Vypeen Island, about 15 km from Cochin. Four ponds were selected for the study : two of which are perennial prawn culture systems (ponds A and B) located within the premises of the prawn culture laboratory of the Central Marine Fisheries Research Institute and the other two are seasonal prawn culture fields (ponds C and D), where prawns and paddy are cultivated during the intermonsoon (October to May) and monsoon (June-September) periods respectively.

Water and sediment samples were collected fortnightly from four fixed sites from each pond for enumeration of the nitrogen cycle bacteria and for monitoring the environmental parameters. Samples collected from the four sites of the same pond were pooled, thoroughly mixed and representative samples were taken. Water samples for bacteriological studies were collected aseptically in sterilized BOD bottles of 300 ml capacity. Sediment samples were collected with a impact corer (100 cm x 4 cm diameter) made of perspex material. Sediment from the upper layer of 10 cm was aseptically removed with a sterile spatula and collected in sterilized glass bottles.

Enumeration of bacterial groups involved in the nitrogen cycle, such as aerobic total heterotrophs, proteolytic, ammonifying, nitrifying (ammonia - oxidizers), denitrifying and nitrogen-fixing, were carried out for two years (October 1982 to September 1984) using standard microbiological methods with a view to study the seasonal variations in their abundance.

Environmental parameters such as temperature, pH, Eh, salinity, nitrite, nitrate, ammonia, dissolved oxygen, organic carbon, total nitrogen and total phosphorus were regularly monitored from all the ponds to study their effect on the distribution and abundance of the bacterial groups in water and sediment. Studies were also carried out on the *in situ* bacterial nitrogen fixation rate fortnightly, using micro-kjeldahl method and the effect of environmental factors on nitrogen fixation was computed.

Linear multiple regression analysis of the data was carried out to determine the influence of the various environmental factors on the distribution of each of the bacterial groups, as well as on the aerobic bacterial nitrogen fixation rate in the water and sediment in the ponds.

Thirty nitrogen fixing *Azotobacter* strains isolated from water and sediment were identified based on their morphological, physiological and biochemical characteristics. Experimental studies were made to elucidate the effect of salinity on the growth and nitrogen fixing ability of thirty isolated strains of *Azotobacter* in the laboratory. Effect of incubation time on nitrogen fixing ability of the thirty strains of *Azotobacter* was also studied by sampling on the 15th, 30th and 45th days of incubation.

Experimental studies were also carried out on nine selected *Azotobacter* strains (*Azotobacter chroococcum* - 3 strains; *A. vinelandii* - 3 strains; *A. beijerinckii* - 3 strains) to evaluate the effect of pH, certain vitamins (cyanocobalamine, biotin, ascorbic acid and thiamine) and trace elements (cobalt - $CoCl_2.6H_2O$; copper-$CuSO_4.5H_2O$; zinc - $ZnSO_4.7H_2O$ and iron - $FeCl_3$) on the growth of the strains. Data obtained from the experiments were statistically analysed, following standard procedures, to ascertain if there were any significant influence of the test parameters on the growth of the bacteria. The optimum requirements for pH, selected vitamins and trace elements for maximum growth of the 9 strains have also been found out.

Results and discussion

Environmental data showed that the physico-chemical conditions, in general, remain more or less steady in both the perennial and seasonal culture systems during the premonsoon period, but major noticeable variations occurred during the monsoon and postmonsoon months as a result of heavy rainfall. The physicochemical parameters, generally, recovered to their premonsoon level by the end of postmonsoon period. Freshwater run-off from catchment areas and local precipitation during monsoon and postmonsoon had enormous dilution effect resulting in decreased salinity of the water.

Water temperature and Eh of the water and the sediment showed decrease during the monsoon and postmonsoon periods. But the quantum of decrease in temperature was considerably higher for the seasonal ponds. The pH of water and sediment was quite stable in the perennial ponds, with a marginal decrease during the monsoon period; whereas considerable increase of pH occurred during monsoon and postmonsoon periods in the seasonal prawn culture systems. The Eh in both the systems behaved in a similar fashion throughout the period of study, except for more reduction in the seasonal prawn culture fields during the premonsoon season. Depletion of dissolved oxygen content was more pronounced during the premonsoon season in the seasonal ponds as compared to the perennial ponds. The considerable increase in nitrite, nitrate and ammonia levels in water observed during the monsoon and early premonsoon periods resulted in increased primary production, and consequently the culture systems became more alkaline as evident from high pH values during the same period.

Total phosphorus and organic carbon showed increased values during the postmonsoon and in the early monsoon, also in certain months of early premonsoon season. Nitrogen level was more in the premonsoon and monsoon than in the postmonsoon season. The dissolved oxygen level showed more or less uniform values, but for a slight decrease during monsoon.

During most part of the investigation, in all the ponds, sediment was found to harbour more number of bacteria than the water. This is attributed to various factors such as gradual deposition of bacteria from the overlying water, increased propagation of the bacteria indigenous to the sediment; settlement of the particulate substrates during the process of sedimentation. In general, seasonal ponds were found to be more bacterial productive than the perennial ponds due to the decomposition of more organic matter and disintegration of paddy-stumps after harvesting the paddy, and also due to the application of organic manure for the paddy crop.

Heterotrophic and proteolytic bacteria occurred in greater numbers during the permonsoon season than the monsoon and postmonsoon seasons, but the ammonifiers were more in the postmonsoon season than the monsoon and the premonsoon seasons in both the culture systems. Nitrifiers (ammonia-oxidizers) did not show any consistent trend during the period of investigation. Denitrifiers were abundant in premonsoon than the postmonsoon and monsoon seasons. In general, total heterotrophs, proteolytic and ammonifiers were more abundant than the nitrifiers, denitrifiers and nitrogen fixers in all the ponds.

The linear multiple regression analysis of the data revealed that water temperature, dissolved oxygen, salinity, nitrate, nitrite, ammonia, water pH, sediment pH and total phosphorus had a significant influence on the distribution of total heterotrophs, proteolytic, ammonifying, nitrifying, denitrifying and nitrogen fixing bacteria in both the sediment and the overlying water of all the ponds, except for minor changes occurred in some of the groups in certain ponds. Water Eh, sediment Eh, organic carbon and total nitrogen had not shown any significant influence on the different groups of nitrogen cycle bacteria, except for *Azotobacter*. Sediment and water Eh and organic carbon had significant influence on the distribution of *Azotobacter* besides other parameters.

The abundance of ammonifiers, nitrifiers and denitrifiers was negatively influenced by water and sediment pH and NH_3-N in both the water and the sediment. Water temperature had a negative influence on the distribution and abundance of denitrifiers in most of the ponds; ammonifiers in ponds B and C, and proteolytic in pond B sediment. An inverse relationship was found for ammonia and heterotrophic, proteolytic, ammonifying and nitrogen fixing bacteria in pond C. Salinity also had a negative influence on the distribution of total heterotrophs, proteolytic and nitrogen fixers only in pond B water and sediment.

Partial regression analysis indicated that the abundance of *Azotobacter* is mostly influenced by nitrogen. Phosphorus and NO_2-N compared to other parameters like dissolved oxygen, water and sediment Eh and water temperature.

Nitrogen fixation in the ponds was more during the premonsoon season than the postmonsoon and monsoon seasons during the first year; but in the second year, more nitrogen fixation occurred in the postmonsoon than the premonsoon and monsoon seasons. Besides, nitrogen fixation was found to be more in the seasonal ponds than the perennial ponds, and nitrogen fixation rates during the first year was superior to that of the second year. Multiple regression analysis showed that bacterial nitrogen fixation in water was significantly influenced by water temperature, water pH, sediment pH, dissolved oxygen, salinity and NO_2-N and in the sediment by factors like sediment pH, salinity, nitrate and total phosphorus in all the ponds.

Morphological, physiological and biochemical characterisation of 30 different free-living aerobic nitrogen fixing *Azotobacter* strains was carried out to identify them upto the species level. Of these : 13 strains belonged to *A. chroococcum* : 9 strains to *A. vinelandii* and 8 strains to *A. beijerinckii*.

Experimental studies were carried out on all the 30 strains to assess their nitrogen fixing capacity for a period of 45 days. Relative nitrogen fixation efficiency of *Azotobacter* strains revealed that good nitrogen fixation occurred even upto the incubation period of 45 days in *A. beijerinckii* and *A vinelandii;* but only in one strain of *A. chroococcum* (Azc6) a decline was noticed in nitrogen fixation after 30 days of incubation. Nitrogen fixation was found to be the highest in two strains belonging to *A. beijerinckii* which fixed 11.74 and 11.42 mg NH_3-N/100 ml after the maximum incubation period of 45 days, followed by two strains belonging to *A. chroococcum* which fixed 10.6 mg NH_3-N/100 ml and 10.70 mg NH_3-N/100 ml of medium.

Salinity showed a significant influence on the growth and nitrogen fixation in all the strains of *Azotobacter*. Salinity leveled above 40 ppt and below 10 ppt in general, resulted in reduced growth of *A. vinelandii* and its nitrogen fixation was limited at salinity levels < 15 ppt and > 50 ppt. The estimated optimum salinity levels for growth ranged from 23.52 to 30.82 ppt and nitrogen fixation from 27.17 to 40.83 ppt for the 9 different strains of *A. vinelandii*. Salinity levels below 5 ppt and above 50 ppt were found to be non-conducive to the growth of *A. croococcum* and its nitrogen fixation was depressed at salinity levels < 10 ppt and > 45 ppt. The estimated optimum salinity levels for growth ranged from 23.98 to 27.85 ppt and for nitrogen fixation from 24.49 to 35.88 ppt for the 13 different strains of *A. chroococcum*. For *A. beijerinckii* salinity level below 5 ppt and above 40 ppt were detrimental and their nitrogen fixation also was very low at salinity levels < 20 ppt and > 50 ppt. The estimated optimum salinity levels for growth ranged from 24.01 to 29.96 ppt and for nitrogen fixation from 30.15 to 39.78 ppt for the 8 different strains of *A. beijerinckii*.

pH showed a significant influence on the growth and nitrogen fixation in all the strains of *Azotobacter*. The estimated optimum pH level for growth varied from 7.75 to 8.82 and for nitrogen fixation from 6.64 to 6.81 in *A. vinelandii* strains. In *A. chroococcum* strains optimum growth was at pH 7.59 to 8.28 and nitrogen fixation at pH 7.79 to 7.91. However, in *A. beijerinckii* strains optimum growth was at pH 7.10 to 7.67 and nitrogen fixation at pH 7.12 to 7.52. In general, pH below 6.5

and above 8.5 resulted in reduced growth and nitrogen fixation of the *Azotobacter*.

The selected trace elements *viz.* zinc, iron, cobalt and copper and vitamins had a significant influence on the growth of *Azotobacter* strains. The optimum levels of trace elements ranged from 219.3 to 391.0 µg/l for cobalt, from 132.32 µg/l to 463.43 µg/l for zinc, from 260.05 to 420.87 µg/l for iron, and from 251.81 to 378.26 µg/l for copper for the nine selected strains. The optimum levels of vitamins for growth ranged from 78.25 to 172.36 µg/l of ascorbic acid, 11.42 to 21.91 µg/l of biotin, 14.38 to 43.39 µg/l of thiamine, and 4.26 to 5.57 µg/l of cyanocobalamine.

Conclusions

The present investigation clearly revealed the considerable variation in the relative abundance of selected nitrogen cycle bacterial groups between ponds, between sediment and water and between the different seasons of the year. A number of environmental variables had significantly contributed to the observed variations indicating that the microbial productivity in these prawn culture systems depends largely upon the physical and chemical conditions of the water and the sediment.

Substantial differences were observed in the efficacy of the thirty *Azotobacter* strains to fix nitrogen. Among the 3 species, strains of *A. beijerinckii* are the most efficient with relatively greater nitrogen fixation rates. Most of the *A. vinelandii* strains preferred a salinity range of 15 to 35 ppt for their optimal growth, though they were obtained from ponds with salinities ranging from 1.28 to 36.48 ppt. This indicates that the genera existed in the brackishwater were comprised of strains originated from the estuarine and marine environments and perhaps include halo-tolerant freshwater forms. In certain strains, while the optimum growth occurred at one salinity level its maximum nitrogen fixation was at another salinity level. These results suggest that growth efficiency and nitrogen fixation efficiency of the *Azotobacter* need not have a direct relationship and that the strains require specific salinity requirements for growth, which is independent of the need for nitrogen fixation.

From the present observations it is clear that the optimum pH for the growth and nitrogen fixation of *Azotobacter* was near or slightly above neutral.

Vitamin and trace element studies revealed that all the strains require optimal levels of cyanocobalamine, thiamine, biotin, vitamin C, cobalt, zinc, iron and copper for normal growth and propagation.