Amino Acids in Marine Environment: Assesing the Role of Bacteria

A Thesis Submitted to Goa University for the Award of

Degree of

Doctor of Philosophy

In

Microbiology

By

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Research Guide

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Goa University

Taleigao Goa

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Certificate

This is to certify that the thesis entitled “Amino acids in marine environment: assessing the role of bacteria” submitted by *Miss Loreta N. Fernandes* for the award of the degree of Doctor of Philosophy in Microbiology is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any university or institution.

Place: Dona Paula

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Statement

As required under the University ordinance 0.19.8 (vi), I state that the present thesis entitled ***"Amino acids in the marine environment: assessing the role of bacteria"*** is my original contribution and the same has not been submitted for any other degree or professional qualification on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been provided to the funding agencies and the suggestions, if any, have been duly incorporated.

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Loreta Fernandes

Dedicated to my Parents

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List of Abbreviations used

AA Amino acid

AFDW Ash Free Dry Weight

Ala Alanine

Arg Arginine

Asp Aspartic acid

β-Ala beta-alanine

BOB Bay of Bengal

BSS Basal salt solution

BSS-GYP Basal salt solution with glycose, yeast extract, and peptone

C/N ratio Carbon/nitrogen ratio

D-AA-C D-amino acid-carbon

D-AA-N D-amino acid-nitrogen

DAPI 4, 6- Diamidino-2-phenylindole

DI Degradation Index

DOM Dissolved organic matter

dw Dry weight

γ-ABA gamma-amino butyric acid

Gly Glycine

Glu Glutamic acid

HCl Hydrochloric acid

HPLC High performance liquid chromatography

IBLC N-Isobutyrl-L-cysteine

Ile Isoleucine

L-AA L-amino acid

L-AA-C L-amino acid-carbon

L-AA-N L-amino acid-nitrogen

Leu Leucine

Lys Lysine

NaOH Sodium hydroxide

N Nitrogen

OM Organic matter

OPA O-phthaldialdehyde

Orn Ornithine

PCA Principal Component Analysis

PGN Peptidoglycan

PG-AA-N Peptidogycan-amino acid-nitrogen

Phe Phenylalanine

POC Particulate organic carbon

POM Particulate organic matter

PON Particulate organic nitrogen

SEM Scanning Electron Microscopy

Ser Serine

SPM Suspended particulate matter

SR Sedimentation rate

TBC Total bacterial count

Thr Threonine

TOC Total organic carbon

TN Total nitrogen

TPN Total particulate nitrogen

Tyr Tyrosine

Val Valine

ZMA Zobell marine agar

Chapter 1

General Introduction

1.1. Introduction

Marine ecosystems are among the largest of Earth's aquatic ecosystems. Marine waters cover approximately 71 % of the Earth's surface with average water depth of 3.8 km with a volume of 1370 x 10^6 km^3. Marine waters are found to generate 32 % of the world's net primary production (Alexander and David, 1999). Primary producer such as phytoplankton's occurring in the euphotic zone are the main source for the organic matter in marine environment termed as autochthonous source. Besides this, allocthonous organic matter brought in by the rivers and estuaries into the coastal region, and in turn transported to deeper regions of marine environment is another important source of organic matter. Aeolian transport is yet another source of particulate organic matter to deep sea environments. Organic matter from these sources is degraded by the heterotrophic bacteria present in the water column and sediment. In order to carry out key ecosystem processes such as primary production and decomposition of organic matter by microorganisms, nitrogen availability as nutrient plays an important role (McCarthy and Carpenter, 1983; Hecky and Kilham, 1988; Anita et al., 1991). However, nitrogen is the limiting nutrient in marine ecosystems. In the biosphere, nitrogen (N) occurs in several different forms and oxidation states, with organic and inorganic forms exhibiting wide range of reaction/transformation/transport pathways (Carpenter and Capone, 1983; Kirchman, 2000a). The earth's atmosphere contains approximately 80 % of nitrogen, thus making it a largest pool of nitrogen. However, the atmospheric nitrogen is of little use, thus leading to the scarcity of usable nitrogen. In order to be useable by the organisms, the atmospheric nitrogen has to undergo numerous transformations in a cyclic manner, termed as nitrogen cycle.

1.2. Marine nitrogen cycle

Nitrogen cycle (N) is composed of multiple transformations of nitrogenous compounds, catalyzed primarily by metabolically diverse range of microorganism (Zehr and Ward, 2002; Hulth et al., 2005). At the ocean surface, the atmosphere nitrogen gas (N_2) present in the environment as "free nitrogen", dissolves into seawater. Thereby, the nitrogen gas is the most abundant form of nitrogen in the ocean; however this free nitrogen cannot be utilized by most *in-situ* organisms. A diverse set of microorganisms, convert dissolved nitrogen gas into a much more useable form, known as ammonium (NH4+) through a process known as "nitrogen fixation" (Postgate, 1982; Young, 1992) (Fig. 1.1). Nitrogen fixation is the most crucially important step in nitrogen cycle, without it, very little nitrogen would be available for thousands of other organisms that live near the ocean surface. Microorganisms mostly consume ammonium, the form of nitrogen, by a process termed as "assimilation" (Fig. 1.1). Similarly, some other marine microorganisms are found to assimilate nitrite and nitrate. Thus, resulting in the incorporation of the nitrogen into the cells of living organisms. However, upon the death and decomposition of these microorganisms, ammonium and tiny particles containing particulate organic nitrogen (PON), and dissolved organic nitrogen (DON) are released into the surrounding seawater termed as "fixed" nitrogen. Some microorganisms present in the seawater convert ammonium to nitrite (NO_2^-) and then nitrite to nitrate (NO_3^-). This two-step process is known as "nitrification" (Fig. 1.1).

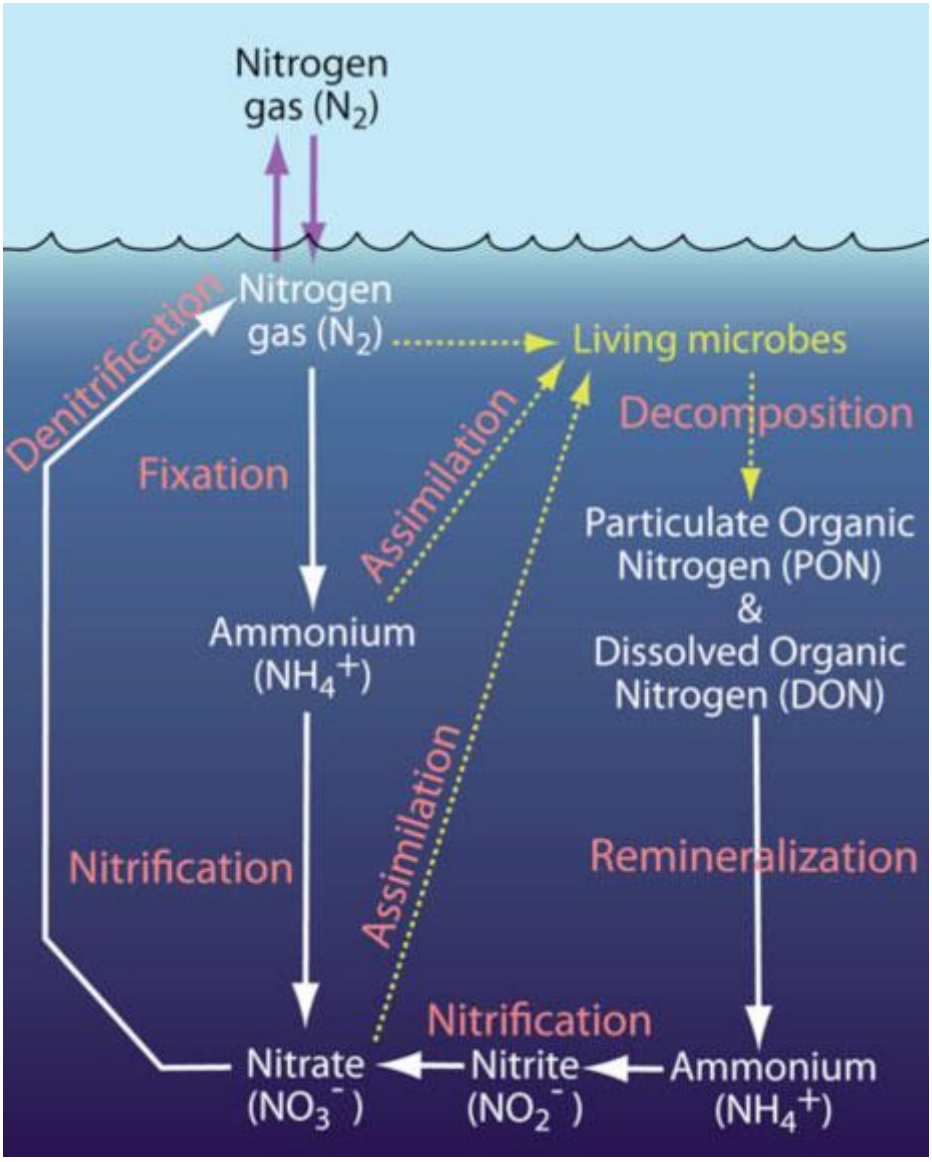

Figure 1.1. Schematic view of marine nitrogen cycle (picture is taken from website cmore.soest.hawaii.edu/cruises/biolines/nitrogen.htm)

The result of this process is that nitrate is released into the ocean. Numerous microorganisms consume particulate organic nitrogen and dissolved organic nitrogen, thereby converting some of the nitrogen back to ammonium through a process called as "remineralization" (Fig. 1.1). "Denitrification" is the process by which some microorganisms convert nitrate and nitrite back to nitrogen gas and thereby, releasing it into atmosphere in complex cyclic manner (Knowles, 1982). Denitrification is generally acknowledged as the main sink for available nitrogen in marine environments (Seitzinger, 1988).In the marine environment, the naturally occurring microorganisms can utilize various forms of nitrogen for their growth. Although, the atmospheric nitrogen is abundant, however its importance as nitrogen source to microorganisms is restricted. This is due to high energy and numerous set of enzymes required for converting the atmospheric nitrogen (Falkowski, 1983; Coffin, 1989; Kirchman, 1994; Kroer et al., 1994; Kirchman, 2000b; Zehr and Ward, 2002). Further, limited group of organisms such as cyanobacteria and some heterotrophic bacteria are able to fix the atmospheric nitrogen (Herbert, 1999). In the marine environment there is abundant amount of fixed nitrogen (Veuger, 2006), elemental constituents of eukaryotic tissues and prokaryotic cell walls, and is an integral component of amino acids, proteins, and nucleic acids. This fixed nitrogen in the form of amino acids requires less energy and thus can be directly utilized by microbial community.

1.3. Amino acids

Amino acids, the building blocks of protein in living organisms, and second largest molecules after carbohydrates present as fixed nitrogen are found abundantly in marine environments. Amino acids are a highly preferred source of nitrogen by the microorganisms since it can be directly incorporated into the microbial biomass with

little or no conversion (Veuger, 2006). Amino acids are molecules containing an amine group (-NH_2), a carboxylic group (-COOH) and a side chain (R) that is specific to each amino acid (Fig. 1.2). The carbon atom next to the carboxyl group is called the alpha–carbon and amino acids with a side-chain bonded to this carbon are referred to as alpha-amino acids.

Amino acids are classified into four groups based on the properties of their side chain (R) (Fig. 1.3):

1) Non polar, hydrophobic: Alanine, Valine, Leucine, Isoleucine, Phenylalanine, Methionine

2) Polar Acidic: Aspartic acid and Glutamic acid

3) Polar Charged: Glycine, Serine, Threonine, Tyrosine

4) Polar Basic: Arginine and Lysine

These primary amino acids are observed in abundant concentration in the organic matter of marine environment.

In nature the amino acids occur in three general structures namely zwitterions, isoelectric point, and isomerism. The later structure is described in more detail below:

Isomerism involves compounds whose molecules differ in the way their atoms are arranged in three-dimensional spaces. Such isomers are refered to as stereoisomers. There are two kinds of stereoisomers, enantiomers and diastereoisomers.

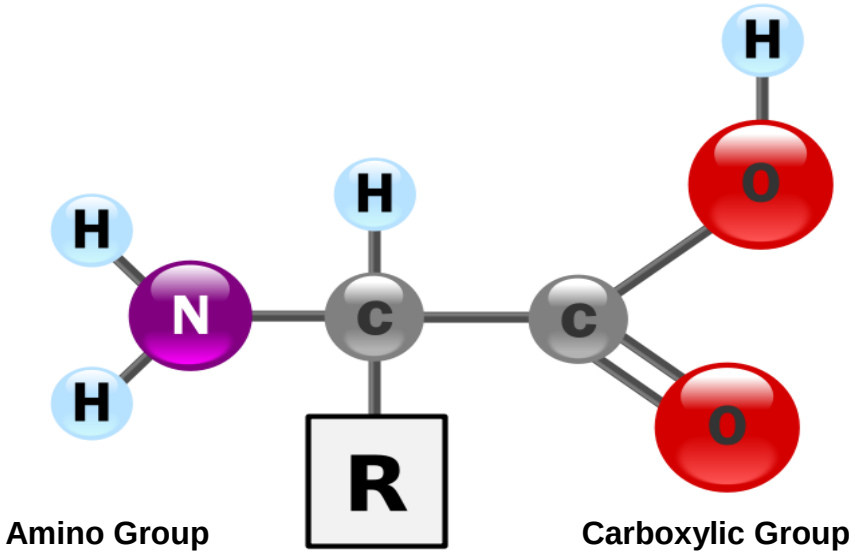

Figure1.2. General Structure of an alpha-amino acid (taken from File: AminoAcidball.svg, From Wikipedia)

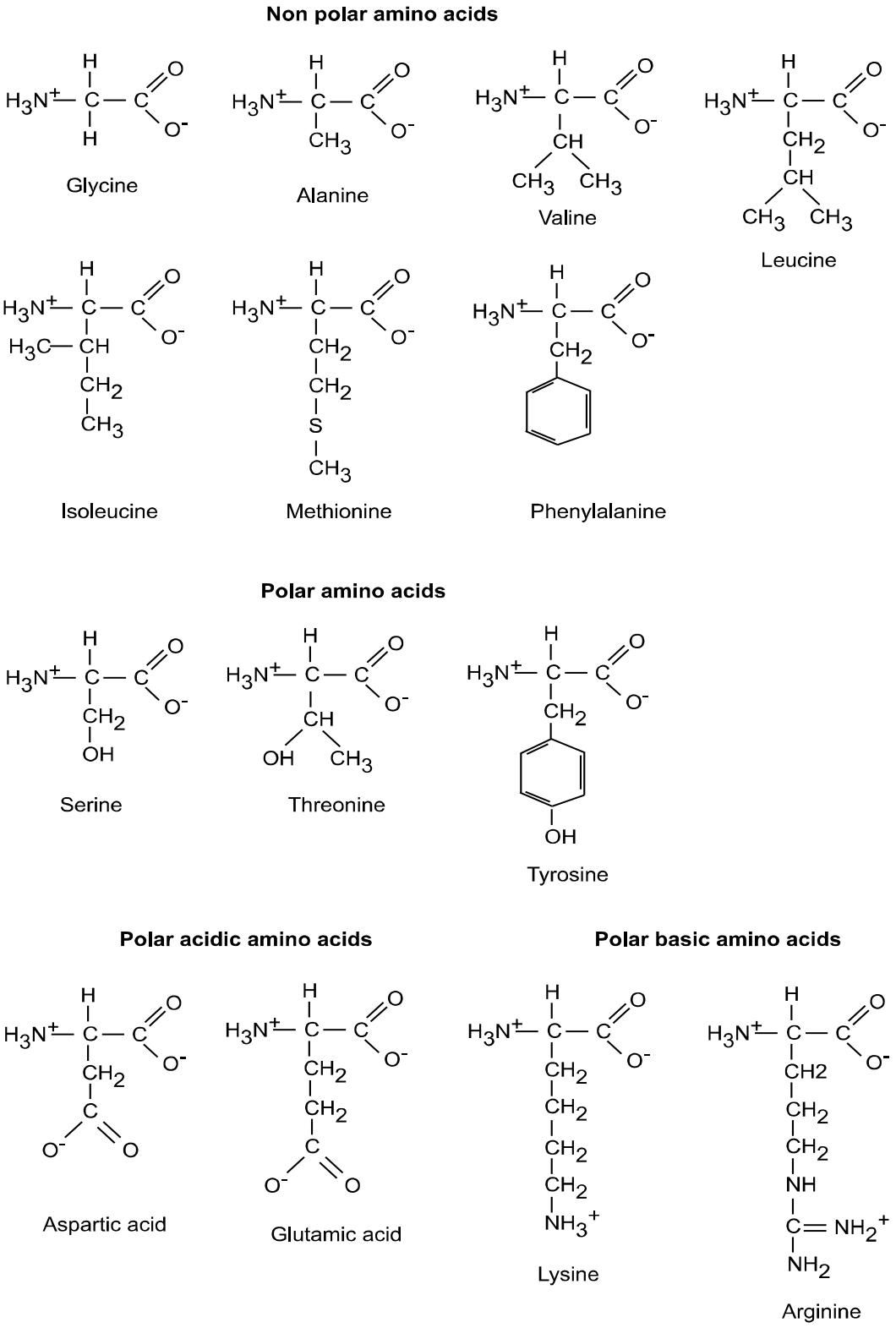

Figure1.3. Structure of primary L-amino acids detected in the organic matter

a) Enantiomers are compounds whose molecules are mirror images of each other and whose mirror images do not superimpose when are laid on top of one another. They differ only in the way they affect plane-polarized light as it passes through the isomers.

b) In contrast, diastereoisomers have different physical properties as they are not mirror images of one another (Garrette and Grisham, 2005).

Each isomer of the pair is capable of rotating plane-polarized light. One isomer rotates light to the right, while the other towards the left for the same number of degrees. Moreover all other physical properties are exactly similar. One enantiomer will be configured right-handed (*R*) and the other will be configured left-handed (*L*). Thus stereoisomers are found to exhibit a property known as chirality. A term chiral is used when the α-carbon is attached to four different groups (Fig. 1.2).

All amino acids present in protein, except glycine, are chiral molecules, having a single asymmentric carbon atom. In nature these amino acids can occur as L- and D-form and known as optical isomers. Moreover, they posses similar chemical and physical properties, but differ in the way they rotate the plane-polarized light in equal and opposite direction (Fig.1.4 and Fig. 1.5). They are the mirror images of each other; however they cannot be superimposed on the other. L-amino acids are found in all proteins, while D-amino acids usually occur in some enzyme, macromolecules, and cell wall of bacteria.

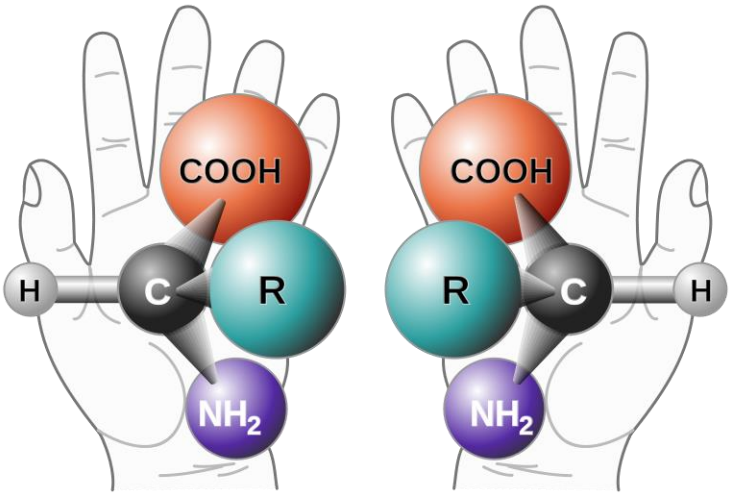

Figure1.4. Stereoisomer of amino acid: L-amino acid and D-amino acid (taken from: www.astrobio.net)

1.4. Sources of amino acids

Amino acids (including proteins, polypeptides, and combined and free amino acids) are the major forms of nitrogen and a component of organic matter of all organisms (Parsons et al, 1977). In nature, amino acid occurs in two forms, L-amino acids (L-AA), and D-amino acids (D-AA), known as stereoisomer. Phytoplankton is the main source for L-amino acids in the marine environments. Nitrogen in L-amino acids accounts for 42–72 % of total nitrogen in marine planktons and 40–65 % of sinking particulate organic nitrogen (Degens and Mopper, 1976; Lee and Cronin, 1982). In the marine environment, L-amino acids account for 31– 80 % of the organic nitrogen (Lee and Cronin, 1982; Cowie and Hedges, 1992; Wang et al., 1998). L-amino acids in living or dead plankton are among the most labile fractions of bulk organic matter and their degradation supports microbial production and regeneration of inorganic nitrogenous nutrients (Middelboe et al., 1995; Burdige and Zheng, 1998; Stepanauskas and Leonardson, 1999). Thus, L-amino acids play a key role in the biogeochemical cycling of organic matter. Moreover, the amino acid composition of most living organisms is remarkably uniform. These highly functionalized substances exhibit contrasting solubilities, charges, and degradation products (Cowie and Hedges, 1992b) as well as distinct compositional trends with degradation (Cowie and Hedges, 1994). Therefore, L-amino acids can be used as proxies to indicate organic matter degradation state.

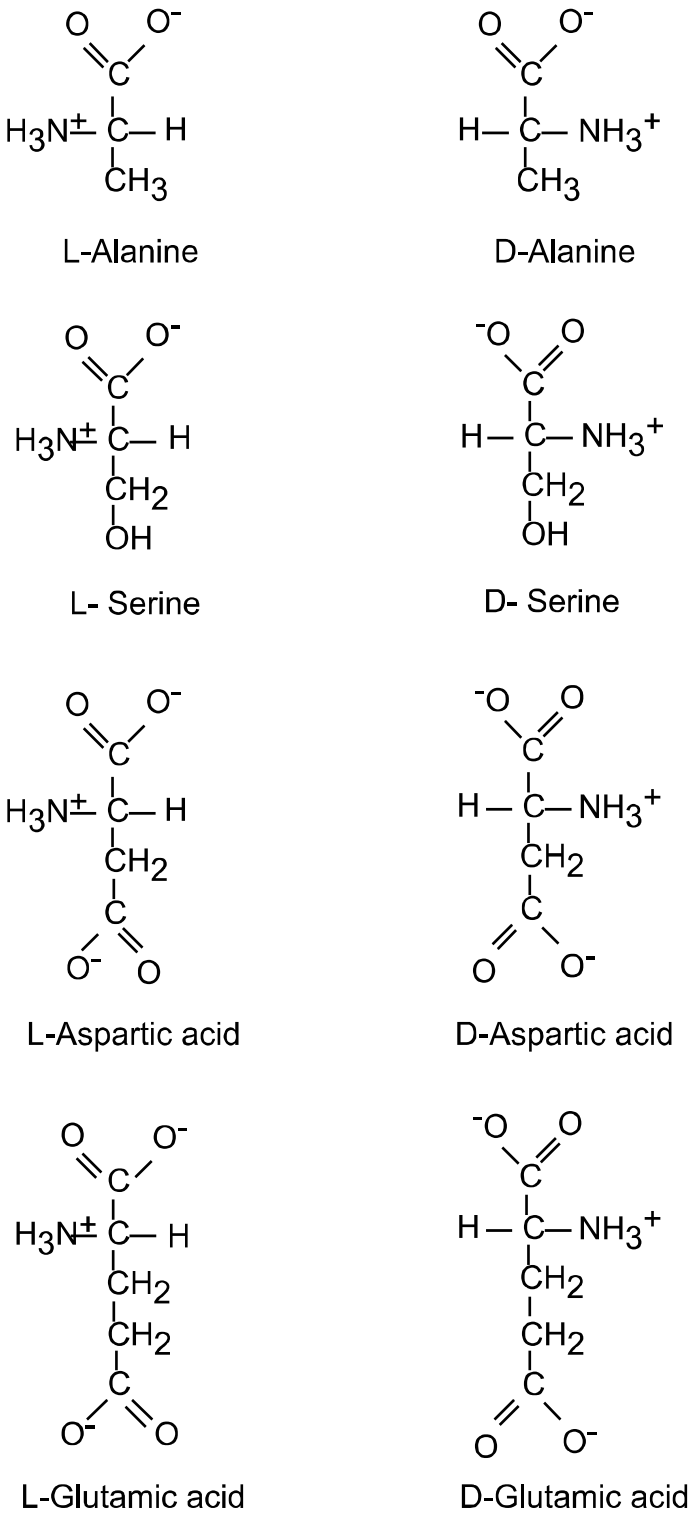

Figure 1.5. Structure of some L-and D-amino acids detected in bacteria and organic matter produced by bacteria

Heterotrophic bacteria play a critical role in the transformation and mineralization of organic matter in aquatic and terrestrial environments. The bacterioplankton plays important roles in cycling of carbon and nitrogen in marine environments (Azam et al., 1983; Cole et al., 1988; Ducklow and Carlson, 1992). Freshly produced organic matter is enriched in labile components, which are readily utilized by heterotrophic microbes (Cherrier et al., 1996; Amon et al., 2001; Meon and Kirchman, 2001). It is estimated that about half of the photosynthetic production in the ocean is processed by heterotrophic bacteria in the microbial loop (Ducklow 2000). As bacterial decomposition progresses, the labile components are selectively removed, resulting in the regeneration of inorganic nutrients and a relative enrichment of less reactive components (Wakeham et al., 1997; Benner, 2003; Lee et al., 2004). In the aquatic environments, heterotrophic bacterial production is often 10–20% of primary production (Cole et al., 1988; Ducklow, 2000). Recent studies have reported that beside the bacterial degradation of organic matter, molecules of bacterial origin have been observed in the degraded organic matter (McCarthy et al., 1998; Nagata and Kirchman, 2001; Benner and Kaiser, 2003). These bacterially derived compounds are found to contribute significantly to the pool of organic nitrogen in marine waters/ environments (Tanoue et al., 1995; McCarthy et al., 1998; Nagata and Kirchman, 2001; Ogawa et al., 2001; Grutters et al., 2002a; Benner and Kaiser, 2003; Nagata et al., 2003; Perez et al., 2003).

In marine environment, there exist two types of bacteria, gram-positive and gram-negative, differentiated based upon the gram staining of the cell wall peptidoglycan. Peptidoglycan is a unique bacterial cell wall heteropolymer. Several studies have indicated that this biopolymer is a ubiquitous constituent of organic

matter of freshwater, estuarine, and open ocean environments (McCarthy et al. 1998; Dittmar et al., 2001; Jones et al., 2005) and sediments (Lomstein et al., 2006). The peptidoglycan layer is unique and essential structural component in the cell wall of most bacteria which imparts to cell its shape, strength, and resistance to osmotic pressure (Holtje, 1998; young, 2006; Vollmer et al., 2008). The cell wall of Gram-positive bacteria consists of a thick and uniform peptidoglycan layer, forming 90 % of the cell wall (Fig. 1.6). In contrast, Gram-negative bacteria have a complex, multilayered cell wall structure with a relatively thin inner peptidoglycan layer (constitutes 10 % of the cell wall) and an outer membrane of lipopolysaccharides and proteins (Schleifer and Kandler, 1972; Koch, 1990; Madigan et al., 2000) (Fig.1.6). Peptidoglycan consists of strands of alternating Peptidoglycan consists of strands of alternating β-1, 4-linked N-acetyl glucosamine and N-acetyl muramic acid units, cross-linked by short peptides containing D-amino acids (Nagata et al., 1998; Madigan et al., 2000) (Fig.1.7). Amino acids in these peptides include D-alanine, D-aspartic acid, D-glutamic acid, and D-serine (Sieradzki and Tomasz, 1996; Nagata et al., 1998; De Jonge et al., 2002; Reynolds and Courvalin, 2005; Veiga et al., 2006; Bellais et al., 2006). Peptide bridges in peptidoglycan are the predominant source of D-amino acids (Kaiser and Benner, 2008). The peptide side chains vary in amino acid composition, length, and position of cross-linking among the bacterial strains (Schleifer and Kandler 1972; Pedersen et al., 2001;

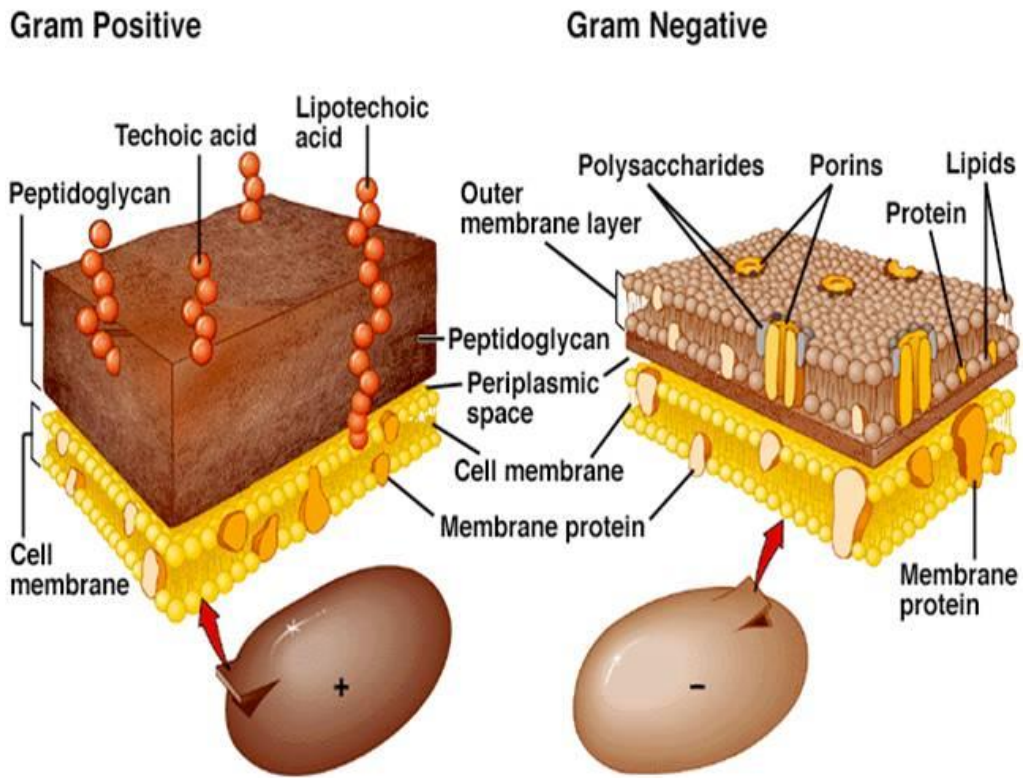

Figure1.6. Cell wall structure of Gram-positive and Gram-negative bacteria(taken from: www.pc.maricopa.edu)

Jorgensen et al., 2003). Peptide-linkages in peptidoglycan of Gram-positive bacteria vary greatly in composition and structural arrangement. D-amino acids that occur in these peptides are not limited to D-glutamic acid and D-alanine as in Gram-negative bacteria, but can also include D-aspartic acid, D-serine, and D-ornithine. Growth conditions can alter the composition of peptide linkages in Gram-positive bacteria (Schleifer and Kandler 1972). A high content of D-alanine (11.67 %) and D-glutamine (22.32 %) was found in Gram-positive bacteria, as compared to their relative amounts in Gram-negative bacteria (Nagata et al., 1998). D-amino acids have been used as markers of peptidoglycan in environmental studies. However, Kaiser and Benner (2008) indicated that D-amino acids are derived from other numerous macromolecules such as teichoic acid, lipopolysaccharides, polypeptides, lipopeptides and siderophores and are not solely from peptidoglycan (Hanby and Rydon 1946; Schleifer and Kandler, 1972; Troy 1973; Vanittanakom et al. 1986; Demange et al., 1990; Morikawa et al. 1993; Hanniffy et al. 1999; Vater et al. 2002; Hashii et al. 2003; Neuhaus and Baddiley, 2003; Kocharova et al. 2004).

1.5. Distribution of Amino acids

Organic matter produced in the surface waters provides nutrients for various organisms in the food web. Approximately 99 % of organic matter is eaten and respired within the water column (Hernes et al., 2001). However, only a small fraction (<1%) of organic matter produced photosynthetically in the ocean surface by the phytoplankton reaches the sediment surface (Suess, 1980; Martin et al., 1987). Organic matter in marine environment is composed of dissolved organic matter (pass through 0.2 μm filter), and particulate organic matter (retained on 0.2 μm filters) in water column, and sediments.

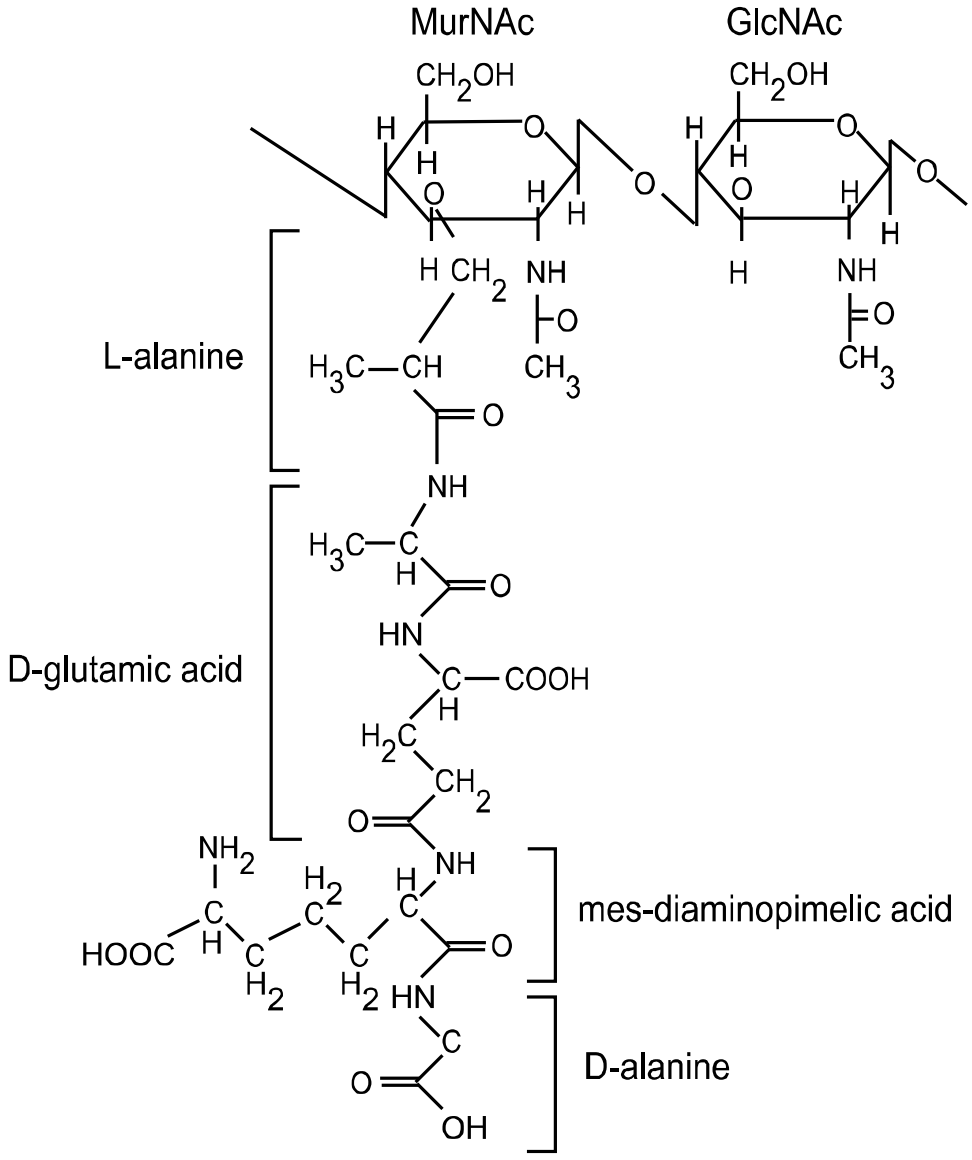

Figure1.7. Structure of peptidoglycan in Bacteria: subunits of N-acetyl glucosamine and N-acetyl mumaric acid linked by short peptide chain

1.5.1. Dissolved organic matter (DOM)

Dissolved organic matter (DOM), is the largest active reservoir of organic carbon and nitrogen in marine environment, and plays a major role in the global carbon and nitrogen cycling (Capone, 2000). The majority of marine DOM has not been characterized on a molecular level. DOM is very resistant to degradation, and appears to be of low molecular weight (< 1000 KD) (Benner et al., 1992; Ogawa and Ogura, 1992; Amon and Benner, 1994). Dissolved organic nitrogen (DON) is one of the major forms of nitrogen in marine environments and is an essential source of nitrogen for heterotrophic bacteria. Most nitrogen in marine DOM resides in amide functional group of amino acids (McCarthy et al., 1997). Dissolved L-amino acids comprise the largest identified component (~15 %) in the bulk DON pool (Keil and Kirchman, 1991; Hubberten et al., 1995). L-amino acids occur in the forms of dissolved free and combined amino acids.

Chemical characterization of a variety of bio-molecules in marine organic matter indicates that bacteria are an important source of marine DOM (Tanoue et al., 1995; Boon et al., 1998; McCarthy et al., 1998). Most of the characterized bio-molecules in DOM are commonly or uniquely found in bacterial cell walls. Peptidoglycan is the dominant cell wall polymer found in bacteria, and existing data suggest that bacterial polymer could be a major component of refractory DOM in the ocean (Boon et al., 1998; McCarthy et al., 1998). Studies have proved that peptidoglycan to be an important source of DON in the open ocean, comparable to that of hydrolyzable protein (McCarthy et al., 1998, Dittmar et al., 2001, Perez et al., 2003) and accounting for 2-5 % of the DON. Kaiser and Benner. (2009) observed a relative abundance of D-amino acids much greater in DOM than in POM or plankton. Several reports have demonstrated that bacterial organic matter is a

major component of non-living organic matter in the oceans (McCarthy et al., 1998, Dittmar et al., 2001, Kaiser and Benner 2008). In contrast, high Mole fractions of D-amino acids do not necessarily indicate advanced diagenesis, as heterotrophic bacteria release bio-reactive DOM during normal growth that is preferentially enriched in D-amino acids compared to their cellular composition (Kawasaki and Benner 2006, Kaiser and Benner 2008). This reflects the selective incorporation of specific bacterial macromolecules into bacterial DOM.

1.5.2. Particulate organic matter (POM) in water column

Particulate organic matter (POM) consists of a complex mixture of living (phytoplankton, heterotrophic bacteria) and non-living organic matter having a broad size, range, form, and reactivity. POM usually contributes less than 10 % of the total organic matter and its concentration does not exceed 0.2 mg CL^{-1} in oligotrophic and 1-2 mg CL^{-1} in eutrophic environments (Thurman, 1985). POM in ocean surface plays an important role as a starting material for various marine biogeochemical processes. The processes include fueling of the food web, the vertical transport of bio-elements to deeper waters and the transfer of newly produced organic matter to the dissolved organic matter pool. Algae and bacteria have been documented as a major component of suspended POM in the oceans (Benner and Kaiser, 2003; Kaiser and Benner, 2008). Significant relationships have been observed between L-amino acids and Chlorophyll *a* and D-amino acids and bacterial abundance in aquatic environments (Lee and Cronin, 1982; Fernandes, 2011). Amino acids liberated from POM during acid hydrolysis form the largest fraction of chemically characterized POM (Wakeham et al., 1997; 2000). Amino acids are important nitrogen source for heterotrophic organisms. Studies on POM have demonstrated that particulate L-amino acids are found to contribute ~ 30 % of

particulate organic carbon (POC) and ~ 50 % of particulate nitrogen (PN) (Handa, 1970; Siezen and Mague, 1978; Liebezeit and Bölter, 1986). Moreover heterotrophic bacteria are found to comprise a significant fraction of the living biomass of POM in ocean waters (Caron et al., 1995; Kirchman, 1997). Ducklow. (1999) observed that the interplay between the bacterial abundance and bacterial secondary production to significantly contribute to organic matter dynamic in oceanic euphotic zone. D-isomers of aspartic acid, glutamic acid, serine, and alanine have been observed in significant amounts in all particulate samples of rivers, estuaries and marine environments (Wu et al., 2007; Tremblay and Benner, 2009; Fernandes, 2011).

1.5.3. POM in sediments

Marine sediments are important zones of global organic matter production, re-mineralization and burial. Particulate organic nitrogenous compounds have been studied extensively in marine sediments because of their labile nature, their abundance in living organisms, and their importance as carbon and energy sources for primary and secondary producers (Henrichs et al., 1984; Lee and Cronin, 1984; Burdige and Martens, 1988; Whelan and Emeis, 1992; Keil et al., 2000; Vandewiele et al., 2009). Amino acids contribute ~ 60 % of the total nitrogen in the marine sediments (Henrichs and Farrington 1987; Cowie et al., 1992; Dauwe and Middelburg, 1998; Lomstein et al., 1998). In marine sediments, heterotrophic activity account for most of organic matter re-mineralization (Jorgensen, 2000). Besides selectively utilizing the bio-reactive organic compounds such as amino acids, bacteria contribute to the pool of living and non-living sediment organic matter in the form of bacterial cell wall and other bacterial macromolecules (Veuger et al., 2006; Lomstein et al., 2009). A fraction of the total amino acid pool consists

of bacterial cell walls in the form of living cells and as dead cell wall fragments. Bottrell et al. (2000) observed active bacteria at sediment depths up to 250 m. At such depths, bacteria are thought to be the dominant contributors of total amino acids because most biological activity probably is restricted to prokaryotic organisms. The bacterial cell walls consisting of peptidoglycan is the main source for the D-amino acids. Vandewiele et al. (2009) observed that living bacteria represented only a small fraction of total sediment D-amino acid pools and the majority of D-amino acid observed were attributed to dead bacteria and their remains. Moreover, only a small proportion of living bacteria may be active (Luna et al., 2002), indicating that actively growing bacteria contribute little to the total sediment D-amino acid pools, supported by the observation of D-amino acids at greater sediment core depths (Lomstein et al., 2009; Loreta et al., under review). In the sediments cores of Bay of Bengal, D-amino acid concentrations varied from 0.04 to 0.76 μmol gdw^{-1} (Loreta et al., under review). However, these concentrations were at lower end when compared to the other regions (Pedersen et al., 2001; Lomstein et al., 2006, 2009; Vandewiele et al., 2009).

1.6. Factors influencing amino acid distribution

Amino acid concentrations in water, particulate matter, and sediment decrease with water column and sediment core depth. The concentrations of amino acids are also influenced by the distance from the shores. Since longer residence time of the organic matter results in their rapid utilization by the *in-situ* organisms during their transport from the euphotic zone to the greater sediment depths. The utilization of the labile amino acids by organisms results into the accumulation of less degradable amino acids in the particulate, dissolved and sediment organic matter.

Further, various factors play a role in the occurrence of amino acids in the organic matter as given below:

1.6.1. Selective preservations

Amino acids are present in proteins of the cell wall, cell membrane, and the cell plasma. Proteins of the cell wall of bacterial or phytoplankton's or both are relatively refractory (Cowie and Hedges, 1992; Keil et al., 2000). Amino acids such as phenylalanine, tyrosine, and glutamic acid, aspartic acid, isoleucine, leucine, and valine present in diatom cell plasma, and are susceptible to degradation, and are abundant in freshly derived marine organic matter (Cowie and Hedges, 1992; Meckler et al., 2004). On the contrary, it is observed that glycine, threonine, serine, and alanine usually found in the cell walls of diatoms and bacteria are believed to accumulate during degradation of organic matter (Dauwe and Middelburg, 1998). Several authors have reported that silica frustules of diatoms are enriched in glycine, threonine and serine (Hecky et al., 1973; King, 1977), and this association may provide these compounds protection from microbial degradation in the water column and in sediments (King, 1974; Ingalls et al., 2003). Dauwe et al. (1999) observed higher proportion of glycine, serine, threonine, and alanine in the highly degraded organic matter.

Several studies have shown that the association of amino acids with the inert mineral phases can protect the amino acids from degradation (Hedges and Hare, 1987; Hedges and Kiel, 1999; Henrichs and Sugai, 1993; Aufdenkampe et al., 2001). Basic amino acids such as arginine and lysine have been seen to concentrate preferentially on the particle surface. Preferential sorption of basic amino acids to mineral grains resulting from the attraction between positively charged amide functional group and the negatively charged minerals (Theng, 1979;

Hedges and Keil, 1995). Strong association of basic amino acids with mineral phases, which in turn have been found to selectively protect them against microbial degradation (Mayer, 1994; Keil and Hedges, 1999).

1.6.2. Formation and accumulation of bacterial matter

A large fraction of total water column and benthic biomass consists of microorganisms, which play an important role in the turnover of organic matter in the water column and deep-sea sediments (Deming and Baross, 1993; Boetius et al., 2000). Bacterial activity plays a central role in the production of uncharacterized molecules and organic matter preservation (Harvey and Macko, 1997; Ogawa et al., 2001; Tremblay and Benner, 2006). Bacterial biomass is rich in D-amino acids (Jørgensen et al., 1999; Asano and Lubbehusen, 2000; Kaiser and Benner, 2008). D-amino acids have been observed in different environmental samples (McCarthy et al., 1998; Dittmar et al., 2001; Lomstein et al., 2009; Vandewiele et al., 2009; Bourgoin and Tremblay, 2010; Fernandes, 2011), indicating a major bacterial contribution to marine organic nitrogen. Bacterial contribution to the organic matter has been observed (Lomstein et al., 2006; Kaiser and Benner, 2008; Bourgoin and Tremblay, 2010; Fernandes, 2011). However, the bacterial contribution is particularly noticeable in nitrogen-poor plant detritus. During bacterial degradation of vascular plant tissues, Tremblay and Benner. (2006) observed incorporation of exogenous nitrogen, which was termed as N-immobilization.

1.7. Amino acids based biological indicators of OM diagenesis

Biological indicators are compounds unique to specific group of organisms. These bio-molecules are part of the organism biomass and makeup a relatively constant fraction of the biomass. Bio-molecules are important for determining the origins and diagenetic state of organic matter in aquatic systems (Wakeham et al., 1997;

Canuel and Zimmerman, 1999; Benner and Opsahl, 2001). Of the many potentially informative bio-molecules, amino acids are dominant components of biomass and comprise the bulk of the molecularly characterized fraction of organic matter (Benner, 2002). Several recent studies have investigated the composition and abundance of amino acids in marine systems (Lee et al., 2000) and illustrated the reactive nature of the amino acids. L-amino acids in the organic matter are largely plankton-derived, and changes in the composition and abundance of amino acids in the particulate and dissolved organic matter have been indicative of the diagenetic state and bioavailability of marine organic matter (Cowie and Hedges, 1992; Dauwe et al., 1999; Amon et al., 2001). While the D-amino acids are the indicators of source and diagenetic state of organic matter. Nevertheless, the biological indicators function most reliably on annual to decadal time scales of organic matter diagenesis.

1.7.1. Amino acid yield

Amino acid carbon or nitrogen yield is defined as the contribution of amino acid carbon or nitrogen to total bulk organic carbon or nitrogen multiplied by 100 (Amino acid-C/TOC x100). Amino acids comprise a major portion of freshly produced marine organic matter. Freshly produced organic matter is usually characterized by higher carbon and nitrogen-normalized yields of amino acids (Cowie and Hedges, 1992, 1994; Benner and Kaiser, 2003). The amino acids carbon and nitrogen are preferentially utilized by *in-situ* microorganisms compared to bulk organic carbon and nitrogen and thereby their proportion decreases during diagenesis resulting in low amino acid yield in degraded organic matter (Cowie and Hedges, 1992; 1994). Therefore, the carbon and nitrogen-normalized yields of the amino acids are good indicators of nutritional quality and diagenetic state of organic matter (Keil et al.,

2000; Amon et al., 2001; Amon and Benner, 2003; Benner, 2002, Benner, 2003; Yamashita and Tanoue, 2003; Davis and Benner, 2005). The vascular plants and terrigenous organic matter are poor in nitrogen compared to marine organic matter, thereby amino acid yield is low (Cowie and Hedges, 1992; Hedges et al., 1997). Bourgoin and Tremblay (2010) observed that amino acid yields are very sensitive to the rapid changes that occur in both marine and terrigenous POM in aquatic systems. Cowie and Hedges (1994) and Davis et al. (2009) demonstrated that amino acid yields were most effective during the early stages of organic matter degradation. Bourgoin and Tremblay. (2010) measured 2.2 to 17.6 % and 4.4 to 7.5 % of amino acid-carbon yield in the St. Lawrence system for the suspended particles and sediment, respectively. In the St. Lawrence system, amino acid-nitrogen yield was 6.8 to 49.2 % and 15.5 to 29.7 % for the suspended particles and sediment, respectively. Further, 4.5 to 20 % of C and 12.5 to 30 % of N were measured for particulate matter and sediments of various oceanic and coastal waters (Henrichs et al., 1984; Burdgie and Martens, 1988; Cowie and Hedges, 1992; Ingalls et al., 2003; Vandewiele et al., 2009).

1.7.2. Amino acid degradation index (DI)

Dauwe et al. (1999) developed a degradation index (DI) based on the changes in amino acid composition during diagenesis in order to rank the stations in terms of organic matter quality using the formula:

$$DI = \sum_i [\frac{\mathrm{var}_i - AVG\ \mathrm{var}_i}{STD\ \mathrm{var}_i}] * fac * coef_i$$

where var_i is the Mole percentage of amino acids i, $AVGvar_i$, and $STDvar_i$ are mean, and standard deviation in the dataset, and $fac.coef._i$ and factor is the factor

coefficient for amino acid i, (Dauwe and Middelburg, 1998). The DI calculated using the formula covers a wide range of degradation states and is found to be uncompromised by source variations. The DI provides a single number that quantifies the degradation state of organic matter. DI varies from values of +1 for newly produced algal material to -2 for extensively degraded deep-sea sediment material (Dauwe and Middelburg, 1998; Dauwe et al., 1999). These amino-acid based DI of Dauwe et al. (1999a) has been successfully applied to many datasets from various ocean basins and even lakes (Gelinas et al., 2001; Meckler et al., 2004; Gaye-Haake et al., 2005; Unger et al., 2005). Dauwe et al. (1999) found DI values smaller than -1 for refractory organic matter in pelagic deep-sea sediments. Gaye-Haake et al. (2005) reported DI values for suspended matter and surface sediments from the northern Indian Ocean, including the Arabian Sea. The DI values ranged from ~ 0.5 in suspended particles to -1.5 in the central, deep Arabian Sea sediments. Similarly, DI values of organic matter in the Pakistan Margin sediments ranged from 0 to -1.5 (Vandewiele et al., 2009).

1.7.3. Non-protein amino acids

Non-protein amino acids such as β-alanine (β-Ala) and γ-amino butyric acid (γ-ABA) are formed during microbial decarboxylation of protein amino acids aspartic acid and glutamic acid, respectively (Lee and Cronin, 1982; Cowie and Hedges, 1992; Suthhof et al., 2000). The non-protein amino acids represent a negligible amount of the total amino acids in the organisms but have been shown to increase in its abundance during degradation of organic matter (Whelan 1977; Cowie and Hedges, 1994). Presence of these non-protein amino acids in the organic matter indicates microbial reworking of organic matter. Cowie and Hedges (1992)

attributed higher Mole % non-protein to the diagenetic origin of these amino acids. Thus, non-protein amino acids are used as the indicators of degradation. β-Ala and γ-ABA are sensitive indicators for intermediate to extensively degraded materials (Dauwe et al., 1999a; Cowie andHedges, 1994). Usually, the Mole % sum of β-Ala and γ-ABA has been used as a diagenetic indicator. These two non-protein amino acids displayed varying dynamics during the decomposition experiments (Davis et al., 2009). Davis et al. (2009) observed a decrease in Mole % β-Ala with the increasing water column depth. In contrast, an increase in Mole % γ-ABA at the same water column depth was observed when these two non-protein amino acids were investigated separately for their diagenetic behavior. Non-protein amino acids were most sensitive in the later stages of degradation/ diagenetic alteration (Davis et al., 2009).

1.7.4. Mol % D-amino acids

D-amino acids are useful tracers for identifying sources and degree of degradation of organic nitrogen. D-amino acids (as Mol %) has been employed as diagenetic indicators for POM and DOM, because D-amino acid concentration increases during diagenesis (Tremblay and Benner, 2009). Laboratory experiments showed that D-amino acids and bacterial DOM are less bio-reactive than algal DOM (Joergensen et al., 1999, Amon et al., 2001) implying microbial alteration reactions could lead to refractory DOM enriched in D-amino acids. D-amino acids (Mol %) may only be an effective indicator of the early stages of diagenesis. Studies have shown that the Mol % D-amino acids increased with depth in sediments (Pedersen et al., 2001; Lomstein et al., 2006) and with plant detritus degradation (Tremblay and Benner, 2006). These results indicate that the Mol % D-amino acids can be a

useful indicator of the diagenetic state of organic matter. Besides being used as diagenetic indicator, increase in the Mol % of D-amino acids can be attributed to greater bacterial contributions and to the lower degradation rates of the bacterial bio-molecules rich in D-amino acids compared with proteins of various origins (Jørgensen et al., 2003; Nagata et al., 2003).

1.8. Aim and scope of present research

Organic matter in marine environment is derived from both the biogenic and terrestrial sources. Microorganisms such as phytoplankton and bacteria are the major sources of organic matter in the marine environments. Amino acids, the most abundant and reactive component of marine organic matter are labile and utilized by the *in-situ* organisms. These amino acids can be used as the proxies to understand the degradation state of the organic matter. Bacteria play a major role in the re-mineralisation of the organic matter. However, components of bacterial origin and unique to the cell wall of bacteria have been observed in the dissolved and particulate organic matter of marine environment. Peptidoglycan is the dominant component of bacteria, and existing data suggest that this bacterial polymer could be a major source of refractory organic matter in Ocean (McCarthy et al., 1998). Moreover, little information is available on whether the peptidoglycan derived cell components are dominant elements of organic matter.

The above literature survey suggests that there are numerous studies on the distribution and cycling of L-amino acids from the temperate environments (Degens and Mopper, 1976; Lee and Cronin, 1982; Cowie and Hedges, 1992; Cowie and Hedges, 1994; Middelboe et al., 1995; Wang et al., 1998; Burdige and Zheng, 1998; Stepanauskas and Leonardson, 1999). Recently, a few studies on the

abundance and cycling of D-amino acids in temperate waters have been reported (McCarthy et al., 1998; Dittmar et al., 2001; Lomstein et al., 2009; Bourgoin and Tremblay, 2010). As compared to these studies, no particular effort has been made to understand the distribution and cycling of organic matter in general and L- and D-amino acids in particular in the tropical waters. Moreover, the importance of bacteria in cycling and preservation of organic matter has not addressed by earlier researchers. In view of this, the major goal of the thesis was to study the distribution and cycling of organic matter, especially L- and D-amino acids and understand the role of bacteria in organic matter cycling and preservation in the tropical environments. The data obtained on L- and D-amino acids was then used to investigate the nature, source, diagenetic changes, and bacterial contribution to organic matter. In order to achieve these goals, the research was planned with the following objectives.

1. To investigate the effect of some factors on the concentration and composition of L- and D-amino acids in some marine bacteria.
2. To examine the degradation of whole cell walls and partially peptidoglycan of bacteria.
3. To assess bacterial contribution to the organic matter of the decaying mangrove leaves.
4. To understand the distribution and cycling of L- and D-amino acids and determine bacterial contribution to organic matter in the estuarine and deep ocean environments.

Chapter 2

Effect of growth, nutrients and starvation on L- and D-amino acids concentration and composition in marine bacteria

2.1. Introduction

In the aquatic environments, heterotrophic bacteria play a central role in biogeochemical cycling of organic matter. Heterotrophic bacteria constitute a large proportion of the planktonic bacterial biomass, and are widely distributed in various environments, including extreme environments (Cho and Azam 1990, Simon et al., 1992, Kawabata and Nakanishi, 1996). Bacteria utilize inorganic and organic nutrients, and produce bacterial biomass, which in turn, is responsible for substantial part of material cycled in aquatic environments (Ichinotsuka et al., 2010). In any given ecosystem, bacteria are differentiated into two types: Gram-positive and Gram-negative based upon the staining of their cell wall peptidoglycan with Gram's stain. Both these types of bacteria are present in the marine environments. However, Gram-negative bacteria appear to dominate the marine bacterial communities (Giovannoni and Rappe, 2002). Bacterial growth is influenced by various factors such as incubation period, temperature, nutrient, carbon source and concentration, grazing and viral lysis (Church et al., 2000; Sala et al., 2002; Hall et al., 2008).

Amino acids, carbohydrates, and lipids are the major biochemical components of the bacterial cells and membrane, and the former compounds are important components of total organic nitrogen pool in aquatic environments. Peptidoglycan is one of the bacterial cell wall components. This bacterial cell wall polymer consists of alternating strands of β-1, 4-linked N-acetylglucosamine and N-acetylmuramic acid units cross-linked by short peptide strand, which is the major source of D-amino acids. Bacterial cell wall remnants such as peptidoglycan and its constituents have been detected in seawater and sediments. D-amino acids and muramic acid are potentially useful to assess bacterial contribution to organic

matter. D-amino acids are also associated with bacterial macromolecules such as teichoic acid, lipopolysaccharides, polypeptides, lipopeptides and siderophores produced by bacteria (Mc Carthy et al., 1998, Dittmar et al., 2001, Lomstein et al., 2006, Kaiser and Benner, 2008). The four D-amino acids D-alanine, D-glutamic acid, D-aspartic acid and D-serine are commonly found in bacteria. Growth conditions and nutrients may influence L-and D-amino acid concentration and composition in the bacteria (Schleifer and Kandler, 1972). Bacteria contribute significantly to the labile as well as refractory organic matter pool in the ocean. The L-amino acids are useful for understanding degradation state, while D-amino acids are useful biomarkers to assess bacterial contribution to bulk carbon and nitrogen. Therefore knowledge, of their abundance and molecular distribution of these L- and D-amino acids in the source organisms, and their sensitivity to growth and environmental parameters is essential to better explain the role of bacteria in organic matter cycling in marine environments. In this chapter, the effect of some growth, nutrient concentrations, and starvation on the concentration and composition of L- and D-amino acid in some marine bacteria is presented.

2.2. Materials and Methods

2.2.1. Source of Bacterial cultures

Two bacterial cultures, BAC-1, and BAC-2 were obtained from the Marine Corrosion and Material Research Division (MCMRD), while the third bacterial culture, BAC-3 was donated by Dr. Saroj Bhosle, Microbiology Department, Goa University, Goa. All the three cultures were grown on Zobell Marine Agar (ZMA) plates and then pure cultures were transferred to ZMA slants and stored at 4°C for further use. The BAC-1 and BAC-2 were isolated from biofilm developed on

stainless steel in the surface waters of Dona Paula Bay, west coast of India (details in D'Souza, 2004).

2.2.2. Bacterial growth Medium

Bacterial cultures were grown in a basal salt solution (BSS) supplemented with 0.1 % glucose, 0.05 % peptone and 0.01 % yeast extract. This medium was designated as BSS-GYP medium. Glucose (10 %) was sterilized separately. A suitable aliquot of glucose was added to each flask so as to obtain desired concentration (i.e. 0.1 %). BSS medium consists of NaCl (25.0 g), KCl (0.75 g), $MgSO_4.7H_2O$ (7.0g), NH_4Cl (1.0 g), K_2HPO_4 (10 %, 7ml), KH_2PO_4 (10 %, 3ml), and 1ml of trace metal solution (TMS) dissolved in 1000 ml of distilled water (D/W). TMS consists of H_3BO_3 (2850 mg), $MnCl_2.4H_2O$ (1800 mg), $FeSO_4.7H_2O$ (2490 mg), sodium tartarate (1770 mg), $CuCl_2$ (26.9 mg), $ZnCl_2$ (20.8 mg), $CoCl_2$ (40.4 mg), $NaMoO_4.2H_2O$ (25.2 mg) in 1000 ml D/W (Bhosle, 1981). pH of the medium was adjusted to 7.5 with 1 N NaOH. The medium was sterilized by autoclaving at 121°C and 15 lb pressure for 15 min.

2.2.3. Bacterial Identification

Bacterial cultures were identified using the conventional physiological and biochemical tests and molecular identification method based on 16S-rDNA sequencing.

2.2.3.1. Conventional Identification method

Bacterial cultures were tested for gram's reaction, cell morphology, physiology and biochemical characteristics following standard methods (MacFaddin, 1980). Morophological, physiological and biochemical characteristics of the cultures were compared with those given in Bergey's Manual of Systematic Microbiology (Kreig

and Holt, 1994). Using these methods, cultures could be identified up to generic level. Further identification was done using molecular methods.

2.2.3.2. 16S rDNA sequencing method

The 16S-rDNA sequencing procedure can be divided into three steps: 1) Bacterial DNA extraction, 2) gene amplification by polymerase chain reaction (PCR) and 3) DNA sequencing and analysis.

1) Bacterial DNA extraction

The cultures were grown overnight in 10 ml nutrient broth (Hi Media, India) and bacterial genomic DNA was isolated using DNA isolation kit (Sigma, USA) following the protocol recommended by the supplier.

2) Polymerase chain reaction

The genomic DNAs of individual bacterial samples were used for PCR amplification. The 16S-rDNA gene fragments were amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-30) (Weisburg et al., 1991). The amplification was carried out in 25 μl of reaction mixture containing DNA template, PCR buffer, dNTP (10 mM), $MgCl_2$ (25 Mm), the primer 27F (10 ppm) and 1492R (10 ppm) respectively, Taq polymerase (Sigma, USA) (2U μl^{-1}) and autoclaved Milli-Q water (Millipore). The amplication was carried out for 35 cycles of 94°C, for 45s, 60°C for 45s and 72°C for 45s, 72°C for 7 min with initial 5 min denaturation at 94°C using a PCR- Express thermal cycler (Hybaid). The DNA sequences of the PCR products were determined using Taq Dye Deoxy terminator cycle sequence kit (Perkin-

Elmer, Applied Biosystems, Foster City, CA, USA) using the protocol recommended by the supplier. Reaction products were sent for sequencing to GeNei™ Bangalore (INDIA).

3) DNA sequencing and analysis

The nucleotide sequencing of the 16S-rDNA genes (about 1400 nucleotides each) were aligned and compared with those available in GeneBank database of National Center for Biotechnology Information (NCBI), USA and searched for sequence similarity analysis of the 16S-rDNA sequence obtained. The full 16S-rDNA sequence of bacterial cultures were deposited to the GeneBank database under the accession numbers HM150643 (BAC-1), HM150644 (BAC-2) and JX155854 (BAC-3).

2.2.4. Growth Curve of the bacterial cultures

Bacterial cultures BAC-1, BAC-2, and BAC-3 present on the slants were sub-cultured twice in 25 ml flask containing 10 ml BSS-GPY medium for 24 h. Subsequently, the cultures were grown individually in 100 ml Erlenmeyer flasks containing 20 ml of the BSS-GYP medium. Erlenmeyer flasks containing the medium were sterilized at 121 °C and 15 lb pressure for 15 min. The Erlenmeyer flasks were cooled and each flask was inoculated individually with 1 % of the bacterial cultures grown as above. The cultures were grown at room temperature (28 ± 2 °C) on a rotary shaker (100 rpm) for 24 h. This step was repeated twice for each culture. Subsequently, three 1 L Erlenmeyer flasks containing 200 ml of the BSS-GYP medium were sterilized at 120 °C and 15 lb pressure for 15 min. One flask was inoculated with 1 % inoculum of BAC-1, second with BAC-2 and third with BAC-3. The flasks were incubated at room temperature (28 ± 2°C) on a rotary

shaker (100 rpm). At specific time intervals, 5 ml culture broth was removed. The growth was monitored by measuring the optical density at 540 nm using a Spectrophotometer (UV-1700 Pharma Spec UV-VIS, Shimadzu).

2.2.5. Effect of growth on concentration and composition of L- and D-amino acid in bacteria

The above bacterial cultures were grown in 250ml BSS-GYP in three individual 1 L flasks as described above over a period of 50 h. At specific time intervals, broth from each culture flasks was sampled. The bacterial cells were harvested by centrifuged at 10,000 rpm for 15 min at 4 °C, while the supernatant was discarded. Cells were washed thrice with 0.2 µm filtered 1.5 % sodium chloride to remove excess nutrients attached to cells. The bacterial cells were centrifuged as above and cells were lyophilized and stored at -20 °C until analyzed for L- and D-amino acids.

2.2.6. Effect of nutrients on concentration and composition of L-and D-amino acid of bacteria

The bacterial culture, BAC-1 was grown in four individual flasks containing BSS-GYP medium prepared as above and supplemented with various concentrations of nitrogen (4.7, 9.3, 14 and 18.7 mM) when other nutrients were kept constant. Similarly, BAC-1 was also grown individually in another four flasks containing BSS-GYP medium, supplemented with various phosphates concentrations (K_2HPO_4 and KH_2PO_4) (1.6, 3.1, 4.7, and 6.2 mM phosphorus). The culture was grown at room temperature for 24h; similar procedure was followed to assess the effect of nutrients on the cultures BAC-2 and BAC-3. The cells were collected following the

procedure described above. Cells were then lyophilized and used for the analyses of L-and D-amino acids.

2.2.7. Effect of Starvation on L- and D-amino acids of bacterial cells and cell free supernatant

BAC-1 and BAC-2 were grown in 200 ml BSS-GPY and processed as above. The bacterial cells thus obtained were then suspended in 200 ml of filtered (0.22 µm) and sterilized aged seawater hereafter termed as culture broth. The flasks were then incubated on a shaker at room temperature (28 ± 2°C) in dark. Samples were removed at 0, 7, 14, 21, and 28 days following inoculation. Cells were separated by centrifugation as above. TBC and L- and D-amino acids, were monitored in both cells and cell free culture broth.

2.2.8. Total bacterial count

Changes in bacterial cells were monitored by enumerating total bacterial cells following the DAPI (4, 6- Diamidino-2-phenylindole) staining method (Porter and Feig, 1980). Hundred micro liter of formalin (2 %) fixed sample was made to 2ml using sterile saline. To this DAPI (final concentration 0.01 %) was added as a fluorescent stain. The sample was allowed to react with stain for 15 min in dark. The sample was subsequently filtered through 0.22 µm black Nucleopore polycarbonate membrane filters. The filter was then placed on a drop of fluorescence free oil, another drop of oil was placed onto it and covered with a cover slip and was then viewed using epifluorescence microscope (Nikon 80i) under 100 X oil immersion lens and a blue filter. About 10 fields containing the bacterial cells were counted per filter and the average field count was used to calculate the total bacterial numbers.

2.2.9. Analysis of L- and D-amino acids:

2.2.9.1. Sample preparation

A known quantity (2 mg) of the lyophilized cells or cell free culture broth (15 ml) was placed in a glass ampoule to which 3ml of 6 N HCl and 20 µl of 11mM ascorbic acid were added and the ampoule was flushed with nitrogen gas and sealed. Bacterial cell or cell free culture broth was then hydrolyzed at 110 ^{o}C for 24 h in an electrical oven. The hydrolysate was cooled and neutralized with 6 N NaOH. The sample was then centrifuged at 1000 rpm for 15 min and the supernatant was collected. The supernatant was then dried under vacuum at 40 ^{o}C, and was dissolved in a known volume of Milli-Q water. An aliquot of the sample was then analyzed for L- and D-amino acids using reverse phase high- performance liquid chromatography (HPLC) as given below.

2.2.9.2. Chromatographic system

The separation of L-and D-amino acids from the samples was carried out using a HPLC system (Shimadzu Model 1) consisting of a quaternary solvent delivery pump, auto-injector, and fluorescent detector. The solvents were degassed using the degasser. The column used for the separation was a reversed phase Shim-Pack HRC-ODS analytical column, 4.6 mm ID, 15 cm length, 5 µm particle size and ODS guard column, 4.6 ID, 4.5 cm length, 10 µm particle size. The temperature of the column was maintained at 35 °C using a column oven. L-amino acid derivatives were monitored by fluorescence detection using excitation at 328 nm and emission at 450 nm. D-amino acid derivatives were monitored using excitation at 335 nm and emission at 450 nm.2.2.9.3. Derivatization and separation of L- and D-amino acid

L-amino acids were analyzed using the method of Lindroth and Mopper (1979) as described by Bhosle et al. (2005). An aliquot of the sample prepared as above was mixed with *O*-phthaldialdehyde (OPA)-mercaptoethanol derivatizing reagent. After 2 min of reaction time a known volume was injected onto the column of the chromatographic system. o-phthaldialdehyde (OPA)-mercapto-ethanol derivatizing reagent was prepared by dissolving 8 mg of OPA in 200 μl methanol to this 8 ml borate buffer (0.4 M) pH 10.5 followed by 10 μl of 2-mercaptoethanol and 30 μl of 30 % Brij solution were added. The volume was made to 10 ml with borate buffer. The reagent mixture was allowed to age at least for 24h prior to use.

A binary solvent system consisting of 50 mM sodium acetate buffer (pH 6.5) containing 3 % of tetrahydrofuran as solvent A and methanol as solvent B was used to separate L-amino acids. The flow rate of 1.5 ml min^{-1} and a multi-step gradient elution of 40 min, beginning with 15 % solvent B, and 85 % solvent A was used for the separation of L-amino acid derivatives (Fig. 2.1).

D-amino acids were analyzed using the modified method of Fitzner et al. (1999) as described in Fernandes (2011). Two mg of OPA and 5 mg of IBLC were dissolved in 100 ml of methanol and made to 1 ml with 0.5 M borate buffer (pH = 9.5) and the reagent was stored at 4 °C. A suitable aliquot (50 μl) of the sample prepared as above was mixed with 50 μl of *O*-phthaldialdehyde (OPA) and *N*-Isobutyrl-L-cysteine (IBLC) derivatizing reagent. OPA-IBLC reagent was prepared fresh every 3rd day (Kaiser and Benner, 2005). After 2 min of reaction time a known volume was injected onto the analytical column of the chromatographic system.

Ternary gradient consisting of 25 mM sodium acetate buffer, pH 7.0 and pH 5.3 as solvent A and C, respectively and HPLC grade methanol as solvent B was used for the elution of D-amino acid derivatives. The flow rate of 1ml min^{-1} and a

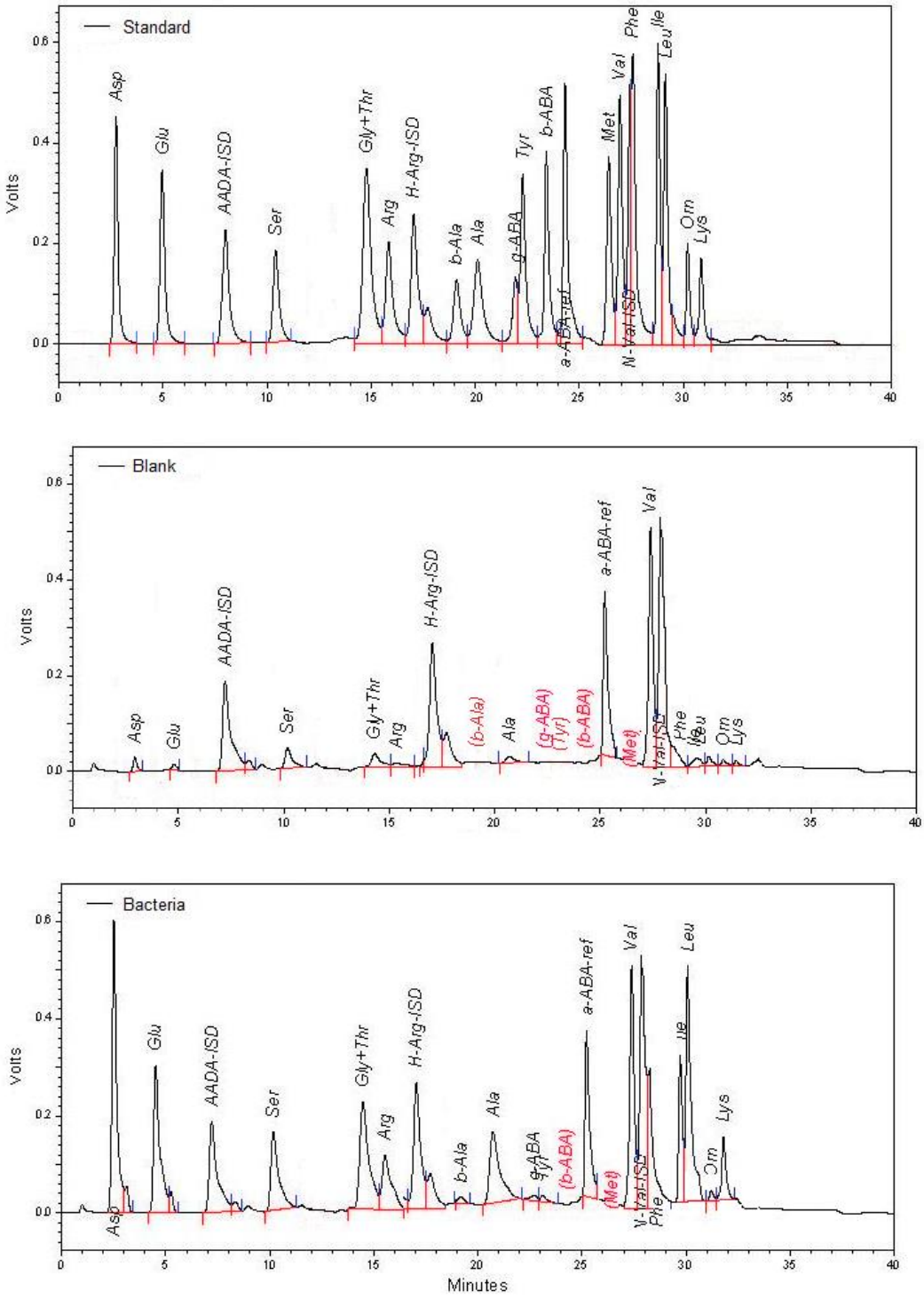

Figure 2.1. Chromatograms showing the seperation of L-amino acid standard, Blank and Bacterial cells. Asp: aspartic acid, Glu: glutamic acid, AADA: alpha amino adipic acid, Ser: serine, Gly+Thr: glycine plus threonine, Arg: arginine, H-Arg: Homo-arginine, b-Ala: bta-alanine, Ala: alanine, g-ABA: gamma-amino butyric acid, Tyr: tyrosine, a-ABA: alpha amino butyric acid, Met: methionine, Val: valine, N-Val-ISD: nor-valine (Internal standard), Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, Lys: lysine

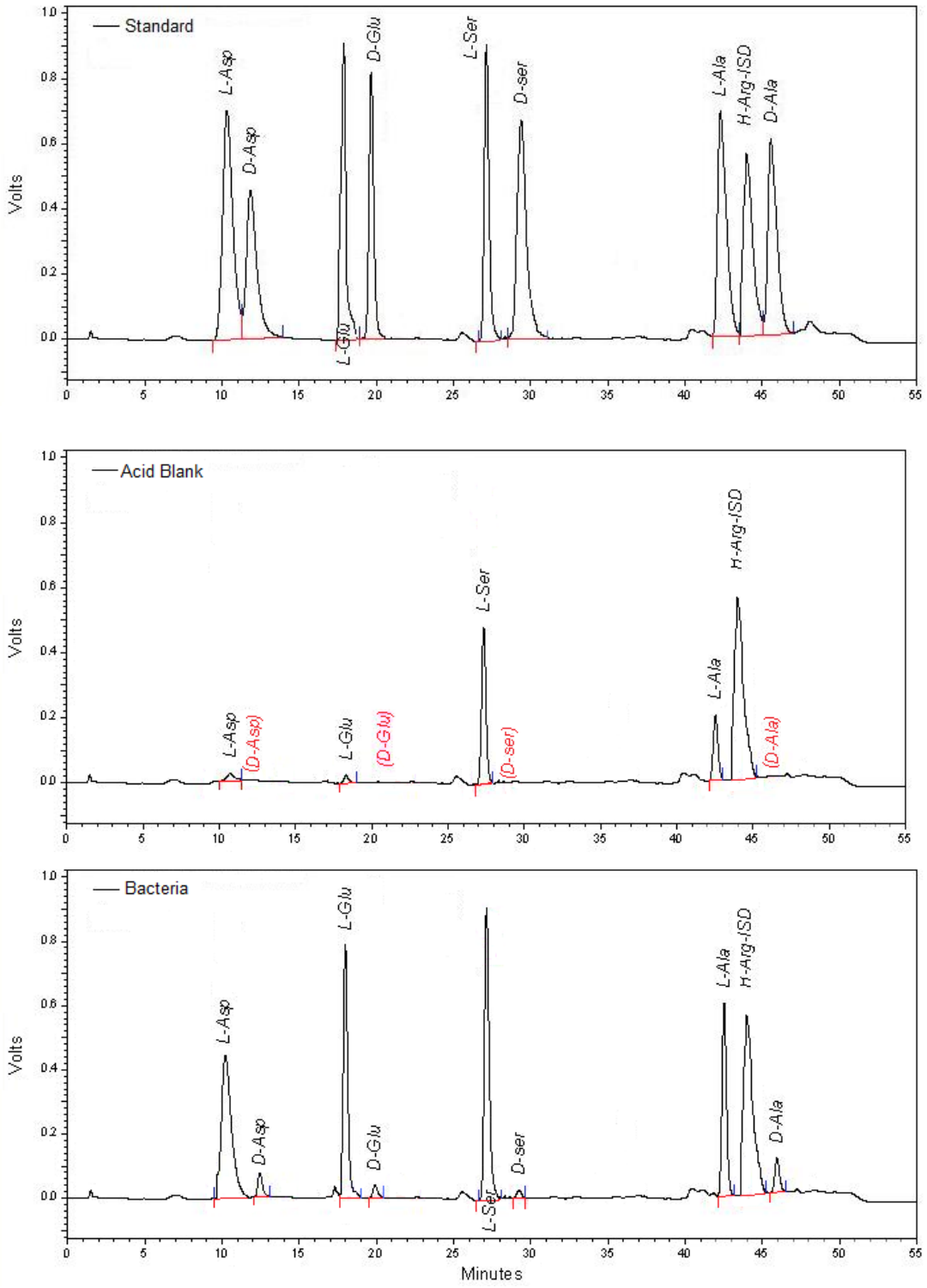

Figure 2.2. Chromatograms showing the separation of L- and D-amino acid isomers of Asp: aspartic acid, Glu: glutamic acid, Ser: serine, Ala: alanine and H-Arg: homo arginine in standard, Blank and Bacterial cells

multi-step gradient elution of 56 min, beginning with 5 % solvent B, 4 % solvent C and 91 % solvent A was used for the separation of D-amino acid derivatives (Fig. 2.2).

2.2.9.4. Identification and quantification of L-and D-amino acids

The identification of individual L- and D-amino acid in the sample was determined separately. Retention time of the sample peaks were compared with those of the standard peaks (Fig. 2.1 and Fig. 2.2). Concentrations of the L- and D-amino acids were calculated from peak area relative to known internal standards and a standard calibration using the data handling system (Shimadzu Class-VP 6.14 SP1) available with the instrument.

2.3.0. Result and Discussion

2.3.1. Morphological, physiological, and biochemical characteristics and rDNA sequencing of Bacterial cultures

Based on Gram's reaction the two bacterial cultures were characterized as Gram-positive (BAC-1 and BAC-2) and third one as Gram-negative (BAC-3). Based on Gram stain, cellular morphology, and physiology and biochemical characteristics the cultures were tentatively identified as *Bacillus* species (BAC-1 and BAC-2) and *Pseudomonas* species (BAC-3) (Table 2.1). Further, identification of the cultures to species level was done using the 16S- rDNA sequences. Bacterial cultures BAC-1, BAC-2, and BAC-3 were closely related to *Bacillus subtilis*, *Bacillus licheniformis*, and *Pseudomonas psychrotolerans*, respectively with similarity value ranging from 97 % to 99 % (Fig. 2.3).

Table 2.1. Morphological, physiological, and biochemical characteristics and tentative identifications of the bacterial cultures

	Bacterial cultures		
	BAC-1	BAC-2	BAC-3
Gram character	Gm +ve	Gm +ve	Gm –ve
Cell morphology	Short rods	Long rods	Small rods
Spore formation	+	+	-
Motility	Motile	Motile	Motile
Pigmentation	Creamy white	Creamy white	Creamy white
Colony shape	Small round	Big round	Small round
Catalase	+	+	+
Citrate	+	+	+
Gelatin	+	+	-
Glucose	Acidic	Acidic	Acidic
Hug leifson	Facultative	Fermentative	Facultative
Indole	-	-	-
Inositol	-	Acidic	-
Lactose	Alkaline	Alkaline	Alkaline
Nitrate reductase	+	+	+
Oxidase	+	+	+
Starch	+	+	+
Sucrose	Acidic	Acidic	Acidic
Genus	*Bacillus* sp.	*Bacillus* sp.	*Pseudomonas* sp.

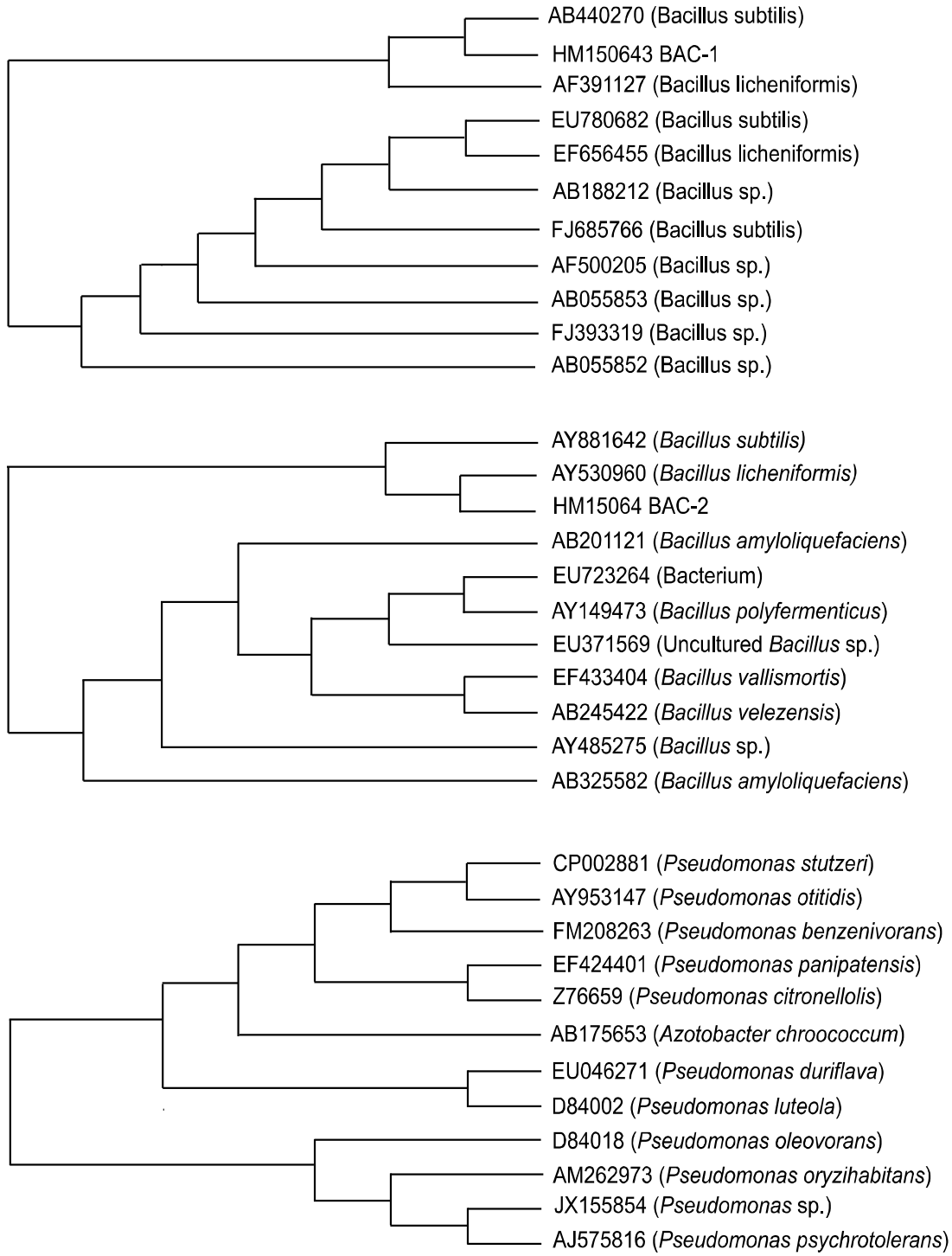

Figure 2.3 Phylogenetic tree showing the relationships of the microbial culture based on 16S -rDNA sequences. The tree was constructed using MEGA 3.1 software using neighbor-joining tree method.

2.3.2. Growth of the cultures

Both the *Bacillus* cultures showed a small lag phase of about 3 h (Fig. 2.4). Thereafter, both the cultures started growing. *Bacillus licheniformis* reached stationary phase after 12 h while, *Bacillus subtilis* took about 28 h to reach stationary phase (Fig. 2.4). *Pseudomonas psychrotolerans* reached a stationary phase after 24 h (Fig. 2.4). After reaching stationary phase, optical density of the culture broth did not vary much over the remaining period of incubation (Fig. 2.4).

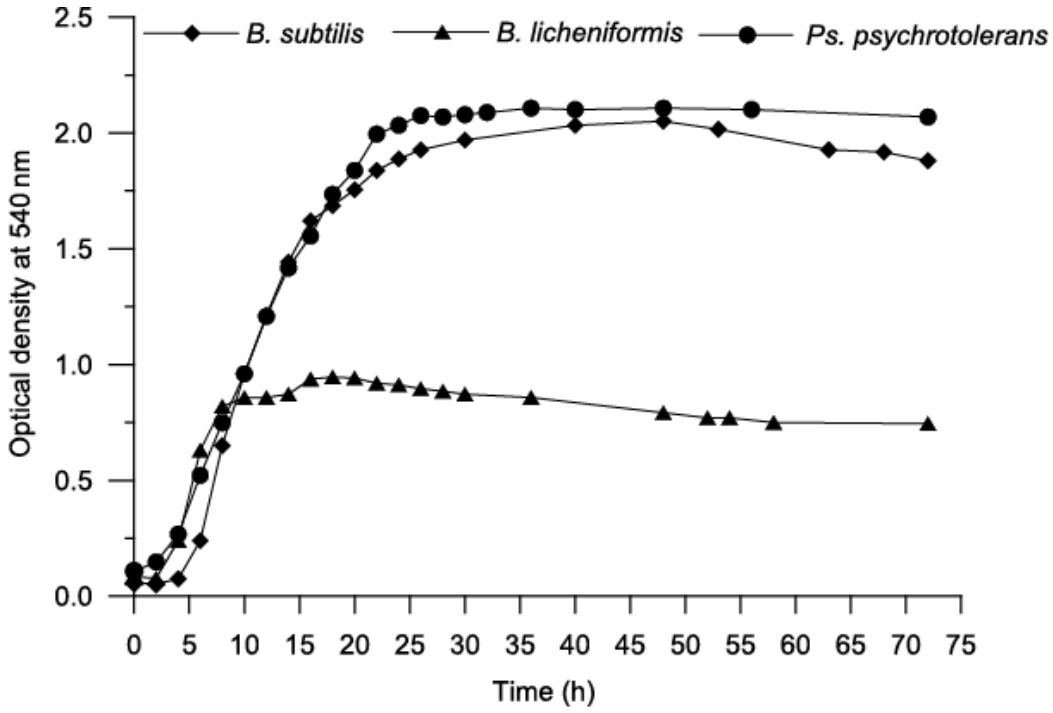

Figure 2.4. Growth curve of Bacillus subtilis, Bacillus licheniformis, and Pseudomonas psychrotolerans when grown on BSS-GYP medium at room temperature

2.3.3. Effect of growth on L- and D-amino acids in the bacterial cells

2.3.3.1. L-amino acid concentration

Total L-amino acid concentration in the cells of three bacterial cultures showed a distinct trend during their growth. The L-amino acid concentrations in the cells varied from 149 to 239 mg gdw^{-1} for *Bacillus subtilis*, 136 to 186 mg gdw^{-1} for *Bacillus licheniformis*, and from 176 to 197 mg gdw^{-1} for *Pseudomonas psychrotolerans* (Fig. 2.5)**.** In *Bacillus subtilis* cells, L-amino acid concentration increased gradually up to 50 h of the growth period (Fig. 2.5). In contrast, in *Bacillus licheniformis* and *Pseudomonas psychrotolerans* cells, the L-amino acid concentration increased and was higher at 20 h and 24 h, respectively, and thereafter the L-amino acid concentrations in cells of these cultures were more or/less constant up to 50 h following inoculation (Fig. 2.5). This indicates that the L-amino acids concentration was influenced by the growth period used for these cultures.

2.3.3.2. Composition of L-amino acids

L-amino acid composition of each of bacterial cultures was influenced by the growth period (Fig. 2.6). In *Bacillus subtilis*, the amino acids glutamic acid, aspartic acid, lysine and glycine plus threonine were abundant (Fig. 2.6). In the cells of *Bacillus licheniformis*, glutamic acid, glycine plus threonine, leucine, lysine and aspartic acid were the dominant amino acids (Fig. 2.6). Mole % of aspartic acid, glycine plus threonine, leucine and lysine increased in the cells of *Bacillus licheniformis* over the period of growth (Fig. 2.6). Alanine, followed by glutamic acid, glycine plus threonine and aspartic acid were relatively more abundant in cells of

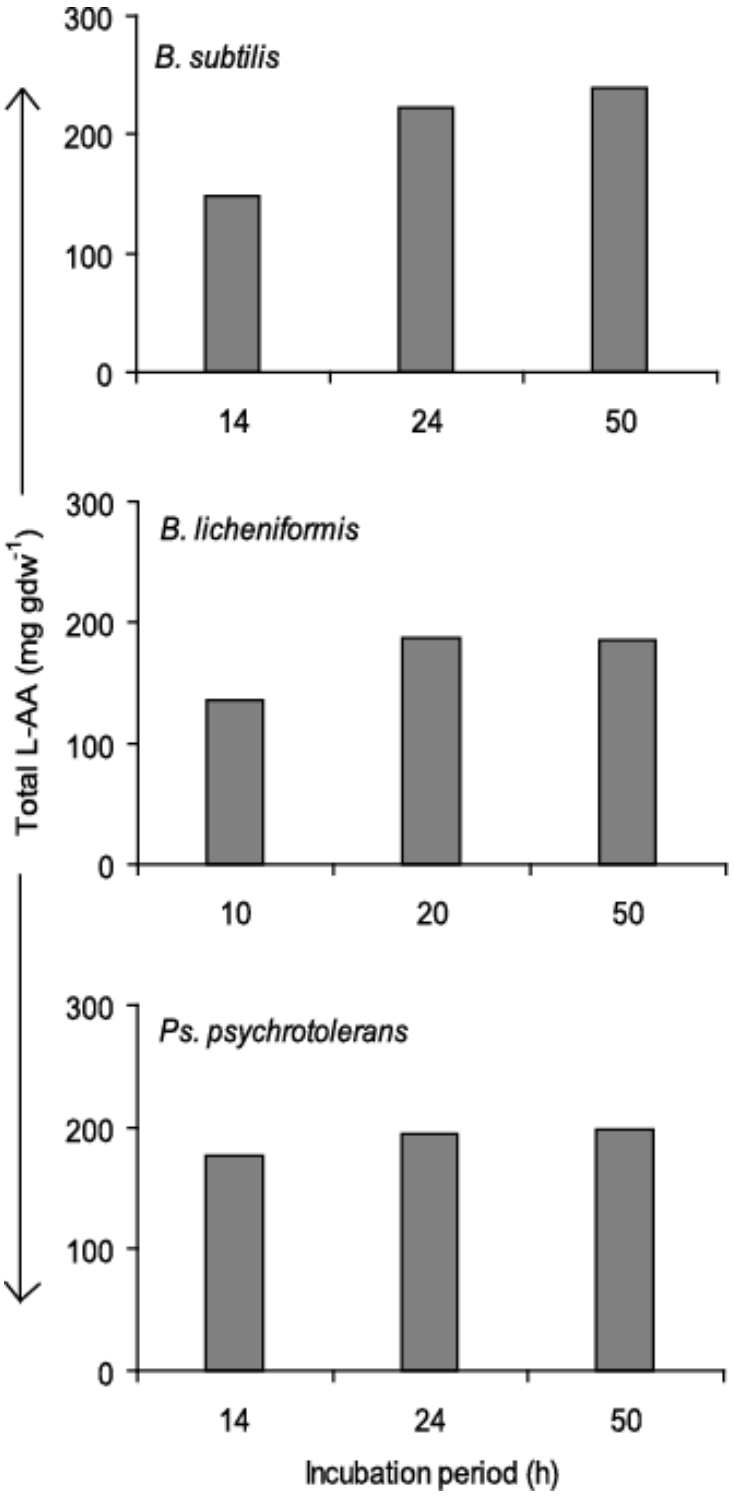

Figure 2.5. Changes in L-amino acid concentration in the cells of Bacillus subtilis, Bacillus licheniformis, and Pseudomonas psychrotolerans when grown on BSS-GYP over a 50 h incubation period.

Pseudomonas psychrotolerans during the incubation period (Fig. 2.6). Amino acids observed in bacterial cells are usually observed in various other species of bacteria (Cowie and Hedges, 1992).

2.3.3.3. D-amino acid concentration

Total D-amino acids concentration in the three bacterial cultures varied over the growth period. The total D-amino acid concentrations were relatively higher in the cells of *Bacillus subtilis* and *Bacillus licheniformis* than in *Pseudomonas psychrotolerans* cells (Fig. 2.7). The D-amino acids concentrations in *Bacillus subtilis*, *Bacillus licheniformis,* and *Pseudomonas psychrotolerans* ranged from 9.1 to 15.3 mg gdw^{-1}, 13.5 to 23.4 mg gdw^{-1}, and 9.2 to 9.7 mg gdw^{-1}, respectively (Fig. 2.7). These D-amino acid concentrations were corrected for racemization during hydrolysis (Kaiser and Benner, 2005). In both *Bacillus* species, there was a gradual increase in concentrations of D-amino acids over the period of growth, and the higher D-amino acid concentrations were recorded at 50 h of the growth period. However, for the *Pseudomonas psychrotolerans*, D-amino acid concentrations were relatively lower than those recorded for the Gram-positive bacteria. Interestingly, in *Pseudomonas psychrotolerans,* D-amino acid concentrations did not vary much over the period of cultivation. The difference observed in the D-amino acids concentrations between the Gram-positive and Gram-negative bacteria was due to difference in their cell wall components. Furthermore, the D- amino acid concentration in the bacterial cell depends upon the growth

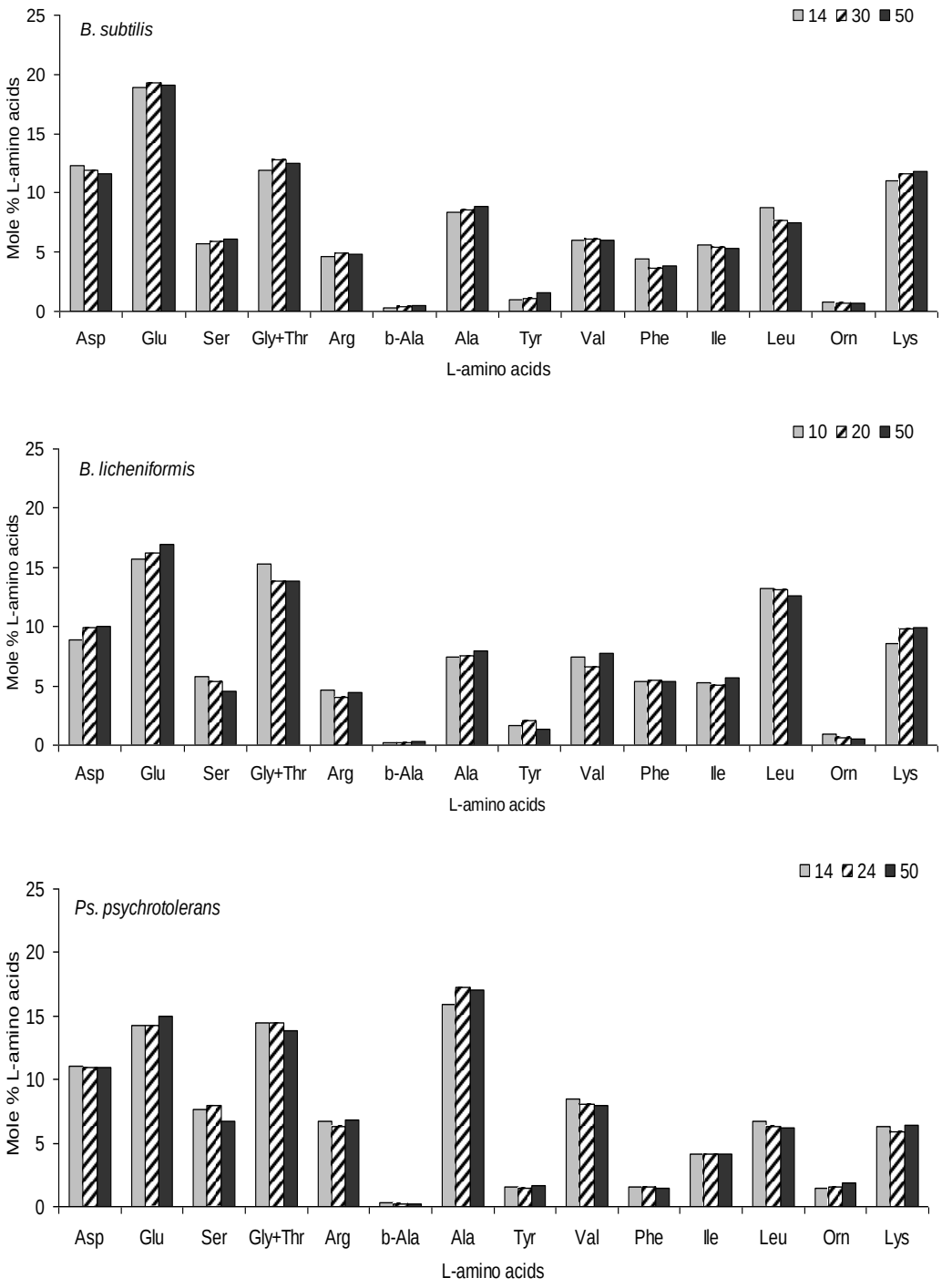

Figure 2.6. Mole percent composition of L-amino acids in the cells of Bacillus subtilis, Bacillus licheniformis, and Pseudomonas psychrotolerans when grown on BSS-GYP over a 50 h growth period. Asp: aspartic acid, Glu: glutamic acid, Ser: serine, Gly+Thr: glycine plus threonine, Arg: arginine, b-Ala: beta-alanine, Ala: alanine, Tyr: tyrosine, Val: valine, Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, Lys: lysine

period and growth conditions. Gram-positive bacteria, particularly the species of the genus *Bacillus* contains large pool of extractable D-amino acids (Bhattacharya and Banerjee, 1974). Moreover, these authors reported that the D-amino acid concentrations in *Bacillus subtilis* varied with culture conditions, and concentrations were maximal when grown at 30°C. Further, they observed greater amounts of D-amino acid when grown for longer incubation periods. In present study higher D-amino acids were observed during the stationary phase of growth for *Bacillus subtilis* and *Bacillus licheniformis.*

2.3.3.4. D-amino acid composition

The D-amino acids D-alanine, D-aspartic acid, D-glutamic acid and D-serine were detected in the cells of *Bacillus subtilis, Bacillus licheniformis* and *P*seudomonas *psychrotolerans* when analyzed by HPLC. The D-amino acid abundance (as Mole %) varied among the three bacterial cultures. The abundance of D-amino acids was higher in the Gram- positive *Bacillus* species, than the Gram-negative *Pseudomonas psychrotolerans* (Fig. 2.8). In the *Bacillus sps*. D-alanine was the most abundant (> 10 %), while D-aspartic acid, D-glutamic acid and D-serine accounted for < 8 % of total L + D- amino acids, respectively (Fig. 2.8). Contribution from D-alanine to total D + L-alanine did not vary much in *Bacillus subtilis during the* growth period, while that of D-alanine was relatively higher for *Bacillus licheniformis* at the 50 h growth period (Fig. 2.8). For *Pseudomonas psychrotolerans*, D-amino acids trend was different from that observed for the *Bacillus* species. D-glutamic acid, D-

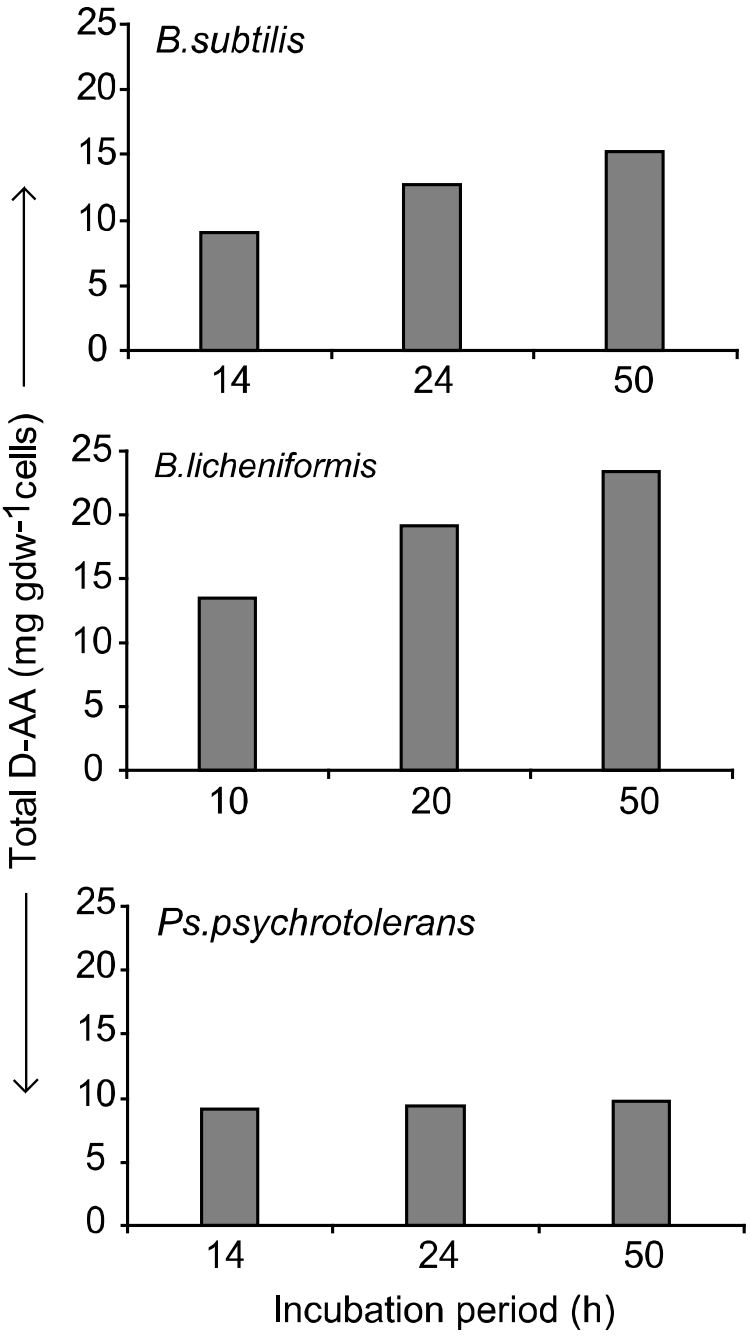

Figure 2.7. Changes in D-amino acid concentration in the cells of Bacillus subtilis, Bacillus licheniformis, and Pseudomonas psychrotolerans when grown on BSS-GYP over a 50 h growth period

aspartic acid and D-alanine were the abundant in the *Psudomonas psychrotolerans* cells over the period of incubation (Fig. 2.8). Moreover, the abundance of all four D-amino acids was relatively constant and/or did not change drastically during the incubation period. Contribution of D-serine was relatively low compared to other D-amino acids. The differences observed in D-amino acid concentration and composition in the *Bacillus* species, and *Pseudomonas s*pecies maybe due to the difference in the cell wall structure.

Gram-positive bacterium has thick cell wall consisting of peptidoglycan and techoic acid. Both these polymers contain D-amino acids. In contrast, Gram-negative bacterium has much thinner cell wall made of peptidoglycan and associated proteins. Moreover, the composition of peptidoglycan may vary among different classes of bacteria (Friedman, 2010). Furthermore, D-amino acid varies in the peptide bridges of gram-positive and gram-negative bacteria (Schleifer and Kandler, 1972; Kaiser and Benner, 2008). Gram-negative bacteria contain D-alanine and D-gutamic acid, while the Gram-positive bacteria besides D-alanine and D-glutamic acid also contain D-serine and D-aspartic acid in the peptide linkages. Bruckner et al. (1993) found highest concentration of D-alanine, D-aspartic acid and D-glutamic acid, while relatively lower but significant amounts of D-serine in several classes of microorganisms. This supports the observed differences in the D-amino acids in the bacterial cultures used in this study. Besides, the abundance of D-alanine in the *Bacillus* species and *Pseudomonas psychrotolerans* can be attributed to variable amount of peptidoglycan present in the cell

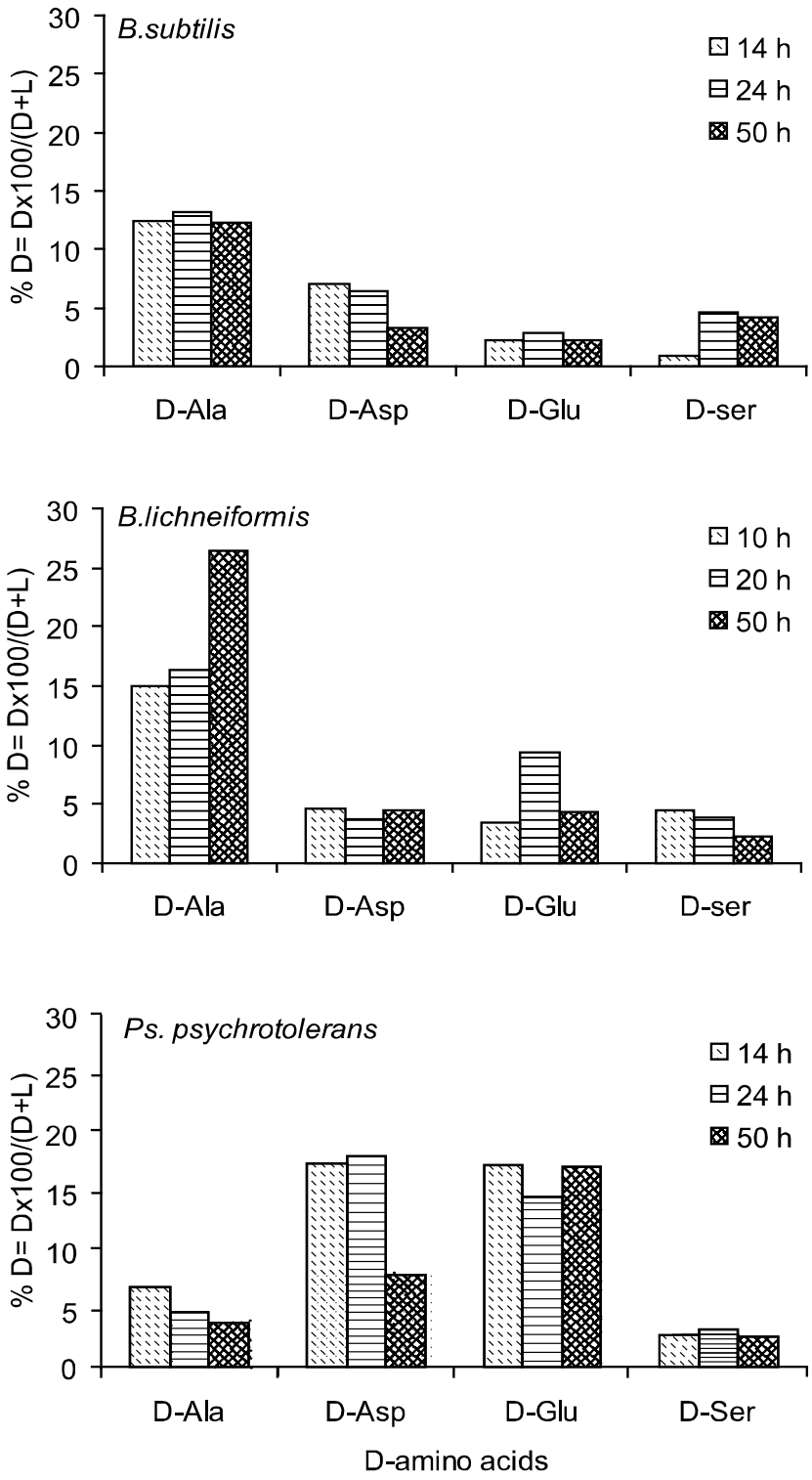

Figure 2.8. Changes in D-amino acids (% D to D+L-amino acid) in Bacillus subtilis, Bacillus licheniformis, and Pseudomonas psychrotolerans when grown on BSS-GYP over a 50 h growthperiod

wall of these two cultures. Further, the relatively lower abundance of D-alanine in the *Bacillus licheniformis* during the initial 24 h incubation period may be due to the release of D-alanine into the culture broth during cell growth and division whereby the peptidoglycan is cleaved and newly synthesized subunits are incorporated into cell wall (Kawasaki and Benner, 2006).

2.3.4. Effect of nutrients (nitrogen and phosphorus) on bacterial cells

2.3.4.1. L-amino acid concentration

Addition of different concentrations of nitrate and phosphates to the growth medium resulted in increased concentrations of total L-amino acids in the bacterial cells of *Bacillus subtilis, Bacillus licheniformis, and Pseudomonas psychrotolerans* (Fig. 2.9). The total L-amino acid concentration increased from 222 to 286 mg gdw^{-1} for *Bacillus subtilis* (*t*-test, $p<0.0001$), 250 to 409 mg gdw^{-1} for *Bacillus licheniformis* (*t*-test, $p<0.001$), and 98 to 127 mg gdw^{-1} for *Pseudomonas psychrotolerans* (*t*-test, $p<0.0001$) when the cultures were supplied with increasing concen- tration of nitrogen (Fig. 2.9). Similarly, an increase in total L-amino acid concentration was observed with increasing concentration of phosphorus in the culture medium. The total L-amino acid concentration increased from 227 to 280 mg gdw^{-1}, 198 to 341 mg gdw^{-1}, and 96 to 122 mg gdw^{-1} in the cells of *Bacillus subtilis* (*t*-test, $p<0.0001$), *Bacillus licheniformis* (*t*-test, $p<0.001$), *and Pseudomonas psychrotolerans* (*t*-test, $p<0.0001$), respectively (Fig. 2.9).

Both nitrogen and phosphorus are the limiting nutrients for primary production in marine environments (Morris and Lewis, 1992; Elser et al.,

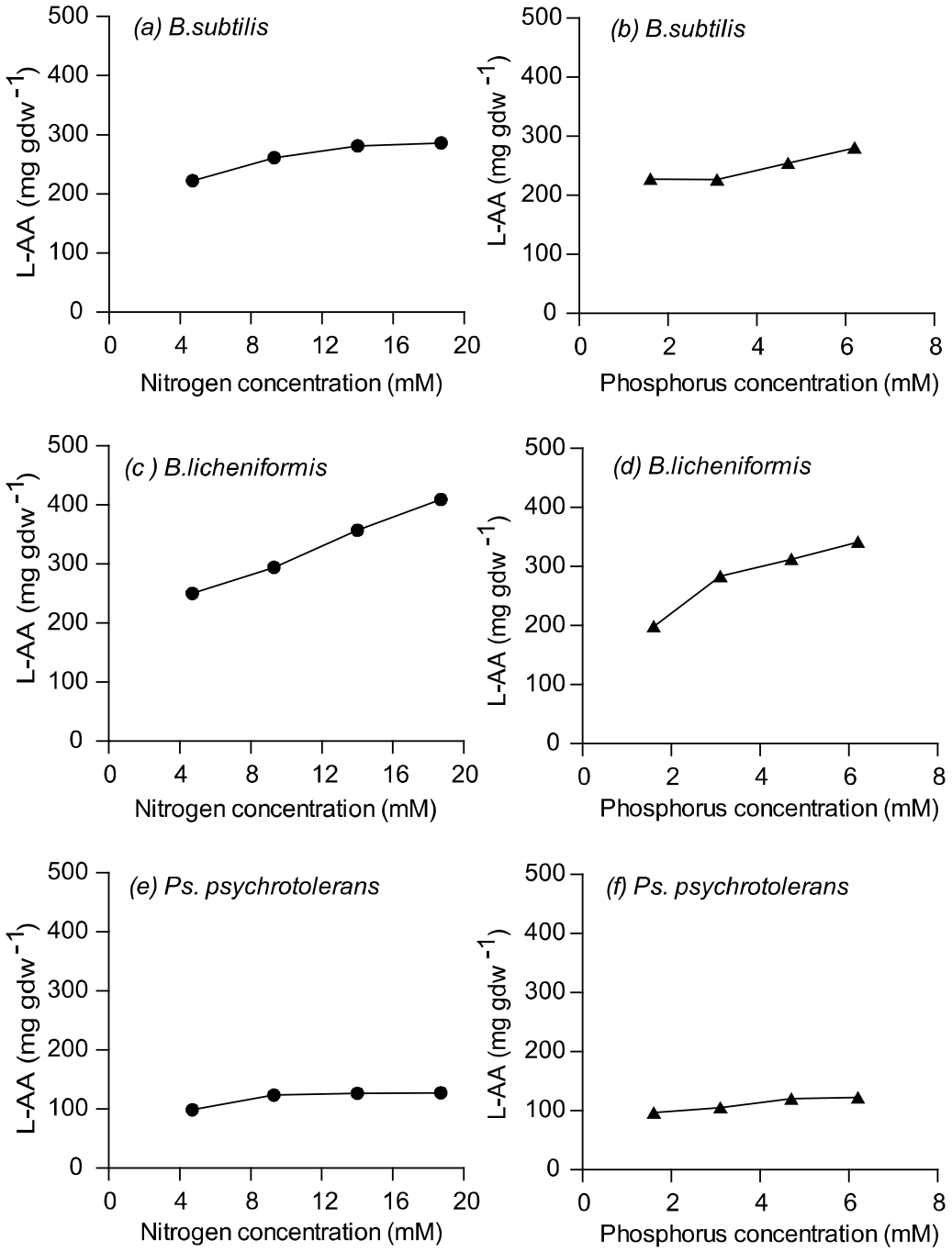

Figure 2.9. Effect of various concentrations of nitrogen and phosphorus on L-amino acids concentration in the cells of Bacillus subtilis (a and b), Bacillus licheniformis (c and d) and Pseudomonas psychrotolerans (e and f).

1995; Pomeroy et al., 1995). Moreover, the nutrient deficiencies can adversely affect bacterial growth and metabolism. Both nitrate and phosphate are essential nutrients for bacteria. These nutrients are assimilated during bacterial cell growth resulting in the production of bacterial cells, biomass, and protein as evident from increasing total L-amino acids concentrations over the period of incubation. Tsukagoshi et al. (1981) observed an increase in protein synthesis in a *Bacillus* species when the concentration of nutrients, especially phosphorus increased in medium.

2.3.4.2. D-amino acid concentration

When the concentration of nitrogen increased in the growth medium, D-amino acid concentration also increased significantly (*t*-test, $p < 0.0001$) in the cells of *Bacillus subtilis* from 14.2 to 18.5 mg gdw^{-1} (Fig. 2.10). A similar significant (*t*-test, $p<0.0001$) increase in D-amino acid concentrations from 15.3 to 21.1 mg gdw^{-1} was observed with increasing phosphorus concentrations in the cells of *Bacillus subtilis* (Fig. 2.10). In the cells of *Bacillus licheniformis,* D-amino acid concentration increased from 8.4 to 9.4 mg gdw^{-1} and 19.6 to 37.1 mg gdw^{-1} (*t*-test, $p<0.005$) when growth medium was supplemented with various concentrations of nitrogen and phosphorus, respectively (Fig. 2.10). The D-amino acid concentration in the cells of *Pseudomonas psychrotolerans* also increased significantly (*t*-test, $p<0.005$) with increase in concentrations of nitrogen and phosphorus**.** D-amino acid concentrations in the cells of *Pseudomonas psychrotolerans* ranged from 38.0 to 44.5 mg gdw^{-1} and 31.6 to 37.6 mg gdw^{-1} when different concentrations of nitrate and phosphorus were used in the growth medium (Fig. 2.10).

Pelagic heterotrophic bacteria play important role in the cycling of carbon and important nutrients such as nitrogen and phosphorus in aquatic ecosystems.

Moreover, bacteria are the major consumers of dissolved nutrients. Bacterial growth and biomass in aquatic ecosystems are regulated by a number of factors, such as temperature (Shiah and Ducklow, 1994), predation (Caron 1991), and substrate supply (organic and inorganic nutrients) (Cole et al., 1988; Rivkin and Anderson, 1997), and viral infections (Proctor and Fuhrman 1992). Bacterial growth in aquatic ecosystems can be limited by the availability of nitrogen and phosphorus rather than the supply of organic carbon (Morris and Lewis, 1992; Elser et al., 1995; Pomeroy et al., 1995).

Nitrogen or phosphorus deficiency can lead to bacterial growth limitation (Carlsson and Caron, 2001). Nitrogen, and phosphorus, or both can play a fundamental role in regulating the growth of bacteria (Zweifel et al., 1993; Elser et al., 1995; Pomeroy et al., 1995; Cotner et al., 1997; Rivkin and Anderson, 1997). Moreover, nitrogen is found to be the constituent of the amino acids, while phosphorus, the constituent of phospholipids, lipopolysaccharides and teichoic acid in the bacterial cell wall. In the present study, the observed variation in D-amino acids concentrations in bacterial cultures in the presence of different concentrations of nitrogen and phosphates may be due to different nutrient requirements of species or variable response to the added nutrients. Bacteria are able to utilize various form of nitrogen, such as org anic nitrogen compound, ammonia, nitrate or molecular nitrogen. The utilization of particular form of nitrogen however, depends on the properties of particular bacteria (Harder and Dijkhuizen, 1983).

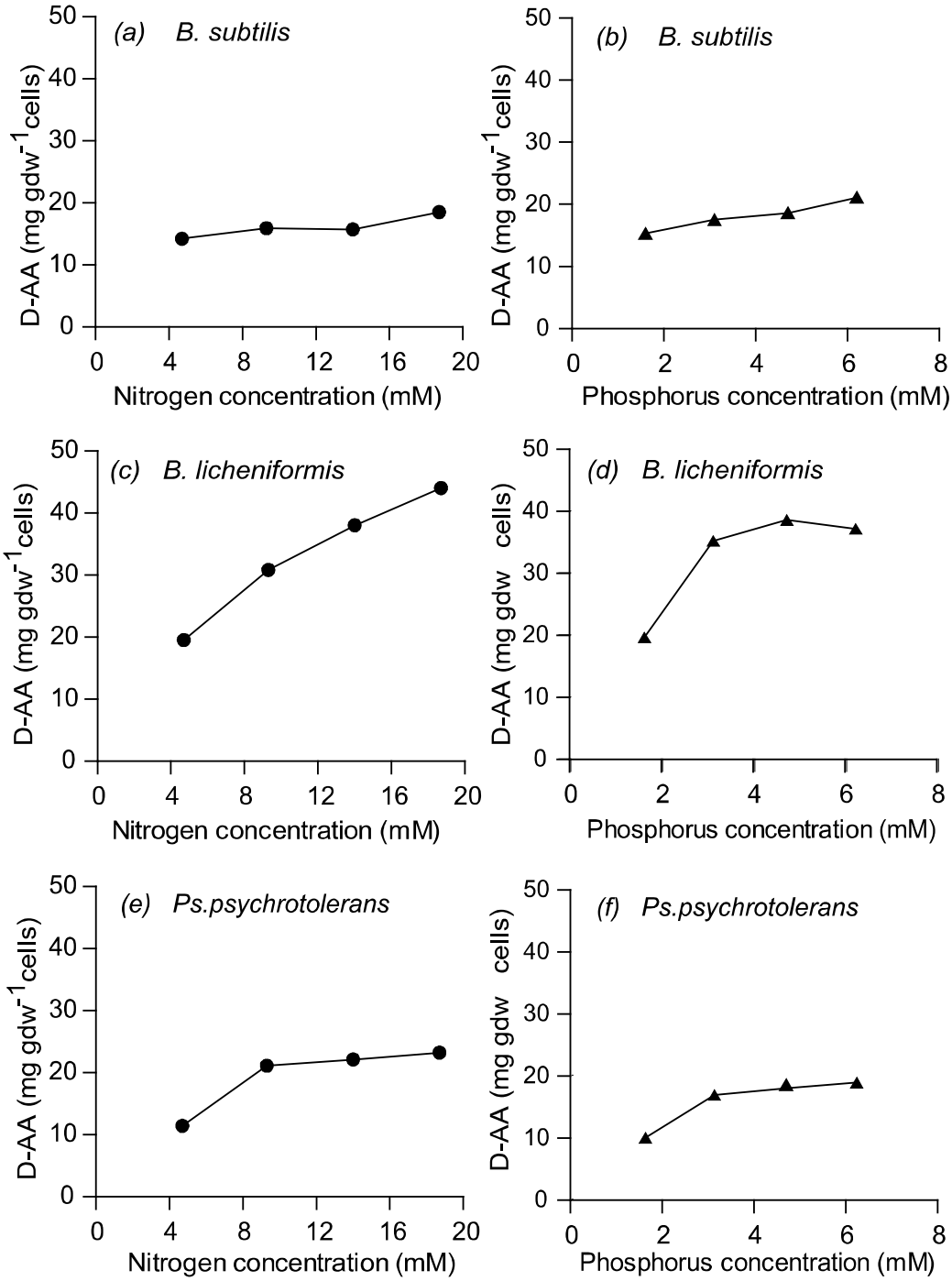

Figure 2.10. Effect of various concentrations of nitrogen and phosphorus on D-amino acid concentration in the cells of Bacillus subtilis (a and b), Bacillus licheniformis (c and d), and Pseudomonas psychrotolerans (e and f)

2.3.5. Effect of starvation on the cell abundance, amino acids in cells and cell free culture broth of Bacillus species.

2.3.5.1. Total bacterial cell count (TBC)

The TBC decreased from 9.6 x 10^8 to 7.0 x 10^8 cells ml^{-1} and 8.6 x 10^8 to 6.8 x 10^8 cells ml^{-1} for *Bacillus licheniformis,* and *Bacillus subtilis*, respectively over the period of starvation (Fig. 2.11).

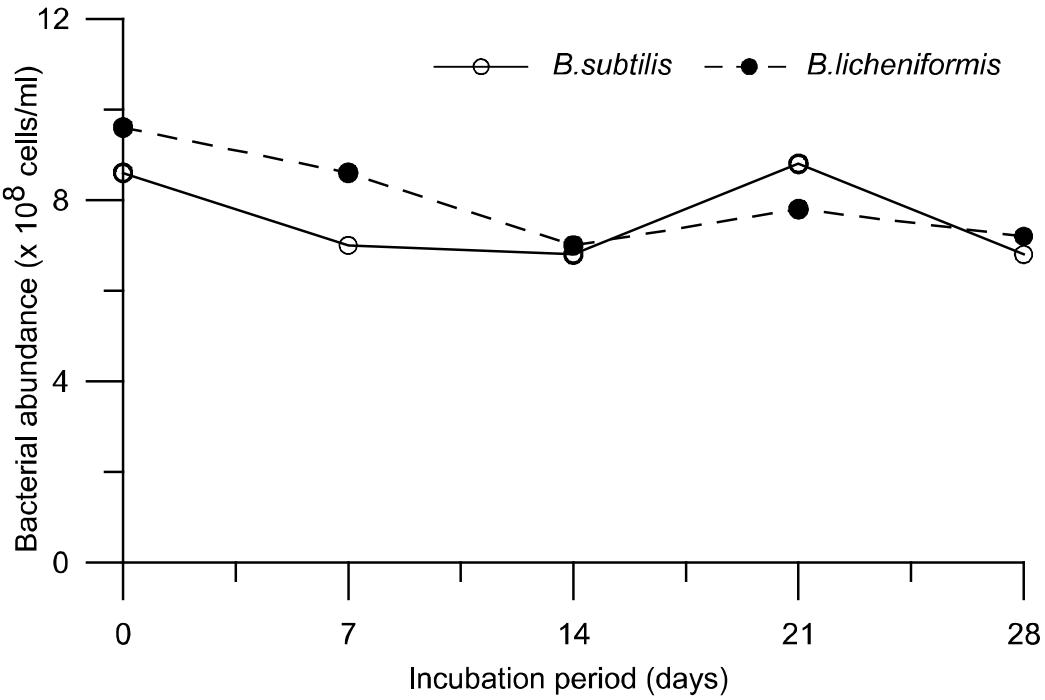

Figure 2.11. Changes in the cells of Bacillus subtilis and Bacillus licheniformis over 28 day period of starvation.

Bacteria in natural environments are often found under nutrient limiting conditions. Many bacteria are found to develop starvation survival strategies that enable them to persist in environment until conditions are favorable. Studies have shown that bacterial cells enter a viable but non culturable state upon starvation, wherein the

cells are metabolically active but incapable of cellular division during starvation (Xu et al., 1982; Kjelleberg et al., 1993; Kaprelyants and Kell, 1993; Oliver, 1993; Kolter et al., 1994; Foster and Spector, 1995).

2.3.5.2. L-amino acid concentration in the cells

During starvation, the total L-amino acid concentrations varied from 273 to 72 µ $molL^{-1}$ and 361 to 164 µ $molL^{-1}$ in the cells of *Bacillus subtilis,* and *Bacillus lichenifomis*, respectively (Fig. 2.12a). A significant difference in L-amino acid concentration was observed for *Bacillus subtilis* (*t*-test, p< 0.01) and *Bacillus lichenifomis* (*t*-test, p<0.001). The total L-amino acid concentration in the bacterial cells was higher at day 0 of the starvation period (Fig. 2.12a). However, as the period of starvation increased total L-amino acid concentrations in cells decreased due to stress. Under nutrient deficient conditions, L-amino acids of the cells are released into starvation medium.

2.3.5.3. D-amino concentrations in the cells

A significant (*t*-test, *p=0.02*) difference in the D-amino acid concentrations in the cells of *Bacillus subtilis* and *Bacillus licheniformis* was observed over 28 day period of incubation. D-amino acids concentration varied from 13.4 to 62.4 µ $molL^{-1}$ and 6.8 to 43.6 µ $molL^{-1}$ in the *Bacillus subtilis* and *Bacillus licheniformis* cells, respectively (Fig. 2.12b). Initially, both *Bacillus licheniformis* and *Bacillus subtilis* had a relatively higher concentrations of the D-amino acids at day 0 of the starvation, which decreased gradually in the cells of both the bacterial cultures during the 28 day period of starvation (Fig. 2.12 b).

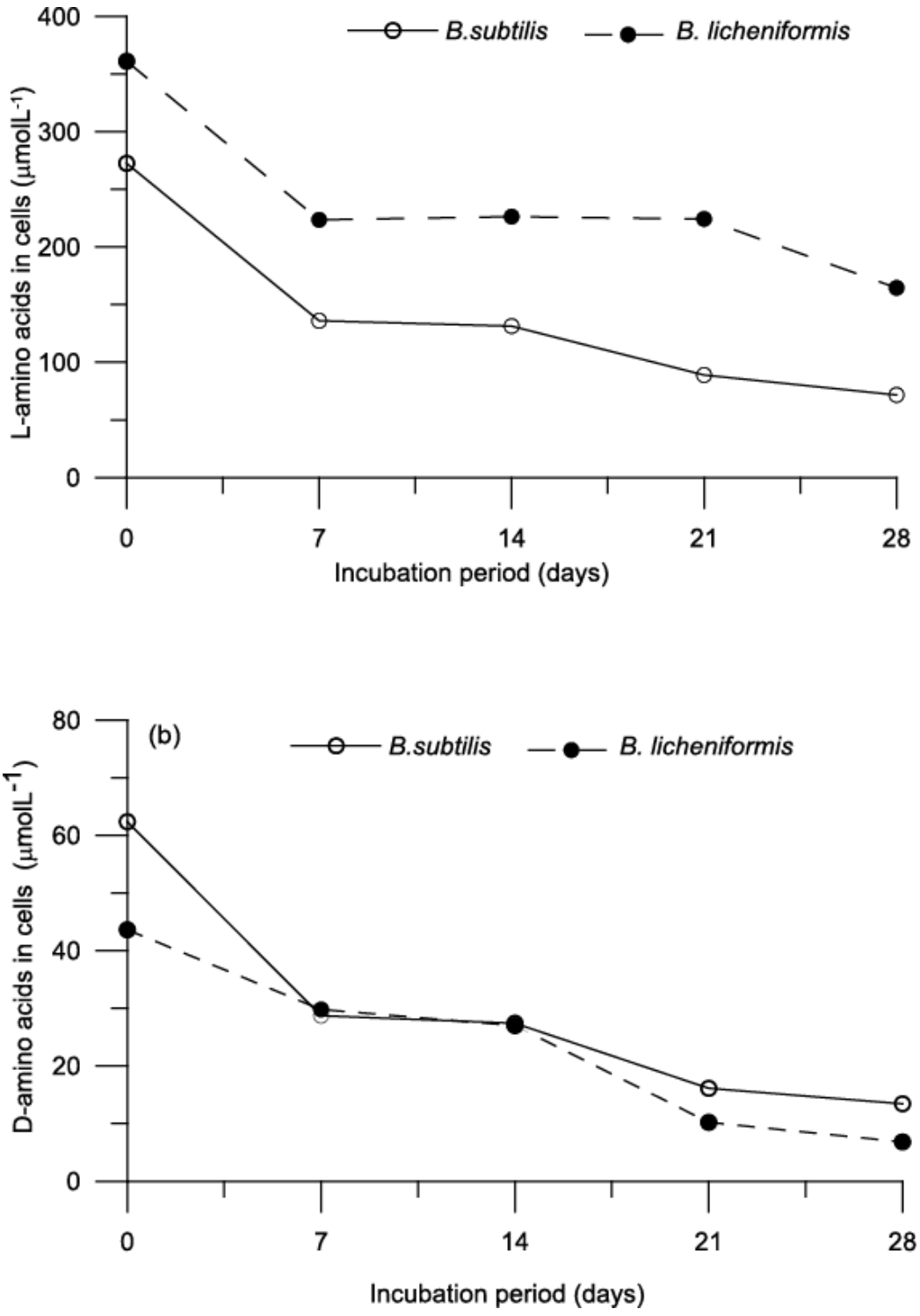

Figure 2.12. Changes in the concentration of L- and D-amino acids in bacterial cells over 28 day period of starvation.

The decrease in D-amino acid concentration in the cells of *Bacillus subtilis* and *Bacillus licheniformis* was due to release of D- amino acids into the starvation medium (seawater) under nutrient deficient condition. Another reason could be lack of or low synthesis of D-amino acids in the cell during nutrient deficient condition. Watson et al. (1998) observed synthesis of peptidoglycan in the bacterial cells of *Staphylococcus aureus* during starvation. However the synthesis of peptidogycan was relatively slower in the starved cells.

2.3.5.4. Release of L-amino acids in culture broth

The total L-amino acid concentrations varied from 20.8 to 11.5 µ molL^{-1} and 55.0 to 35.9 µ molL^{-1} in culture broth of *Bacillus subtilis* and *Bacillus licheniformis,* respectively (Fig. 2.13). The observed differences in the L-amino acid concentrations were highly significant (*t*-test, $p< 0.001$) for the *Bacillus* sp. In the culture broth of *Bacillus subtilis* and *Bacillus licheniformis*, relatively higher L-amino acid concentrations were observed at day 0 which decreased at day 14 of the starvation (Fig. 2.13). L-amino acids are relatively labile in nature and hence are utilized by the bacterial culture as nutrient source, thereby resulting in their decrease in the culture broth. Ryan (1951) observed recycling of nutrients derived from dead cells for the maintenance of the surviving bacterial cells in the medium. However, after 14 day period of starvation, total L-amino acid concentrations remained relatively constant in the culture broth of *Bacillus subtilis* (Fig. 2.13). This may be due to the balance between the release and uptake of L-amino acids by the bacterial cells. However, in the culture broth of *Bacillus licheniformis*. L-amino acids increased after 14 day period of starvation presumably suggesting their release from bacterial cells or degradation of bacterial cells.

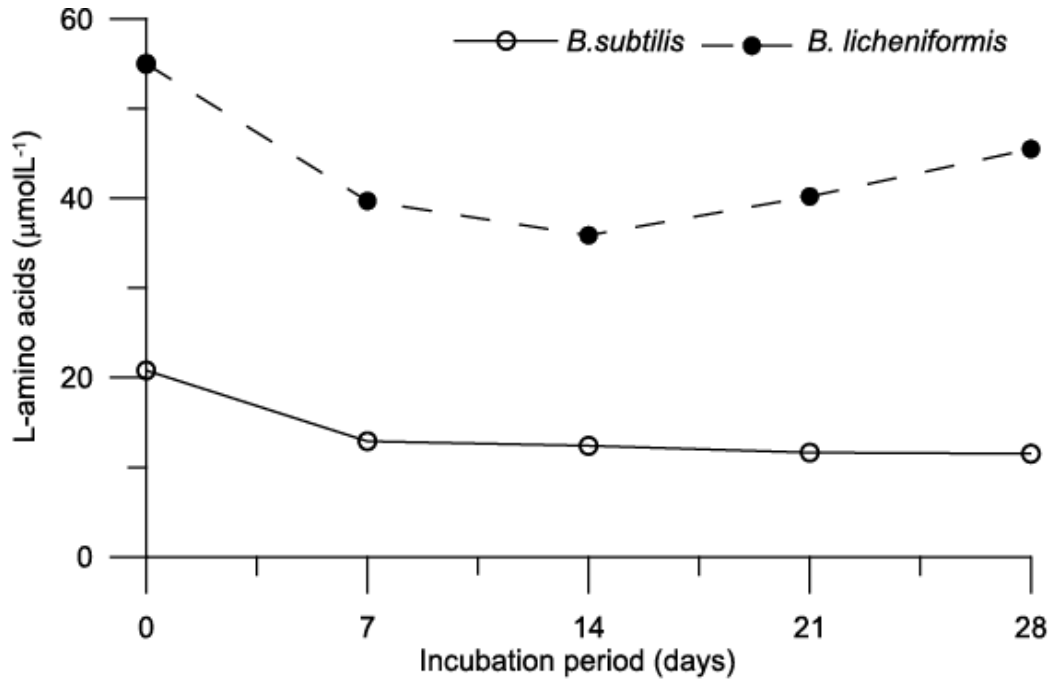

Figure 2.13. Changes in the concentration of L-amino acids in culture broth over 28 day period of starvation.

2.3.5.5. Composition of L-amino acids in culture broth

The Mole % of the individual L-amino acids varied in the cell free culture broth of *Bacillus subtilis* and *Bacillus licheniformis* over the incubation period (Fig. 2.14). In the cell free culture broth of *Bacillus subtilis*, alanine, glutamic acid, aspartic acid and lysine were the most abundant amino acids and they contributed more than 10 Mole % to total amino acid pool (Fig. 2.14). In the cell free culture broth of *Bacillus subtilis*, aspartic acid, glutamic acid, alanine, and glycine plus threonine increased, while arginine, valine, isoleucine, leucine and lysine decreased with the increasing starvation period (Fig. 2.14). In the cell free culture broth of *Bacillus licheniformis*, alanine, leucine, aspartic acid, glutamic acid, valine, and lysine were abundant amino acids during 28 day starvation period (Fig. 2.14). Aspartic acid, glutamic acid, alanine, valine, phenylalanine, leucine, iso- leucine, and lysine decreased during the starvation period in the cell free culture broth of *Bacillus licheniformis.* In contrast in the cell free culture broth of *Bacillus licheniformis* serine and glycine plus threonine increased during the incubation period (Fig. 2.14).

Amino acids such as valine, phenylalanine, arginine, leucine, isoleucine, and lysine are found in the freshly produced labile organic matter in marine environment (Hecky et al., 1973). It has been observed that the Mole % of these amino acids decrease during the degradation of organic matter. In contrast, the serine and glycine plus threonine are found to increase in the degraded organic matter. Increasing Mole % of glycine plus threonine in the cell free culture broth indicates that the organic matter from the bacterial cells was relatively degraded with increasing starvation period.

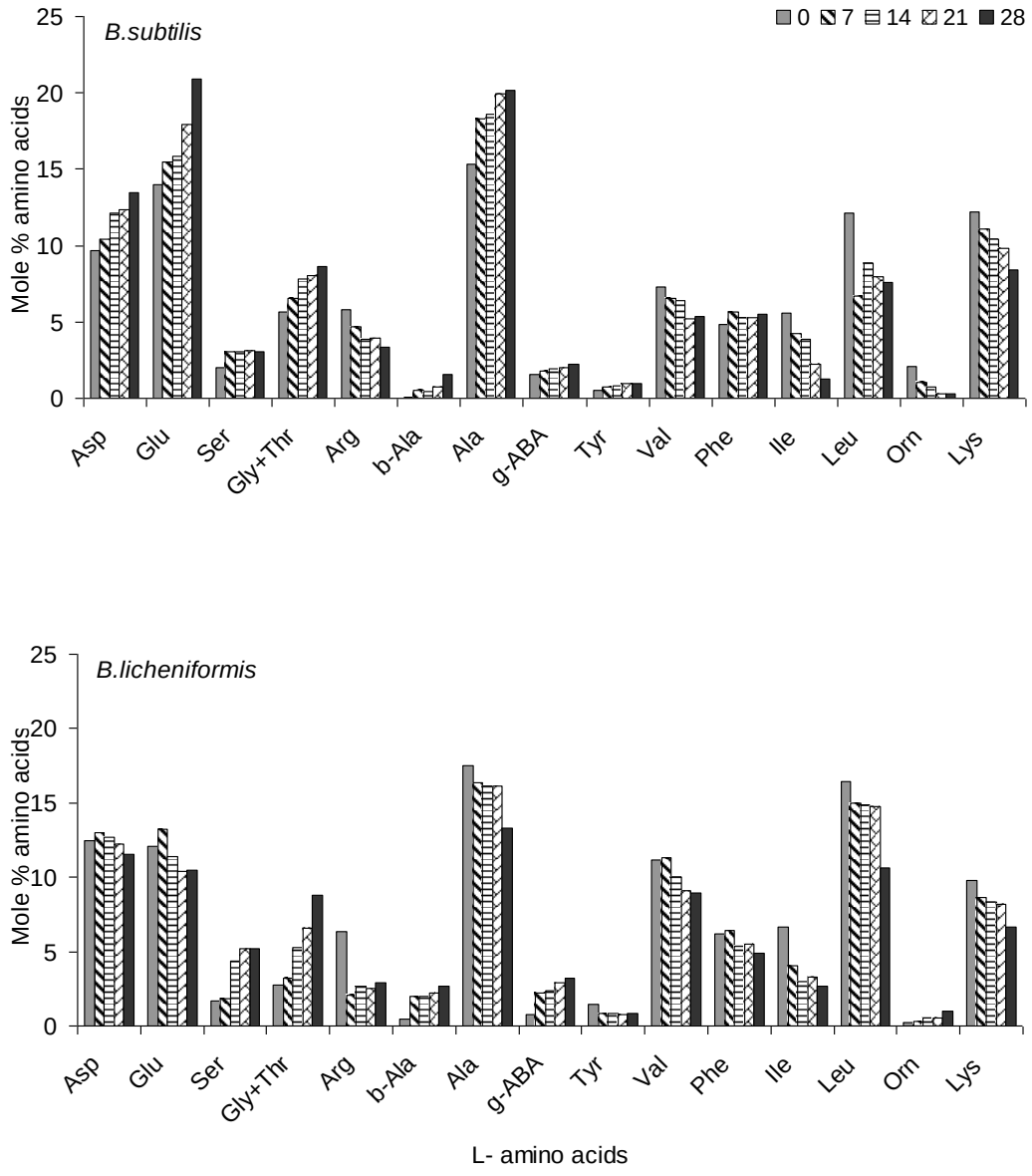

Figure 2.14. Mole percent composition of L-amino acids in the cell free broth of Bacillus subtilis and Bacillus licheniformis over 28 day period of starvation. Asp: aspartic acid, Glu: glutamic acid, Ser: serine, Gly+Thr: glycine plus threonine, Arg: arginine, β-Ala: bta-alanine, Ala: alanine, γ-ABA: gamma-amino butyric acid, Tyr: tyrosine, Val: valine, Phe: phenylalanine, Ile: isoleucine, Leu: leucine, Orn: ornithine, Lys: lysine

The non-protein amino acids such as β-alanine (β-Ala) and γ-amino butyric acid (γ-ABA) are the bacterial decarboxylation product of aspartic acid and glutamic acid (Cowie and Hedges, 1992). During the incubation period in the cell free culture broth the *Bacillus* species., non-protein amino acids varied from 0.1 to 2.7 and 0.8 to 3.2 Mole % for β-Ala and γ-ABA, respectively (Fig. 2.14). Moreover, the Mole % non-protein amino acids increased during the incubation period (Fig. 2.14). The presence of non-protein amino acids and their increase in the cell free culture broth during the starvation indicates degradation of bacterial biomass.

2.3.5.6. Release of D-amino acids in culture broth

During the starvation, both *Bacillus* cultures released D-amino acids in culture broth. Highly significant (*t*-test, $p<0.0001$) differences were observed for the D-amino acid concentration in the *Bacillus* sp. The D-amino acids concentration in the cell free culture broth varied from 3.0 to 4.2 μ molL^{-1} and 8.0 to 11.1 μ molL^{-1} in the *Bacillus subtilis* and *Bacillus licheniformis* amended culture broth, respectively (Fig. 2.15).

The *Bacillus subtilis* and *Bacillus licheniformis* showed distinct trends for the D-amino acids released in the culture broth. The D-amino acid concentration in the cell free culture broth of *Bacillus subtilis* did not vary much with the increasing starvation period. However, in the cell free culture broth of *Bacillus licheniformis* substantial increase in D-amino acid concentrations during the 28 day starvation period was observed (Fig. 2.15).

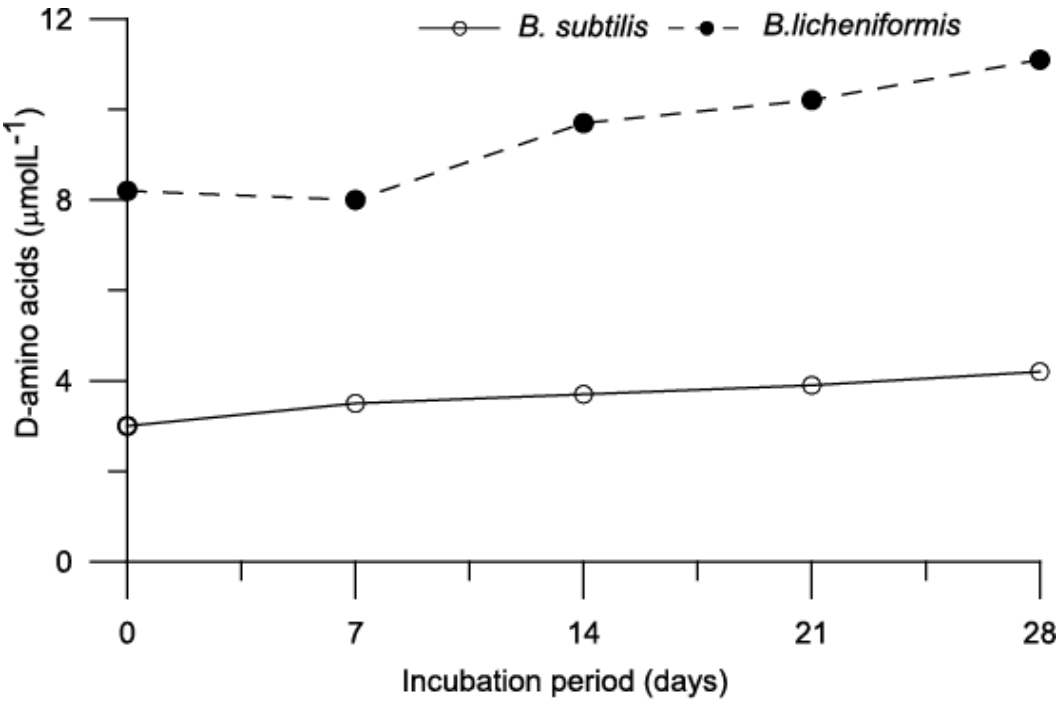

Figure 2.15. Changes in the concentration of D-amino acids in culture broth over 28 day period of starvation.

The D-amino acid released into the culture broth accounted for 18 to 23 %, and 22 to 27 % of total amino acids for *Bacillus subtilis* and *Bacillus licheniformis*, respectively (Fig. 2.16). Contribution of D-amino acids to the total amino acids showed a small increase over the period of incubation for the *Bacillus subtilis* and *Bacillus licheniformis* (Fig. 2.16).

In aquatic environments, especially marine environments a large fraction of dissolved organic matter is derived from bacteria (Nagata, 2000; Ogawa et al., 2001). Bacterial cell wall (McCarthy et al., 1998) and outer membrane components (Tanoue et al., 1995) have been observed in surface and deep seawater. The abundance of the D-amino acids in the starvation medium of both the cultures indicates that bacterial cell wall components are the integral part of the dissolved

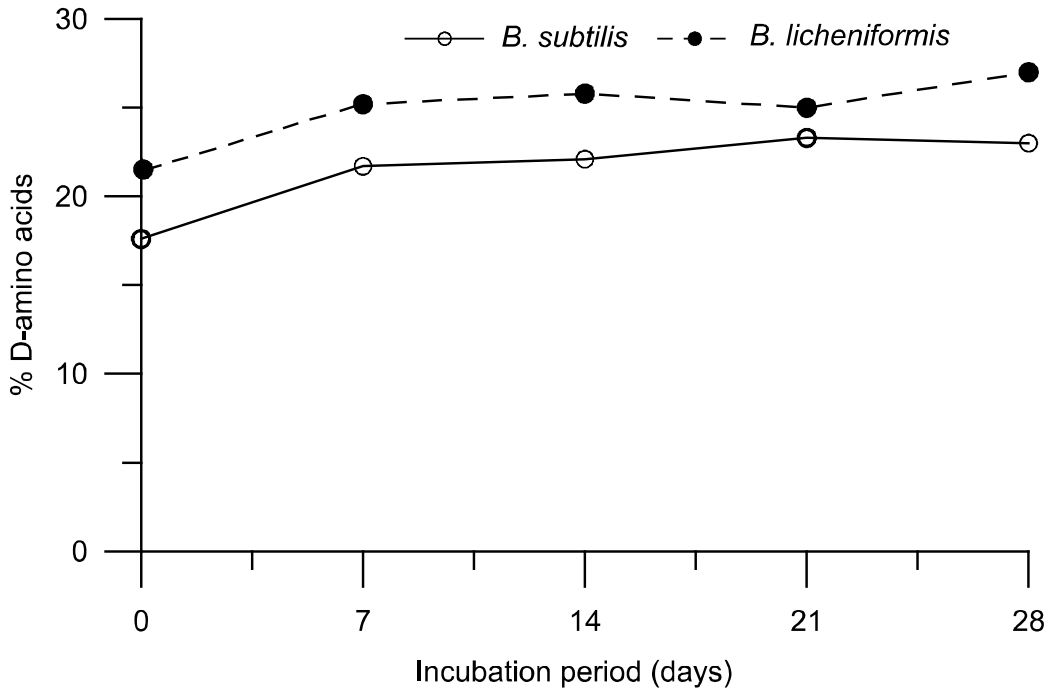

Figure 2.18. Changes in the contribution of D-amino acids in the culture broth over 28 day period of starvation.

fraction, and thus influence the chemical composition of the dissolved fraction of seawater in the marine environment. The difference observed in the composition of the D-amino acids of the two bacterial cells indicate that different bacteria react differently to the environmental stress. Moreover, the difference in the abundance of D-amino acid in dissolved fraction could be influenced by microbial community structure as well as rates and mechanism of D-amino acid release and utilization (Kawasaki and Benner, 2006). Cava et al. (2011) observed release of extra cellular D-amino acids during nutrient scarcity in the environment. Moreover, the present study revealed that diverse bacteria released, various D-amino acids into the aquatic environments during starvation .

2.3.5.7. D/L-amino acid ratios

Information on D-amino acids in the seawater is often presented as D/L-amino acid

ratios (McCarthy et al., 1998; Jorgensen et al., 1999). It has been observed that bacteria (whole cell) have relatively low D/L-amino acid ratios, which reflects dominance of protein derived amino acids, whereas, peptidoglycan has high D/L-amino acid ratios. In the present starvation study for the culture broth low average D/L-amino acid ratio of 0.25 for *Bacillus subtilis* and 0.3 for *Bacillus licheniformis* culture broth was observed at day 0 of starvation (Fig. 2.17). The D/L-amino acid ratio increased from 0.25 to 0.3 at day 7 for *Bacillus subtilis* and 0.3 to 0.4 for *Bacillus licheniformis* (Fig. 2.17). Thereafter, no changes were observed in

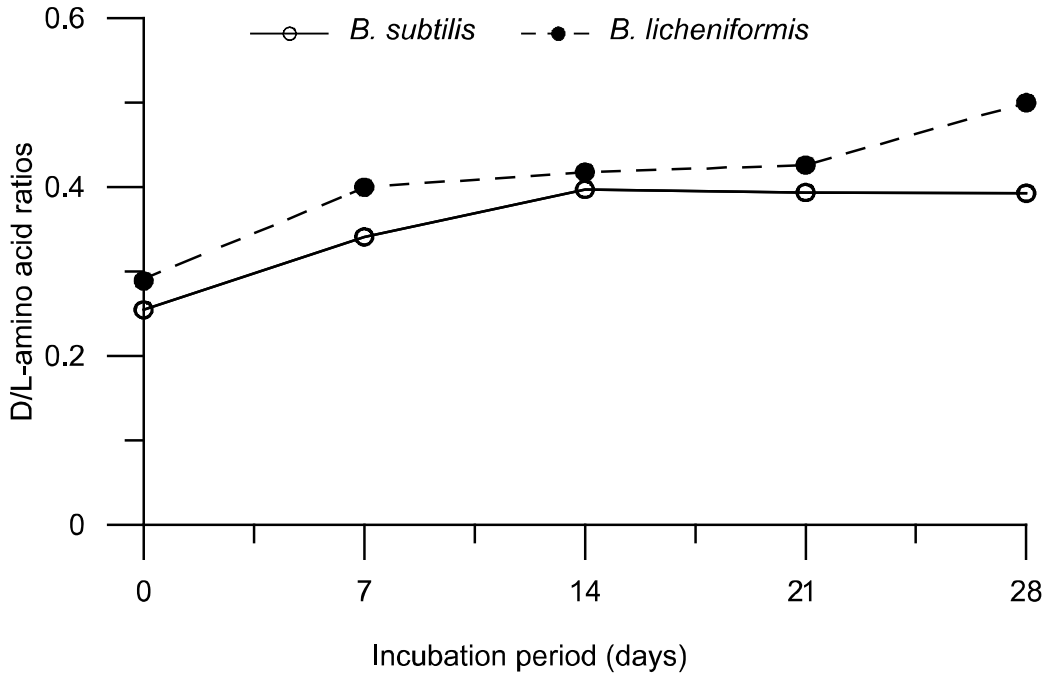

Figure 2.19. Changes in D/L-amino acid ratio in culture broth over 28 day period of starvation

D/L-amino acid ratios for the culture broth of *Bacillus subtilis*. However, in *Bacillus licheniformis* D/L-amino acid ratio increased to 0.5 at day 28 of starvation period (Fig. 2.17).

Jorgensen et al. (1999) reported a shift in D/L-amino acid ratios from a low value in the fresh material to higher value in diagenetically altered dissolved organic matter. The calculated D/L-amino acid ratios indicate that dissolved fraction derived from *Bacillus* cultures was relatively fresher at day 0, however with subsequent starvation of the cultures in the nutrient devoid seawater resulted in substantial alteration of the freshly produced dissolved organic matter.

2.3.5.8. Relative contribution of PGN-nitrogen to amino acid pool

The observed changes in the D/L-ratios can be explained by the input of peptidoglycan to the culture broth of starvation medium. The peptidoglycan input (PG-N) to TN pool can be quantified by assuming an average peptidoglycan structure as: PG-N= 5.7 x D-Ala-N (Roger, 1983). and total amino acid nitrogen (AA-N) as AA-N= 12 x Ala-N (Cowie and Hedges, 1992). Thus the percentage contribution of peptidoglycan to amino acids (% PG-AA-N) was calculated as % PG-AA-N= PG-N/AA-N x 100 (Mc Carthy et al., 1998). In the present study, the % PG-AA-N varied between 11 to 52 % and 14 to 43 % in *Bacillus subtilis* and *Bacillus licheniformis*, respectively (Fig. 2.18). Further, the % PG-AA-N was several folds higher by day 14 when compared to day 0 in both the starvation medium (Fig. 2.18). Further increase in starvation of the *Bacillus* cultures, resulted in small increase in % PG-AA-N contribution from 48 to 52 % and 37 to 39 % of total nitrogen for *Bacillus subtilis* and *Bacillus licheniformis*, respectively (Fig. 2.18).

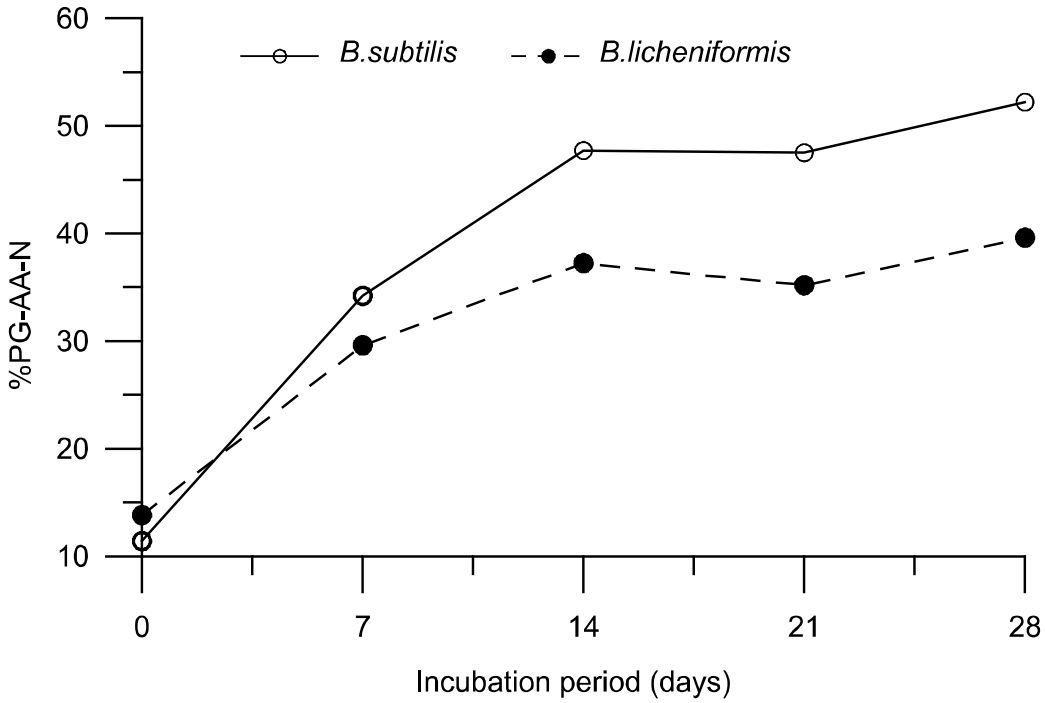

Figure 2.110. Changes in the abundance of PGN-amino acids to tolal nitrogen in culture broth over 28 day period of starvation

The increasing contribution of PG-AA-N in the cell free culture broth indicates that peptidoglycan contributed significantly to the pool of dissolved organic nitrogen.

The experiments in the chapter 2 indicate that environmental factors influence the L- and D-amino acids concentration and composition in the laboratory grown bacterial cultures. Moreover, components derived from bacterial cell wall constitute a major fraction of the dissolved and particulate organic matter in marine environments. A further laboratory experiment was carried out to assess degradability of structurally and compositional different whole bacterial cell walls and purified peptidoglycan from Gram-positive and Gram-negative bacteria. These experiments are presented and discussed in chapters 3.

Chapter 3

Microbial degradation of bacterial cell wall and peptidoglycan

3.1. Introduction

Bacteria play a central role in ecological and biogeochemical processes in marine environment. Bacteria degrade organic matter, the bacterial biomass thus developed forms an important source for the active pool of organic matter (McCarthy et al., 1998; Nagata and Kirchman, 2001; Jorgensen et al., 2003; Kawasaki and Benner, 2006; Kaiser and Benner, 2008). Bacterial cell death produces bacterial remnants that are prone to varying degree of degradation. Bacteria will degrade some of the labile components, and the more resistant components get accumulated and persists in nature for indefinite period. Bacterial cell wall components such as membrane proteins (Tanoue et al., 1995, Yamada and Tanoue, 2003), lipopolysaccharides (Wakeham et al., 2003), and peptidoglycan (Lee and Bada, 1977; McCarthy et al., 1998, Dittmar et al., 2001, Benner and Kaiser, 2003, Perez et al., 2003, Kitayama et al., 2007) have been found in marine dissolved organic matter and sediments. Observation of the bacterial cell wall components in the natural environment indicates them as an important component of recalcitrant organic matter.

Bacterial cell wall component, peptidoglycan makes up a major fraction of recalcitrant organic matter in the ocean (McCarthy et al., 1998; Nagata et al., 2003). Peptidoglycan is found in the cell walls of virtually all species of bacteria, with the exception of mycoplasma and archaebacteria (Schleifer and Kandler, 1972). Laboratory studies on the degradation of peptidoglycan by bacterial cultures (Jorgensen et al., 2003), natural seawater samples (Nagata et al., 2003; Kitayama et al., 2007) and sediment (Veuger et al, 2006) showed that peptidoglycan is semi labile, and degraded at slower rate than proteins resulting in its accumulation during degradation.

Bacterial peptidoglycan, made up of alternating strands of β-1, 4-linked N-acetylglucosamine and N- acetylmuramic acids units cross-linked by short peptide strand (Schleifer and Kandler, 1972), which is the major source of D-amino acids. Peptide strand vary with bacterial species. Amino acids in these peptides include D-enantiomers of D-alanine, D-glutamic acid, D-aspartic acid and D-serine. The occurrence of D-amino acids which is a characteristic of peptidoglycan is used as the molecular biomarker for bacteria.

Chemical composition of Gram-positive and Gram-negative bacteria is different. So it was interesting to find out if chemical differences in cell walls of these two groups of bacteria influence the degradation of their cell walls in natural seawater (0.7 μm filtered). In order to address this question degradation of bacterial cell walls and partially purified peptidoglycan isolated from Gram-positive and Gram-negative bacteria was evaluated in this chapter.

3.2. Materials and Methods

3.2.1. Preparation of bacterial inoculum

The bacterial cultures, *Bacillus subtilis, Bacillus licheniformis and Pseudomonas psychrotolerans* present on the Zobell marine agar slants were sub-cultured individually in 25 ml Erlenmeyer flasks each containing 10 ml Zobell marine broth. The cultures were grown at room temperature (28 ± 2° C) on a rotary shaker (100 rpm) for 24 h. This above procedure was repeated twice before the cells were used as inoculum for the experiments described below.

3.2.2. Harvesting of bacterial cells

Each bacterial culture was grown individually in a separate 5 L Erlenmeyer flask containing 2 L sterile Zobell marine broth on a rotary shaker (100 rpm) at room

temperature (28 ± 2° C) for 48 h. Bacterial cells were then harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. Bacterial cells pellet was then washed thrice with 0.2 µm filtered phosphate-buffered saline (PBS; 10 mM NaH_2PO_4 in 0.9 % NaCl at pH 7.5) in order to remove excess nutrients attached to cells (Jorgensen et al., 2003). In order to kill the cells, they were then dried in the oven at 50 °C. One portion of the dried bacterial cells was used to study the degradation of whole bacterial cells, while the other portion was used for the isolation of peptidoglycan.

3.2.3. Isolation of peptidoglycan

Isolation of peptidoglycan from bacterial cell wall was carried out by the method described by Pelz et al. (1998). A lysis buffer containing 35 ml sodium dodecyl sulfate (SDS) to a final concentration of 2.5 % in EDTA-Tris buffer (0.15mM EDTA, 1.5 mM Tris-HCl, pH 8) was added to the cell pellet (0.8 g dry weight). This mixture was vortexed and then boiled in a water bath for 1 h. The lysate containing the peptidoglycan was centrifuged at 48000 X g for 45 min at room temperature. The resulting pellet, containing the peptidoglycan complex, was then washed thrice with de-ionized water to remove the SDS. The pellet was then suspended in 10 ml 0.1 M phosphate buffer (pH 7.8), and sonicated for 1 min on ice bath. The suspended pellet was then treated with 2 mg trypsin (Sigma) at 37°C for 3h, in order to remove membrane-associated proteins from the peptidoglycan (Braun et al., 1970). The peptidoglycan was recovered by centrifuging at 48000 X g for 45 min. Further, the peptidoglycan was washed once with 0.1 M phosphate buffer (pH 7.0) and twice with de-ionized water. Lipids were removed from the pelleted peptidoglycan following the method of Vasstrand et al. (1987) by treating with ethanol/ethylether (3:1, v/v) followed by chloroform/methanol (2:1, v/v), chloroform/hexane (1/1, v/v);

and hexane. Each solvent treatment step was performed for 15 min at room temperature, after which the solvents were discarded. The pellet thus obtained was considered purified peptidoglycan and was used for further studies as described below.

3.2.4. Degradation of whole bacterial cell

Two hundred milligrams of the dried bacterial cells of each culture prepared as above were suspended individually in a conical flask (2 L) containing 1000 ml seawater (0.7 µm filtered) containing 3 x 10^7 cells ml^{-1} of natural bacterial population. The flasks were then incubated at room temperature (28 ± 2°C) in dark on the rotary shaker at ~ 100 rpm. Samples for total bacterial count (1ml) and D-amino acids (10 ml) were withdrawn at day 0, 3, 12, 24, 48, 60, and 75 days following inoculation. A flask containing only the filtered seawater (0.7 µm) was used as a control. Control flask was also sampled at the same time periods that were used for the experimental flasks.

3.2.5. Degradation of purified peptidoglycan

One-liter seawater (0.7 µm filtered) containing 0.46 x 10^8 cells/ml of bacteria was transferred to three flasks (2 L) individually. To each flask, 1 mg of the partially purified peptidoglycan of either *Bacillus subtilis,* or *Bacillus licheniformis,* or *Pseudomonas psychrotolerans* was added. The flasks were then incubated at room temperature (28 ± 2°C) in dark on the rotary shaker (100 rpm). Samples for total bacterial count (1 ml) and D-amino acids analyses (15ml) were removed at day 0, 3, 6, 9, 16, 25, 105, 107, 112, and 116 days following inoculation. The collected sample was filtered through 0.22 µm Millipore filter and the filtrate defined as dissolved fraction was used for the analysis of D-amino acids. At day 105 following

inoculation, 50 µl L-broth (10 g tryptone, 5 mg yeast extract, 10 g NaCl and 1 g glucose in 1000ml Milli-Q water) was added to each flask in order to stimulate bacterial growth in an attempt to promote degradation of any residual peptidoglycan in the seawater medium.

3.2.6. Total bacterial count

Changes in bacterial cells were monitored by enumerating total bacterial cells following the DAPI (4, 6- Diamidino-2-phenylindole) staining method (Porter and Feig, 1980) as given in chapter 2.

3.2.7. Analysis of D-amino acid in cell free culture broth

Culture free supernatant filtered through 0.22 µm is defined here as dissolved fraction. Samples of dissolved fraction collected during the experiments described above were used for D-amino acid analysis. Dissolved fraction (15 ml) was hydrolyzed and analyzed for D-amino acids following the method described in chapter 2.

3.3. Result and Discussion

3.3.1. Degradation of whole bacterial cells

3.3.1.1. Total bacterial count (TBC)

The TBC varied from 0.2 to 0.6 x 10^8, 3.2 to 7.1 x 10^8, 3.7 to 7.7 x 10^8 and 3.9 to 8.0 x 10^8 cells/ml in the control and in flasks amended with dead cells of *Bacillus subtilis, Bacillus licheniformis and Pseudomonas psychrotolerans*, respectively (Fig. 3.1). The bacterial cell numbers increased from day 0 to day 24 of the incubation period (Fig. 3.1) in the flasks containing dead cells of *Bacillus subtilis, Bacillus licheniformis,* and *Pseudomonas psychrotolerans*. This increase in cell numbers

implies utilization of dead bacterial cells as a source of nutrient by the bacterial population present in natural seawater. After an initial increase in bacterial abundance, a drastic decrease was observed at day 48 following inoculation of dead cells of *Bacillus subtilis* and *Bacillus licheniformis*. Further, increase in incubation period to 75 day showed small increase in TBC in flasks containing dead cells of *Bacillus subtilis*. Conversely, TBC generally decreased in flasks containing dead cells of *Bacillus licheniformis* and *Pseudomonas psychrotolerans*.

3.3.1.2. D-amino acid concentration in culture medium

In the seawater culture medium, the D-amino acid concentrations decreased from 15766 to 1470 nM, 14314 to 1454 nM, and 9041 to 3520 nM over the 75 day period of incubation when enriched with the whole dead cells of *Bacillus subtilis, Bacillus licheniformis,* and *Pseudomonas psychrotolerans*, respectively. D-amino acid concentration in the control

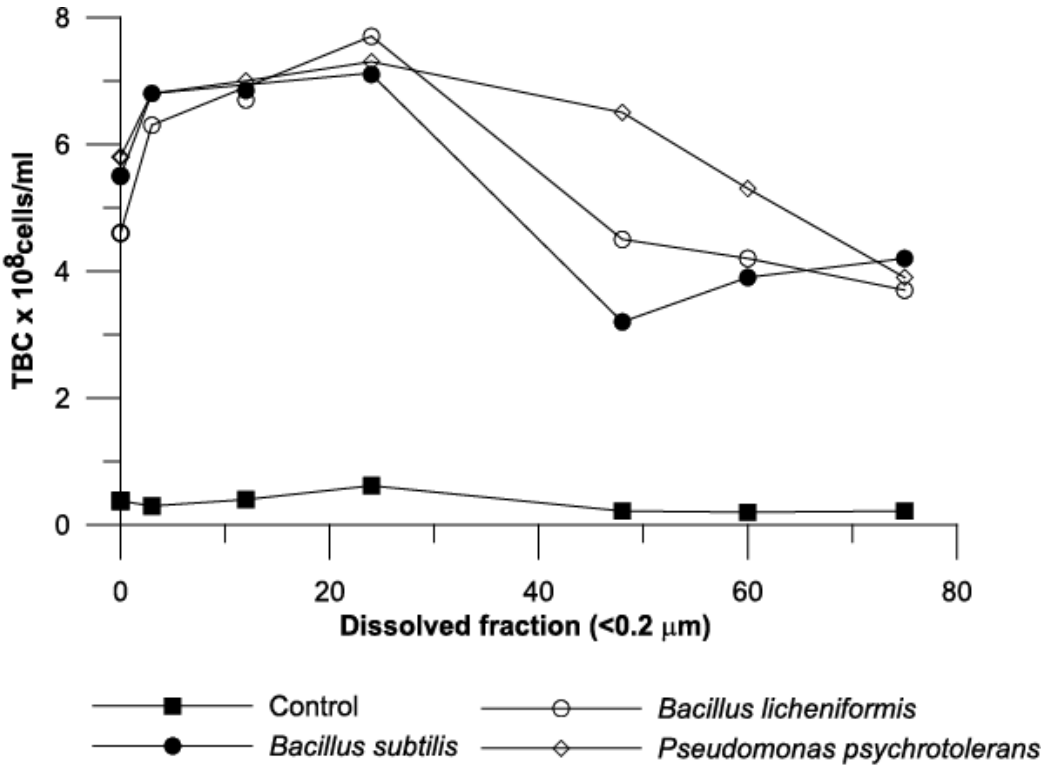

Figure 3.1.Changes in the total bacterial count (TBC) during degradation of dead cells of Bacillus subtilis, Bacillus licheniformis and Pseudomonas psychrotolerans in natural filtered (0.7 µm) seawater over the 75 day period of incubation.

was 156 nM at day 0 which decreased to 124 nM at day 75 following incubation periods (Fig. 3.2). The initial high D-amino acid concentration in the culture medium following the addition of whole cells of *Bacillus subtilis, Bacillus licheniformis,* and *Pseudomonas psychrotolerans* at day 0 was probably due to the damage to cell wall as a result of oven drying of the cells (Fig. 3.2). However, bacterial population present in natural seawater degraded 62 to 90 % of peptidoglycan D-amino acids *of the Bacillus subtilis, Bacillus licheniformis*, and *Pseudomonas psychrotolerans* over the 75 day period of incubation (Fig. 3.2). Degradation of whole cell walls of *Pseudomonas psychrotolerans* was relatively less than that recorded for the whole cell walls of *Bacillus* species. Jorgensen et al. (2003) observed 72 % and 51 % reduction in the peptidoglycan D-amino acids from the cell wall material of *Bacillus* species and *Pseudomonas* species, respectively by natural bacterial population over the 8 day period of incubation. The morphological changes using the immune fluorescence reaction, suggest that the natural bacterial population rapidly degrade the cell wall of Gram-positive bacteria, but did not degrade gram-negative bacterial cell wall to a similar extent (Jorgensen et al., 2003). These results indicate that the peptidoglycan derived from Gram-negative cell wall was more resistant to degradation than the Gram-positive cell wall and thus may accumulate in the marine environment. Kitayama et al. (2007) observed 1.1 % of petidoglycan remaining in the incubation medium at the end of 240 day incubation period, indicating that peptidoglycan released from the bacterial cells were degraded by the bac terial population. Hagstrom et al. (2000) observed bacterial 'ghosts' or ne-

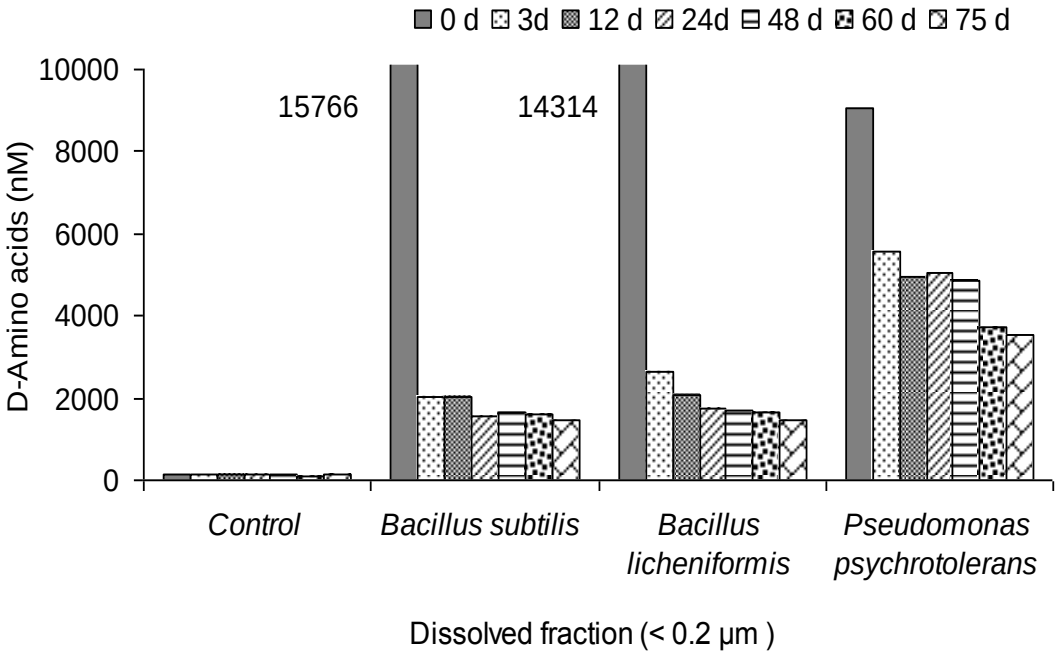

Figure 3.2. Changes in D-amino acids in cell free culture growth medium during degradation of whole dead cells of Bacillus subtilis, Bacillus licheniformis and Pseudomonas psychrotolerans in natural seawater over the 75 day period of incubation.

ucleiod-less bacteria contributing 61 to 88 % of the total bacterial numbers in marine waters. Similarly, Moriarty and Hayward (1982) observed empty 'cell sacs' in marine sediments. Therefore, from the results presented here, it can be concluded that Gram-negative bacteria may contribute substantially to the empty 'cell sacs' or 'ghost' population in the marine environment as their cell wall is relatively more resistant to degradation as compared to the gram-positive bacterial cell wall. This may be due to differences in peptidoglycan structure between Gram-positive and Gram-negative bacterial cells (Jorgensen et al., 2003). Moreover, the Gram-negative bacteria predominate in the marine environment (Giovannoni and Rappe, 2000).

3.3.2. Degradation of peptidoglycan

3.3.2.1. Changes in bacterial abundance (TBC)

The addition of partially purified peptidoglycan in the seawater culture medium notably stimulated growth of natural bacterial population. Bacterial numbers increased from 0.54 to 1.10 x 10^7, 0.52 to 1.05 x 10^7 and 0.67 to 1.18 x 10^7 cells/ml at day 9 following incubation when grown on the partially purified peptidoglycan isolated from *Bacillus subtilis, Bacillus licheniformis*, and *Pseudomonas psychrotolerans*, respectively (Fig. 3.3). The relatively higher bacterial numbers at day 9 in the experimental flasks than in the control flask indicates that the added purified peptidoglycan sustained greater bacterial growth. Subsequently, in control flask, bacterial cell numbers decreased gradually from day 9 to day 75 of the incubation period. Gradual decrease in the bacterial numbers from day 16 to day 25 of incubation period was also recorded for

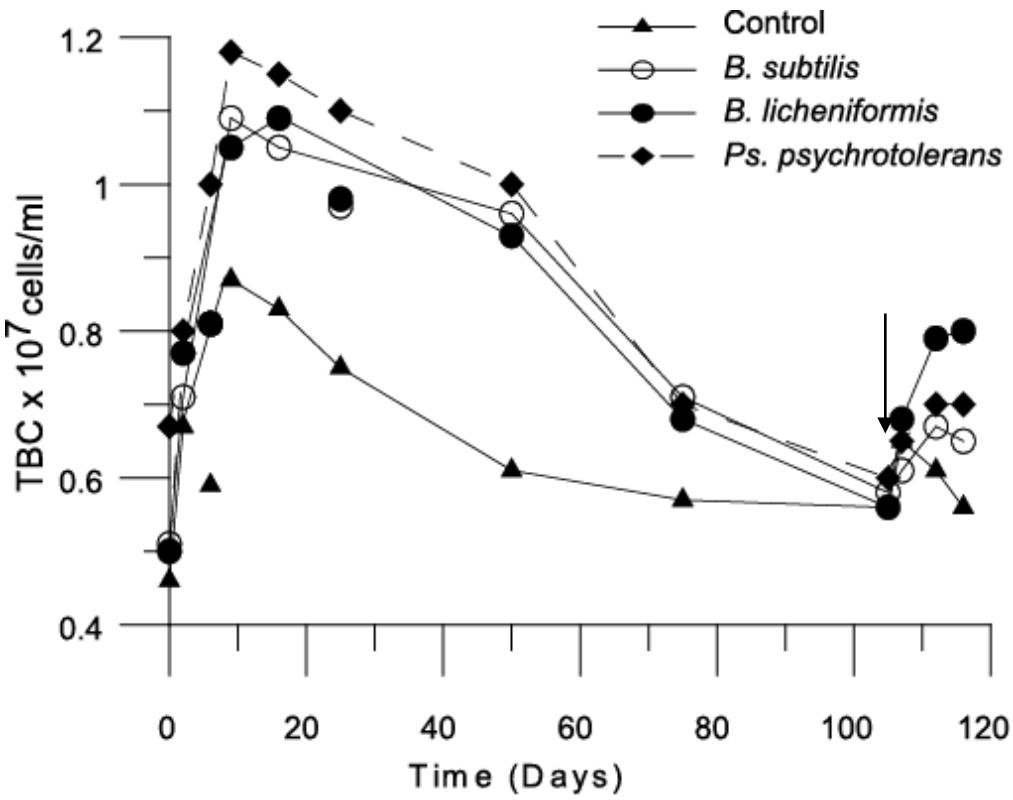

Figure 3.3. Changes in the total bacterial count (TBC) during the degradation of partially purified peptidogycan obtained from Bacillus subtillis, Bacillus licheniformis and Pseudomonas psychrotolerans. Arrow at day 105 indicates addition of L-broth.

the experimental flasks containing purified peptidoglycan of *Bacillus subtilis, Bacillus licheniformis* and *Pseudomonas psychrotolerans*. Subsequently, the bacterial numbers decreased drastically in the three experimental flasks on day 105 of the incubation period.

At day 105 following inoculation, bacterial cell number was 0.56 x 10^7, 0.58 x 10^7, 0.56 x 10^7 and 0.6 x 10^7 cells ml^{-1} in control, and in the flasks containing partially purified peptidoglycan *of Bacillus subtilis*, *Bacillus licheniformis* and *Pseudomonas psychrotolerans*, respectively. Subsequently, on day 105 of the incubation period, fresh growth medium was added to the control and experimental flasks. Addition of growth medium i.e. L- broth, resulted in increase in the bacterial cells from 0.56 x 10^7 to 0.65 x 10^7 cells ml^{-1} at day 107 of the incubation period in control. A similar re-growth of natural bacterial population from 0.58 x 10^7 to 0.67 x 10^7 cells ml^{-1} was observed for *Bacillus subtilis* when incubated until 112 days, and thereafter the bacterial cell numbers decreased. On the contrary, bacterial cell numbers increased gradually until 116 day of the incubation period, during the degradation of peptidoglycan isolated from *Bacillus licheniformis* and *Pseudomonas psychrotolerans* (Fig. 3.3). The Paired *t*- test indicated that the differences observed between control and experimental flasks with *Bacillus subtilis*, *Bacillus licheniformis* and *Pseudomonas psychrotolerans* were statistically significant (*t*- test, *p= 0.001).*

3.3.2.2. Changes in peptidoglycan component during incubation with natural bacterial population

Peptidoglycan appears to be one of the components of recalcitrant dissolved fraction of natural seawater. The components of peptidoglycan such as D-amino acids are distributed widely in the marine waters (Lee and Bada, 1977; McCarthy et

al., 1998; Dittmar et al., 2001b; Benner and Kaiser, 2003, Perez et al., 2003). D-amino acids decreased from 190 nM at day 0 to 129 nM at day 25 in the control flask containing no added partially purified peptidoglycan (Fig. 3.4). In the flasks, containing partially purified peptidoglycan of *Bacillus subtilis* and *Pseudomonas psychrotolerans*, the D-amino acid concentrations decreased from 587 to 202 nM and 589 to 212 nM at day 6, respectively. However, for flasks enriched with partially purified peptidoglycan of *Bacillus licheniformis*, D-amino acid concentrations decreased from 625 nM at day 0 to 321 nM at day 3 of the incubation period (Fig. 3.4). There was 33 %, 38 %, and 26 % decrease in D-amino acids in the flasks enriched with partially purified peptidoglycan of *Bacillus subtilis*, *Bacillus licheniformis* and *Pseudomonas psychrotolerans,* respectively*.* The reduction in D-amino acids of the partially purified peptidoglycan reflects bacterial uptake. However, the actual uptake of D-amino acids cannot be determined as the culture medium contained added partially purified peptidoglycan as well as the peptidoglycan released by the natural bacterial population in growth medium. At day 9 of the incubation, the D-amino acid concentrations increased to 308 nM and 457 nM for *Bacillus subtilis,* and *Bacillus licheniformis*, respectively (Fig. 3.4). Studies in the natural environments have observed presence of higher D-amino acids associated with higher bacterial biomass (Jorgensen et al., 1990; Fernandes, 2011). Kawasaki and Benner (2006) observed a relatively higher concentration of D-amino acid such as D-alanine a biomarker for peptidoglycan in the dissolved fraction during exponential growth phase.

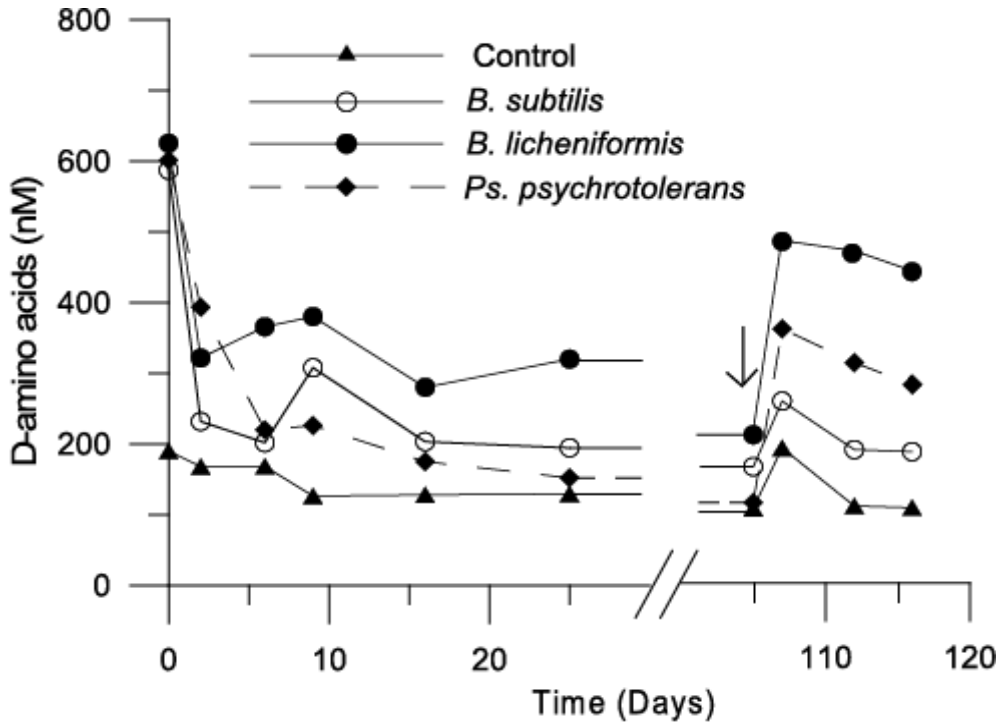

Figure 3.4. Changes in D-amino acid concentration during the degradation of partially purified peptidoglycan isolated from Bacillus subtilis, Bacillus licheniformis and Pseudomonas psychrotolerans. Arrow at day 105 indicates addition of growth medium.

The selective release of D-alanine occurred during cell growth and division when peptidoglycan is cleaved and newly synthesized subunits are incorporated into the cell wall via transpeptidation. Similar release of D-amino acids in the culture medium was observed in other studies during the exponential growth phase (Goodell and Schwarz, 1985; Kitayama et al., 2007). The subsequent decrease in D-amino acid concentrations from 16 to 105 day of incubation period in *Bacillus subtilis, Bacillus licheniformis* and *Pseudomonas psychrotolerans* indicates rapid utilization of the released bacterial cell wall D-amino acids by the natural bacterial population.

Addition of the growth medium at day 105 of the incubation period resulted in significant ($p>0.001$, *t*- test) increase in bacterial cell numbers along with increase in D-amino acid concentrations at day 107 of incubation period in the control flask and flasks containing partially purified peptidoglycan of *Bacillus subtilis, Bacillus licheniformis* and *Pseudomonas psychrotolerans* (Fig. 3.4). However, the D-amino acid concentration in growth medium decreased gradually until day 116 of the incubation period in both control and experimental flasks ($p> 0.001$, *t*- test). Overall, the D-amino acid concentration differed significantly ($p> 0.001$) in control and the three experimental flasks. The observed residual concentration of D-amino acids after 105 days of incubation period following the addition of growth medium were relatively lower than that present at day 0, implying that the added partially purified peptidoglycan or produced dissolved peptidoglycan by the naturally occurring bacterial population was substantially degraded during the incubation. Further, the occurrence of significant amount of D-amino acids in the growth medium by day 116 of the incubation period, indicates that although portion or constituents of peptidoglycan exhibits a bio-reactive property as they were used quickly, a small

portion was still resistant to further microbial degradation and remained in dissolved form for a long time (Kitayama et al., 2007). This may be due to its polymeric structure wherein the D-amino acids are protected from bacterial enzymes (Jorgensen et al., 2003). In the natural environment, peptidoglycan can be released into the seawater during viral lysis and grazing along with the complex matrix surrounding the peptidoglycan, these complexes could substantially impede degradation of peptidoglycan by natural bacterial population (Nagata and Kirchman, 1999). Moreover, studies have demonstrated that labile proteins associated with membranes and phospholipid vesicles are less degradable than the freely dissolved protein (Nagata et al., 1998; Borch and Kirchman, 1999). A similar mechanism could explain the preservation of peptidoglycan in natural environment. Overall, the Paired *t*- test indicates the differences observed in the D-amino acid concentrations in case of control flask and flasks containing peptidoglycan of *Bacillus subtilis* (*t*-test, $p=0.005$), *Bacillus licheniformis* and *Pseudomonas psychrotolerans* were statistically significant (*t*- test, $p< 0.001$).

The above experiments with the whole bacterial cell walls and purified peptidoglycan indicate that peptidoglycan derived component are not completely degraded and that they are important in shaping the composition and reactivity of organic matter. Therefore, a subsequent laboratory study was carried out using nitrogen poor mangrove leaves to estimate the L-and D-amino acids concentration, composition and bacterial contribution in mangrove leaf material.

Chapter 4

Evaluation of bacterial carbon and nitrogen contribution to degraded mangrove organic matter

4.1. Introduction

Living biomass consisting of vascular plants and their detritus forms a dominant component of reactive organic matter in both terrestrial and aquatic environments. Vascular plant detritus is important source of carbon, nitrogen and other nutrients to the aquatic organisms and an ancestor to much of the organic matter buried in the recent sediments. Mangrove forests are the most dominant, productive inter-tidal ecosystem in tropical coastlines (Bhosle et al., 1976; Boto and Bunt, 1981; Saenger, 2002) that exports organic matter to support a variety of organisms (Lee, 1989; Rivera-Monroy et al., 1995; Wafar et al., 1997) and enhances phytoplankton production (Pant et al., 1980; Rivera-Monroy et al., 1998). In the mangrove ecosystems, the total net primary production has been estimated at 218±72 TgC/yr (Bouillion et al., 2008; Twilley et al., 1992). As such, the mangrove plants are considered to be of low nutritional values, since it contains low nitrogen and has high carbon to nitrogen ratio (> 15) (Mfilinge et al., 2002). Moreover, the presence of higher concentration of polyphenolic compounds such as tannin makes the mangrove leaves non-utilizable as food source by higher organisms (Steinkie et al., 1993). However, upon decomposition of mangrove leaves in the seawater, the molecular composition of original plant material is highly altered, thus resulting in the presence of some unidentified molecules in the organic matter of soil and sediments (Hedges et al., 2000). Therefore, the study on the decomposition of plant material is important in understanding the biogeochemistry of organic matter.

During decomposition of leaf material, there exist two phases, the initial short leaching phase, wherein tannins are leached out resulting in net loss of nitrogen from leaf matter. This phase is followed by a longer and more extensive decomposition phase carried out by microorganisms (Benner et al., 1990; 1991;

Rice and Tenore, 1981; Wafar et al., 1997; Tremblay and Benner, 2006). During decomposition phase incorporation of exogenous nitrogen, termed as nitrogen immobilization or nitrogen enrichment by the microbial biomass occurs on mangrove leaf material. Process of nitrogen enrichment onto leaf material is of great ecological importance as nitrogen is a limiting nutrient in many ecosystems.

Microorganisms play an important role in the decomposition process, and are the source for the exogenous nitrogen. However, if microorganisms are responsible for the observed exogenous nitrogen on leaf detritus matter, then the bio-molecules specific to microorganisms should increase during the decomposition of the detritus organic matter. Specific amino acids observed in bacteria have been used to trace the presence and abundance of microbially derived organic matter (McCarthy et al., 1998; Benner and Kaiser, 2003). The D-enantiomers of amino acids (D-AA), D-alanine, D-aspartic acid, D-glutamic acid and D-serine found exclusively in the bacterial cell wall peptidoglycan and other bacterial macromolecules but absent in higher plants (Brock et al., 1994) have been used as bacterial biomarkers. Tremblay and Benner. (2006) observed these bacterial biomarkers in highly decomposed plant material during a degradation study of mangrove leaves.

Since mangroves are important sites of primary production and contribute considerable quantity of organic matter, primarily to adjacent waters and sediments (Benner et al 1986), thereby constituting a large available source of carbon and nitrogen. Therefore, the objectives of this chapter were to 1) investigate the concentration and composition of L- and D-amino acids in the leaves of the mangrove *Rhizophora mucronata*. 2) determine the changes in L- and D-amino

acids during decomposition of mangrove leave. 3) Assess the contribution of bacterial carbon and nitrogen into leaf detritus organic matter during decomposition.

4.2. Materials and methods

4.2.1. Collection of leaves and experimental setup

The senescent leaves from the mangrove tree, *Rhizophora mucronata* growing along the banks of the Mandovi estuary were handpicked. The leaves were cut into equal size (8cm x 8cm) and were air-dried at 25-30 °C. Eight grams each of leaf material was placed in eight individual 1 L conical flasks containing 0.8 L unfiltered seawater. The flasks were covered with aluminum foil, and placed in dark in order to prevent algal growth and photochemical transformation. The seawater used in the experiment was collected on 26th June, 2008 from Dona Paula Bay. Seawater had a salinity 30 psu and temperature 30 °C. The seawater from the flask was changed every 24 h. Each flask was sampled at fixed intervals of time over the 168 days period of incubation. The leaves were removed from the flask and rinsed in distilled water to remove salt. A sub-sample of the leaf material (1cm x 1cm) was placed in a vial containing saline and preserved with 2 % formalin for total bacterial count and in 2.5 % glutraldehyde for scanning electron microscopic observation, while the remaining leaf material was removed and oven dried at 45 °C for 3 days and weighed. Subsequently, the dried leaf material was ground in a motor and pestle and filtered through a 45-µm sieve, and stored at -20°C until analysis.

4.2.2. Ash determination of sample

Ash content was determined by combusting a known amount of leaf material (200 mg) at 550 °C in a muffle furnace for 6h. The sample was cooled and weighed on a microbalance (Mettler AT20), so as to determine the ash content of the sample.

4.2.3. Total bacterial count (TBC)

Total bacterial count was estimated following the DAPI (4, 6- Diamidino- 2-phenylindole) staining method (Porter and Feig, 1980). Leaf sample (1x1 cm) in triplicate was vortex in 5 ml phosphate buffered saline (PBS buffer) for 15 min to detach the bacteria from the leaf and preserved using 3 % formalin. The formalin fixed 2 ml sample was then stained with DAPI (final concentration 0.01 %) for 15 min in dark. The sample was then filtered through 0.2 µm black Nucleopore polycarbonate membrane filter. The filter was then placed on a drop of fluorescence free oil, another drop of oil was placed onto it and covered with a cover slip, and was then viewed using epifluorescence microscope (Nikon 80i) under 100 X oil immersion lens and a blue filter. About 10 fields containing the bacterial cells were counted per filter and the average field count was used to calculate the total bacterial numbers.

4.2.4. Scanning Electron Microscopy (SEM)

Leaf material containing the attached bacteria was fixed with 2.5 % glutraldehyde in filtered seawater (0.22 µm pore size) for 2 hours. Then the leaf sample was dehydrated using ethanol gradient (beginning with 20 %, 40 %, 60 %, 80 % and 100 % ethanol). The samples were then air-dried, sputter coated with gold using a Sputter Coater, and then observed with Joel SEM 5800-LV-Microscope.

4.2.5. Determination of total organic carbon, total nitrogen, Stable organic carbon, and nitrogen

Leaf material (1 mg) was packed into a tin foil and analyzed for organic carbon (OC) and total nitrogen (TN) using Thermo CHN analyzer. Atropine ($C_{17}H_{23}NO_3$)

was used as a standard. Coefficient of variation for C and N analysis was 1.05 % and 0.3 %, respectively.

The bulk abundance of $\delta^{13}C_{oc}$ and $\delta^{15}N$ in the leaf sample was determined using an elemental analyzer (EA, Thermo Electron Model-112) coupled online via a conflo interface with an isotopic ratio mass spectrometer (EA-IRMS< Delta V Plus, Thermo). Isotopic ratios reported in standard (δ) notation and expressed as per mil (‰) differences from a standard reference material as given below.

$$\delta X\ (‰) = [(R_{sample}/R_{standard})^{-1}] \times 100$$

Where, X is the $^{13}C_{oc}$ or, ^{15}N, R is the corresponding ratio of $^{13}C/^{12}C$, or $^{15}N/^{14}N$, in a sample or standard (PDB for carbon and atmospheric N_2 for nitrogen). Precision of $\delta^{13}C_{oc}$ and $\delta^{15}N$ measurement was ± 0.04 ‰ and ± 0.1 ‰ respectively based on replicate analysis of IAEA (International Atomic Energy Agency) standard.

4.2.6. Analysis of plant material for L- and D- amino acids

Leaf material (10 mg) was placed in a glass ampoule. To which 2 ml 6 N HCl was added and the ampoule was flushed with nitrogen gas and sealed, and the sample was hydrolyzed at 110 ^{o}C for 24 hours in oven. The hydrolysate obtained was then neutralized with 6 N NaOH and centrifuged and the supernatant was collected. The supernatant was then dried under vacuum, and was made to a known volume using Milli-Q-UV- water. An aliquot of the sample was then analyzed for L- and D-amino acids using reverse phase high-performance liquid chromatography (HPLC) following the method of Bhosle et al. (2005) and Fitzner et al. (1999) as described in chapter 2 and 3A.

4.3. Results and Discussion

4.3.1. Bulk parameters

4.3.1.1. Dry weight loss

Bacterial contribution to the leaf detritus of *Rhizophora mucronata* was determined over a short-term period of 168 days in a laboratory, under controlled conditions. Mangrove forests have been proposed to play an important role in the carbon and nitrogen cycling in tropical coastal ecosystems. In the present study, the net weight loss from the leaf material was about 32.4 % by the end of the 168 days of incubation period. The loss of organic matter from the leaves is defined as decomposition. The % ash free dry weight ranged from 81.5 to 57.7 % by day 168 of incubation (Table 4.1). Early weight losses during the first 10 days of incubation were mostly attributed to leaching. Mangrove leaves lost 12 % of their ash-free dry weight during the initial leaching phase (1.2 % day^{-1}). Further, during the decomposition phase (day10 to day 168), rates of weight loss was relatively low and ranged between 0.17 to 0.03 % day^{-1}.

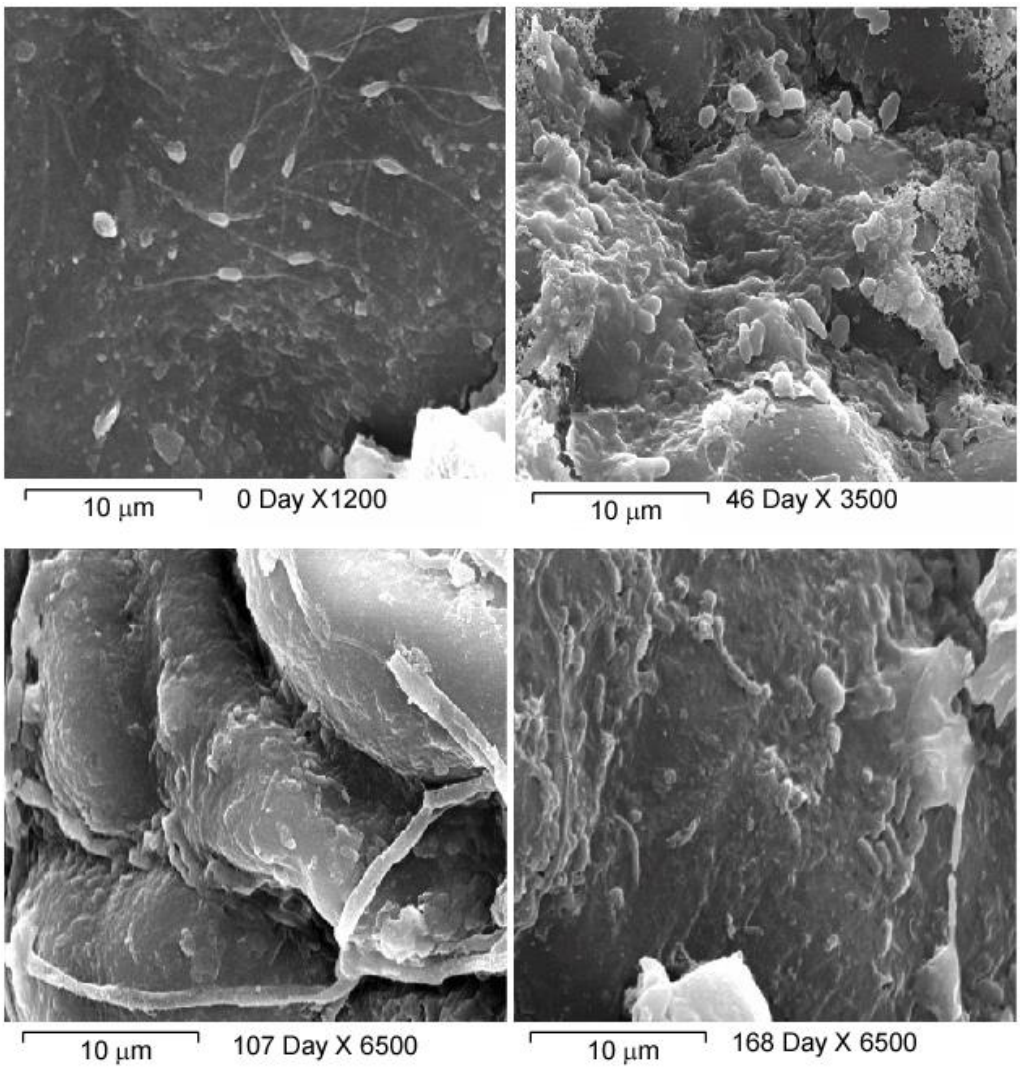

Figure 4.1. Scanning electron microscopic (SEM) observations of the bacterial growth on the Rhizophora mucronata leaves at 0, 46, 107 and 168 day period of incubation in the seawater.

4.3.1.2. Scanning Electron Microscopy (SEM)

The SEM micrographs of degraded mangrove leaf material clearly showed the presence of bacteria over the period of incubation (Fig. 4.1). At day 0, very few bacteria could be seen on the leaf material. By day 46 following incubation, abundance of bacteria increased. As the incubation period further increased bacterial abundance also increased at day 107 and day 168 following the inoculation (Fig. 4.1).

4.3.1.3. Total bacterial count

Bacterial numbers on the degraded leaves of *Rhizophora mucronata* ranged from 0.11 x 10^8 to 2.94 x 10^8 cells cm^{-2} over the 168 days of incubation (Fig. 4.2). During the initial first 10 days of incubation period, the bacterial numbers ranged from 1.1 x 10^7 to 2.2 x 10^7 cells cm^{-2}. However, with the onset of decomposition at day 20 of the incubation period, the bacterial abundance rapidly increased several folds (2.2 x 10^8 cells cm^{-2}); thereafter the increase was relatively low over the 168 days period of decomposition period. Abundance of bacteria observed on the leaf material was also supported by the scanning electron microscopic examination of the leaf material, wherein higher bacterial cells were observed with progressive decomposition of leaf material.

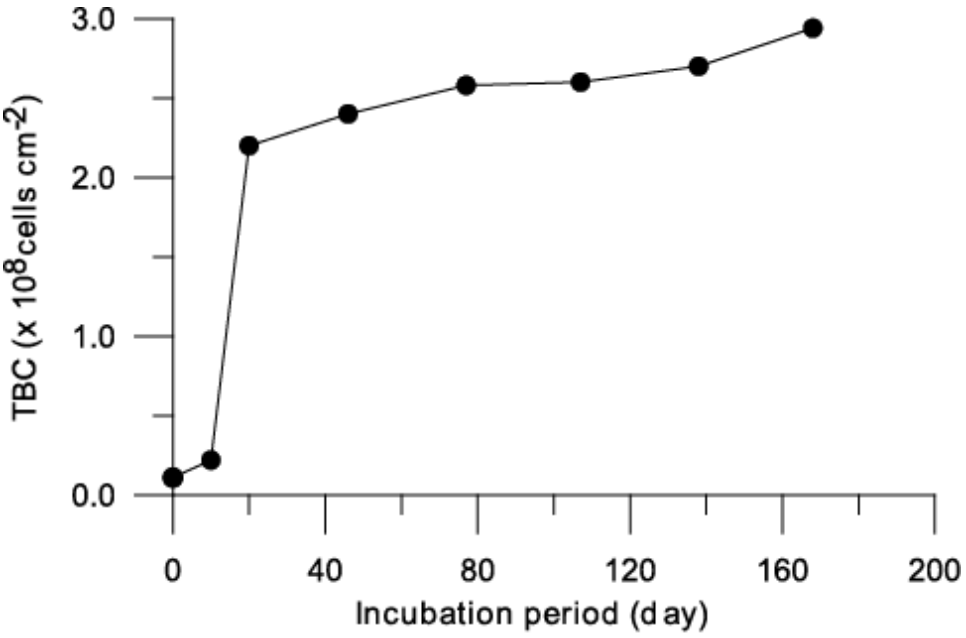

Figure 4.2. Changes in bacterial abundance on the Rhizophora mucronata leave over 168 day period of incubation.

4.3.1.4. Changes in carbon, nitrogen, and C/N ratio

Mangrove litter is generally characterized by high total organic carbon, C/N ratio, and low total nitrogen and highly depleted $\delta^{13}C_{oc}$ values. During decomposition, the total organic carbon and nitrogen varied from 40 to 46.3 % and 0.4 to 1.2 %, respectively (Table 5.1). Both, the carbon and nitrogen content of degraded leaf material increased gradually over the 168 days of incubation. Jennerjahn and Ittekkot. (2002) have estimated that inputs from mangrove forests could account for 11 % of the total input of terrestrial carbon into the ocean and 15 % of the total carbon accumulating in modern marine sediments. A significant positive correlation between TOC and TN ($r=0.7257$, $n=8$, $p<0.05$), suggests common origin for these compounds.

The C/N ratio of the mangrove litter has been considered as an indicator of its nutritional value (Fell et al., 1984). The C/N ratio during decomposition varied in the ranged from 39 to 100 (Table 4.1). It has been well documented that the nutritional value of mangrove litter increased through nitrogen enrichment during microbial decomposition (Fell et al., 1984; Mann and Steinke, 1992; Woitchik et al., 1997). A large decrease in C/N ratio from 100 at day 0 to 39 at day 168 was observed during the decomposition. Rao et al. (1994) observed a tenfold decrease in C/N ratio from senescent leaves (C/N = 200) to the sediments (C/N = 22), suggesting significant nitrogen enrichment during leaf decomposition.

Table 4.1. Changes in AFDW, TOC, TN, C/N ratio, δ^{13}Coc and δ^{15}N, L-AA-C and L-AA-N, and D-AA-C and D-AA-N yields during the decomposition of Rhizophora mucronata leaves in seawater over a period of 168 day incubation

Sampling time (days)	AFDW (%)	TOC (Wt. %)	TN (Wt. %)	C/N ratio	$\delta^{13}C_{oc}$ (‰)	$\delta^{15}N$ (‰)	L-AA-C (%)	L-AA-N (%)	D-AA-C (%)	D-AA-N (%)
0	81.5	40.0	0.4	100	-32.9	3.9	1.6	44.9	0.02	0.6
10	69.8	40.2	0.5	86.3	-33.5	3.4	1.6	38.2	0.02	0.5
20	68.1	41.0	0.8	51.3	-33.2	3.4	1.9	27.7	0.05	0.8
46	66.7	42.9	1.0	42.9	-32.8	3.0	2.1	25.6	0.06	0.8
77	65.8	45.4	1.0	45.4	-34.6	3.2	3.2	41.0	0.06	0.9
107	63.4	45.6	1.1	41.5	-33.6	3.2	3.2	38.0	0.06	0.8
138	59.9	46.0	1.1	41.8	-34.5	3.3	3.6	42.8	0.06	0.8
168	57.7	46.3	1.2	38.6	-34.3	3.4	3.7	40.0	0.08	0.9

4.3.1.5. $\delta^{13}C_{oc}$ and $\delta^{15}N$

Stable carbon and nitrogen isotopic ratios varied from -34.6 to –32.8 ‰ and 3.05 to 3.90 ‰, respectively (Table 4.1). $\delta^{13}C_{oc}$ signatures of mangrove leaves obtained in present study was in the range of values reported earlier for mangrove leaves (-35 ‰ to -22 ‰) (Bouillon et al., 2008; Nyunja et al., 2009). A significant inverse relationship was observed between $\delta^{13}C_{oc}$ and OC ($r = -0.8340$, $n=8$, $p<0.01$), the shift in $\delta^{13}C_{oc}$ values in relation to OC content can be explained by the mechanisms of microbial re-mineralization (Tue, et al., 2012). The depleted $\delta^{13}C_{oc}$ values observed during degradation experiment is mainly attributed to rapid bacterial growth on the mangrove leaves. Plant tissues comprises typically of lignin and polysaccharides cellulose and hemicelluloses (Sjostrom, 1981). The bacteria utilize the reactive polysaccharides thereby resulting into the accumulation of more refractory lignocelluloses. Lignocelluloses are isotopically depleted compared to bulk $\delta^{13}C_{oc}$ (Benner et al, 1987, 1990).

$\delta^{15}N$ observed for *Rhizophora mucronata* was relatively lower than the values reported for the mangrove from other areas (Prasad and Ramanathan, 2009). Different microbial interactions may be the reason for the differences in the observed $\delta^{15}N$ in this study. Purvaja et al (2008) reported that variability in the availability of nitrogen is mainly due to nitrogen immobilization, mediated by microbial conversion of inorganic nitrogen to organic form during decomposition of plant material. Moreover, the changes in the $\delta^{15}N$ were generally small or the $\delta^{15}N$ showed small increase after initial leaching period (Table 4.1). Tremblay and Benner

(2006) observed a 0.7 ‰ decrease in $\delta^{15}N$ during 189 day period of decomposition, which they attributed to leaching of leaf material. The observed changes in the $\delta^{15}N$ are mainly due to microbial immobilization or new N production (Caraco et al., 1998). The magnitude and direction of the $\delta^{15}N$ change will depend upon number of factors such as the inorganic nitrogen substrate, the importance of nitrogen fixation (Woitchik et al., 1997), the $\delta^{15}N$ of the added nitrogen, and the degree of fractionation during immobilization (Bouillon et al., 2008).

4.3.2. L-amino acid in Mangrove detritus

4.3.2.1. L-amino acid concentration

The L-amino acid concentrations in *Rhizophora mucronata* ranged from 104 to 280 µmol gdw^{-1} during the decomposition period (Fig. 4.3). Hernes et al. (2001) reported that amino acids can represents up to 9 % of mangrove leaf biomass. Zieman et al. (1984) reported 833 µmol gdw^{-1} of total amino acids concentration in *Rhizophora* leaves. A two-fold increase in the L-amino acid concentration was observed from day 0 to day 168 of the incubation period (Fig. 4.3, Table 4.1). The L-amino acids concentration increased gradually from 104 µmol gdw^{-1} at day 0 to 280 µmol gdw^{-1} at day 168 of the incubation period. The observed increase in amino acid concentrations during the incubation period can be attributed to the microbial enrichment on the decomposed mangrove leaves (Hernes et al., 2001; Tremblay and Benner, 2006), and thereby contributing to the pool of organic matter. A significant positive relationship ($r=0.8028$, $n=8$, $p= 0.01$; Fig. 4.3) was recorded between L-amino acids and bacterial cell

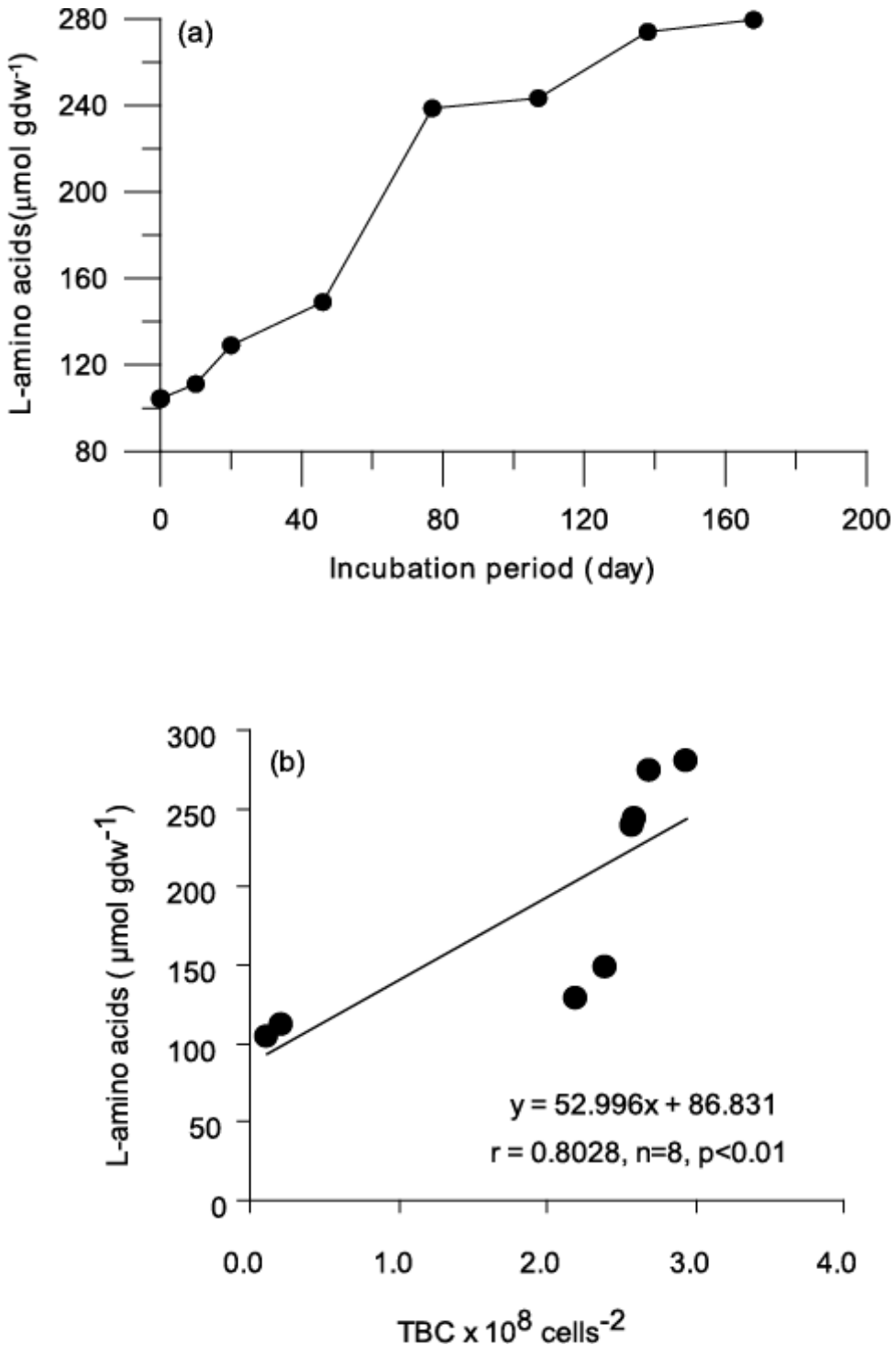

Figure 4.3. Changes in L-amino acid concentration (a) and relationship of L-amino acids with total bacterial count (TBC) (b) in the Rhizophora mucronata leaves during 168 day period of incubation.

numbers. This indicates that L-amino acids were derived from bacteria.

4.3.2.2. Contribution of L-amino acid in Mangrove detritus

L-amino acids (L-AA) account for a substantial amount of freshly produced organic matter, and are relatively labile compared to bulk organic carbon and nitrogen (Ittekkot and Arain 1986; Cowie and Hedges, 1994). L-amino acid yields (measured as % L-AA-C/OC and % L-AA-N/TN) decrease during organic matter degradation. Therefore, yield was used to assess the degradation state of organic matter (Cowie and Hedges, 1992; Davis et al., 2009). The L-AA-C and L-AA-N yield varied from 1.6 to 3.7 % and 26 to 51.2 %, respectively (Table 4.1). Such low L-AA-C and L-AA-N yields are expected in vascular plants tissues (Cowie and Hedges, 1992). L-AA-C yield was low at day 0, which increased with progressive increase in degradation of mangrove leaves. In contrast, the L-AA-N yield was higher initially and decreased with progressive decomposition of plant material by day 46. The decrease in the L-AA-N yield during the incubation period indicates that the mangrove leaf material have undergone extensive degradation. However, the L-AA-N yield increased from day 46 to day 77 and thereafter remained more/or less constant (Table 4.1). The increase in L-AA-C and L-AA-N yield following decomposition indicates incorporation of freshly produced organic matter in the degraded leaf material.

4.3.2.3. Composition of L-amino acid in Mangrove detritus

The glycine plus threonine followed by the alanine, glutamic acid, aspartic acid, and Lysine were the most abundant amino acids observed in mangrove leaves during the incubation period (Fig. 4.4). Zieman et al.

(1984) observed more than 10 % contribution to total amino acids from glycine, glutamic acid, and leucine in the *Rhizophora* leaves.

Amino acids are present in the proteins, in cell walls, cell membrane, and cell plasma (Cowie and Hedges, 1992; Keil et al., 2000). During the incubation of *Rhizophora mucronata* leaves, a decrease in Mole % of arginine, alanine, valine, phenylalanine, leucine, and serine was observed from day 0 to day 168 of incubation period. These amino acids are mostly observed in freshly produced organic matter, and their proportions to total amino acids are found to decrease during the organic matter decomposition. The reason for decrease is that these amino acids are present in diatom cell plasma and since cell plasma is relatively susceptible to microbial degradation compared to the other cell components and thereby these amino acids being labile are readily utilized by microorganisms (Hecky et al., 1973; Cowie and Hedges, 1992; Dauwe and Middelburg, 1998; Meckler et al., 2004). Therefore, the presence of these amino acids is indication of relatively fresh organic matter.

In contrast, increase in the Mole % of glycine plus threonine, aspartic acid, glutamic acid, and lysine was observed in the *Rhizophora mucronata* leaves during 168 days of the incubation period (Fig. 4.4). The amino acids aspartic acid, glutamic acid, and lysine are usually found in greater amounts in the vascular plants and bacteria (Cowie and Hedges, 1992). Several studies have observed progressive enrichment of glycine in a highly degraded organic matter and with the increasing sediment depths (Burdige and Martens, 1988; Dauwe et al., 1999; Vandewiele et

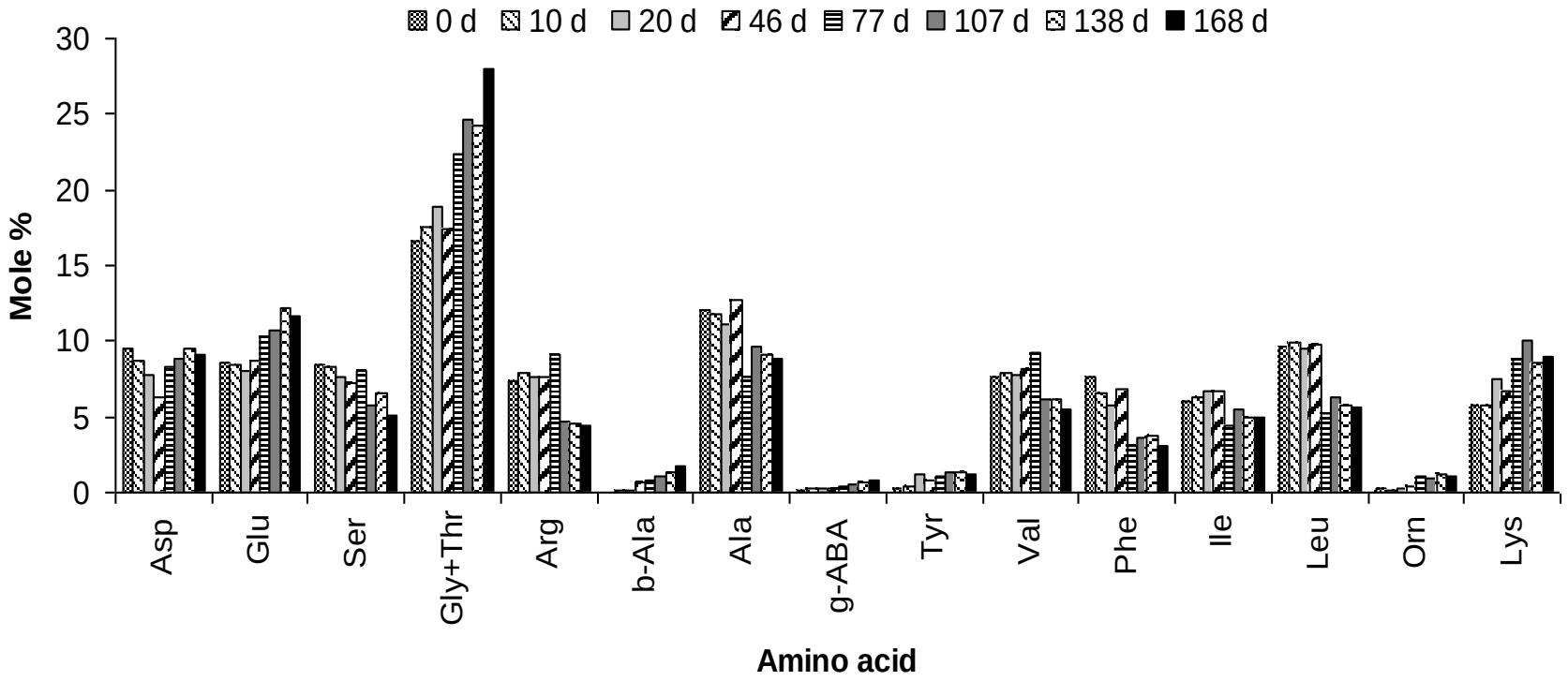

Figure 4.4. Changes in L-amino acid composition (as Mole %) during the degradation of senescent leaves of Rhizophora mucronata in natural seawater over a period of 168 days. Asp: aspartic acid; Glu: glutamic acid; Ser: serine; Gly+Thr: glycine plus threonine; Arg: arginine; β-ala: beta alanine; Ala: alanine; γ-ABA: gamma amino butyric acid; Tyr: tyrosine; Val: valine; Phe: phenylalanine; Ile: isoleucine; Leu: leucine; Orn: ornithine; Lys: lysine

al., 2009, Fernandes, 2011). Glycine plus threonine are enriched in the cell wall of plants. The association of glycine plus theronine with the cell wall renders these amino acids less degradable by bacteria, thereby resulting into their accumulation in the degraded organic matter. Another possible reasons for the enrichment of glycine maybe its occurrence as short chain amino acid, thereby having minor food value. Further, glycine is also synthesized from other amino acids during heterotrophic metabolism.

Further, enrichment of non-protein amino acids such as β-alanine, and γ-amino butyric acid was observed in *Rhizophora mucronata* leaves during the 168 days of decomposition period (Fig. 4.4 and Fig. 4.5). Initially at day 0, the contribution of non-proteins amino acids to total L-amino acids was relatively low (~ 0.1 Mole %), however, the contribution increased to 1.7 and 0.7 Mole % of β-alanine and γ-amino butyric acid, respectively (Fig. 4.5 a). The aspartic acid/ β-alanine (Asp/β-ala) ratio decreased from 176 at day 0 to 5 by day 168 of incubation period (Fig. 4.5 b). Similar decrease in ratio from 54 to 16 was observed for glutamic acid/ γ-amino butyric acid (Glu/ γ-Aba) ratio (Fig. 4.5 b). The non-protein amino acids are absent in higher organisms and plants. β-alanine, and γ-amino butyric acid are produced by bacteria as byproducts of decarboxylation of protein amino acids aspartic acid, and glutamic acid, respectively during degradation and their contribution to total organic pool is found to increase with more degraded organic matter (Cowie and Hedges, 1992). Thus, increasing abundance of the non-protein amino acids over the period of

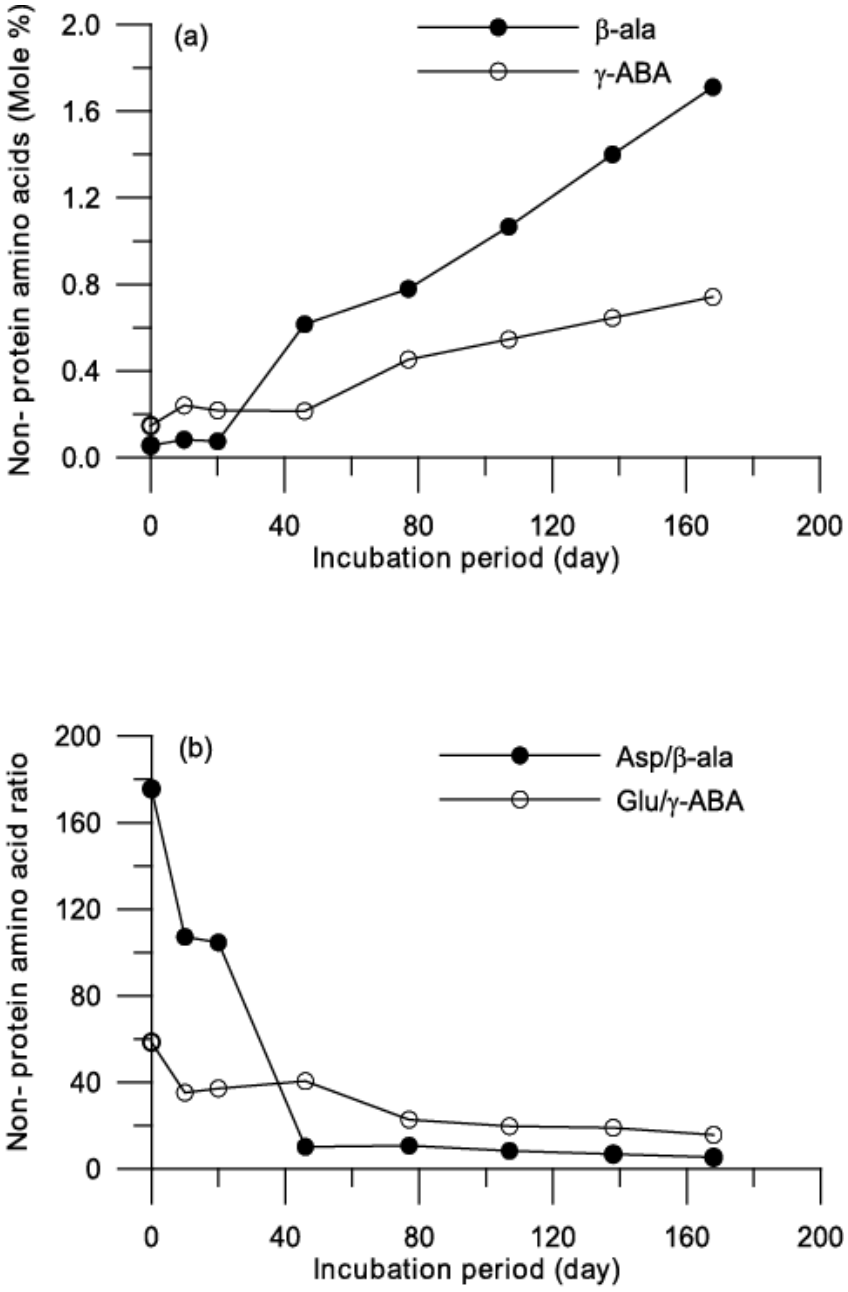

Figure 4.5. Changes in the abundance (as Mole % of total amino acids) of non-protein amino acids β-alanine (β-Ala) and γ-amino butyric acid (γ-Aba) (a) and changes in the ratios of aspartic acid/β-Ala and glutamic acid/γ-Aba (b) during degradation of leaves of Rhizophora mucronata over the 168 day incubation in seawater.

incubation indicates bacterial reworking on the degraded leaves of *Rizophora mucronata*.

4.3.3. D-amino acids in mangrove detritus

4.3.3.1. Concentration and contribution of D-amino acids

The D-amino acid concentrations varied from 1.6 to 8.0 µmol gdw^{-1} in the leaves of *Rhizophora mucronata* during the incubation period of 168 days (Fig. 4.6a). The D-amino acid concentration observed at 0 day was mainly due to the presence of bacteria on senescent leave. This observation was confirmed by the TBC and SEM studies of leaf at day 0. Highly significant relationship (r= 0.9665, n= 8, p= 0.001) was observed between D-amino acid concentration and total bacterial count (Fig. 4.6b). This indicates bacterial biomass is the source for the D-amino acids in the mangrove detritus.

Yields of D-amino acid-carbon (D-AA-C) (i.e. yield =D-AA-C/TOC x 100) and D-amino acid-nitrogen (D-AA-N) (i.e. yield =D-AA-N/TN x 100) varied from 0.02 to 0.08 % and 0.6 to 1.1 %, respectively (Table 4.1). The D-AA-C and D-AA-N yield increased with the progressive increase in the decomposition of *Rhizophora mucronata* leaf material. Since D-amino acids are relatively less degradable or recalcitrant, they get accumulated in degraded organic matter. For example, peptidoglycan and other macro-molecules, which accumulated in the organic detritus during decomposition.

The Mol % of D-amino acids increased from ~ 1.5 % to 3.8 % during the course of decomposition of *Rhizophora mucronata* leaves (Fig. 4.7 a). D-amino acids (as Mol %) are useful biomarkers to assess the deg-

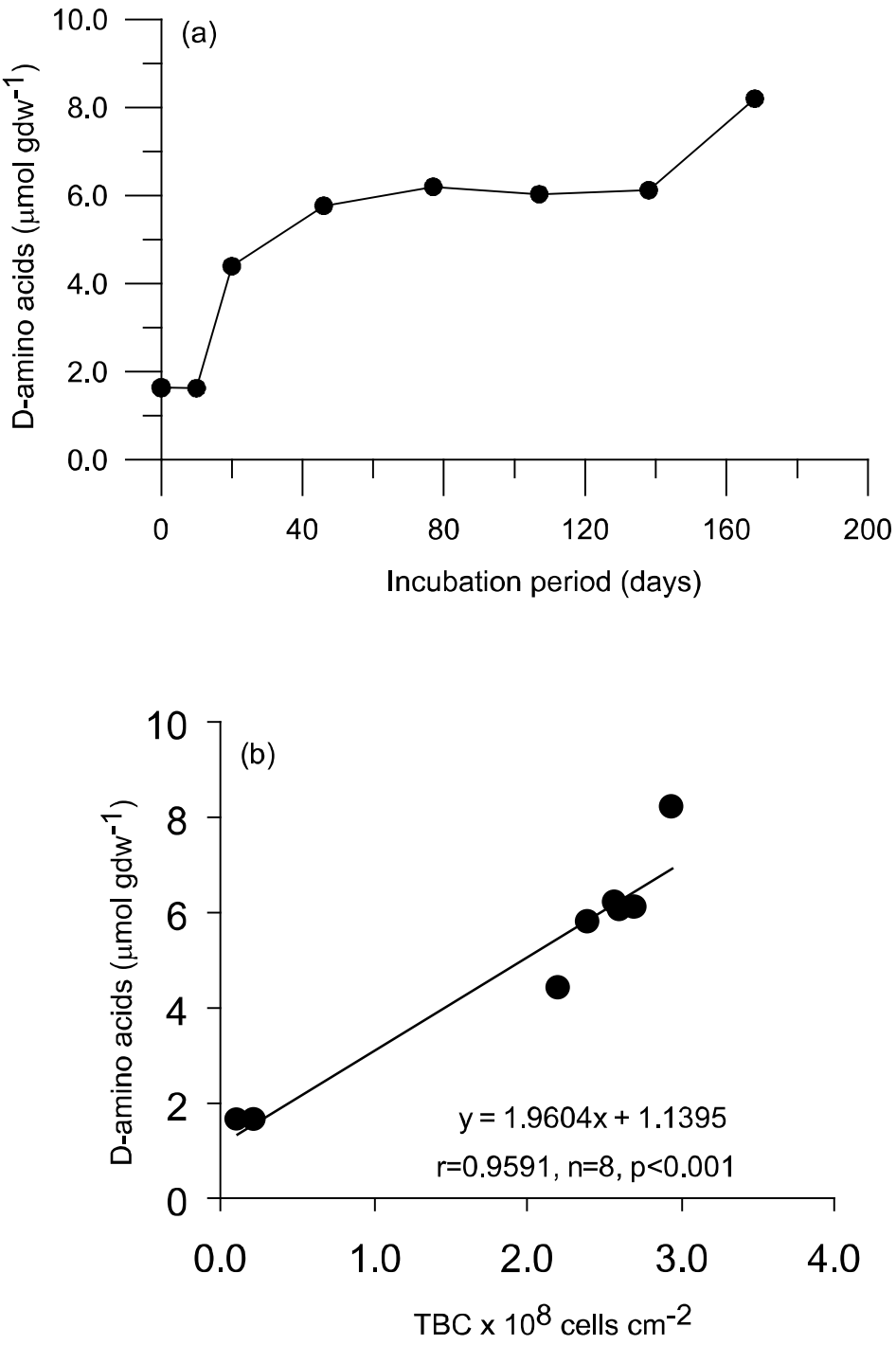

Figure 4.6. Variations in D-amino acid concentration (a) and relationship between D-amino acid concentration with total bacterial count (b) during the degradation of leaves of Rhizophora mucronata over 168 day period of incubation

radation of organic matter (Jorgensen et al., 2003, Tremblay and Benner, 2009). A highest Mol % D- amino acid was observed at day 20 and day 46 following incubation, indicating that mangrove leaf material has undergone extensive degradation due to increase in bacterial biomass as supported by total bacterial count. This was supported by the significant relationship ($r= 0.7477$, $n= 8$, $p< 0.02$) between Mol % D-amino acids and bacterial abundance (Fig. 4.7b).

4.3.3.2. Proportion of D-amino acids

The presence of bacterial origin organic matter on the *Rhizophora mucronata* leaf material was confirmed by the occurrence of biomarker prevalent in bacteria. The four D-amino acids detected in the degrading leaves of mangrove according to their abundance were D-aspartic acid, D-alanine, D-glutamic acid, and D-serine (Fig.4.8). These D-amino acids have been reported in bacterial peptidoglycan, bacterial antibiotics and other bacterial macromolecules (Schleifer and Kandler, 1972; Yang et al., 2003, Kaiser and Benner, 2008). The contribution of individual D-amino acids to the sum of D-amino acid + L-amino acid was calculated using the equation:

$$\text{e.g. } \%\text{ D-Ala} = \frac{100 \text{ X D-Ala}}{(\text{D-Ala} + \text{L-Ala})}$$

The contribution of D-aspartic acid, D-alanine, D-glutamic acid and D-serine varied from 12 to 23 %, 17 to 36 %, 3 to 11 % and 1 to 4 %, respectively over the period of incubation (Fig. 4.8). The proportion of the D-amino acids in the degrading leaves of mangrove increased during the

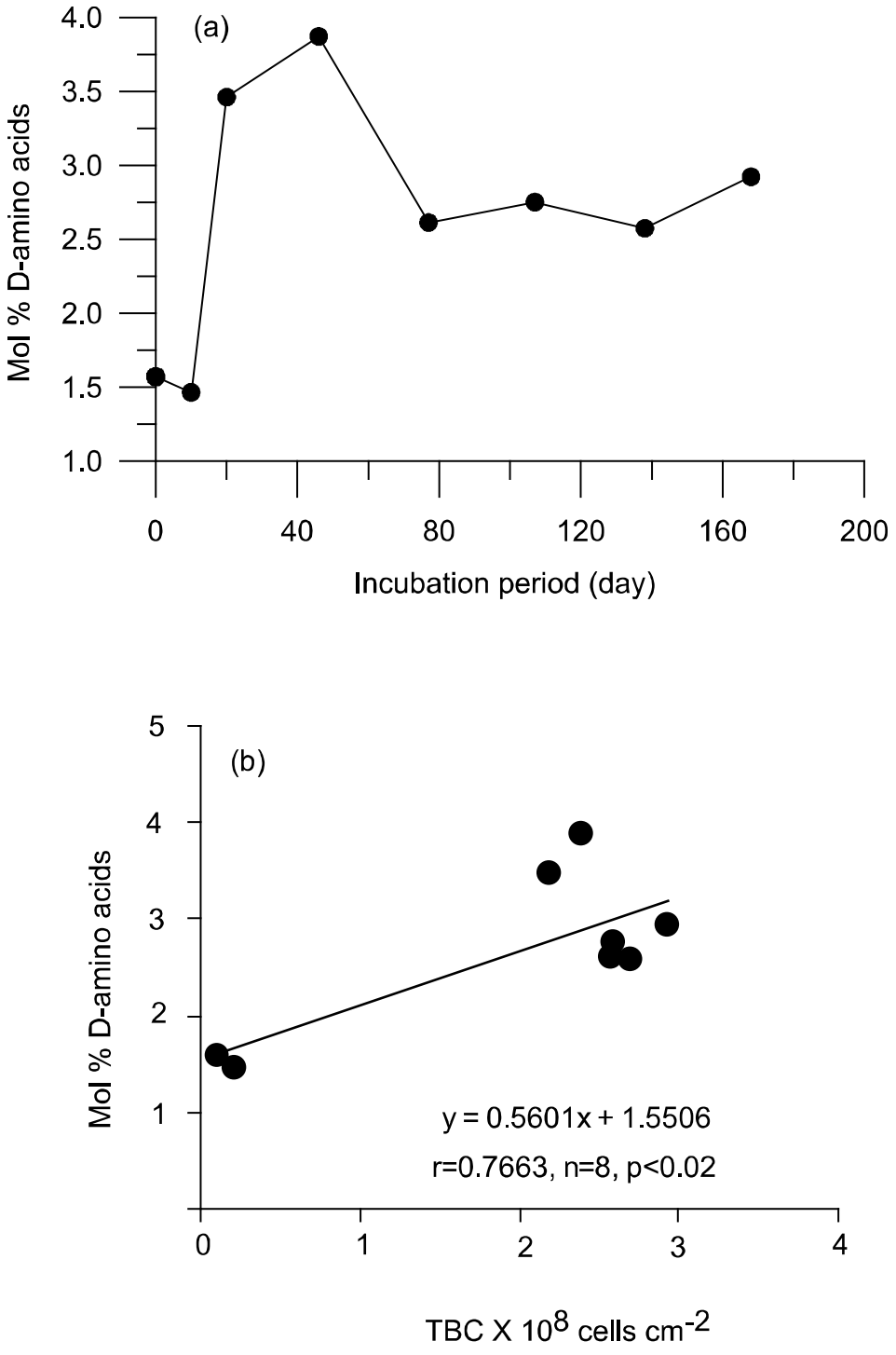

Figure 4.7. Variation in Mol % D-amino acid (a) and relationship between Mol % D-amino acids and total bacterial count (TBC) (b) during degradation of the leaves of Rhizophora mucronata over the 168 day period of incubation.

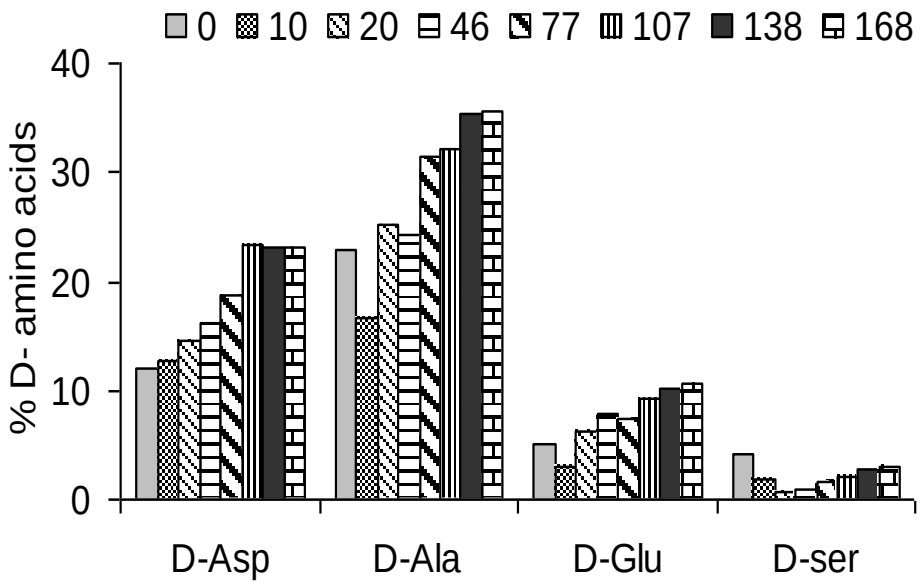

Figure 4.8. Changes in Mole percentages of D-enantiomers (Dx100/(D+L)) in the leaves of Rhizophora mucronata over the 168 day period of incubation.

period of incubation. Abundance of D-alanine has been observed in the bacterioplankton (Mc carthy et al., 1998; Amon et al., 2001). The increasing proportion of D-alanine in the degraded leaves of *Rhizophora mucronata* further confirms increasing abundance of bacterial biomass over the period of incubation.

4.3.3.3. D/L-amino acid ratios

In aquatic systems, the abundance of the D-amino acids is usually reported relative to their respective L-stereoisomers as D/L-amino acid ratios. The D/L–amino acid ratios ranged from 0.17 to 0.29 during the 168 days degradation period for the leaves of *Rhizophora mucronata* leaves. The D/L- amino acid ratios increased with the decomposition of the *Rhizophora mucronata* leaves (Fig. 4.9a). Presence of a mixture of D- and L-amino acids indicates microbial biosynthesis, and increasing proportions of the D-amino acids over the incubation period indicates bacterial growth on *Rizophora mucronata* leaves (Zieman et al., 1984). This observation was supported by the increasing bacterial count and SEM observations.

4.3.3.4. Contribution of peptidoglycan to amino acid nitrogen

The peptidoglycan input to the total amino acid nitrogen was quantified by assuming an average peptidoglycan structure as: PG-N= 5.7 x D-Ala-N (Roger, 1983) and total amino acid nitrogen (AA-N) as AA-N= 12 x Ala-N (Cowie and Hedges, 1992). Thus the percentage contribution of peptidoglycan to amino acids (% PG-AA-N) was calculated as % PG-AA-N= PG-N/AA-N x 100 (Mc Carthy et al., 1998). The % PG-AA-N calculated during the decomposition of the plant detritus ranged from 10 to 28 % of the total amino acid nitrogen (Fig. 4.9 b).

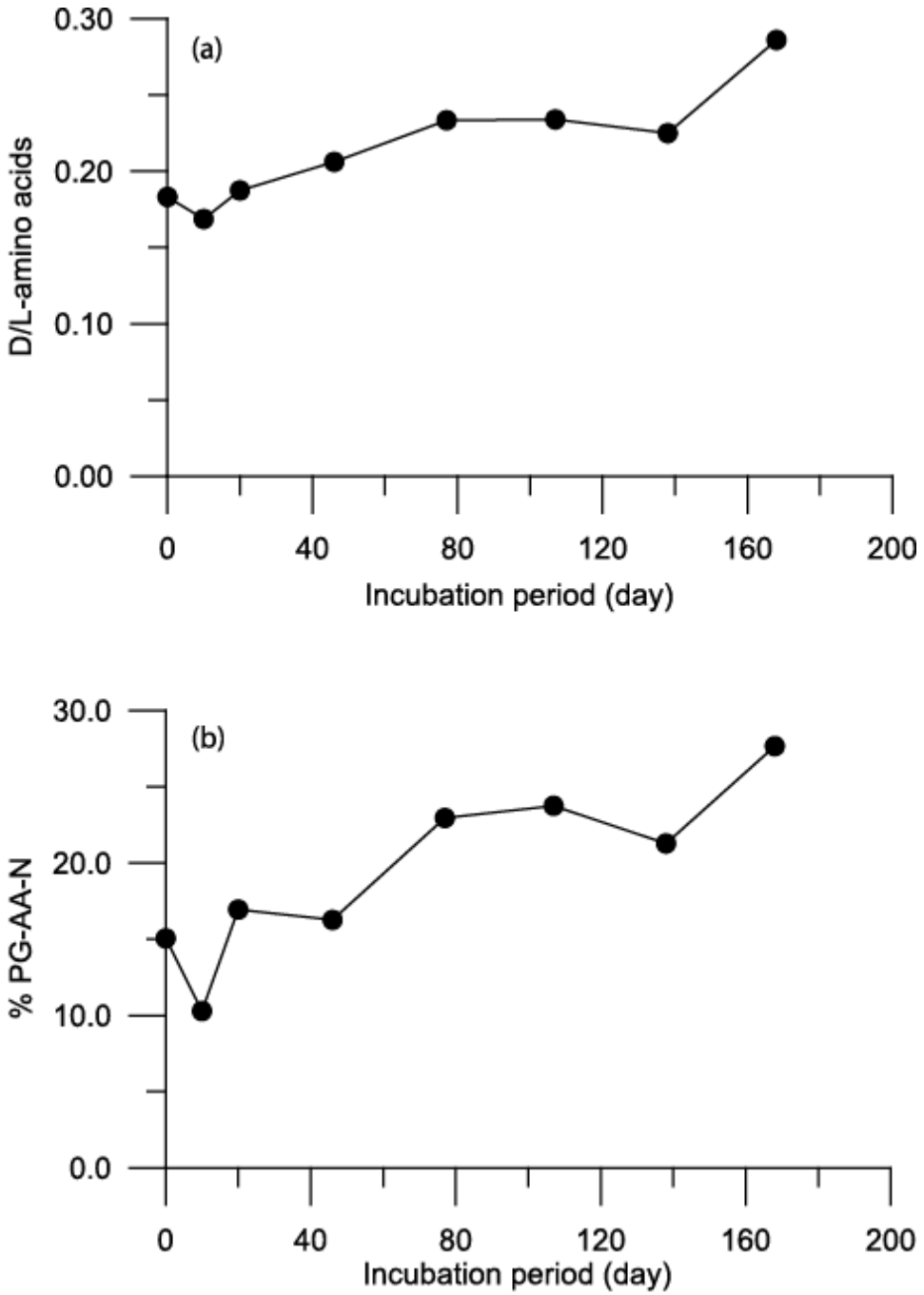

Figure 4.9. Changes in D/L-amino acid ratio (a) and the contribution of peptidoglycan-N (% PG-AA-N) to total amino acid nitrogen (b) of degrading leaves of Rhizophora mucronata in natural seawater for 168 day period of incubation.

4.3.4. Proportion of immobilized N and Bacterial C and N on Rizophora mucronata

Two independent approaches were used to quantify the percentage of detrital N derived from N-immobilization and from bacteria as given by Tremblay and Benner (2006)

In the first approach, the percentage of immobilized N ($\%N_{immob}$) was calculated using the following equation:

$$\%N_{immob} = 100(N_{sample}-N_{plant})/N_{sample} \quad (1)$$

Where, N_{sample} is the nitrogen content of the detritus sample and N_{plant} is the nitrogen from the original plant tissue. The N_{plant} from the senescent tissue (time 0) remaining at time *t* was determined assuming that N_{plant} had same reactivity as bulk C during decomposition:

$$N_{plant,t} = N_{plant,0}(C_t/C_0) \quad (2)$$

Where, C_0 and C_t is the carbon content in plant tissue at time 0 and remaining at time *t.*

Assuming that the nitrogen contribution from senescent plant tissue followed bulk carbon, the percentage immobilized N during the 168 day of decomposition varied in the range from 20 to 68 % (Fig. 4.10).

The second approach was based on the yield of D-alanine in the detritus. D-alanine is a bacterial biomarker found in bacteria (Brock et al., 1994). Yield of D-alanine was assumed to be proportional to bulk carbon and nitrogen in the detritus.

$$\%\ C, N_{immob} = 100[biomarker]_{sample}/[biomarker]_{microb} \quad (3)$$

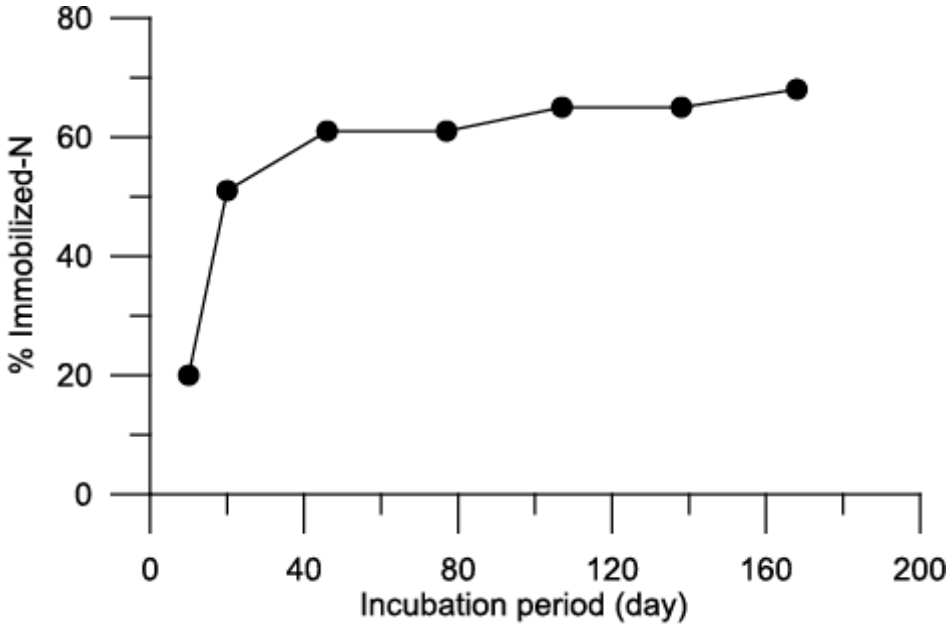

Figure4.10. Changes in percentage of immobilized nitrogen in the degrading laeves of Rhizophora mucronata detritus during 168 day of incubation period. Values were calculated using Eqs (1) and (2) (See text).

Where, $[biomarker]_{sample}$ and $[biomarker]_{microb}$ are the C- or N- normalized yields of D-alanine in the detritus and in heterotrophic bacteria.

The D-alanine yield for the heterotrophic bacteria was taken as 38.0 nmol/mgC and 193.6 nmol/mgN. This yield was calculated by using the mixture of 95 % Gram negative and 5 % Gram-positive heterotrophic bacteria (Giovannoni and Rappe, 2000) based on culture and coastal natural populations (Benner and Kaiser, 2003; Kaiser and Benner, 2008). The Bacterial–C increased from 2.0 to 11.0 % of OC after the 168 days of incubation of *Rizophora mucronata* leaves (Fig. 4.11). The estimates of the bacterial contribution to nitrogen followed the same patterns as for carbon but were much greater and increased from 31.4 to 86.1 % of TN (Fig. 4.11). Vascular plants mostly contain a relatively lower nitrogen, the relatively higher nitrogen observed during the incubation period indicates bacteria to be the source for the observed exogenous nitrogen. This was also supported by the lower C/N ratio observed in *Rhizophora mucronata* leaves during the incubation period (Table 4.1).

The above experiments carried out in the laboratory showed the importance of bacteria in the degradation of terrestrial organic matter such as mangrove leaves. Similar processes are unknown in the natural tropical environments. Therefore, the cycling, distribution, and fate of L-and D-amino acid in estuarine POM of the Mandovi estuary and deep sea sediments of the Bay of Bengal were investigated in chapter 5A and chapter 5B, so as to understand the role of bacteria in cycling and preservation of organic matter.

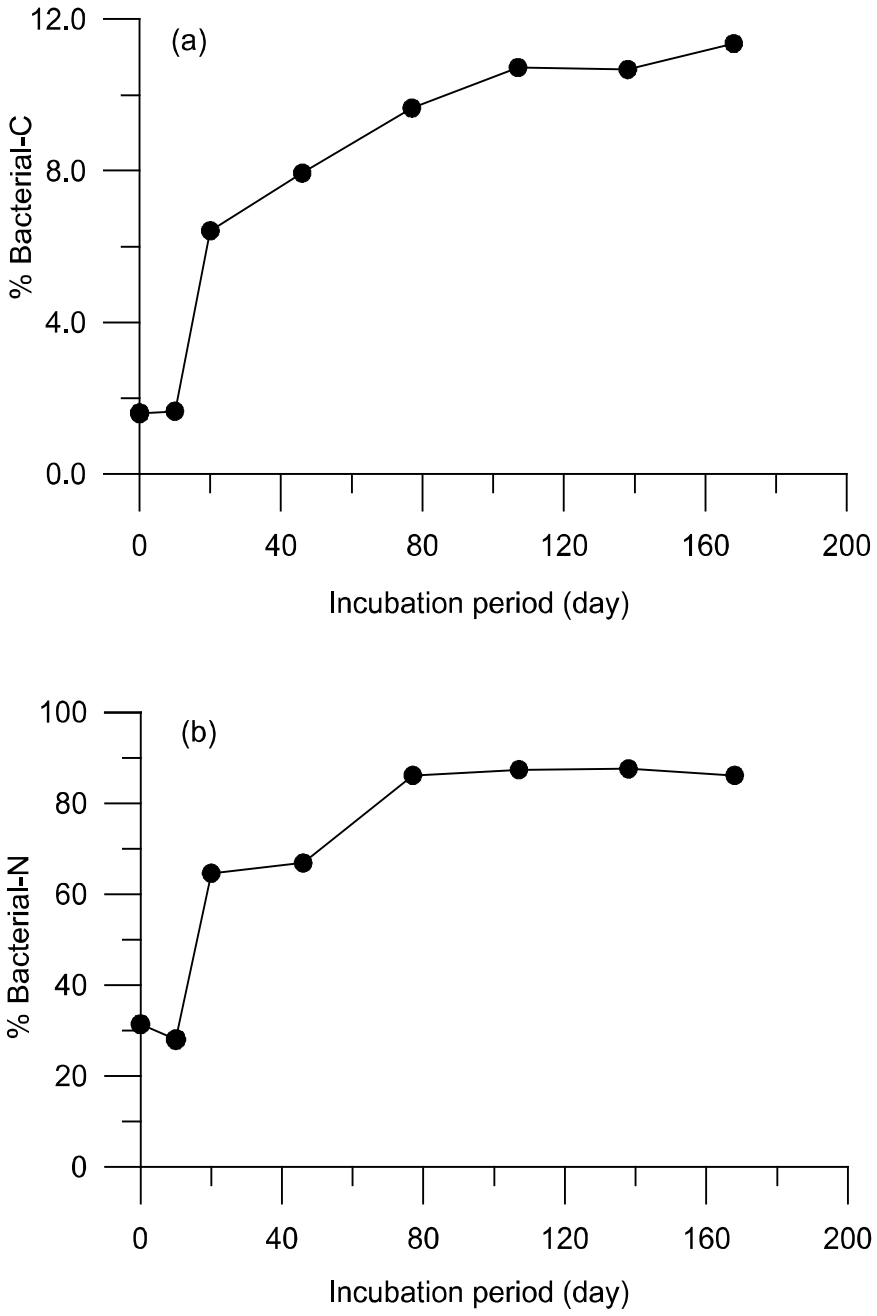

Figure4.11. Percentages of bacterial carbon (a) and nitrogen (b) in Rhizophora mucronata during 168 day of incubation period. Values were calculated using Eq. (3) and the C- or N-normalized yields of D-alanine measured in a mixture of heterotophic bacteria.

Chapter 5A

Origin and biogeochemical cycling of particulate nitrogen in the Mandovi estuary

5A.1. Introduction

Each year rivers transport via estuaries ~ 4 x 10^{14} g of terrigenous organic carbon to continental margin (Schlesinger and Melack, 1981). Estuaries are also active sites for high biological production and heterotrophic metabolism. Biochemical and physical processes in estuaries may modify the quantities and characteristics of the organic matter (OM) delivered to the ocean. Activities of heterotrophic microorganisms exert strong influence on the amount and the composition of OM (Findlay et al., 1992; Tremblay and Benner, 2009). Therefore, estuaries are ideal sites to study the biogeochemistry of OM derived from terrigenous and marine sources.

OM in the estuaries and coastal waters is derived from several sources that can be divided into allochthonous and autochthonous. Moreover, OM from both sources may be degraded or transformed by various groups of microorganisms. As a result, it is difficult to unequivocally identify the sources of OM in estuarine samples (Canuel et al., 1995; Cloern et al., 2002). Despite this, there are several instances wherein C/N ratio and $\delta^{13}C_{POC}$ have been employed previously to distinguish inputs and cycling of particulate organic matter (POM) in rivers, estuaries, and marine systems (Zhang et al., 1997; Wu et al., 2007a; Bouillon et al., 2008). Moreover, a better understanding of the processing of OM in estuarine systems is required to identify the biogeochemical fate of OM as it is transported through the estuary to the coastal sea (Lee et al., 2004; Bourgoin and Tremblay, 2010).

Nitrogen is a principal limiting nutrient in marine production. Quality and source of particulate nitrogen determines its impact on coastal

processes. Amino acids, the building blocks of proteins, are the major forms of nitrogen in both terrestrial and aquatic organisms. These compounds represent important constituents of living and dead OM. Their natural occurrence and geochemical behavior have been evaluated in several types of samples (Cowie and Hedges, 1992; Gupta and Kawahata, 2000; Vandewiele et al., 2009). The contribution of amino acids carbon to organic carbon and amino acid nitrogen to organic nitrogen and relative proportion of individual amino acids are useful diagenetic indicators (Cowie and Hedges, 1994; Dauwe and Middelburg, 1998; Dauwe et al., 1999; Keil et al., 2000; Chen et al., 2004). Amino acid composition of source organisms is relatively similar. However, upon degradation the amino acid composition undergoes marked differences. Glycine, serine and threonine are accumulated while tyrosine, phenylalanine and glutamic acid are depleted during degradation of OM. Highly degraded, refractory OM of marine and terrestrial origin exhibits significantly different amino acid composition pattern, which reflect the different diagenetic processes involved in the formation and preservation of marine and terrestrial OM (Dittmar et al., 2001b). Amino acids are selectively utilized by heterotrophic microorganisms, which in turn contribute to the preserved OM (Ogawa et al., 2001; Tremblay and Benner, 2006). Such microbial contribution is especially found in nitrogen poor plant detritus by incorporation of exogenous nitrogen, termed N-enrichment, where inorganic nitrogen can be converted into organic microbial molecules (Tremblay and Benner, 2006). Bacterial activity plays a central role in the production of uncharacterized molecules and OM

preservation (Harvey and Macko, 1997; Tremblay and Benner, 2006). Quantification of microbial contribution, to the detrital OM is difficult considering highly heterogeneous and reworked nature of material. However, to estimate microbial contribution specific biomarkers such as D- amino acids are employed to identify sources and extent of degradation of OM (Hedges and Prahl, 1993; Pedersen et al., 2001; Grutters et al., 2002; Lomstein et al., 2009; Kaiser and Benner, 2008; Tremblay and Benner, 2009; Vandewiele et al., 2009; Bourgoin and Tremblay, 2010). This is because bacterial biomass is rich in D-amino acids, whereas, phytoplankton and most other primary producers contain almost exclusively L-enantiomers (Jorgensen et al., 1999).

In this study, we applied a combined tracer approach of stable carbon isotope and amino acid enantiomers to identify the major sources of particulate organic nitrogen and the bacterial contribution to OM in the Mandovi estuary during the monsoon and the pre-monsoon season.

5A.2. Materials and methods

5A.2.1. Area of study

The Mandovi estuary is a tropical, tide dominated, interstate river basin located between the Sahyadris hills and the Arabian Sea along the west coast of India (Fig. 5A.1). The estuary is ~75 km long and has a drainage area of about ~1, 895 sq km (Qasim and Sen Gupta, 1981). The mouth of the estuary is 3.2 km in width and it progressively narrows to 0.25 km towards the upstream end. The estuary receives 660 cm/yr of rainfall (Shetye et al., 2007). During the southwest monsoon season (June to

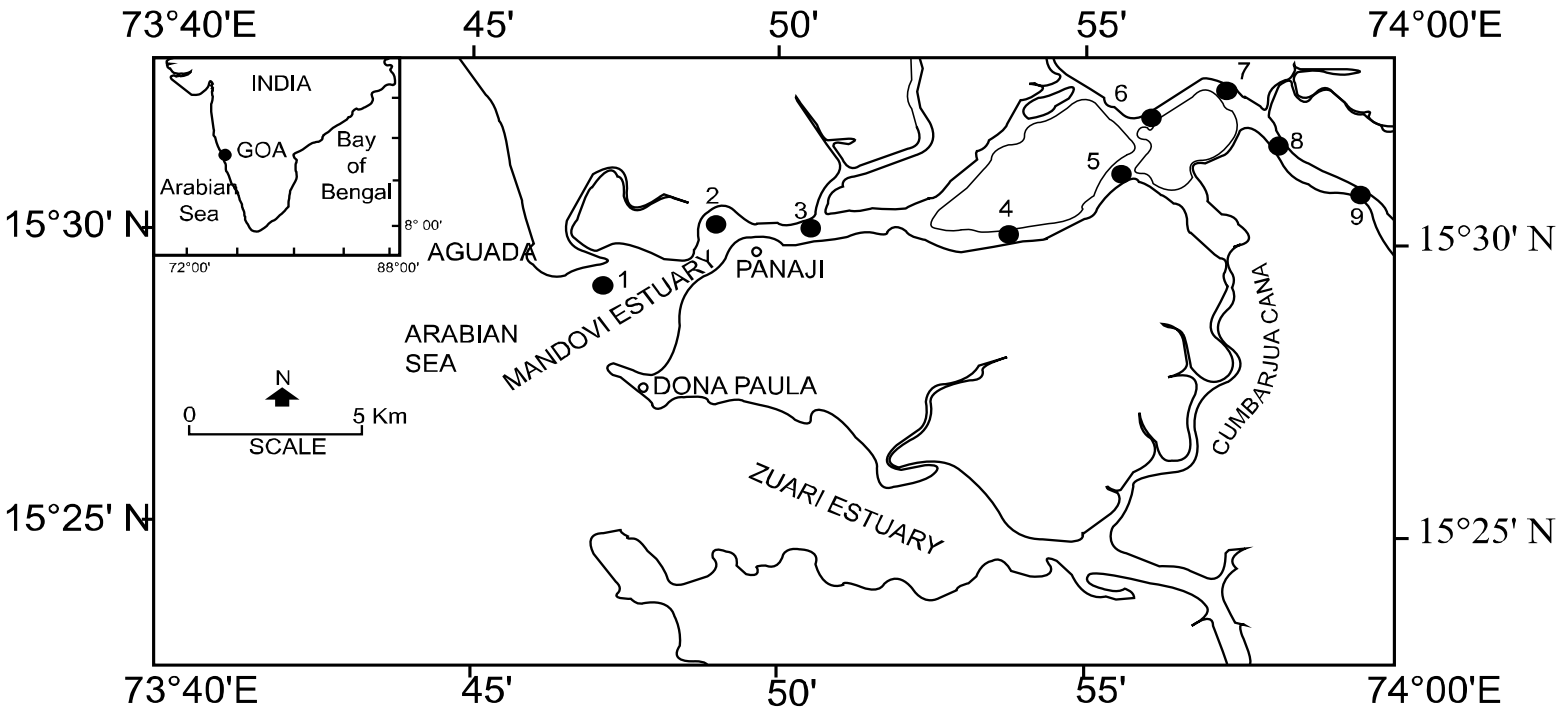

Figure 5A.1. Map showing sampling site and stations sampled in the Mandovi estuary, west coast of India.

September) the estuary receives large amount of fresh water run-off, while the run-off is nearly absent during the dry season (October to May), and is regulated by semi-durinal tides. The residence time of water is usually of 5 to 6 days during the monsoon season and about 50 days during dry season (Qasim and Sen Gupta, 1981). Clay minerals such as smectite and kaolinite are abundant near the mouth and the upstream stations of the estuary, respectively. Mangroves are present on the banks of estuary. The Mandovi estuary is mostly used for navigation of fishing vessels, transport of ores and people and for recreation activities. Recently, physical, chemical, and biological characteristics of the estuary have been compiled (Shetye et al., 2007).

5A.2.2. Sample collection

Surface (~ 1 m) water samples were collected using a 5L Niskin sampler from various locations in the estuary during the summer monsoon (August 2005), and the pre-monsoon season (March 2007) (Fig. 5A.1). Immediately, after collection, the water was passed through 200 µm mesh to remove large zooplankton, and then filtered through pre-ashed (450 ^{o}C, 4h) GF/F filters (47mm, 0.7 µm pore size) to collect suspended particulate matter (SPM), for the analysis of chlorophyll *a* (Chl *a*), particulate organic carbon (POC) and total particulate nitrogen (TPN), $\delta^{13}C_{POC}$, and total hydrolysable amino acids (L- and D-amino acids). After filtration, GF/F filters containing SPM were transferred into clean screw cap vials and stored at -20 ^{o}C. In order to estimate total bacterial count (TBC), seawater sample (20 ml) was fixed with glutaraldehyde (final concentration 2 %) at 4 °C until analysis.

5A.2.3. Analytical methods

5A.2.3.1. Hydrographic and Bulk chemical parameters

Surface water temperature and surface salinity were measured by the portable conductivity/temperature/depth (CTD) (Model SBE 19 plus, SeaBird Electronics, USA) Instrument. The GF/F filter containing SPM was washed with 10 ml distilled water, and the filter was dried at 40 ^{o}C, and then weighed on a microbalance (Mettler Model AT20). SPM weight was obtained by subtracting the weight of empty GF/F filter from the weight of GF/F filter containing SPM. Chlorophyll *a* (Chl *a*) was estimated by the fluorometric method (Boto, 1978; Parsons et al., 1984). In order to estimate TBC, a known volume of seawater (2–5 ml) was stained with acridine orange (final concentration 0.01 %) for 5 min, filtered onto 0.22 µm black Nuclepore polycarbonate filter. Bacterial cells were counted in at least 25 randomly selected fields using 100 X objective, and an epifluorescence microscope (Nikon). Average cell number per field was calculated, and used to estimate total bacterial cells following the procedure described by Parsons et al. (1984).

5A.2.3.2. Carbon and Nitrogen Analysis

POC was analyzed spectrophotometrically by the wet oxidation of carbon with acid dichromate (Parsons et al., 1984). TPN was estimated following the method of Raimbault and Slawyk. (1991). Coefficient of variation of analytical methods used for POC and TPN was 4.9 % and 8.8 %, respectively. Stable isotope ($\delta^{13}C_{POC}$) was analyzed using the Thermo Finnigan Flash 1112 elemental analyzer, linked with a Thermo Finnigan Delta V plus Isotope Ratio MS using the following equation

$$\delta^{13}C_{POC} = \{(^{13}C/^{12}C)_{Sample} / (^{13}C/^{12}C)_{PDB} - 1\} \times 1000$$

The analytical precision for the method was ± 0.04‰ for $\delta^{13}C_{POC}$. All isotopic compositions are reported as, per-mil (‰) relative to variation (*∂*) from the PDB standard.

5.3.3. Analysis of total hydrolysable L- and D- amino acids

GF/F filter containing the SPM was hydrolyzed and analyzed for L-and D-amino acids as described in chapter 2. The precision of the method based on the replicates samples ranged between 0.4 to 14 % for L-amino acids and 6 to 10% for D-amino acids (Table 5A.1).

5A.2.3.4. Bacterial contribution to POM

The average C- and N-normalized yields of D-amino acids, observed during the monsoon and pre-monsoon seasons in the estuary were compared with yields calculated in different assemblage of bacteria (Bourgoin and Tremblay, 2010). The proportion of bacterial- C and N was calculated using the equation

$$\% \text{ Bacterial C or N} = 100[\text{biomarker}]_{sample}/[\text{biomarker}]_{bacteria}$$

Where $[\text{biomarker}]_{Sample}$ and $[\text{biomarker}]_{bacteria}$ are the C- or N- normalized yields of a specific biomarkers (e.g. D-alanine, D-aspartic acid and D-glutamic acid) in the SPM (sample) of Mandovi estuary and different assemblage of bacterial cells (bacteria).

Table 5A.1.Precision of L- and D-amino acid method based on replicate samples

L-Amino acids	Sample replicates					Ave	SD	%CV
	1	2	3	4	5			
Asp	4.6	4.7	4.7	5.3	4.7	4.8	0.3	5.3
Glu	4.5	4.6	4.6	3.4	4.5	4.3	0.5	11.8
Ser	4.4	4.4	4.3	4.3	4.3	4.3	0.1	1.3
Gly	10.6	10.6	10.7	10.6	10.7	10.6	0.0	0.4
Arg	3.5	3.4	3.5	3.5	3.4	3.5	0.0	0.9
b-ala	0.1	0.1	0.1	0.1	0.1	0.1	0.0	7.2
Ala	5.5	5.6	5.7	5.7	5.4	5.6	0.1	2.0
g-aba	0.4	0.4	0.4	0.4	0.4	0.4	0.0	4.2
Tyr	0.2	0.2	0.2	0.3	0.2	0.2	0.0	7.7
Val	4.0	4.0	4.0	4.0	4.0	4.0	0.0	0.6
Phe	2.3	2.3	2.4	2.4	2.3	2.4	0.0	1.2
Ile	2.8	2.8	2.8	2.9	2.8	2.8	0.0	1.2
Leu	3.9	4.1	4.1	4.1	4.0	4.0	0.1	2.1
Orn	0.8	1.0	1.0	1.1	0.8	0.9	0.1	14.2
Lys	5.4	5.4	5.3	5.3	5.4	5.4	0.0	0.9

D-Amino acids	Sample replicates					Ave	SD	%CV
	1	2	3	4	5			
L- Asp	14.3	13.8	13.7	13.8	15.7	14.3	0.8	5.9
D- Asp	2.6	2.5	2.6	2.8	2.9	2.7	0.2	6.5
L- Glu	10.2	9.3	8.0	8.1	9.4	9.0	0.9	10.5
D- Glu	0.9	0.8	0.8	0.7	0.8	0.8	0.1	9.6
L- Ser	8.3	6.9	8.3	7.6	7.0	7.6	0.7	8.9
D-Ser	ND	ND	ND	ND	ND	ND	ND	ND
L- Ala	5.9	5.1	5.2	4.7	5.3	5.2	0.5	8.6
D-Ala	0.8	0.8	0.7	0.7	0.8	0.8	0.1	7.9

ND: Not detected

5A.2.3.5. Statistical methods

A simple regression analysis was performed to assess the relationships between parameters using excel program. One–way analysis of variance (ANOVA) was used to evaluate the significance of seasonal differences in amino acids. The degradation Index (DI) of OM was estimated using Mole % L-amino acid composition and calculated using the formula proposed by Dauwe et al. (1999) as given in Chapter 1.

5A.3. Results and Discussion

5A.3.1. Hydrographic and bulk parameters

Mandovi estuary experiences distinct environmental conditions during the monsoon and pre-monsoon season. The estuary receive heavy river run-off during southwest monsoon. However, during the pre-monsoon season there is negligible water discharge and the estuary experiences marine condition due to intrusion of seawater several kilometers upstream from the mouth of the estuary. The surface salinity decreased from station 01 to 09 during both the seasons, and was higher during the pre-monsoon season. Average surface water temperature in monsoon was lower (~28 ± 0.3°C) than the pre-monsoon (~31 ± 0.4°C) (Table 3A.2). Higher concentrations of SPM, Chl *a*, POC, and TPN were observed at downstream stations (stations 01, 02 and 03) and that decreased towards upstream station 08 during the monsoon (Table 5A.2). These distribution trends recorded for Chl *a*, POC, and TPN were inversed during the pre-monsoon. SPM trend observed in the Mandovi estuary was different from those observed for other estuaries wherein, SPM decreased from freshwater end to seawater end (Grabemann et al., 1997, Chen et al.,

2004). During monsoon, the higher SPM concentration at the stations near the estuary mouth was the result of re-suspension of bottom sediments due to presence of strong tidal currents, westerly winds, and waves (Qasim and Sen Gupta, 1981).

5A.3.2. Sources of organic matter

C/N ratio varied from 5.8 to 22 and $\delta^{13}C_{POC}$ from -32 to -25 ‰ during the monsoon. For the pre-monsoon, C/N and $\delta^{13}C_{POC}$ ranged from 4.1 to 6.1 and -29.6 to -21.1 ‰, respectively (Table 5A.2). In estuaries and rivers, OM is derived from various sources including autochthonous and allochthonous sources. The average C/N ratio for the fresh living phytoplankton and bacteria is 7 and 4, respectively (Redfield et al., 1963; Lee and Furhman, 1987), while for the terrestrial OM, it varied from 12 to 200 (Hedges and Man, 1979) which decreases during degradation due to incorporation of exogenous nitrogen known as N-enrichment (Temblay and Benner, 2006). The higher and lower C/N ratio during the monsoon and pre-monsoon season, respectively, suggests the presence of degraded terrestrial and relatively fresh OM of phytoplankton origin during the former and latter season, respectively. However, C/N ratios during the monsoon were much lower than those recorded for terrestrial OM, thereby suggesting N-enrichment due to bacterial contribution to the organic nitrogen (Tremblay and Benner, 2006).

$\delta^{13}C_{POC}$ signatures of the various carbon sources are often different and therefore useful tracer to carbon inputs in various environments. In the aquatic ecosystems, the $\delta^{13}C_{POC}$ varied broadly for terrestrial C3 plants (-30 ‰ to – 26 ‰), marine phytoplankton (-22 ‰ to –18 ‰), and

mangrove leaves (-35 ‰ to -22 ‰) (Cifuentes, 1991; Dittmar et al., 2001a; Wu et al., 2007a; Bouillon et al., 2008). $\delta^{13}C_{POC}$ values of POM in the present study area were depleted (-32 ‰ to -25 ‰) during the monsoon than those recorded for the pre-monsoon season (-29.6 ‰ to -21.1 ‰) (Table 5A.2). The $\delta^{13}C_{POC}$ values of SPM of Mandovi estuary are similar to those recorded for terrestrial C3 plants and mangrove leaves in the monsoon season and phytoplankton $\delta^{13}C_{POC}$ during the pre-monsoon season (Smith and Epstein, 1971; Dittmar et al., 2001a; Wu et al., 2007a; Bouillon et al., 2008). $\delta^{13}C_{POC}$ data suggest that POM was derived from terrestrial and mangrove sources during the monsoon and from marine sources during the pre-monsoon.

5A 3.3. Distribution of L- amino acids (L-AA)

L-amino acid concentration decreased from downstream station 01 to upstream station 08 during the monsoon, and increased from the downstream station 01 to upstream station 09 (Fig. 5A.2a) during the pre-monsoon season. L-amino acid values observed in the Mandovi estuary are in the range of values observed for other rivers, estuaries, and coastal region (Ittekkot and Zhang, 1989; Hedges et al., 1994; Chen et al., 2004; Duan and Bianchi, 2007; Wu et al., 2007b). During monsoon, higher L-amino acid concentrations at the downstream stations (Station 01 to 03) maybe due to hydrodynamic sorting of SPM (Duan and Bianchi, 2007). During low discharge period there is rapid settling of large suspended particles, which are temporarily stored in the riverbed. Moreover, in the Mandovi estuary during the monsoon season, a sand bar develops at the mouth of the estuary, wherein suspended particles are trapped,

Table 5A.2. Distribution of hydrographic and bulk parameters during the monsoon and pre-monsoon season in the Mandovi estuary

Stations	Temperature (°C)	Salinity	SPM (mg/l)	Chl a (µg/l)	POC (µM C)	TPN (µM N)	C/N ratio	$\delta^{13}C_{POC}$	TBC (x 10^8 cells/l)
Monsoon season									
Stn1	27.4	16.34	16.9	4.5	107.8	4.9	22.0	-25.1	29.1
Stn2	27.6	14.58	17.4	4.7	59.1	7.8	7.6	-32.1	12.3
Stn3	27.6	13.40	18.4	4.3	98.2	7.6	12.9	-26.1	28.1
Stn4	28.1	1.03	9.6	2.1	29.1	5.0	5.8	-27.7	5.4
Stn5	27.5	0.14	4.0	1.3	19.8	2.1	9.4	-28.7	9.3
Stn6	27.7	0.09	3.5	1.0	15.5	4.1	11.9	-29.5	6.1
Stn7	27.4	0.09	4.1	6.0	29.9	3.4	8.8	-29.5	6.3
Stn8	28.3	0.06	2.9	6.5	30.6	3.8	8.1	-28.5	6.0
Mean	27.7 ± 0.3	5.7 ± 7.5	9.6 ± 6.9	3.8 ± 2.1	48.8 ± 36	4.8 ± 2.0	10.8 ± 5.1	-28.4 ± 2.2	12.8 ± 10

Continued

Pre-monsoon season									
Stn1	30.8	34.58	7.2	9.2	40.6	7.7	5.3	-24.8	20.3
Stn2	30.9	34.06	8.6	6.3	44.2	8.7	5.1	-22.0	24.2
Stn3	30.7	33.86	10.8	5.8	57.8	10.7	5.4	-23.8	17.1
Stn4	30.8	32.65	7.3	14.7	86.0	14.1	6.1	-29.6	24.3
Stn5	31.5	30.19	8.8	14.0	64.1	14.3	4.5	-23.2	33.6
Stn6	31.5	29.13	10.9	13.9	74.1	15.3	4.8	-22.4	42.1
Stn7	31.3	24.44	14.1	11.0	65.3	13.9	4.7	-22.4	22.8
Stn8	31.2	21.33	7.6	17.2	85.1	15.0	5.7	-21.1	19.8
Stn9	31.8	17.46	6.3	9.3	71.7	14.0	5.1	-24.4	41.5
Mean	31.2 ± 0.4	28.6 ± 6.2	9.1 ± 2.5	11.3 ± 3.9	65.4 ± 16	12.6 ± 2.8	5.2 ± 0.5	-23.7 ± 2.5	27.3 ± 9.4

and stored (Qazim and Sen Gupta, 1981). In the monsoon due to the combined effect of higher river run-off and physical forcing, amino acid rich bottom sediments are re-suspended. Therefore, higher L-amino acid concentration at the stations 01 to 03 (Fig. 5A.2a) was probably due to re-suspension of the trapped POM (Duan and Bianchi, 2007; Wu et al 2007b). The intense river flow during the monsoon is counteracted by the strong tidal and wind induced currents (Rao et al., 2011), which may have led to the lateral advection or transport of L-amino acids from station 01 to 03. The low L-amino acids observed at station 04 to 08 during the monsoon was due to the presence of degraded terrestrial OM or poor growth of phytoplankton at these stations due to greater turbidity and poor light conditions. This was also supported by a poor relationship ($r = 0.264$, $n=8$; Fig. 5A.3a) between Chl *a* and L-amino acid suggesting the phytoplankton biomass did not Influence L-amino acid concentration during the monsoon. In contrast, during the pre-monsoon, although the SPM levels were high, improved light conditions favored the growth of *in-situ* phytoplankton resulting into higher L-amino acid concentrations (Fig. 5A.2a). This was also supported by a positive relationship between L-amino acid and Chl *a* ($r = 0.6804$, $n=9$, $p < 0.02$; Fig. 5A.3b). Abundance of amino acid in the Mandovi estuary was significantly affected by the change in the season ($F = 24$, $p< 0.0001$).

During the monsoon, L-amino acid yields (measured as % L-AA-C/POC and % L-AA-N/TPN), decreased from station 01 to 08, except for station 07, where L-AA yields were slightly greater than those recorded at

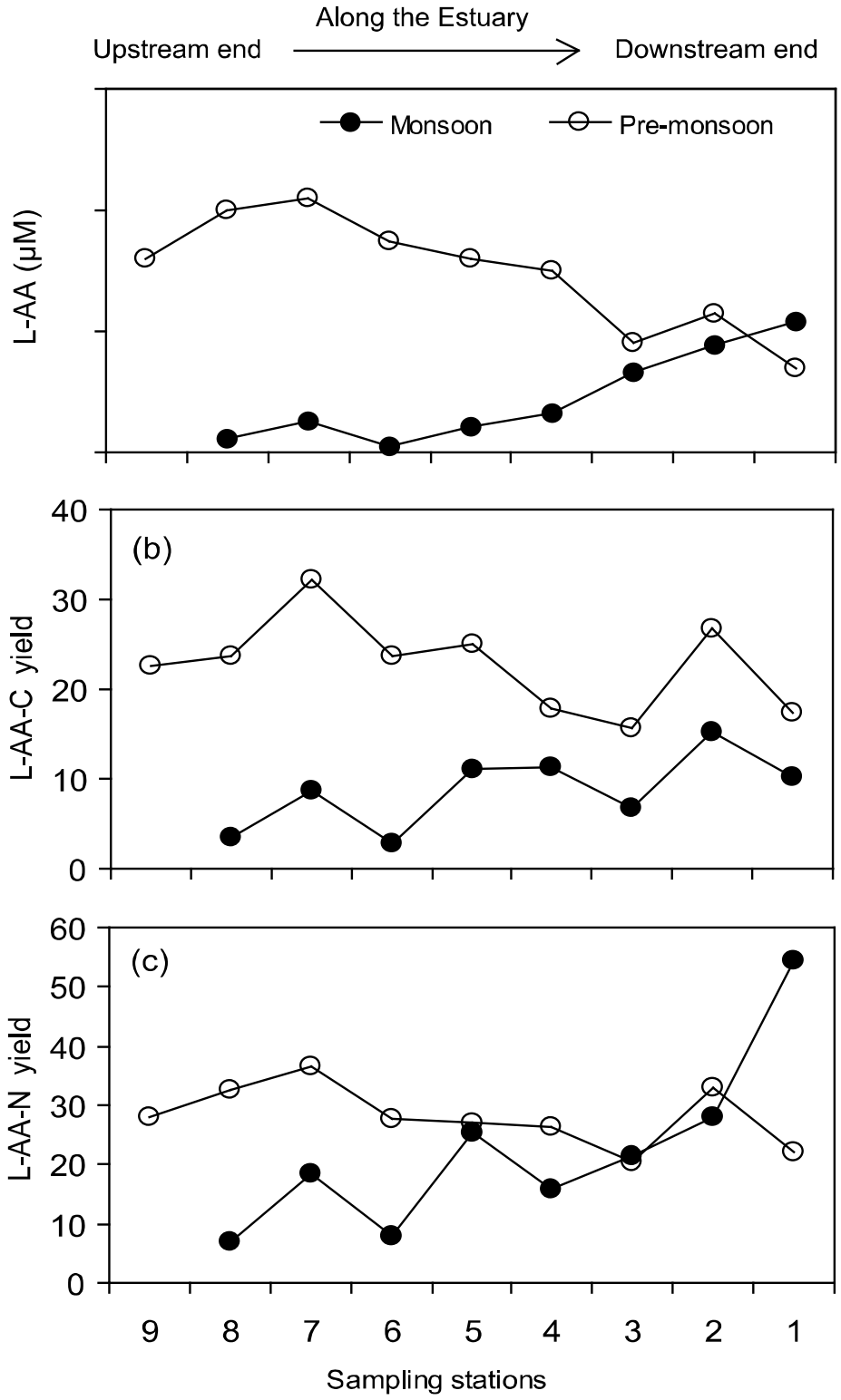

Figure 5A.2. Distribution of L-amino acid concentration (a), and % L-AA-C yield (b), and % L-AA-N yield (c), in the Mandovi estuary during the monsoon and pre-monsoon seasons.

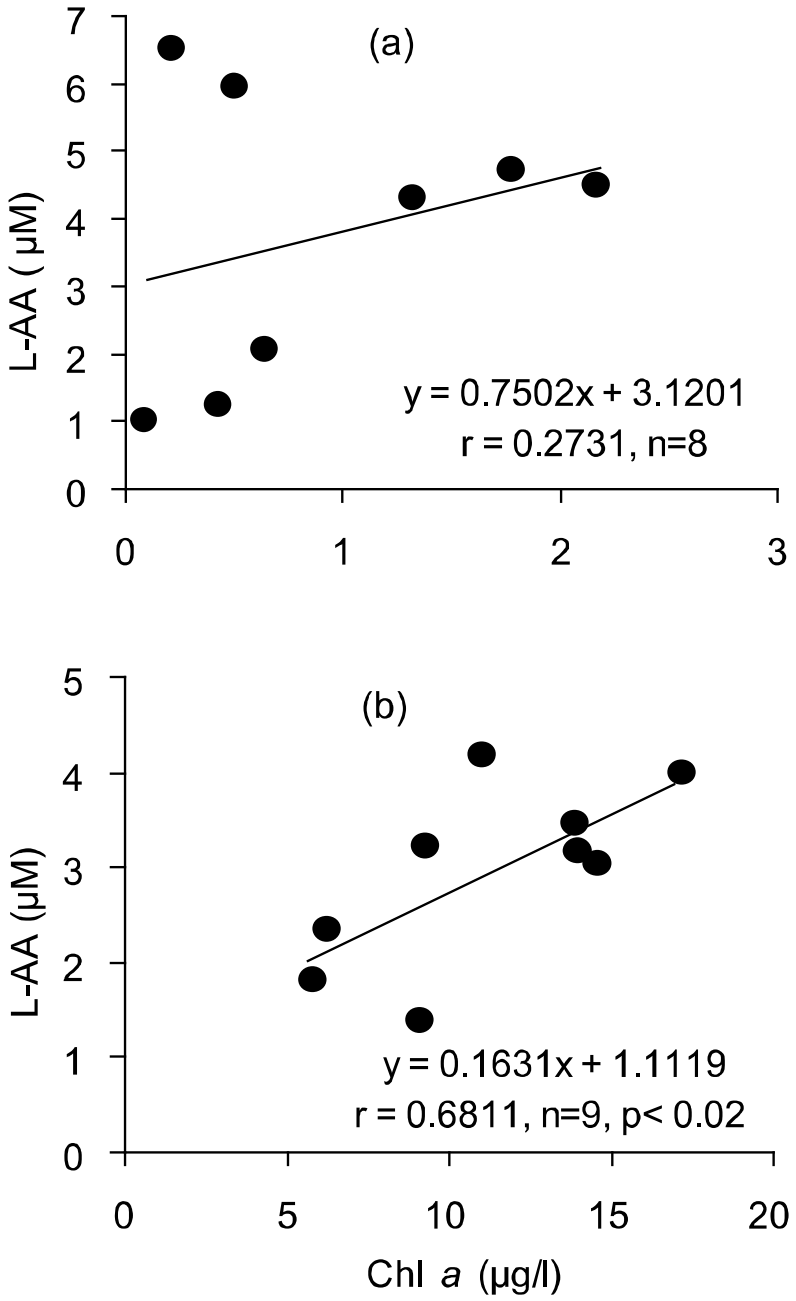

Figure 5A.3. Relationship between total bacterial count (TBC) with L-amino acids during the monsoon and pre-monsoon season in the Mandovi estuary.

station 01 (Fig. 5A.2 b, and c). In the pre-monsoon, L-AA yields increased from station 01 to station 09 (Fig. 5A.2b, and c). L-amino acid s account for a major fraction of the freshly produced OM, and are relatively labile compared to bulk OM (Ittekkot and Arain, 1986; Cowie and Hedges, 1994). L-AA yields decrease during OM degradation and therefore, used to assess the degradation state of OM (Cowie and Hedges, 1992; Davis et al., 2009). During the monsoon season, L-AA yields were lower than that of freshly derived phytoplankton OM. Moreover, the L-AA yields decreased from station 01 to 08. Low L-AA yields indicate that the OM was substantially degraded during the monsoon (Fig. 5A.2b, and c). Another reason for the low L-AA yield was the presence of terrestrial OM, which contains lesser amino acids than phytoplankton (Cowie and Hedges, 1992; Hedges et al., 1997). Ittekkot and Arain (1986) suggest a concept wherein the microbially re-worked OM from the flood plains is transported into the main channels during the high sediment discharge period. The processes occurring in the flood plains and the introduction of the re-worked OM control the composition of the POC carried by these rivers. Ittekkot and Arain (1986) observed that POC associated with high sediment discharge period contains much less labile constituents such as amino acids. This concept may also hold true for the Mandovi estuary during the monsoon season where low contribution of L-amino acid to POC was observed during the high sediment discharge period. Conversely, during the pre-monsoon season, the L-AA yields were greater and generally increased from station 01 to station 09. This suggests that

POM was relatively fresh and of phytoplankton origin during pre-monsoon season.

5A.3.4. Molecular composition of amino acids

The molecular composition of L-amino acid did not show strong spatial differences during both the seasons and hence the Mole % data were averaged (Fig. 5A.4). In the monsoon, the most abundant amino acids were alanine, aspartic acid, leucine, serine, arginine, and threonine. In contrast, glutamic acid, glycine, valine, lysine, and isoleucine were the dominant amino acids during the pre-monsoon season (Fig. 5A.4).

Amino acid composition in source organisms is relatively constant. However, a change in the amino acid composition of suspended particles is mainly due to the alterations as a result of biodegradation and different reactivities of individual amino acid. Thus, amino acids are useful indicators to identify OM degradation state (Cowie and Hedges, 1994; Dauwe and Middelburg, 1998; Dauwe et al., 1999; Keil et al., 2000; Chen et al., 2004). In the Mandovi estuary, the molecular composition of L-amino acid did not, show strong spatial differences during both the seasons and hence Mole % data was averaged (Fig. 5A.4). In general, L-amino acid composition of the Mandovi estuary agrees well with L-amino acid composition of SPM from other world rivers (Hedges et al., 1994; Chen et al., 2004; Duan and Bianchi, 2007; Wu et al., 2007b; Bourgoin and Tremblay, 2010), estuaries, and coastal environment (Cowie and Hedges, 1992; Dauwe and Middelburg, 1998).

The changes in the amino acid composition during OM decomposition depend primarily on the amino acids and their association

with cell wall, cell membrane or cell plasma, and/or sorption onto mineral surfaces (Aufdenkampe et al., 2001). The observed higher amount of non-protein amino acid, β-alanine, and cell wall amino acids serine, threonine and alanine indicates that the OM was relatively degraded during the monsoon season (Fig. 5A.4). In contrast, enrichment of glutamic acid, valine, isoleucine, and lysine, along with depletion in Mole % serine, threonine, alanine, and β-alanine indicates the presence of relatively fresher OM during the pre-monsoon season (Fig. 5A.4).

Glycine and the hydroxyl amino acids, serine and threonine are enriched in diatom cell walls (Hecky et al., 1973). The association of these amino acids with the cell wall protects them from degradation, resulting in their accumulation in degraded OM (Dauwe and Middelburg, 1998). However, the presence of glycine, serine, and threonine in the structural matrices of diatoms and bacteria, cannot be the only possible reason for their enrichment in OM. Ogren and Chollet (1982) observed the release of glycine and serine by growing algal cells during photorespiration. Enrichment of glycine during pre-monsoon may be also because it is a short chain amino acid, has minor food value, and is synthesized from other amino acids during heterotrophic metabolism. Dauwe et al. (1999) observed higher proportion of glycine and alanine in the highly degraded OM. Therefore, the higher abundance of alanine along with serine and threonine during the monsoon indicates presence of highly degraded OM. The observed higher Mole % of aspartic acid, serine, arginine, alanine and leucine and low Mole % of glycine during the monsoon than the pre-monsoon season, maybe due to the presence of terrestrial OM during the

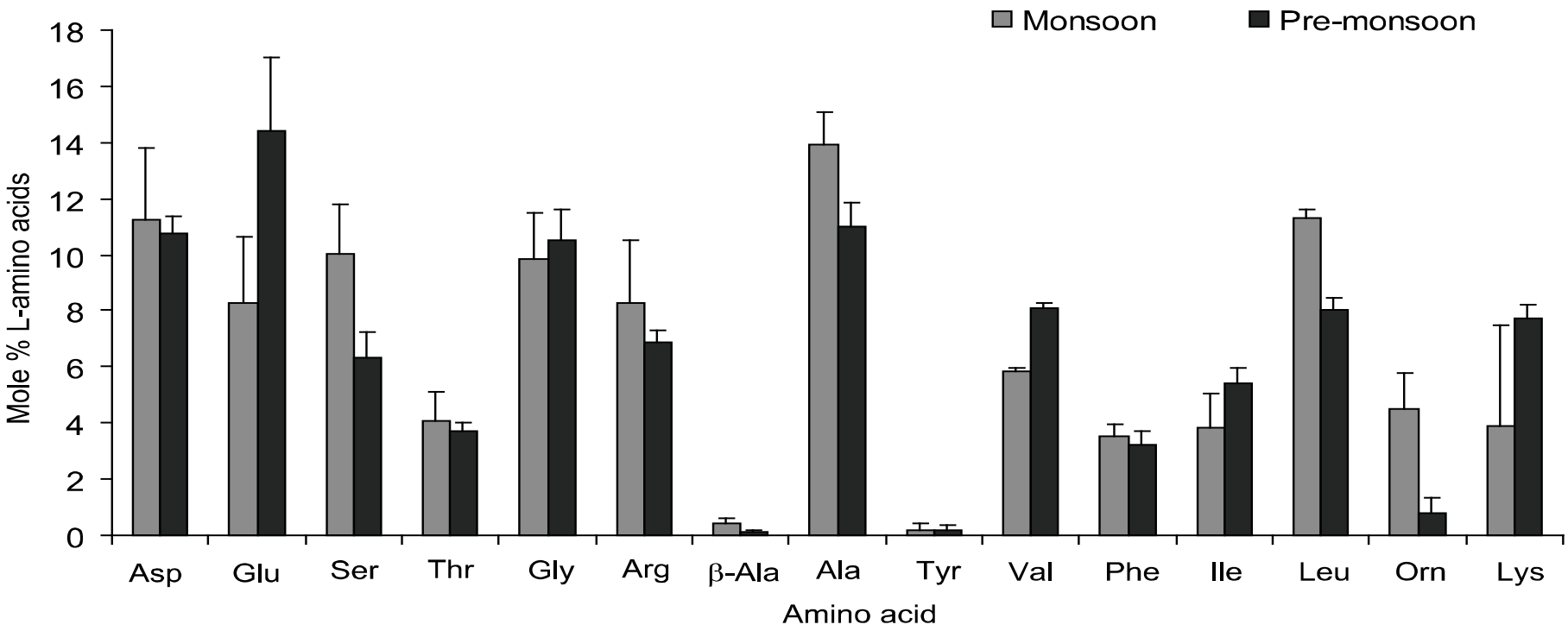

Figure 5A.4. Average Mole percentages of total L- amino acids (L-AA) in the Mandovi estuary, bar indicates standard deviation. Asp: aspartic acid; Glu: glutamic acid; Ser: ser; Gly+Thr: glycine plus threonine; Arg: arginine; β-Ala: beta-alanine; Ala: alanine; γ-ABA: gamma-amino butyric acid; Tyr: tyrosine; Val: valine; Phe: phenylalanine; Ile: isoleucine; Leu: leucine; Orn: ornithine; Lys: lysine.

former season. These amino acids are found to be more abundant in vascular plant tissues as compared to phytoplankton (Cowie and Hedges, 1992). Wu et al. (2007b) observed abundance of alanine, threonine, arginine, and leucine in the terrestrial OM of Yangtze riverine OM. Amino acids such glutamic acid, aspartic acid, isoleucine, valine, tyrosine, and phenylalanine are generally concentrated in diatom cell plasma (Hecky et al., 1973; Dauwe and Middelburg, 1998) and are found to be easily susceptible to degradation and are abundant in freshly derived marine OM. These amino acids show strong depletion with increasing state of decomposition.

The non-protein amino acid, β-alanine was the minor component and accounted for 0.4 ± 0.2 Mole % and 0.1 ± 0.04 Mole % of total L-amino acid during the monsoon and pre-monsoon season, respectively (Fig. 5A.4). The non- protein amino acids, β-alanine, is the bacterial degradation by-product of protein amino acid aspartic acid. This non-protein amino acid is generally absent or present at trace level in living organisms, however Mole % of β-alanine increases with OM degradation (Cowie and Hedges, 1992). β-alanine was more abundant in OM during monsoon than pre-monsoon suggesting the presence of degraded OM during monsoon.

Amino acid degradation index (DI) values ranged between -1.8 and -0.4 during the monsoon season. Except for station 02, the DI values became more negative towards the upstream end stations 07 and 08 (Fig. 5A.5). In the pre-monsoon, DI values were positive and varied between +0.5 and +1.3 (Fig. 5A.5). DI is another proxy for assessing the

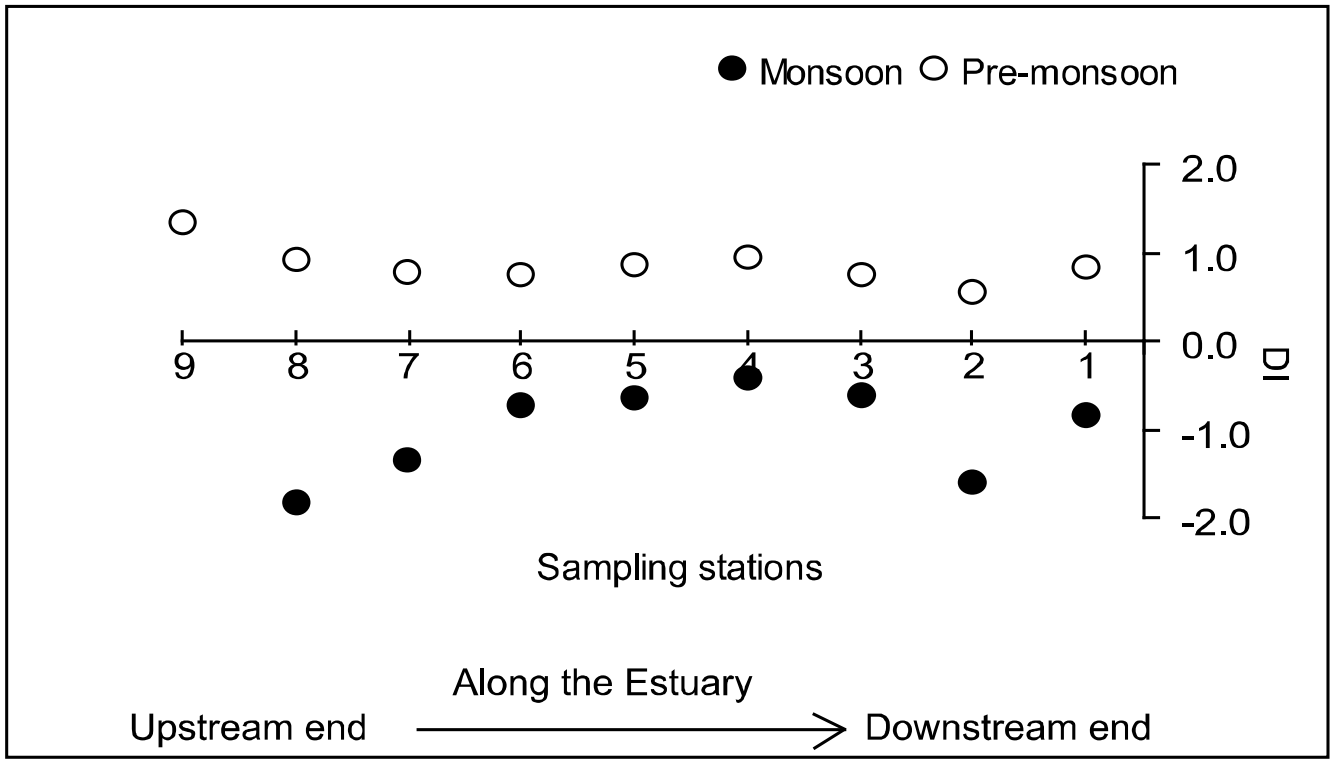

Figure 5A.5. Variation of Degradation index (DI) in the Mandovi estary.

degradation state of OM. In the Mandovi estuary, the low and negative values of DI's during monsoon season indicate that the OM at most of the stations in the estuary was relatively degraded (Fig. 5A.5). This conclusion was also supported by high C/N ratio, and Mol % D-amino acids, and low L-AA yields. Conversely, greater and positive DI (Fig. 5A.5), higher % L-AA yields, and lower C/N ratio and Mole % D-amino acids all indicate the presence of relatively fresh OM during the pre-monsoon season.

5A.3.4. Distribution and composition of D-amino acids

D-isomers of alanine, glutamic acid, aspartic acid, and serine were detected in the Mandovi estuary. D-amino acid concentrations were low (9.0 to 25.3 nM) during the monsoon as compared to the pre-monsoon season (22.1 to 36.7 nM) (Table 5A.3). Heterotrophic bacteria are the primary agent of decomposition of terrestrial and planktonic OM in aquatic environments. D-amino acids are derived from bacterial cell wall, and bacterial macromolecules but are absent in phytoplankton or vascular plants (Kaiser and Benner, 2008). In the Mandovi estuary, a shift in the ecosystem production from net heterotrophy to net autotrophy occurs during the pre-monsoon season (Khodse et al., 2010). The higher D-amino acid concentration observed during the pre-monsoon season (Table 5A.3) indicate accumulation of bacterial remnants due to diagenetic processes was not the reason for the observed higher D-amino acids concentrations in the Mandovi estuary, since other diagenetic indicators such as DI, non-protein amino acid, and L-AA yields, indicates an increased bacterial biomass (McCarthy et al., 2004). In surface waters, TBC varied from 5.4×10^8 to 42.1×10^8 cells/l (Table 5A.2). TBC was

lower during the monsoon than the pre-monsoon season (Table 5A.2). This was also supported by a significant positive relationship between the total bacterial count (Table 5A.1) and D-amino acids concentrations (r =0.6993, n= 9, p< 0.02; Fig 3A. 6).

Total D-amino acids accounted for 1.1 to 11.5 Mol % and 1.5 to 2.8 Mol % of L-AA during monsoon and pre-monsoon season, respectively (Table 5A.3). Spatially, Mol % D-amino acid increased towards the upstream end, with highest contribution at station 06 (11.5 Mol %) (Table 5A.3). D-amino acids (as Mol %) are useful biomarkers to assess OM

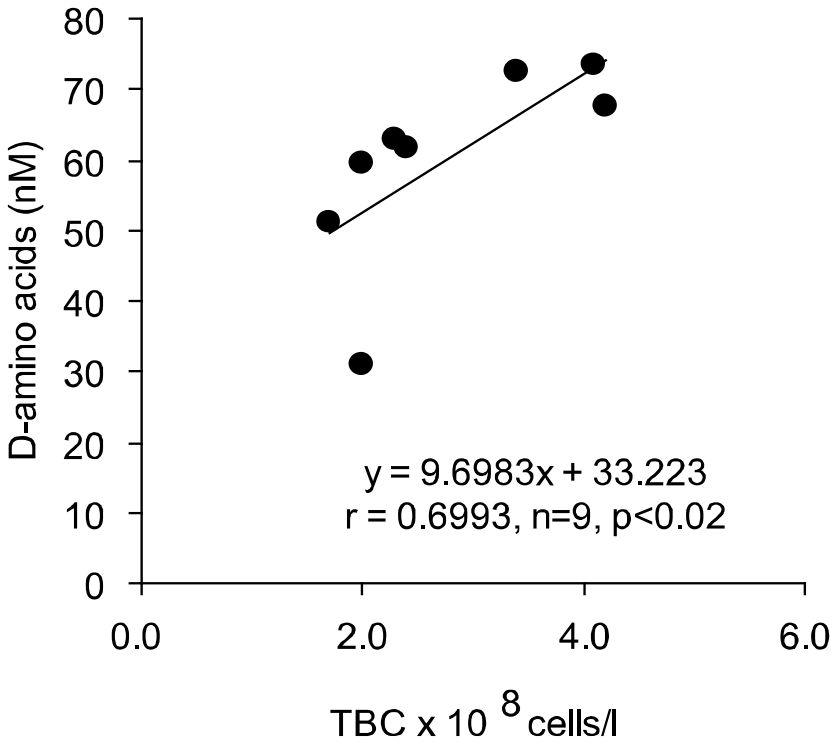

Figure 5A.6. Relationship of total bacterial count (TBC) with D-amino acids during the pre-monsoon in the Mandovi estuary.

degradation (Jorgensen et al., 2003; Nagata et al., 2003; Wu et al., 2007b; Tremblay and Benner, 2009). In the Mandovi estuary, D-amino acids accounted for 1.1 to 11.5 Mol % of total L-amino acid, indicating greater bacterial contribution to the OM during the monsoon season. Further, the Mol % D-amino acids increased towards the upstream end, and highest contribution of D-amino acid to L-amino acid was observed at station 06 (11.5 Mol %) (Table 5A.3). Mol % D-amino acids observed during pre-monsoon were relatively low (1.5 to 2.8 Mol %). D-amino acids were highest at station 06 suggesting the presence of degraded OM.

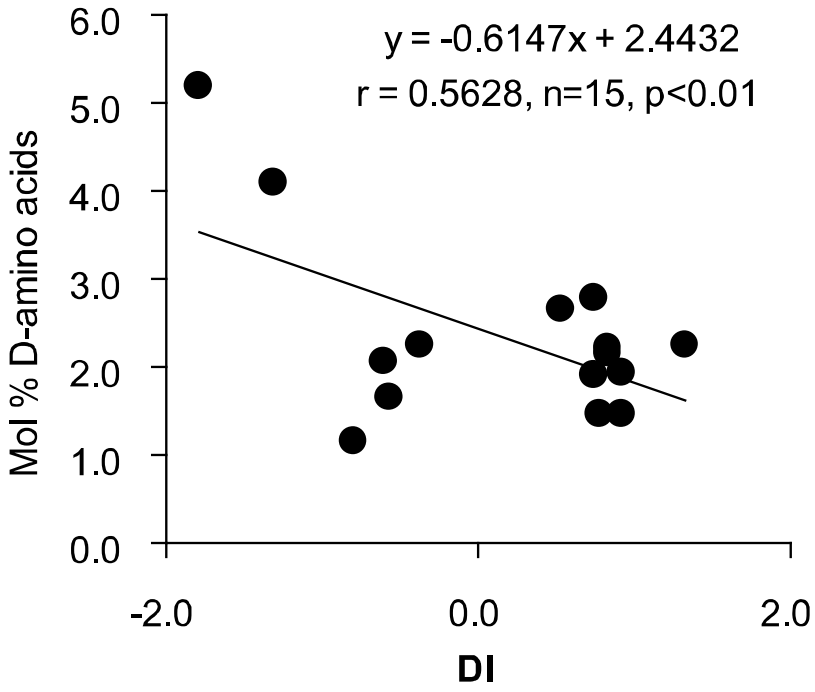

Figure 5A.7. Relationship of total bacterial count (TBC) with Mol % D-amino acids during monsoon and pre-monsoon season in the Mandovi estuary.

Table 2A. 3. Distribution of D-amino acids, Mol % D-amino acid to total amino acids, D-amino acid normalized C and N yields and Bacterial-C and -N contribution at various locations during the monsoon and pre-monsoon seasons in the Mandovi estuary

Stations	D-AA	D-AA [a]	D-Glu	D-Ala	D-Asp	D-Ser	D-Glu	D-Ala	D-asp	D-Ser	Bacterial	
	(nM)	(mol %)	nmol mg C^{-1}				nmol mg N^{-1}				% C	% N
Monsoon season												
Stn1	25.3	1.2	11.1	5.7	1.8	0.9	208.7	107.6	34.4	17.5	16.4	70.2
Stn2	20.1	1.1	10.4	14.5	1.7	1.7	67.8	94.1	11.2	11.2	23.6	34.6
Stn3	22.0	1.7	9.1	6.8	2.2	0.6	101.1	75.0	23.9	7.1	16.0	40.0
Stn4	14.5	2.2	15.0	22.7	2.3	1.4	74.9	113.1	11.7	7.1	24.1	29.1
Stn5	9.0	2.1	16.5	18.3	1.0	2.2	133.3	147.6	8.2	17.7	21.6	42.2
Stn6	9.7	11.5	17.2	33.3	0.2	1.3	175.3	340.1	2.2	13.2	30.5	75.5
Stn7	20.9	4.1	33.7	22.9	0.1	1.4	254.2	172.9	1.1	10.3	34.3	62.4
Stn8	11.1	5.2	7.5	21.2	0.1	1.4	52.1	146.4	0.9	9.8	17.4	29.1
Mean	16.6 ± 6.3	3.6 ± 3.5	15.1 ± 8.3	18.2 ± 9.1	1.2 ± 0.9	1.4± 0.5	133.4 ± 73.1	149.6 ± 83.3	11.7 ± 12	11.7 ±4.1	23.0 ± 6.7	47.9 ± 18.7

Continued

Pre-monsoon season												
Stn1	30.3	2.2	16.6	21.1	24.4	1.6	75.0	95.5	110.5	7.1	38.3	40.4
Stn2	60.9	2.6	26.9	49.8	38.0	1.5	117.4	216.8	165.4	6.7	77.8	79.2
Stn3	50.3	2.8	21.7	27.0	23.9	1.2	100.3	124.8	110.7	5.7	49.3	53.3
Stn4	58.5	1.9	10.7	28.4	17.5	0.8	56.2	148.5	91.4	4.1	39.7	48.5
Stn5	71.4	2.2	14.9	53.2	24.7	1.6	57.3	204.6	94.8	6.2	69.1	62.1
Stn6	66.4	1.9	18.5	25.2	30.9	1.4	77.1	104.7	128.3	5.7	44.4	43.1
Stn7	61.7	1.5	21.4	26.5	30.8	1.4	86.3	106.8	123.9	5.7	48.7	45.8
Stn8	58.2	1.5	16.6	13.4	27.1	1.4	80.7	65.0	131.5	6.8	30.4	34.5
Stn9	72.3	2.3	24.1	27.7	32.3	1.3	105.6	121.5	141.9	5.8	52.5	53.8
Mean	57.2 ± 12.5	2.1 ± 0.5	19.1 ± 5.0	30.3 ± 13	27.7 ± 6.0	1.4± 0.2	84.0 ± 21	132.0 ± 50.1	122.1 ± 23.3	6.0± 0.9	50.0 ±15	51.2 ± 13.3
Other studies												
Sargasso Sea[b]											~ 25	~ 50
Amazon River[c]											4 - 17	17 – 37
St. Lawrence system[d]											~ 20	~ 40

D-AA[a] = D-AA X 100/ L-AA, Sargasso Sea[b]: Kaiser and Benner 2008; Amazon River [c]: Tremblay and Benner, 2009; St. Lawrence system [d]: Bourgoin and Tremblay, 2010.

This conclusion is well supported by low L-AA yield and high C/N ratio of POM at this station. A significant inverse ($r = -0.5628$, $n=15$, $p< 0.05$; Fig. 5A.7) relationship was observed for Mol % D-amino acid and DI, suggesting that D-amino acids indicate degradation of OM.

5A.3.5. Bacterial contributions to the POM

Based on biomarker yields, bacteria accounted for 16 to 34 % (23.0 ± 6.7 %) of POC and 29 to 75 % (47.9 ± 18.7 %) of TPN during monsoon, and 30 to 78 % (50.0 ± 15 %) of POC and 34 to 79 % (51.2 ± 13.3 %) of TPN during the pre-monsoon season (Table 5A.3). In an estuary, activities of heterotrophic bacteria play an important role in determining the quality and quantity of OM. In view of this, assessing bacterial contribution to OM in estuaries is extremely important and challenging. D-amino acids are useful proxies to assess bacterial contribution. This is because D-amino acids are produced by bacteria and not by phytoplankton. It is assumed that D-amino acid yields in cultured bacteria are representative of natural assemblages, and their reactivities are representative of bulk carbon and nitrogen reactivites (Kaiser and Benner, 2008; Tremblay and Benner, 2009; Bourgoin and Tremblay, 2010). In this study, we used D-amino acids yields to calculate bacterial contribution to OM using average bacterial yields taken from published literature on bacterial assemblages typically found in soils and freshwaters and estuarine system (Bourgoin and Tremblay, 2010). We used yields of D-alanine and D-glutamic acid to calculate the bacterial contribution to bulk OM as D-aspartic acid and D-serine yields were highly variable and greater than those recorded for bacteria (Kaiser and Benner, 2008). Estimates of bacterial-C to POC

varied from 16 to 34 % for monsoon and 30 to 78 % for pre-monsoon season (Table 5A.3). Similarly, estimates of bacterial-N contribution to TPN ranged between 29 to 75 %, and 34 to 79 % for monsoon and pre-monsoon season, respectively (Table 5A.3). The estimate of bacterial- C and N obtained for the Mandovi estuary is in the range of values reported by others Kaiser and Benner, 2008; Temblay and Benner, 2009; Bourgoin and Tremblay, 2010). Bacteria accounted for ~ 25 % of C and ~ 50 % of N in marine POM from the Sargasso Sea in North Atlantic Ocean and North Pacific Ocean (Kaiser and Benner, 2008). Tremblay and Benner. (2009) estimated 4 - 17 % of the C and 17 - 37 % of the N in the POM of Amazon River, whereas bacterial OM accounted for ~ 20 % of POC and ~ 40 - 70 % of TPN in the St. Lawerence system (Bourgoin and Tremblay, 2010). Bacterial–N contribution to TPN in monsoon appears to be similar to that observed for pre-monsoon (Table 5A.3). $\delta^{13}C_{POC}$ suggests that the OM in monsoon was mostly derived from terrestrial sources. If the OM was of terrestrial origin, then we would expect higher C/N ratio (> 15) since terrestrial OM is nitrogen poor. However, C/N ratios of OM were lower (<13) with the exception of one value than those recorded for terrestrial OM. In view of this, the lower C/N ratio strongly suggests the enrichment of bacterial–N on the terrestrial OM. It is documented that biodegradation of terrestrial OM results in bacterial enrichment (Tremblay and Benner, 2006). Such enrichment of bacterial–N on terrestrial OM may have reduced the differences in bacterial–N contribution in monsoon and pre-monsoon season.

Chapter 5B

Amino acids biogeochemistry and bacterial contribution to organic matter of the sediments of the Bay of Bengal

5B.1. Introduction

Marine sediments are important zones of global organic matter (OM) production, re-mineralization and burial. Large proportion of newly produced and imported euphotic zone OM settles at the water-sediment interface. Burial of organic carbon in the sediments is estimated to be between 126 and 160 Mt C yr^{-1} (Benner, 1982; Hedges and Keil, 1995; Baumgart et al., 2010; Bourgoin and Tremblay, 2010). High sedimentation rates and biological productivity in the overlying water column influence burial of organic carbon (Cowie and Hedges, 1992; Burdgie, 2007). OM buried in the marine sediments forms a major link between the "active" surface pools of carbon and inactive and/or slow cycling carbon pools (Burdige, 2007). In the deep ocean, OM is mostly of marine origin; however influence of terrestrial OM due to advection from the shelf to deep sediments by rivers and aerial transport of aeolian particles has been observed (Hedges and Keil, 1995; de Haas et al., 2002).

Amino acids, the building blocks of proteins, are the major forms of nitrogen in both terrestrial and marine organisms. These compounds are the important constituents of living and dead OM (Cowie and Hedges, 1992), and represent a significant fraction of bioactive OM preserved in the marine sediments (Keil et al., 2000; Vandewiele et al., 2009). Moreover, natural occurrence and geochemical behavior of amino acids have been evaluated in several types of samples (Cowie and Hedges, 1992; Gupta and Kawahata, 2000; Vandewiele et al., 2009). The contribution of amino acids carbon to total organic carbon and amino acid nitrogen to total nitrogen and relative proportion of individual amino acids

are useful diagenetic indicators (Cowie and Hedges, 1994; Dauwe et al., 1999; Davis et al., 2009).

Heterotrophic microorganisms play an important role in the ecological and biogeochemical processes in the marine sediments. Heterotrophic activity account for most of OM re-mineralization in marine sediments (Jorgensen, 2000). Besides selectively utilizing the bioreactive OM including amino acids, bacteria contribute to the pool of living and non-living sediment OM in the form of bacterial cell wall and other bacterial macromolecules (Veuger et al., 2006; Lomstein et al., 2009). D-amino acids and several other macromolecules of bacterial origin contribute significantly to detrital OM of marine sediments (Pedersen et al., 2001; Grutters et al., 2002; Lomstein et al., 2006; Kaiser and Benner, 2008). Initially the quantification of microbial carbon and nitrogen, to the detrital OM was difficult due to the highly heterogeneous and reworked nature of OM by different assemblage of bacteria (i.e. soil vs. freshwater vs. seawater). However, recently there have been a few efforts to quantify the bacterial contribution to detrital OM (Kaiser and Benner, 2008; Tremblay and Benner, 2009; Bourgoin and Tremblay, 2010).

In the Bay of Bengal, both the quantity and quality of OM is affected by the fluvial input of sediments and biological production in overlying waters (Ittekkot et al., 1991). Moreover, there is little knowledge about the L-amino acids distribution and cycling in the marine sediment of Bay of Bengal (Bhosle and Dhople, 1988; Unger et al., 2005). Further, no study is available about the D-amino acid distribution, cycling and bacterial contribution in the surface and at greater depths of sediment cores of Bay

of Bengal. In the view of this, the aims of the present study were to 1) evaluate the distribution of L- and D-amino acids 2) investigate composition of L-and D-amino acids 3) determine the nature of organic matter 4) use biomarker approach to trace bacterial remnants and 5) provide estimate of bacterial contribution to marine organic matter.

5B.2. Materials and Methods

5B.2.1. Study area

The Bay of Bengal (BOB) is one of the largest fresh water input sites of the world ocean (Fig. 5B.1). The annual fresh water discharge into the Bay exceeds 1.625 x 10^{12} m^{-3} yr^{-1}. The suspended sediment discharge into the Bay is estimated to be 1.382 x 10^{9} tones (Subramanian, 1993). Because of the freshwater, the mean salinity of the waters reduces by about 7 ‰ in the northernmost region. The major input of the suspended particulate matter (2.0 x 10^{15} g) into the Bay is mainly through the Himalayan Rivers- the Ganga and Brahmaputra, and peninsular Indian rivers such as Mahanadi in the north, Godavari and Krishna in the central region, and Irrawady, Penner and Cauvery in southern region (Rao, 1985). About, 0.6 x 10^{12} mol C_{org} yr^{-1} is brought into the Bay by the Ganga - Brahmaputra system (Galy et al., 2007). The warm sea surface temperature (SST's) and the low salinity in the BOB lead to strong stratification of the water column, which prevents the transport of nutrient rich bottom waters into the surface. Moreover, the weak winds prevailing in the BOB are unable to break the thermocline layer. The hydrography of the bay is also seasonal.

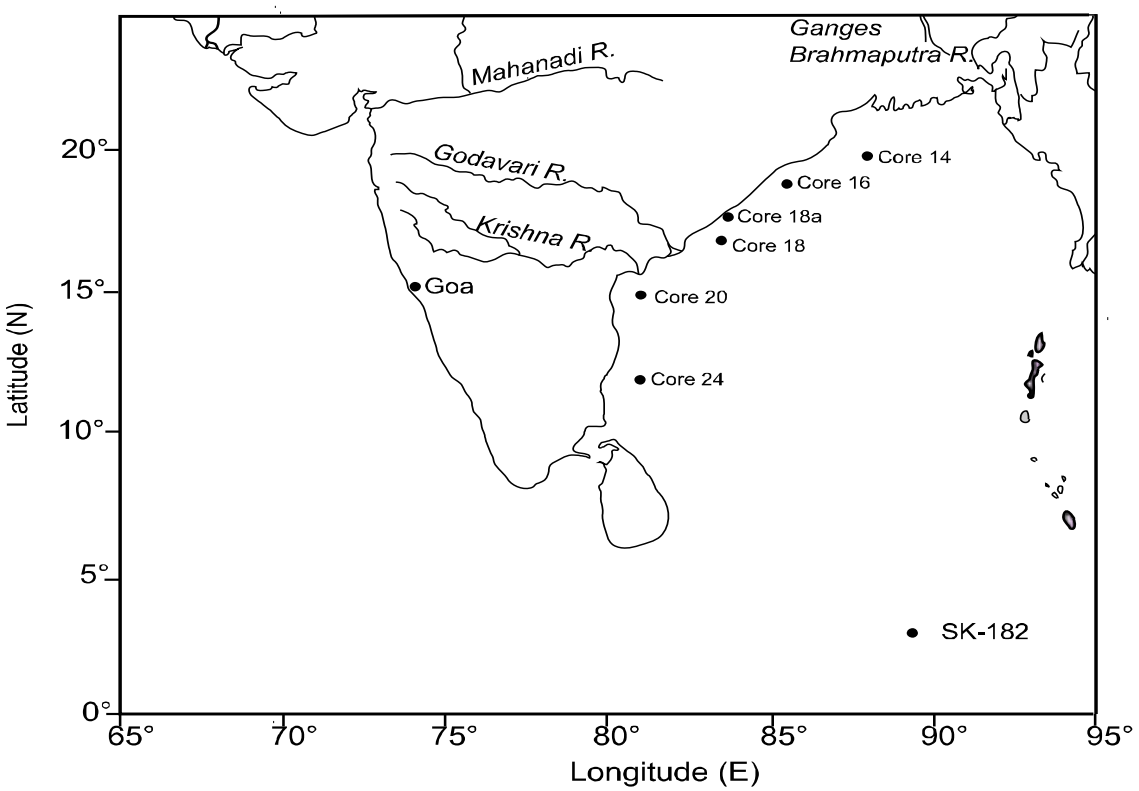

Figure 5B.1. Map showing sampling stations in the Bay of Bengal.

Frequently, occurring cyclones enrich the surface waters with nutrients, resulting in a localized algal bloom (Vinayachandran and Mathew, 2003). Moreover, the western boundary of the Bay is characterized by intense western boundary currents, East India Coastal Currents, and eddy activity (Nuncio and Prasanna Kumar, 1012). Upwelling has been observed along the western boundary of the Bay (Shetye et al., 1991). Although the BOB has a low biological productivity, there is a wide range of spatial and temporal variability in the biological productivity in the Bay (Prasanna Kumar et al., 2010). The river run-off in the northern region besides freshening water column also adds nutrients. The upwelling in the western boundary during south west monsoon results in high chlorophyll *a* (Fernandes et al., 2009) and primary production (Prasanna Kumar et al., 2010). Moreover the frequently occurring cyclones and eddies enhance biological productivity several folds in the BOB. Clastic sediments are recorded at the inner shelf and continental slope, while calcareous relict sediments are present at the outer shelf. Off the river mouth the shelf is covered by fine-grained terrigenous sediments (Rao, 1964; Khodse et al., 2008).

5B.2.2. Collection of samples

Six sediment cores were collected during the fall inter-monsoon (FIM) cruise SK-182 of the ORV Sagar Kanya in the BOB from 14 September to 12 October 2002 (Fig.5B.1). Immediately after collection, the cores were sectioned at 2 cm intervals, and sectioned sediments were dried at 40 °C in an electrical oven. Sediments were ground to a fine powder using an agate pestle and mortar, and stored at -20 °C until analysis.

Table 5B.1. Details of location, sampling year, water depth, core length, and sedimentation rate of the sediment cores of the Bay of Bengal

Stations	Latitude (N)	Longitude (E)	Sampling year	Water Depth (m)	Core length (cm)	SR (cm/yr)
18a	17.27	83.13	2002	70	25	0.272
16	19.00	85.50	2002	680	25	0.199
14	20.00	88.00	2002	930	25	0.191
18	17.00	83.50	2002	2500	25	0.347
20	15.00	81.50	2002	2695	25	0.275
24	11.00	81.00	2002	3500	25	-

SR = sedimentation rate

5B.2.3. Estimation of bulk geochemical parameters

Dry combustion was used to estimate total carbon using Shimadzu TOC 5000-A analyzer. To estimate inorganic carbon 100 mg of sample was treated with 0.5 ml orthophosphoric acid, and heated to 200°C in the presence of oxygen. The evolved CO2 was measured using the Shimadzu TOC 5000-A analyzer. Organic carbon (OC) was calculated as the difference between total carbon and inorganic carbon. Organic nitrogen (ON) was estimated following the method of Raimbault and Slawyk (1991).

5B.2.4. Sedimentation rates

^{210}Pb in the sediment core section was determined following the procedure described by Ram et al., (2009). The sedimentation rate was calculated from the $^{210}Pb_{exc}$ activity. The $^{210}Pb_{exc}$ activity decreased with depth, due to decay of the radioactive compound with time. Hence, the estimation of the sedimentation rate becomes possible. The sedimentation rates have been estimated from the depth profile of $^{210}Pb_{exc}$ in a core assuming the constant initial concentration (CIC) models (Koide et al., 1972; Krishnaswami and Lal, 1978). The apparent ages of the sediment layers have been calculated using linear sedimentation rate

5B.2.5. L- and D-amino acids analysis

Sediment sample (50 mg) was hydrolyzed and analyzed for L-and D-amino acids as given in chapter 2. The precision for 5 replicate samples

Table 5B.2. Precision of L- and D-amino acid method based on replicate samples

L-amino acids	Replicate sediment samples				Ave	SD	%CV
	1	2	3	4			
Asp	6.57	6.51	6.12	7.59	6.70	0.63	9.35
Glu	6.83	7.42	6.26	6.04	6.64	0.62	9.31
Ser	4.17	5.15	4.85	5.05	4.80	0.44	9.13
Gly+Thr	17.84	20.41	17.69	20.65	19.15	1.60	8.36
Arg	4.34	4.65	4.95	5.36	4.83	0.43	9.01
b-Ala	0.87	0.98	0.84	0.98	0.91	0.07	7.88
Ala	8.25	8.63	7.71	9.29	8.47	0.66	7.81
g-ABA	0.55	0.52	0.51	0.59	0.54	0.03	6.01
Try	0.10	0.09	0.10	0.09	0.10	0.01	9.38
b-ABA	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Val	4.91	5.38	5.96	5.18	5.36	0.45	8.32
Phe	2.47	2.71	2.44	3.02	2.66	0.27	10.04
Ile	3.14	3.42	3.13	2.93	3.15	0.21	6.51
Leu	3.90	3.26	3.84	3.83	3.71	0.30	8.16
Orn	1.95	1.90	1.97	1.67	1.87	0.14	7.41
Lys	8.13	9.14	9.99	8.36	8.91	0.84	9.45

D-amino acids	Replicate sediment samples					Ave	SD	%CV
	1	2	3	4	5			
L- Asp	13.30	13.82	11.68	13.79	15.69	13.7	1.4	10.5
D- Asp	2.58	2.50	2.58	2.74	2.92	2.7	0.2	6.3
L- Glu	10.20	9.30	7.89	9.19	9.76	9.3	0.9	9.4
D- Glu	0.92	0.83	0.80	0.70	0.84	0.8	0.1	9.5
L- Ser	8.29	6.93	8.31	7.64	6.97	7.6	0.7	8.9
D-Ser	0.33	0.41	0.37	0.43	0.40	0.4	0.0	10.1
L- Ala	5.93	5.08	5.20	4.69	5.34	5.2	0.5	8.6
D-Ala	0.60	0.64	0.65	0.56	0.70	0.6	0.1	8.7

was between 6 and 10 % for L-amino acid and 8 and 10 % for D-amino acids (Table 5B. 2). The concentrations of D-amino acids were corrected for racemization as suggested by Kaiser and Benner (2005).

5B.2.6. Statistical analysis

A simple regression analysis was performed to assess the relationship between various parameters using excel program. A Principal Component Analysis (PCA) was performed to evaluate the organic matter quality at various locations using Statistica 6 following Dauwe et al. (1999) as described in Chapter 1.

5B.2.7. Bacterial contribution to OM

The proportion of bacterial-C and N was calculated using the equation given by Bourgoin and Tremblay (2010).

$$\% \text{ Bacterial C or N} = 100[\text{biomarker}]_{\text{sample}}/[\text{biomarker}]_{\text{bacteria}}$$

Where $[\text{biomarker}]_{\text{sample}}$ and $[\text{biomarker}]_{\text{bacteria}}$ are the C- or N-normalized yields of a specific biomarkers (e.g. D-alanine, and D-glutamic acid) in the sediment (sample) of BOB and different assemblage of bacterial cells (bacteria).

5B.3. Results and Discussion

5B.3.1. Sedimentation rate and estimated age

The results of $^{210}Pb_{total}$ and $^{210}Pb_{exc}$ activities are shown in Table 5B.3. Downcore exponential decrease was observed for $^{210}Pb_{exc}$, when $^{210}Pb_{exc}$ was plotted against sediment depth (Fig. 5B.2). Thus the sedimentation rates (SRs) for the sediments was calculated from these profiles for the five cores collected from the Bay of Bengal (Fig. 5B.2). $^{210}Pb_{exc}$ derived

sedimentation rates were found to vary between 0.191 to 0.347 cm/yr. Higher SR values were observed for cores collected at station 18, 20, and 18a. Lower values were observed for cores of station 14 and 16. Assuming that the SR rates are constant for the cores, the age and calendar year were assigned to the sediment depths (Table 5B. 3). The dating of sediment in the upper 25 cm core by ^{210}Pb provided coverage of time period spanning between 62 to 118 yr (Table 5B. 3). Core at station18 was relatively younger with time span of 62 yr (1999 - 1937 AD) compared to core at station 18a which provided with much longer time coverage of 113 yr (1997 – 1884 AD) (Table 5B. 3).

5B.3.2. Organic carbon, organic nitrogen, and C/N ratio

Distribution of OC, ON and C/N ratio in the sediments of BOB are shown in Table 5B.4. OC and ON concentration varied from 8.1 to 19.6 mg gdw^{-1} (0.8 to 1.96 wt %), and 0.54 to 1.63 mg gdw^{-1} (0.054 to 0.163 wt %), respectively (Table 5B.4). A decrease in both OC and ON concentration was observed with the sediment core depth for most of the sediment cores (Table 5B.4). However, at few of the stations (14, 16, and 18) relatively higher ON concentrations were recorded with the increasing sediment core depth. Suthhof et al. (2000) observed 0.3 to 2.9 wt % and 0.1 to 0.4 wt % of OC and ON in the surface sediments of Pakistan. Similarly, 0.7 to 3.9 wt % OC and 0.10 to 0.45 wt % ON was recorded across Pakistan margin (Vandewiele et al., 2009). Niggemann et al. (2005) observed 1.9 to 20.2 wt % OC for sediments off Peru. In the North Atlantic Ocean, sediments OC varied from 1.3 to 3.2 mg g^{-1} and ON varied from 0.5 to 1.3 mg/g (Horsfall and Wolff, 1997). Dauwe and Middelburg (1998) observed

Table 5B.3. Concentration of $^{210}Pb_{total}$, $^{210}Pb_{exc}$, age and calender year of the sediment cores of the Bay of Bengal

Stations	Depth (cm)	^{210}Pb (dpm/g)	^{210}Pb exc (dpm/g)	Age (yr)	Calender year (AD)
18a	0-2	23.57 ± 2.66	22.57 ± 2.60	5	1997
	2-4	20.56 ± 1.23	19.56 ± 1.17	16	1986
	4-6	14.85 ± 1.26	13.85 ± 1.21	26	1976
	6-8	13.78 ± 1.18	12.78 ± 1.12	37	1965
	8-10	13.44 ± 0.85	12.44 ± 0.80	47	1955
	10-15	7.32 ± 0.45	6.32 ± 0.39	65	1937
	15-20	3.89 ± 0.27	2.89 ± 0.21	92	1910
	20-25	3.31 ± 0.26	2.31 ± 0.21	118	1884
16	0-2	15.14 ± 0.80	14.14 ± 0.74	5	1997
	2-4	11.30 ± 0.47	10.30 ± 0.41	15	1987
	4-6	9.42 ± 0.67	8.42 ± 0.61	25	1977
	6-8	8.68 ± 0.81	7.68 ± 0.75	35	1967
	8-10	5.54 ± 0.35	4.54 ± 0.29	45	1957
	10-15	3.44 ± 0.22	2.44 ± 0.16	63	1939
	15-20	1.98 ± 0.13	0.98 ± 0.07	88	1914
	20-25	1.59 ± 0.12	0.59 ± 0.06	113	1889
14	0-2	7.78 ± 0.54	6.78 ± 0.48	4	1998
	2-4	5.53 ± 0.31	4.53 ± 0.25	11	1991
	4-6	3.62 ± 0.29	2.62 ± 0.23	18	1984
	6-8	3.32 ± 0.50	2.32 ± 0.44	26	1976
	8-10	2.86 ± 0.18	1.86 ± 0.12	33	1969
	10-15	3.05 ± 0.24	2.05 ± 0.18	46	1956
	15-20	2.91 ± 0.20	1.91 ± 0.14	64	1938
	20-25	2.60 ± 0.16	1.60 ± 0.10	83	1919
18	0-2	25.94 ± 1.18	24.94 ± 1.12	3	1999
	2-4	19.44 ± 1.47	18.44 ± 1.41	9	1993
	4-6	21.43 ± 2.03	20.43 ± 1.97	14	1988
	6-8	8.38 ± 0.56	7.38 ± 0.51	20	1982
	8-10	20.36 ± 1.68	19.36 ± 1.62	26	1976
	10-15	12.26 ± 0.88	11.26 ± 0.82	36	1966
	15-20	6.21 ± 0.39	5.21 ± 0.32	50	1952
	20-25	4.22 ± 0.32	3.22 ± 0.26	65	1937
20	0-2	12.85 ± 1.12	11.85 ± 1.06	4	1998
	2-4	12.63 ± 0.95	11.63 ± 0.08	11	1991
	4-6	11.93 ± 0.92	10.93 ± 0.86	18	1984
	6-8	8.55 ± 0.63	7.55 ± 0.57	25	1977
	8-10	8.12 ± 0.57	7.12 ± 0.51	33	1969
	10-15	5.12 ± 0.42	4.12 ± 0.36	45	1957
	15-20	4.07 ± 0.32	3.07 ± 0.26	64	1938
	20-25	1.99 ± 0.21	0.99 ± 0.15	82	1920

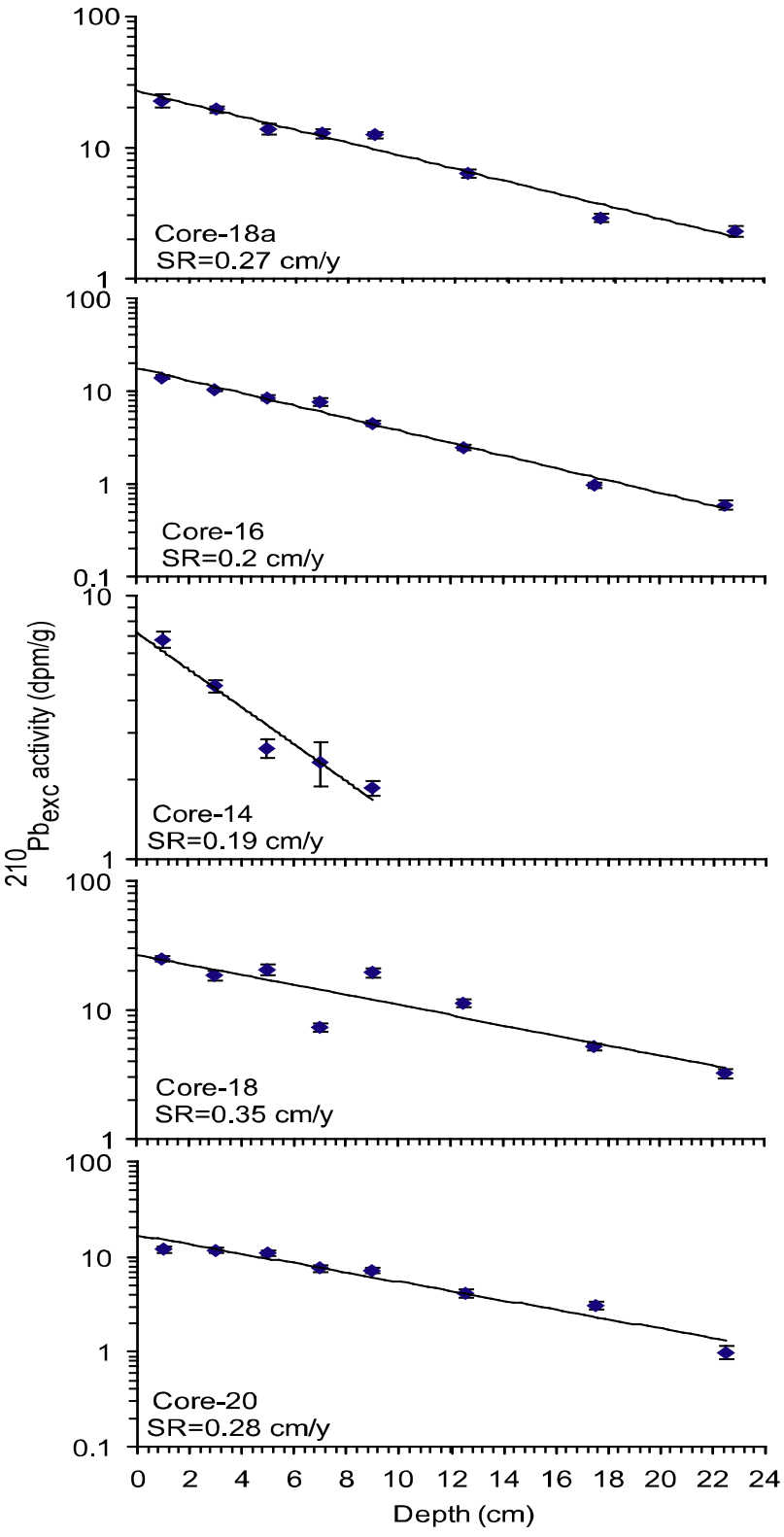

Figure 5B.2. Profile of logarithmically plotted $^{210}Pb_{exc}$ activity against sediment depth for sediment cores of Bay of Bengal.The sedimentation rates (SR) were determined from the plot slope.

0.23 to 24.95 mg g^{-1} and 0.06 to 2.62 mg g^{-1} of OC and ON in the sediments of North Sea.

Terrestrial OM is generally regarded as being less reactive towards microbial degradation than the marine OM (Hedges et al., 1988). Thus, the OM source is an important control on the rates and extent of OM degradation (Niggemann et al., 2007). In the present study, C/N ratio varied from 7.0 to 22.0 (Table 5B.4), indicating a predominantly marine origin, with some influence of terrestrial OM to the sediment OM of BOB. Freshly produced plankton OM, typically rich in protein, has C/N-ratios of 5-7, preferential degradation of N-containing compounds during early diagenesis results in values up to 12 (Emery and Uchupy, 1984). In contrast, terrestrial OM dominated by nitrogen-free bio-macromolecules such as cellulose and lignin has significantly higher C/N ratios of 20–500 (Hedges et al., 1986). Increasing C/N ratios with increasing sediment core depth indicate that in the sediments of BOB, C/N ratios were primarily controlled by ongoing degradation and there was no evidence for significant input of terrestrial OM.

5B.3.3. Amino acid concentration

L-amino acids (L-AA) concentrations varied from 2.6 to 13.0 µmol $gdw^{-}1$ (0.34 to 1.71 mg g^{-1}) in the sediment cores of the BOB (Fig. 5B.3a and 4). A spatial variation in the surface L-amino acid concentrations was observed (Fig. 5B.3a and 4).The amino acid concentrations in the BOB sediments were lower than those observed in sediment cores from other regions (Lee et al., 2000; Grutters et al., 2002; Lomstein et al., 2006).

Table 5B.4. Distribution of sediment age, TOC, TN, C/N ratio, Mole % individual amino acids, and asp/b-ala ratio in the sediment cores of the Bay of Bengal

Station	Depth	Age	OC	ON	C/N	Mole %															
	(cm)	yr	mg/g	mg/g		Asp	Glut	Ser	Thr	Gly	Ala	Val	Ile	Leu	Lys	Arg	Phe	Try	b-Ala	Orn	Asp/b-ala
18a	0-2	3.7	13.6	0.53	25.6	6.5	6.7	5.0	6.8	17.0	11.0	7.6	5.0	6.9	3.5	4.1	4.2	0.18	1.0	0.47	6.4
	2-4	11.1	13.5	0.82	16.5	7.4	7.3	4.8	6.4	16.2	10.4	7.3	4.7	6.5	4.2	5.1	4.0	0.99	1.0	0.51	7.2
	4-6	18.5	13.5	0.96	14.0	7.2	7.0	8.7	7.6	20.1	14.7	9.9	6.5	8.0	5.3	4.5	4.8	0.03	1.2	1.12	6.0
	6-8	25.9	13.3	0.86	15.4	7.0	6.9	8.1	6.4	16.3	11.8	8.0	5.2	6.7	4.4	4.1	3.9	0.04	1.1	1.07	6.5
	8-10	33.3	13.5	0.77	17.5	7.8	8.0	8.9	6.8	16.2	12.2	7.9	5.1	6.3	4.4	4.4	3.7	0.21	1.0	1.17	7.5
	10-15	46.3	11.9	0.68	17.5	6.7	6.3	8.3	6.2	16.5	10.6	7.0	4.7	6.4	5.5	4.8	3.9	1.09	0.9	0.71	7.5
	15-20	64.8	12.9	0.89	14.5	6.7	5.1	10.3	7.1	18.7	13.9	9.2	6.0	7.0	4.6	4.7	4.3	0.15	0.9	1.06	7.8
	20-25	83.3	13.2	0.89	14.8	5.6	5.5	4.3	6.1	16.0	10.5	6.9	4.5	6.1	5.3	4.0	3.8	0.45	0.8	0.88	7.1
16	0-2	5.0	19.6	1.21	16.2	9.3	9.6	4.8	6.4	21.3	9.3	7.4	4.6	5.4	7.3	3.6	3.4	0.18	2.3	1.01	4.1
	2-4	15.1	18.1	1.54	11.8	5.3	4.7	4.9	6.8	24.1	10.4	8.4	5.3	6.1	8.5	4.1	3.5	0.05	2.5	0.96	2.2
	4-6	25.1	18.5	1.56	11.9	10.5	10.3	3.2	6.8	18.7	8.5	7.7	5.2	6.3	6.2	7.2	4.0	0.07	2.1	0.78	5.0
	6-8	35.2	17.5	1.46	12.0	8.1	8.0	10.2	6.3	17.5	12.6	8.0	5.0	6.0	4.3	3.7	3.5	0.13	1.6	0.90	4.9
	8-10	45.2	17.5	1.49	11.8	9.3	7.7	11.3	6.9	16.3	11.4	7.4	4.5	5.3	4.9	4.4	3.3	0.13	1.6	0.87	5.9
	10-15	62.8	18.4	1.47	12.5	9.9	10.2	3.0	6.9	19.3	8.7	8.5	5.6	6.6	5.1	6.6	4.1	0.05	2.2	0.67	4.5
	15-20	87.9	19.4	1.42	13.6	4.0	1.2	7.3	7.7	24.8	10.1	8.9	5.8	6.6	5.6	9.4	4.4	0.41	1.0	1.39	4.0
	20-25	113.1	19.3	1.23	15.7	5.8	4.9	3.6	8.0	21.5	10.0	9.5	6.4	7.4	5.8	6.7	4.6	0.06	2.4	0.69	2.4
14	0-2	5.2	16.7	1.22	14.9	10.9	8.8	8.6	5.0	15.8	9.6	6.7	4.3	5.0	8.2	5.7	3.2	0.31	2.6	1.89	4.2
	2-4	15.7	16.0	1.58	10.1	9.8	8.5	6.3	6.0	18.2	10.8	7.5	4.9	5.8	6.5	4.2	3.7	0.06	2.0	0.96	5.0
	4-6	26.2	16.1	1.63	9.9	4.2	2.5	7.9	7.6	20.1	11.3	7.7	5.1	6.0	9.7	6.3	3.8	0.12	1.6	1.43	2.6
	6-8	36.6	15.8	1.56	10.1	7.5	3.5	10.0	7.5	18.3	11.1	7.5	4.5	4.6	6.0	7.2	3.6	0.42	1.7	3.37	4.5
	8-10	47.1	15.6	1.47	10.6	7.2	6.8	7.0	6.0	17.8	10.7	8.0	5.2	5.8	7.3	5.8	3.7	0.09	1.9	1.13	3.8
	10-15	65.4	14.8	1.34	11.0	7.5	3.2	11.1	5.8	17.6	11.5	8.1	5.0	5.2	6.0	6.2	3.5	0.19	1.5	1.95	5.2
	15-20	91.6	15.2	1.26	12.1	3.7	1.9	6.1	7.1	21.2	10.5	8.6	5.6	6.0	11.0	4.3	4.2	0.16	1.4	1.79	2.7
	20-25	117.8	15.1	1.12	13.5	3.2	1.1	9.6	5.9	24.9	10.1	6.9	4.4	5.1	10.8	4.7	3.5	0.45	1.3	2.59	2.5

Continued

18	0-2	2.9	9.5	0.90	9.4	5.7	3.3	5.7	6.7	17.8	12.1	8.2	5.6	6.3	9.5	8.2	0.6	0.01	3.1	1.48	1.8
	2-4	8.6	9.5	1.17	8.1	3.5	0.9	5.7	6.9	18.2	11.6	7.7	4.9	5.9	15.0	10.7	2.9	0.01	1.9	1.86	1.8
	4-6	14.3	9.3	1.14	8.2	6.8	3.9	14.2	5.0	17.1	10.8	4.6	3.2	4.3	7.8	7.2	2.8	0.56	1.7	3.59	3.9
	6-8	20.0	9.3	1.12	8.3	4.1	2.5	4.1	7.2	19.4	11.8	7.2	4.8	6.1	9.7	7.2	3.6	0.00	3.0	1.69	1.4
	8-10	25.7	9.5	1.10	8.6	3.1	3.1	9.3	6.3	20.7	11.9	6.8	4.3	5.4	7.5	7.2	3.4	0.34	1.8	1.84	1.7
	10-15	35.7	8.5	1.25	6.8	5.6	4.4	4.3	7.1	18.5	11.5	7.7	4.7	5.9	6.9	6.1	3.6	0.01	3.2	1.09	1.7
	15-20	50.0	8.1	1.29	6.3	6.9	6.5	6.7	6.3	19.7	11.8	7.4	4.5	5.5	6.5	5.6	3.4	0.21	2.9	1.28	2.4
	20-25	64.3	8.6	1.17	7.4	3.9	2.1	11.0	6.5	21.0	11.8	6.6	4.0	5.1	6.7	7.9	3.2	0.69	2.9	1.91	1.4
20	0-2	3.6	10.7	0.90	14.8	7.7	7.0	6.5	5.3	18.3	10.4	7.6	4.6	5.8	6.2	3.4	3.7	0.03	2.7	0.94	2.8
	2-4	10.7	10.9	0.81	13.5	16.3	11.3	3.9	4.3	11.6	9.8	8.1	5.2	6.1	6.3	4.9	3.6	0.30	2.6	2.54	6.3
	4-6	17.9	9.4	0.72	13.0	7.3	4.9	11.0	5.0	19.9	12.8	8.0	4.9	5.8	1.9	2.2	3.6	0.61	2.2	0.46	3.3
	6-8	25.0	8.8	0.66	13.4	11.4	7.8	8.4	4.4	17.2	11.7	7.8	4.6	5.5	2.6	3.3	3.3	0.20	2.5	0.61	4.6
	8-10	32.1	9.1	0.58	15.7	18.1	12.7	4.9	4.4	11.8	9.9	7.4	3.9	4.7	3.9	8.4	2.5	0.05	3.1	0.90	5.9
	10-15	44.6	10.3	0.57	18.0	6.5	6.0	5.3	4.2	19.3	10.7	6.7	4.0	4.9	9.8	4.4	3.4	0.1	3.4	1.66	1.9
	15-20	62.5	10.3	0.54	19.0	6.2	4.3	5.0	4.8	18.9	10.3	6.5	3.9	4.6	10.2	5.4	3.1	0.1	3.2	1.39	1.9
	20-25	80.4	11.2	0.62	18.0	14.4	10.0	6.9	3.0	19.5	10.0	6.1	3.4	3.9	4.0	4.1	2.3	0.13	3.3	0.84	4.3
24	0-2	-	17.2	1.10	20.4	9.5	8.0	5.0	5.9	19.8	11.0	7.6	4.4	5.0	4.3	3.0	3.2	0.08	4.3	0.81	2.2
	2-4	-	16.2	1.05	15.4	8.7	7.4	4.3	5.9	20.2	11.0	7.8	4.6	5.1	4.6	3.1	3.4	0.07	4.8	0.86	1.8
	4-6	-	17.1	0.94	18.2	8.8	7.4	3.8	6.2	16.6	11.0	8.4	5.3	6.2	5.6	5.1	3.8	0.07	4.5	1.07	2.0
	6-8	-	17.4	0.94	18.5	7.6	6.9	3.5	6.9	17.4	11.3	8.9	5.5	6.6	5.3	4.7	3.9	0.03	4.3	1.03	1.8
	8-10	-	16.3	0.85	19.2	8.9	7.0	3.9	6.4	17.6	11.0	8.3	5.2	6.2	4.9	4.5	3.7	0.07	4.6	0.86	1.9
	10-15	-	14.6	0.84	17.4	7.1	5.8	5.1	7.3	17.8	11.4	8.3	5.0	6.2	5.1	4.7	3.8	0.18	4.3	0.81	1.7
	15-20	-	17.8	0.93	19.1	7.3	6.4	3.9	6.3	17.8	11.3	8.6	5.5	6.6	5.2	4.5	3.9	0.15	4.6	0.88	1.6
	20-25	-	16.0	0.89	18.0	9.3	7.0	3.4	6.5	17.1	10.6	8.3	5.1	6.1	5.2	4.9	3.6	0.32	4.6	0.87	2.0

Figure 5B.3. Variation of L-amino acid concentration (a) and yields (b and c) in the surface sediments (0-2 cm) as a function of water column depth in the Bay of Bengal.

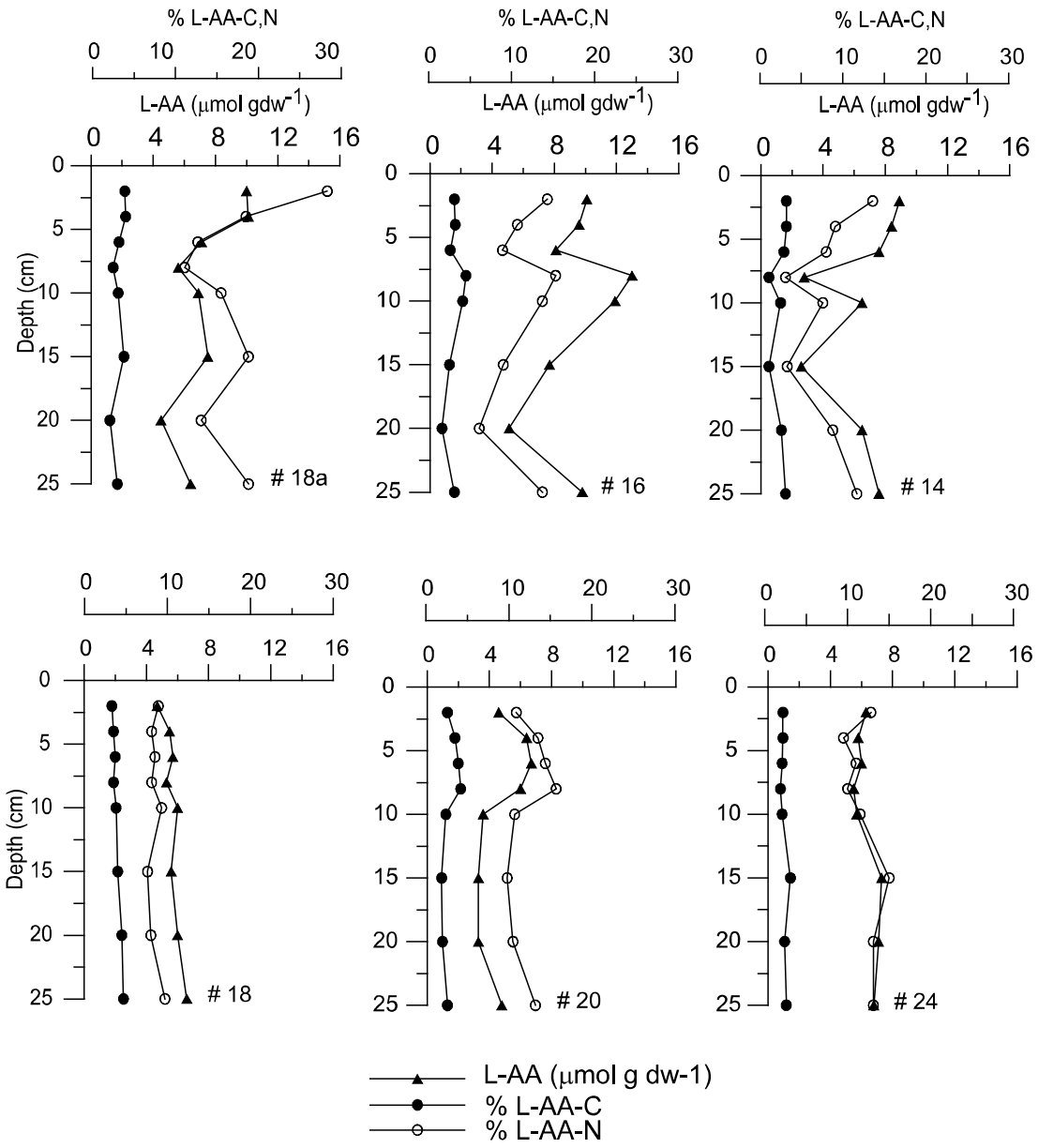

Figure 5B.4. Variation of L-amino acid concentration and yields in the sediment core depths of the Bay of Bengal.

However, the L-amino acid values in the present study were higher than those reported previously for the surface sediments of BOB (Bhosle et al., 1988). Unger et al. (2005) observed slightly higher L-amino acid concentration (1.57 and 3.37 mg g^{-1}) for the surface sediments of BOB. L-amino acid concentrations in marine sediments were depleted by a factor of 2 or more than those observed in either plankton and sinking particles, likewise higher L-amino acid concentrations were observed in surface sediment of the shelf (station 18a) and slope (station 16) sediments than those in deep sea sediments of stations 18, 20, and 24 (Fig. 5B.3a). Generally, the concentrations of L-amino acid decreased with both water column and sediment core depth (Fig. 5B.3a and Fig. 5B.4). These observed trends indicate that the L-amino acid concentrations were influenced by the distance off shore, water column depth, and sediment core depth. In the Bay, water column overlying the surface sediments is usually oligotrophic. However, the frequently occurring cyclonic eddies enriches the surface waters with nutrients, which in turn induce primary production (Prasanna kumar et al., 2007). The high Primary Productivity (350 mg C $m^{-2}d^{-1}$) (Gauns et al., 2005; Prasanna Kumar et al., 2007) coupled with ballasting of the lithogenic material presumably hastened the removal of the organic material from the surface water column to the bottom sediments (Jacob et al., 2008). Such a process may account for higher L-amino acid concentrations at shelf (18) and slope (14 and 16) stations. Fernandes et al. (2009) observed higher SPM load at the northern stations during the FIM, moreover a shelf to slope

transport of SPM was also recorded. However, phytoplankton biomass and surface primary production were lower at slope station 14. OM brought in by the rivers by the advection of the strong currents may also have influenced the concentration of amino acids at the station 14. Further, during FIM, the southern region recorded a 2-fold increase in primary productivity (513 mg C $m^{-2}d^{-1}$) which in turn resulted into higher POC and Chl *a* in the overlying water column. Greater surface primary production may account for the increase in L-amino acid concentration at stations 18 to 24 in the deep-water region (Prasanna Kumar et al., 2007). Prasanna Kumar et al. (2007) observed enhancement of nitrate and silicate along the western boundary during the FIM towards 20°N and 11°N, this is the region wherein, stations 16 and 24 are located. The observed higher silicates towards the north further supports the fact that river discharge to be another source for OM (Sardessai et al., 2007). Sardessai et al. (2007) also observed surface circulation southwards from the head of Bay along the western boundary than in the offshore region; hence, the observation supports the presence of higher amino acid concentration at slope station 16 than at station 14 in the offshore region. Similarly, at deep-water station 24, the runoff from the southern river Cauvery, may be associated with enhanced nutrients, Chl *a*, and amino acids. The relatively smaller decrease, observed in the L-amino acid concentration with the sediment depth at deep water stations 18 and 20 (Fig. 5B.4), indicates that the OM has undergone extensive degradation prior to its settling to the ocean floor, and as a result is less reactive. Similar trends in

the L-amino acid concentrations with the sediment depth were observed for the sediments collected from the other regions (Dauwe and Middelburg, 1998; Pantoja and Lee, 2003; Lomstein et al., 2006). The vertical decrease observed at 6 and/8 cm and also at 15 and/or 20 cm in sediment cores (Fig. 5B.4) can be attributed to the utilization of L-amino acid by the benthic macro- and microorganisms. Presence of sub-surface L-amino acid maxima, at depth 6 and 15 cm at stations 16 and 18a, respectively and at 8 cm depth at station 20 and at greater sediment core depth (25 cm) (Fig. 5B.4), may be due to a varying supply of L-amino acid through time (Henrichs et al., 1984; Haugen and Lichtentaler, 1991) or by *in-situ* L-amino acid production by the active benthic microbial community or by bacterial re-working of sediment OM.

Accumulation flux is the indicator of the organic matter quality. The flux is calculated by multiplying the amino acid concentration with sedimentation rate and density.

$$\text{Accumulation flux (mg/cm.yr}^{-1}) = AA_{conc} \times \omega \times \rho$$

Wherein, AA_{conc} = L-amino acid concentration (mg g^{-1}), ω = sedimentation rate (cm/yr) and ρ= grain density (gm/cm^2)

A significant positive relationship was observed between L-amino acid concentration ($r=0.7538$, $n=40$, $p < 0.001$), with accumulation flux. This implies better preservation of labile organic matter with greater flux (Fig. 5B.5).

5B.3.4. Amino acid yields

L-amino acids are labile as compared to bulk C and N. As a result L-AA yield

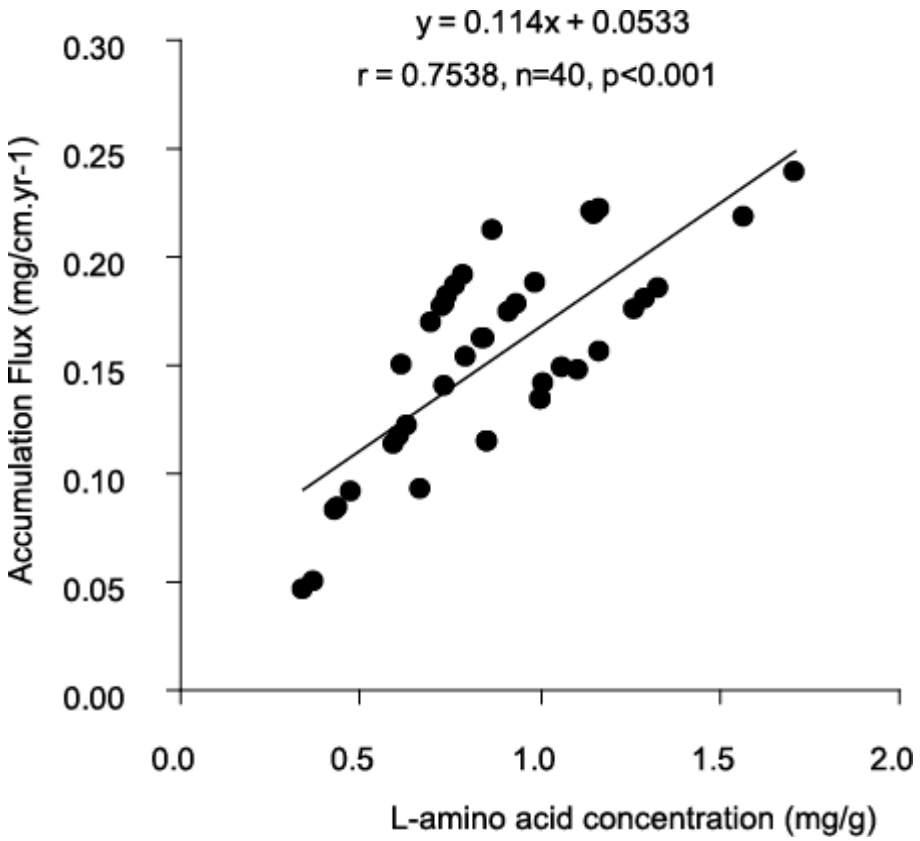

Figure 5B.5. Relationship of L-amino acid concentration with accumulation flux yields in the Bay of Bengal.

(% L-AA-C and % L-AA-N) are often used to assess the quality and degradation status of the sediment OM (Cowie and Hedges, 1992; Keil et al., 2000; Grutters et al., 2001; Lomstein et al., 2006; Vandewiele et al., 2009). L-AA-C and L-AA-N yield varied from 1.1 to 4.7 % and 3.1 to 28.3 %, respectively, in the sediment cores of the BOB. A spatial variation in the surface L-AA yields was observed (Fig. 5B.3b and c, Fig. 5B.4). L-AA-C and L-AA-N yield followed a distribution trend similar to that of L-amino acid concentrations in the BOB. L-AA yields were higher at shelf station (18a), than at the deep water station 24 (Fig. 5B.3b and c). L-amino acid concentrations and yields decreased from shelf (18a) to slope (16 and 14) and to deep water station 18. Thereafter a two-fold increase in L-amino acid concentration and L-AA-N yield was observed at deep water station 24 (Fig. 5B.3b and c, Fig. 5B. 4) indicating presence of fresh OM produced by the *in-situ* microbial organisms. The L-AA yields observed in the present study were at the lower range of values reported for sediments from other regions (Degens and Mopper, 1976; Suthof et al., 2000; Pantoja and Lee, 2003; Lomstein et al., 2006; Vandewiele et al., 2009; Bourgoin and Tremblay, 2010). Moreover, the L-AA-C (1.1 to 4.7 %) and L-AA-N (3.1 to 28.3 %) yields in sediment cores of the BOB were relatively lower than those reported for typical continental margin systems (% L-AA-C = 12 % and % L-AA-N = 30 %) (Keil et al., 2000). L-amino acid account for substantial portion (> 60 %) of particulate organic nitrogen (PON) in marine sediments (Henrichs and Farrington 1987; Cowie et al., 1992; Dauwe and Middelburg, 1998; Lomstein

et al., 1998; Pedersen et al., 2001). The low values in the BOB sediment cores (Fig. 5B.3b and c, 4) indicate that the OM has undergone substantial degradation and/or dilution by inorganic or amino acid poor soil material. This observation was supported by higher C/N ratios (7.0 to 22.0) and lower sedimentation rates of 0.2 to 0.3 cm yr^{-1} for the Bay. In contrast, presence of relatively higher L-AA yields in the surface sediments of shelf station (18a) indicates the presence of relatively fresher OM at surface sediment (Fig. 5B.3b and c, Fig. 5B.4). Lower surface L-AA yields were observed at northern slope stations 14, and 16 (Fig. 5B.3b and c, Fig. 5B.4), lower yield may be a result of dilution of sediment OM with terrigenous material brought in from the surrounding flood plains by Ganges-Brahmaputra and Godavari rivers into the BOB. The rivers flowing through the Indian sub-continents, introduce ~1387 x 10^6 tonnes/y^{-1} in the Bay (Subramanian, 1993). The terrigenous material is found to exert a strong influence on the biogeochemical and hydrological characteristics of the Bay (Jacob et al., 2008). The L-AA yields indicate no preferential degradation of L-amino acid relative to the bulk OM at station 18. Moreover, it seems that the OM was substantially degraded prior to its settlement into the sediment of BOB (Grutters et al., 2001; Lomstein et al., 2006); as supported by higher C/N ratios at the deeper sediment depths. A significant relationship was observed between decomposition/ accumulation flux and % L-AA-C yield ($r=0.7937$, $n=40$, $p< 0.001$) and % L-AA-N yield ($r=0.5533$, $n=40$, $p< 0.001$) indicating presence of fresh OM due to enhanced accumulation flux of OM (Fig. 5B.6).

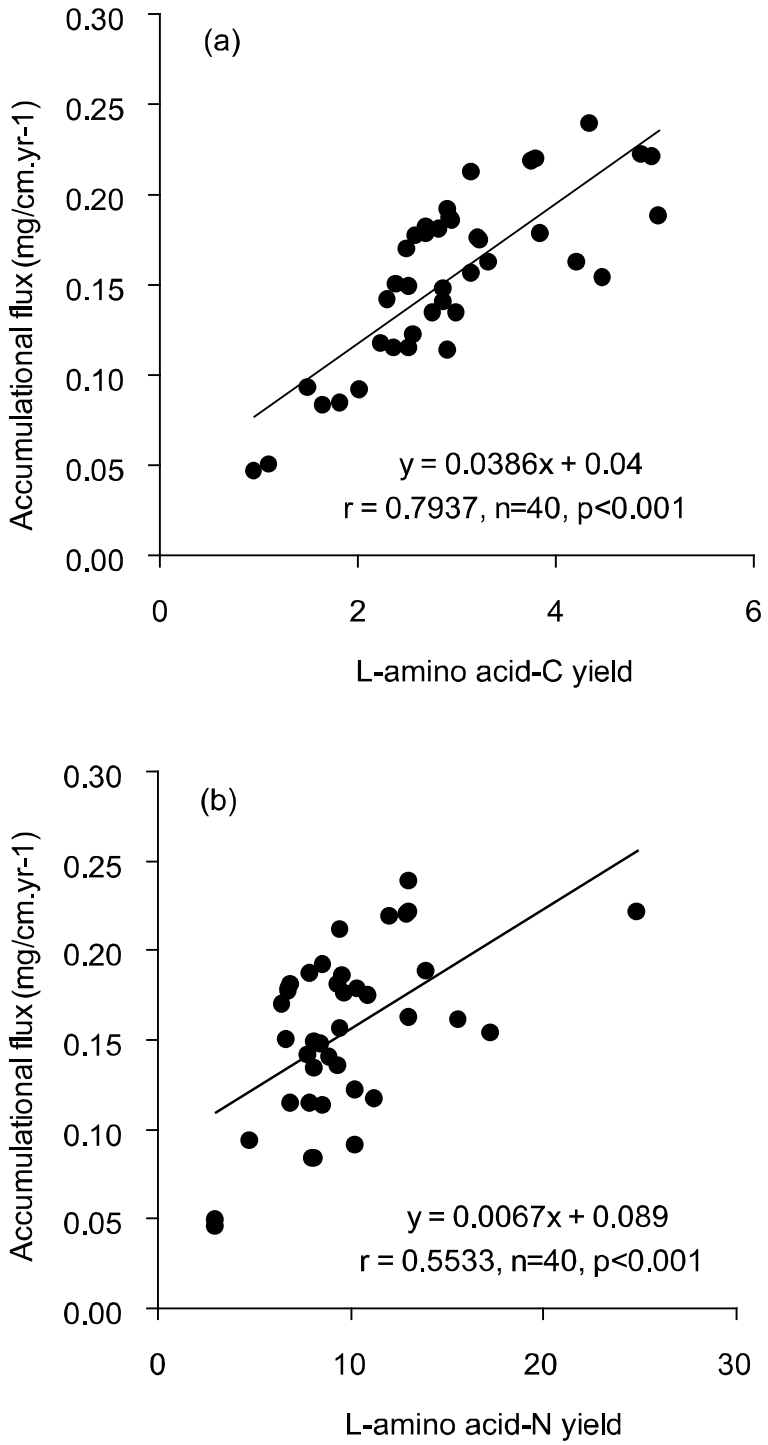

Figure 5B.6. Relationship of L-amino acid-C (a) and L-amino acid-N (b) yield with accumulation flux yields in the Bay of Bengal.

5B.3.5. L-amino acid composition

Amino acid composition in the source organisms is relatively similar. However, the biological activity and diagenetic processes are found to modify the composition of L-amino acid in the marine sediments. Thus, individual L-amino acids are useful indicators to identify the OM degradation state (Cowie and Hedges, 1992; Dauwe and Middelburg, 1998; Dauwe et al., 1999; Keil et al., 2000; Pedersen et al., 2001; Grutters et al., 2001; Vandewiele et al 2009). In the sediments of BOB, glycine, alanine, aspartic acid, glutamic acid and lysine dominated the pool of OM (Table 5B.4). Molecular composition observed in the present study area agrees well with L-amino acid composition reported for the sediments of other regions dominated with planktonic inputs (Haugen and Lichtentaler, 1991; Cowie et al., 1992; Cowie and Hedges, 1992; Dauwe and Middelburg, 1998; Lomstein et al., 1998, 2006; Pedersen et al 2001; Vandewiele et al., 2009). Aspartic acid, glutamic acid, glycine, alanine and lysine are abundant in pure cultures of marine phytoplankton, zooplankton and bacteria (Cowie and Hedges 1992, Horsfall and Wolff, 1997). Moreover, the accumulation of certain amino acids predominant over the other amino acids in the degraded samples depends primarily on the association of the amino acids with structural components of the source organisms (Cowie and Hedges, 1992; Keil et al., 2000; Vandewiele et al 2009) and / or sorption to mineral surfaces (Henrichs and Sugai, 1993). In the BOB sediment samples, the Mole % glycine increased with increasing sediment core depth at slope stations (14 and 16) and deep

water stations (18 and 20) (Table 5.B4). The increase in the Mole % glycine, and serine, was associated with decrease in Mole % valine, isoleucine, leucine, aspartic acid and glutamic acid at stations 14, 18 and 20 (Table 5B.4). The abundance of glycine, and serine, their enrichment with sediment core depth accompanied by the concurrent decrease in cell plasma amino acids such as valine, isoleucine, leucine aspartic acid, and glutamic acid has been observed for other environments (Hecky et al 1973). Glycine has been often found to increase with sediment depth (Haugen and Lichtentaler, 1991; Cowie and Hedges, 1992). Glycine, serine, and threonine accumulate during diagenesis because they are protected from degradation due to their close association with the biogenic silica in the diatom cell wall and frustules (Heckly et al., 1973; Cowie and Hedges, 1992; Dauwe and Middelburg, 1998; Keil et al., 2000). Another reason for increase in glycine in the sediment core samples could be its low nutritional value and/or its synthesis by the microorganisms. Amino acids such as valine, isoleucine, leucine, aspartic acid, and glutamic acid present in diatom cell plasma are relatively labile. These amino acids are utilized by the *in-situ* organisms thereby reducing their abundance during OM degradation. Dauwe et al. (1999) observed higher proportion of glycine and alanine in the highly degraded OM. These amino acids are also found in bacterial cell wall. The observed trend of increasing Mole % of glycine and serine as well as alanine accompanied by decreasing cell plasma amino acids with the sediment depth (Table 5B.4) indicates the

presence of highly degraded sedimentary OM at the greater depths of the sediment cores of BOB (Cowie et al., 1992).

Conversely, at station 24, Mole % glycine decreased with increasing sediment core depth (Table 5B.4). Amino acids composition of the deep sea sediment core (Station 24) suggests the presence of relatively fresher OM at greater depths than at the surface of core. Lomstein et al. (2006) had a similar observation, wherein the Mole % glycine increased with the increasing depth for a sediment core collected off Antofagasta in the Chilean sediments. The non-protein AAs, β-alanine (β-Ala) and γ–amino butyric acid (γ-ABA) are the bacterial degradation byproduct of protein-AA aspartic acid and glutamic acid, respectively (Cowie and Hedges, 1992; Suthhof et al., 2000). These non-protein AAs are generally absent or present at trace level in living organisms and tend to accumulate in older sediments. Cowie and Hedges (1992) attributed the abundance of these non-protein AAs to diagenetic origin, resulting into their enrichment in degraded sedimentary OM. The non-protein amino acid, β-Ala ranged from 1.0 to 5.0 Mole % (Table 5.B.4). The higher Mole % β-Ala and lower Asp/β-Ala ratio in BOB at deeper water column stations (18, 20 and 24) and with increasing sediment core depth indicates a progressive diagenesis of OM at the sampled stations in the BOB (Table 5B.4). Since during degradation, in sediment OM, amino acid composition is subjected to changes. The amino acid based degradation index (DI) by Dauwe et al. (1999) was used to assess the degradation state of the OM. PCA was applied to amino acid, data from the BOB (Fig. 5B.7). It

has been observed that DI covers a wide range of degradation states and it is supposed to be uncompromised by source variations. The DI in the OM varies from ~ +1 for the newly produced algal material to -2 for extensively degraded deep-sea sediment material (Dauwe and Middelburg, 1998; Dauwe et al., 1999). The DI observed in the BOB sediments varied from -2.6 to 1.7. DI indicated presence of relatively fresher OM in the surface sediments of stations of shelf (18a) and slope (14 and 16), compared to deep-water stations 20 and 24. This observation was supported by higher L-AA yields, high Asp/β-Ala and low C/N ratios at station 18a, 14 and 16. Pantoja and Lee (2003) observed a less degraded OM in the surface sediments of the near shore stations, while a degraded OM at deep-water stations. At greater sediment core depths the observed negative DI values, with corresponding lower Asp/β-Ala and higher C/N ratios indicated presence of extensively degraded OM. Degraded OM was observed for deep-water stations (20 and 24), wherein the surface sediment DIs were negative. In contrast, positive DI values were observed at greater core depths (6 to 25 cm) of station 24, thereby indicating the presence of freshly derived OM. This conclusion was also supported by high L-AA yield, and the ratios of low C/N at deeper depth of station 24. Lomstein et al. (2006) observed positive DI values, deeper in the sediment cores for Off Antofagasta region in Chilean sediments. The negative DI values at surface than at greater core depths were due to the extremely higher Mole % glycine (~ 21 Mole %) in the surface as compared to greater core depths (17 – 19 Mole %, Lomstein et al., 2006). Lomstein et al.

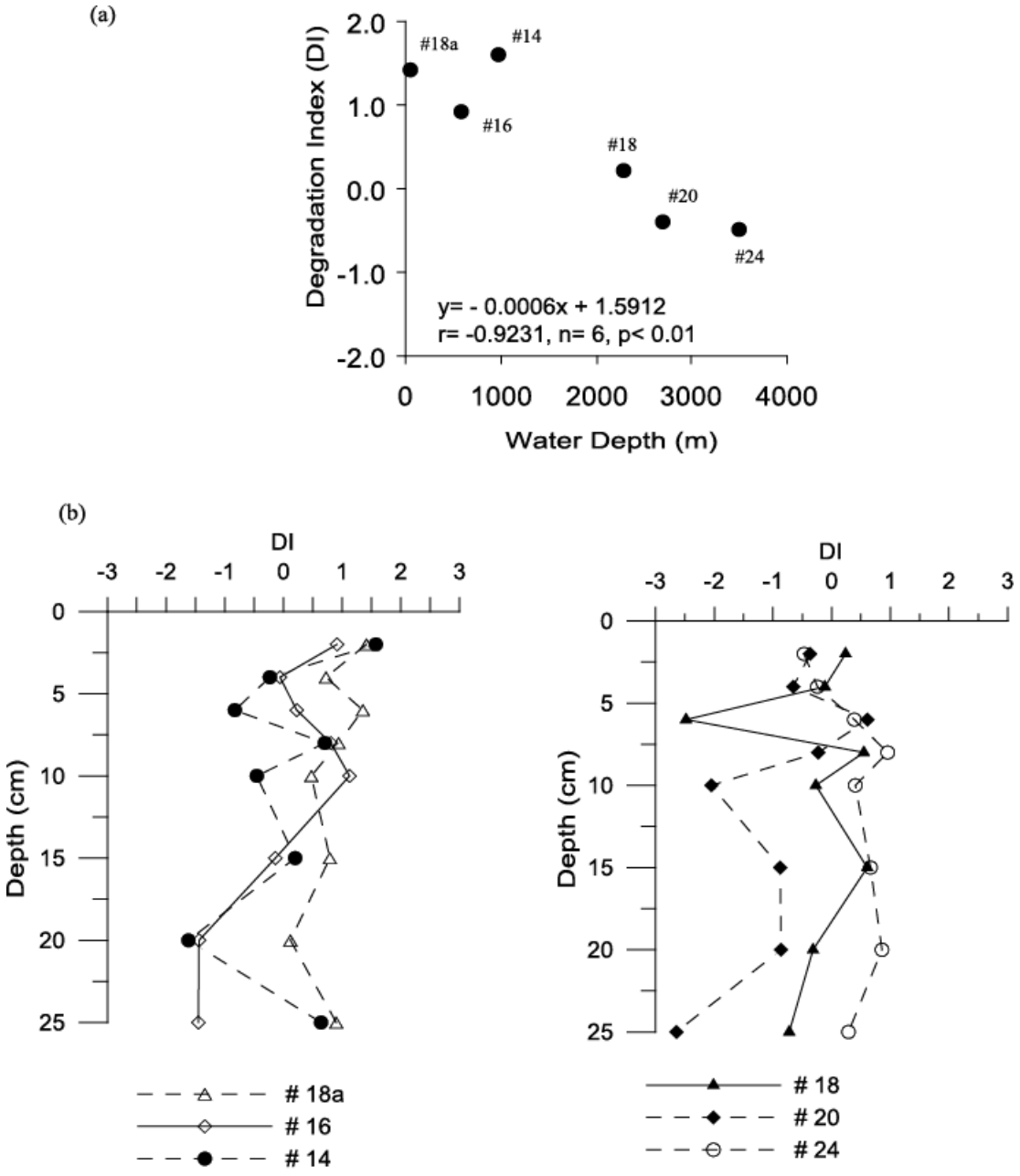

Figure 5B.7. Variation of the degradation index (DI) in the surface sediment as a function of water column depth (a) and sediment core depth (b) in the Bay of Bengal.

(2006) observed high abundance of glycine-rich empty diatom cells which produced glycine rich organic matter in surface sediments of a station. High proportion of glycine due to glycine-rich plankton was also associated with low DI values in the sediments of Southern Ocean (Ingalls et al., 2003).

5B.3.6. D-amino acid concentration and composition

D-amino acids are the major components of bacterial cell wall and are not produced by phytoplankton or vascular plants. Bacteria are the primary agent of decomposition of OM in aquatic environment. Bacterial cell walls of living and/or dead bacteria and the other bacterial macromolecules are the sources of the D-amino acids (Kaiser and Benner, 2008). Bottrell et al. (2000) observed active bacteria occurring up to 250 m sediment depths. In the BOB sediments, D-amino acids such as, D-aspartic acid, D-glutamic acid, D-alanine and D-serine were abundant and detected at all the sampled stations and sediment core depths. The concentration of D-amino acids, in the sediments core of BOB varied from 0.04 to 0.76 μmol gdw^{-1} (Fig. 5B.8a and b). The total D-amino acids concentration observed for the BOB sediments were at lower end when compared to those reported for the sediments of other regions (Pedersen et al., 2001; Lomstein et al., 2006, 2009; Vandewiele et al., 2009). Surface D-amino acid concentrations were relatively higher at slope stations (14, and 16) compared to shelf (18a) and deep-water stations (18, 20, and 24) (Fig. 5B.8a and b). Further, at the slope stations (14, and 16), decrease in D-amino acid concentration was observed with the increasing sediment core depths. In contrast, higher D-amino acid concentr-

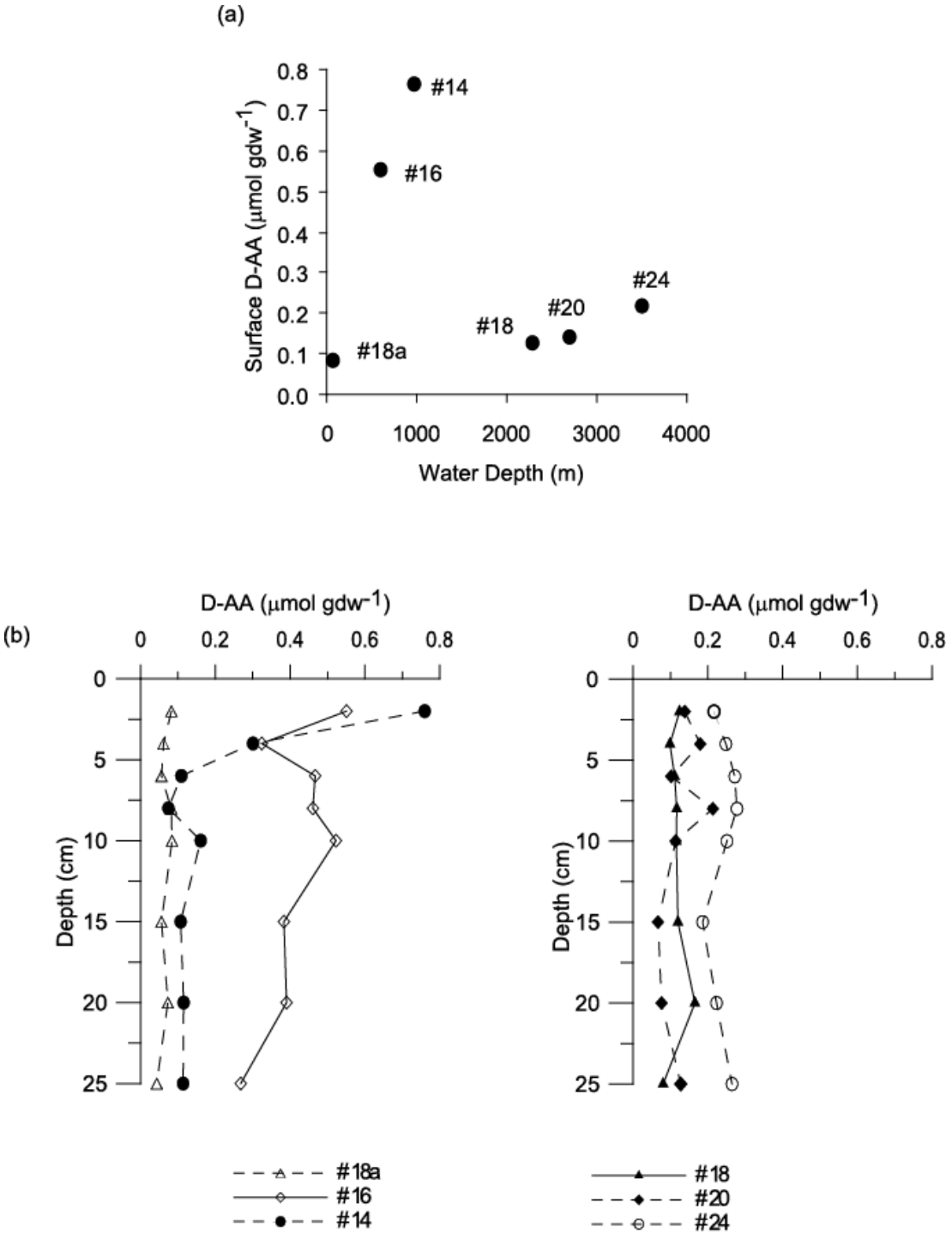

Figure 5B.8. Variation of total D-amino acids in the surface sediment as function of water column depth (a) and sediment core depth (b) in the Bay of Bengal.

ations were observed at greater core depths of station 24 (Fig. 5B.8a, and b). The presence of D- amino acids in the surface and as well as at deeper sections of sediment cores of BOB indicates presence of microbial biomass and fast turnover of phytoplankton derived OM (Dittmar et al., 2001).

The D-amino acids (Mol %) has been used to assess the degradation state of OM (Tremblay and Benner, 2009; Bourgoin and Tremblay, 2010). The Mol % D-amino acid in the sediments core of BOB varied from 0.3 to 8.5 Mol % (Table 5B.5). In the surface sediment, Mol % D-amino acid followed a trend similar to D-amino acid concentration, wherein higher values were observed for slope stations (14, and 16) as compared to shelf (18a), and deep water stations (18, 20 and 24) (Fig.5B. 4 and Table 5B.3). Lower Mol % D-amino acid at shelf station 18a, indicates the presence of relatively fresher OM at this station (Table 5B.3). The relatively higher surface Mol % D-amino acid observed at the slope stations (14, and 16) indicates presence of highly degraded OM. However, the biological indicators such as % L-AA yields, DI and Asp/β-Ala ratios points towards fresher OM at these stations. The enrichment of bacterial biomarker, Mol % D-amino acids along with higher L-AA yield in the surface sediments of slope stations (14, and 16), indicate increase in *in-situ* benthic bacterial biomass rather than the bacterial degradation (Bourgoin and Tremblay, 2010). Jorgensen et al. (1990) reported higher abundance of D-amino acids at a region that had high bacterial density. Increase in the D-amino acids, due to increase in bacterial biomass

also was observed in the suspended matter of the Mandovi estuary (Fernandes, 2011).

5B.3.7. Contribution of peptidoglycan D-amino acid to total L-amino acids

The relative contribution of peptidoglycan amino acids to L-amino acid (% D-AApep/ L-AA) was calculated from the measured D-amino acids and the average L/D ratio of peptidoglycan from the Gram-positive *Staphylococcus aureus* given in Pedersen et al. (2001). The D-AApep/ L-AA values (5.3 to 55.0 %) in the surface sediments of BOB were in the range of values obtained for other areas. Lomstein et al. (2006) estimated 18- 5 % of D-AApep/L-AA in the coastal Chilean sediments. At ~ 40 %, contribution of D-AApep/L-AA was observed for coastal surface sediments of Aarhus Bay, Denmark (Pedersen et al., 2001), and 46 % in the sediments from Atlantic Ocean (Grutters et al., 2002). The surface D-AApep/L-AA was lower at shelf station (18a) and higher at slope stations (14, and 16) (Fig. 5B.9a and b), indicates that bacteria and cell wall remains in source material were degraded at slower rate than other proteinaceous material during its descend through water column. However, this was not supported by the L-AA yields, and DI, indicating that the observed higher D-amino acids concentration, Mol % D-amino acids, and D-AApep/L-AA values were a result of increased living bacterial biomass and not due to degradation of OM at slope stations 14 and 16. Contribution of D-AApep/L-AA increased gradually from shelf (18a), to deep-water stations (18, 20 and 24). Moreover, the D-AApep/L-AA decreased

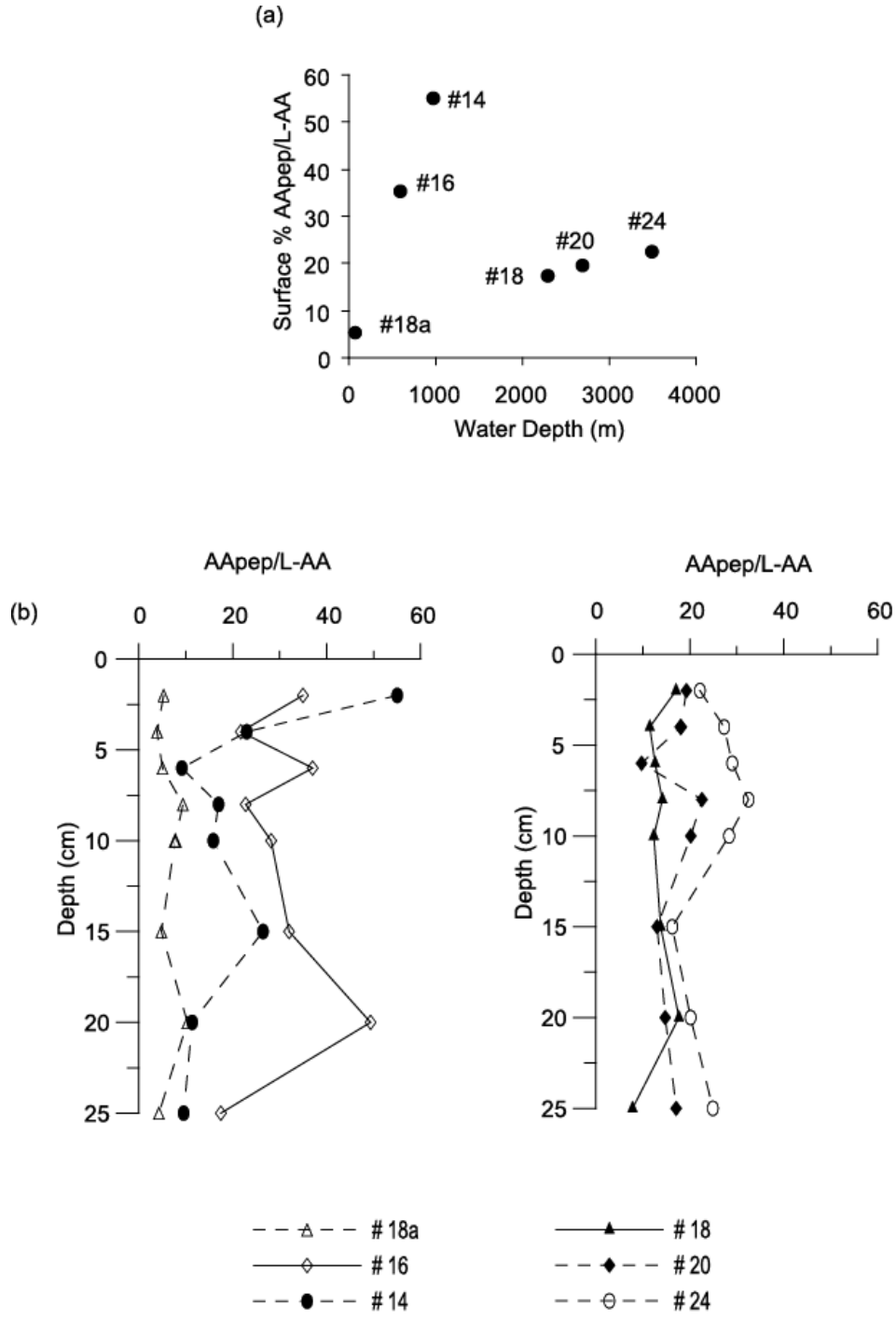

Figure 5B.9. Contribution of D-amino acid peptidoglycan to amino acidsin the surface sediment as a function of water column depth (a) and sediment core depth (b) of the Bay of Bengal

below surface and thereafter did not change drastically with the increasing sediment core depth (Fig. 5B. 9a and b). The occurrence of peptidoglycan derived D- amino acids indicates that bacterial cell wall components become increasingly important in the degraded OM.

5B.3.8. Bacterial contributions to OM

In marine sediments, the quality and quantity of OM is mainly determined by the activities of the *in-situ* heterotrophic bacteria. Bacteria are the source for the D-amino acids. It is assumed that D-amino acid yields in cultured bacteria are representative of natural assemblages, and their reactivities are representative of bulk carbon and nitrogen reactivites (Kaiser and Benner, 2008; Tremblay and Benner, 2009; Bourgoin and Tremblay, 2010). In this study, we used D-amino acids yields to calculate bacterial contribution to OM using average bacterial yields taken from published literature on bacterial assemblages typically found in seawater (Bourgoin and Tremblay, 2010). We used yields of D-alanine and D-glutamic acid to calculate the bacterial contribution to bulk OM as D-aspartic acid and D-serine yields were highly variable than those recorded for bacteria (Kaiser and Benner, 2008). Based on the D-alanine and D-glutamic acid yields, bacteria contribute 1.1 to 14.5 % of OC and 3.8 to 45.4 % of ON to the sediments of BOB (Table 5B.5). Bourgoin and Tremblay. (2010) observed between 12 and 38 % of OC and 40 % and 60 % for ON for the sediments collected from St. Lawrence Estuary and Saguenay Fjord. This approach indicates that bacteria contributed to the bulk OM of BOB. The terrestrial OM brought in by

Table 5B.5. Distribution of Mol % D-amino acids, carbon and nitrogen normalized yields of individual D-amino acids and contribution of Bacterial-C and -N in the sediments of the Bay of Bengal

Core	Depth (cm)	Mol %D	nmol mgC				nmol mgN				Bacterial	
			D- Asp	D- Glu	D-Ala	D-Ser	D- Asp	D- Glu	D-Ala	D-Ser	% C	% N
18a	0-2	0.7	3.1	1.2	1.1	0.6	80.0	31.3	27.7	16.3	2.3	14.0
	2-4	0.6	2.2	0.8	1.1	0.4	36.8	13.5	17.3	7.1	1.9	7.3
	4-6	0.8	2.0	0.7	0.9	0.5	28.5	9.9	12.5	7.1	1.6	5.3
	6-8	2.3	1.9	1.0	2.9	0.4	29.1	14.8	44.9	6.0	3.9	14.1
	8-10	1.6	2.4	1.2	2.3	0.3	41.8	21.7	41.0	4.4	3.6	14.9
	10-15	0.3	0.7	2.8	0.8	0.5	11.5	48.5	14.0	8.1	3.6	14.8
	15-20	1.4	0.7	2.6	1.9	0.5	9.4	37.6	28.0	6.9	4.6	15.5
	20-25	0.7	1.9	0.6	0.4	0.3	28.4	9.3	6.6	4.2	1.1	3.8
16	0-2	5.5	16.2	6.6	4.1	1.1	263.2	107.0	66.5	18.4	10.9	41.1
	2-4	3.4	7.5	4.1	4.7	1.7	87.8	48.4	54.7	19.5	8.9	24.4
	4-6	5.8	13.8	5.9	3.7	1.8	164.1	69.8	43.7	21.7	9.7	26.9
	6-8	3.6	13.3	7.9	3.7	1.4	159.3	94.4	44.6	17.3	11.8	32.9
	8-10	4.4	16.8	7.8	3.6	1.6	197.3	91.8	42.5	19.1	11.6	31.8
	10-15	5.0	9.3	7.0	3.2	1.3	117.0	87.9	39.7	16.2	10.3	30.2
	15-20	3.6	8.4	4.4	4.5	2.9	114.5	60.0	60.9	39.6	9.0	28.6
	20-25	2.7	6.6	2.8	2.9	1.6	103.4	44.6	44.8	25.0	5.8	21.2
14	0-2	8.5	17.7	8.6	3.0	1.5	242.6	118.0	40.7	21.0	11.8	37.6
	2-4	3.6	9.8	5.4	2.6	1.0	99.6	54.2	26.7	9.7	8.1	19.2
	4-6	1.2	1.5	1.8	2.4	1.1	14.8	17.4	24.0	10.6	4.2	9.8
	6-8	1.5	0.9	0.9	2.4	0.4	9.3	9.5	24.3	4.5	3.4	8.0
	8-10	2.5	4.6	2.4	2.6	0.7	49.1	25.8	27.2	7.2	5.1	12.6
	10-15	1.5	0.8	3.9	2.1	0.4	8.7	42.9	23.5	4.7	6.1	15.7
	15-20	0.9	1.0	4.3	1.6	0.7	12.1	51.7	19.1	8.5	5.9	16.8
	20-25	0.9	0.9	3.5	2.2	0.9	12.0	46.5	30.2	12.7	5.8	18.2
18	0-2	2.7	4.9	3.5	3.4	1.3	52.0	37.0	35.9	13.3	7.0	17.3
	2-4	1.8	2.2	0.8	5.8	1.6	17.8	6.6	46.7	13.3	6.7	12.6
	4-6	1.1	3.3	1.3	5.6	1.7	26.6	11.1	46.2	13.6	7.1	13.6
	6-8	2.2	4.2	2.1	4.8	1.5	35.1	17.0	39.6	12.7	6.9	13.4
	8-10	1.9	4.5	3.4	3.0	1.3	38.3	29.6	26.0	11.1	6.6	13.2
	10-15	2.2	6.1	2.7	4.0	1.3	41.5	18.4	27.4	8.5	6.9	10.9
	15-20	2.8	9.9	5.2	4.0	1.2	62.2	32.9	25.4	7.6	9.4	13.8
	20-25	1.1	2.5	2.0	3.1	1.7	18.3	15.0	23.0	12.3	5.2	9.0
20	0-2	3.0	6.3	3.2	2.6	0.8	74.2	38.4	30.9	10.0	5.9	16.4
	2-4	0.9	7.5	3.3	4.2	1.5	100.8	44.2	57.0	19.6	7.6	24.0
	4-6	0.5	3.9	2.4	3.5	1.1	50.5	31.3	45.6	13.7	6.0	18.2
	6-8	5.0	8.7	4.6	9.8	1.1	116.8	61.0	130.7	14.8	14.5	45.4
	8-10	1.5	1.8	7.2	2.0	1.4	28.3	114.1	31.1	22.6	9.4	34.4
	10-15	0.9	1.4	3.1	1.1	0.9	25.3	56.3	19.7	15.5	4.3	18.0
	15-20	1.3	1.2	2.6	1.2	2.4	23.8	48.6	23.4	45.5	3.8	17.1
	20-25	2.4	1.8	1.4	7.5	0.8	31.6	25.9	134.6	13.5	9.0	38.0
24	0-2	3.5	6.2	3.2	2.0	1.2	96.2	50.4	30.7	19.5	5.3	19.2
	2-4	4.3	9.0	3.6	2.0	0.8	137.9	55.2	30.4	12.9	5.6	20.3
	4-6	4.5	10.0	3.4	1.9	0.6	181.6	61.3	35.2	11.3	5.4	22.9
	6-8	5.1	9.9	3.5	1.9	0.7	182.5	65.2	34.9	13.1	5.5	23.7
	8-10	4.4	9.0	3.8	1.8	0.7	172.8	73.7	35.1	13.7	5.8	25.8
	10-15	2.5	6.5	3.2	2.1	1.0	112.1	55.5	37.0	16.9	5.4	21.9
	15-20	3.1	6.5	3.5	1.7	0.8	125.0	67.5	32.2	15.3	5.3	23.6
	20-25	3.9	9.6	4.3	1.8	0.8	172.9	78.1	31.9	14.5	6.2	26.1

the rivers mostly influences the northern slope stations 14, and 16. The presence of higher bacterial-N indicates accumulation of exogenous nitrogen over the nitrogen deficit terrestrial OM. Since terrestrial OM is poor in nitrogen, the C/N ratio of terrestrial organic matter is usually higher than 20 (Hedges et al 1986), however it was observed the C/N ratio at these stations was ~ 14 to 15, thereby further supporting the observed enrichment of bacterial-N in the OM.

Chapter 6

Summary

❖In marine environments, organic matter is derived from several sources including biogenic and terrestrial. Phytoplankton and bacteria are generally the major sources of organic matter in marine environment. Amino acids are abundant and have been observed in the suspended particulate matter, dissolved organic matter, and sediments of the estuarine/marine environments. Phytoplankton has been recognized as the main source for the L-amino acids in the organic matter. Microorganisms present in the water column and sediment utilize the amino acids as they are relatively bio-available. Bacteria play an important role in the degradation and transformation of organic matter. However; it is observed that during the degradation some of the components of bacterial origin are introduced into the pool of organic matter. D-amino acids unique to bacteria and present in peptide linkage of peptidoglycan, a component of bacterial cell wall are frequently observed in marine organic matter. These bacterial D-amino acids are found to contribute substantially to the organic nitrogen pool in marine waters. Since nitrogen is the limiting nutrient in the marine environment, bacterial D-amino acids form an extra source of nitrogen to the total organic nitrogen pool. Moreover, the bacterial contribution to the organic matter can be observed in the nitrogen poor organic matter such as mangrove detritus. L-amino acids can be used as the proxies of organic matter quality and degradation state. Besides D-amino acids can also be used to trace source as well as degradation state of organic matter. Most of the studies on the amino acid abundance, distribution and cycling have been carried out in

temperate environments. However, little is known about the amino acid cycling and bacterial contribution to the organic matter from the tropical waters. Therefore, to understand distribution, cycling and degradation state of organic matter and also to investigate bacterial contribution to organic matter in tropical environments some aspects of L- and D-amino acids were addressed in this thesis.

❖Chapter 2 deals with the effect of some environmental factors on the L- and D-amino acids concentration and composition in marine bacteria. For this study, three bacterial cultures (BAC-1, BAC-2, and BAC-3) were used. These bacterial cultures were identified using physiological and morphological, biochemical characteristics and by 16-rDNA sequencing as *Bacillus subtilis* (BAC-1), *Bacillus lichineformis* (BAC-2), and *Pseudomonas psychrotolerans* (BAC-3). The effect of growth, phosphate, nitrate, and starvation on L-and D-amino acid concentration and composition in these cultures was investigated. These studies suggest that L- and D-amino acid concentration and composition were influenced by these factors in all the cultures. It was observed that both the L- and D-amino acid concentrations in *Bacillus subtilis*, *Bacillus lichineformis*, and *Pseudomonas psychrotolerans* increased over the period incubation. The concentration of D-amino acids was higher in *Bacillus subtilis*, and *Bacillus lichineformis* as compared to *Pseudomonas psychrotolerans*. The observed differences were presumably due to the difference in their cell wall components. Gram-positive bacteria contain a relatively thicker peptidoglycan layer compared to much thinner

peptidoglycan layer in gram-negative bacteria. Therefore, higher D-amino acids were mainly attributed to the higher peptidoglycan content in the *Bacillus subtilis*, and *Bacillus lichineformis*.

As the concentrations of phosphate and nitrate increased, L- and D-amino acid concentration increased in all three cultures. In the marine environment, concentrations of these nutrients vary seasonally. That means L- and D-amino acid concentration and composition will be strongly influenced by changes in phosphate and nitrate concentration.

In order to study the effect of starvation on L-and D-amino acids in the bacterial cells and cell free culture broth, cultures of *Bacillus subtilis*, and *Bacillus lichineformis,* were incubated in a sterile aged seawater for a period of 28 days. The cells and cell free culture broth (0.2 μm filtered) was sampled at 0, 7, 14, 21, and at 28 day of incubation period. It was observed that, the cells of *Bacillus subtilis*, and *Bacillus lichineformis* had higher concentrations of L- and D-amino acids at day 0, which decreased with increase in the incubation period, thereby indicating the liable nature of both L- and D-amino acids. Subsequently, concentrations of L- and D-amino acids increased in the seawater incubation medium. The decrease in L- and D-amino acids in bacterial cell wall and its presence in the incubation medium indicate that bacteria released L- and D-amino acids from the cell wall into the culture medium. Thus release of L- and D- amino acids by bacteria may influence the composition of dissolved organic matter, especially dissolved organic N in the marine environments.

❖In chapter 3, microbial degradation of whole cell wall and purified peptidoglycan of bacteria was studied. Bacteria play a central role in various ecological and biogeochemical processes in marine environment. Bacteria degrade and utilize the organic matter in the marine environment. The bacterial biomass thus developed forms an important source for the active pool of organic matter. For this purpose whole cell wall and purified peptidoglycan isolated from *Bacillus subtilis, Bacillus licheniformis* and *Pseudomonas psychrotolerans* were incubated in natural seawater containing the natural bacterial community and were sampled at fixed time periods. Peptidoglycan–derived D-amino acids are released in the growth medium i.e. in natural seawater. This implies that D-amino acids are important components of dissolved organic matter of natural seawater. Decrease in the D-amino acids in the incubation medium, indicates that large fractions of the D-amino acids from the peptidoglycan is semi-labile and are assimilated and/or degraded by the natural bacterial population. The D-amino acids from the whole cell walls and partially purified peptidoglycan *of Bacillus subtilis* and *Bacillus licheniformis* were relatively more degraded than *Pseudomonas psychrotolerans*. This presumably suggests that cell wall components of Gram-positive bacteria were relatively less resistant to degradation than Gram-negative bacteria. At the end of the incubation period, substantial amounts of D-amino acids were present in the growth medium indicating that some components of peptidoglycan are degraded while others are partially degraded.

❖Decomposition of leaves of mangrove *Rhizophora mucronata* and bacterial contribution to the degraded organic matter of the mangrove leaves were evaluated and the data are presented in chapter 4. Mangrove forests are the most dominant and a productive inter-tidal ecosystem. Mangrove leaves contribute considerable amount of organic matter, primarily to adjacent waters and sediments, thereby providing a large available source of carbon and nitrogen to indigenous microbial populations. Leaves of *Rhizophora mucronata* collected from the banks of the Mandovi estuary were submerged in natural seawater and incubated in dark over the 168 day period of incubation. During the incubation period, bacterial abundance, TOC, TN increased, while the C/N ratio and AFDW decreased during the 168 days of incubation. Similarly, the L-amino acids and D-amino acids concentration, and Mol % of D-amino acids also increased over period of incubation. Significant correlations of total bacterial counts with L-and D-amino acids indicate that bacteria were the source for these amino acids. This observation was also supported by the low C/N ratio, high D/L-amino acid ratios, and % PG-AA-N to total amino acids-N, indicating bacteria, and their cellular components contributed significantly to mangrove leaf detrital organic matter. The presence of bacterial organic matter in the mangrove detritus was confirmed by the presence of D-amino acids in degraded leaf material. During these studies, bacteria accounted for 20 to 86 % of TN and 2 to 11 % of TOC of the degraded mangrove leaves. This strongly implicates the importance of bacteria as nitrogen source for mangrove leaves utilizing organisms.

The above experiments carried out in the laboratory provided useful information on the importance of bacteria as a nitrogen source in degraded terrestrial material such as mangrove leaves. However, it is not known if similar process takes place in the natural environment. Therefore, this experimental information about the cycling of L- and D-amino acids and bacterial contribution to organic matter was further extended to field studies in estuarine and deep sea environments of Mandovi estuary and the sediment cores of the Bay of Bengal, respectively. These results are presented in chapters 5A and 5B.

❖In chapter 5A distribution, cycling, nature, and sources of organic matter were assessed using L- and D-amino acids in suspended particles of the Mandovi estuary. An effort was made to identify bacterial contribution to the particulate carbon and nitrogen. During these studies, a combined tracer approach consisting of stable carbon isotope and amino acid enantiomers was used. For this purpose, eight stations in monsoon season and nine stations in the pre-monsoon season were sampled in the Mandovi estuary. At these stations, suspended particulate matter was collected and analyzed for POC, TPN, C/N ratio, $\delta^{13}C_{POC}$ and L-and D-amino acids. C/N and $\delta^{13}C_{POC}$ ratios indicated that particulate organic matter was mostly derived from terrestrial sources during the monsoon season and from phytoplankton during the pre-monsoon season in the Mandovi estuary. Biological processes to a greater extent governed the distribution of the amino acids in the Mandovi estuary. Bacteria played an important role in deciding the quality and quantity

of organic matter in the estuary. Amino acid composition and organic matter degradation indicators including C/N ratio, amino acid yields, Mol % D-amino acids, and degradation index suggest that the organic matter was substantially degraded during the monsoon season, and was relatively fresher during the pre-monsoon season. Amino acids such as alanine, aspartic acid, leucine, serine, arginine, and threonine in monsoon and glutamic acid, glycine, valine, lysine, and isoleucine in pre-monsoon were relatively abundant. Substantial concentrations of bacterial biomarkers, D-amino acids in the SPM of the Mandovi estuary during monsoon and pre-monsoon seasons signify important contribution of bacteria to the estuarine detrital organic nitrogen pool. Relatively higher concentrations of D-amino acids during the pre-monsoon season indicate higher bacterial biomass and not the degraded organic matter. This conclusion was also supported by higher L-amino acids, low C/N and POC/Chl *a* ratios of the SPM. Further, the bacteria accounted for 16 to 34 % of POC and 29 to 75 % of TPN during monsoon, and 30 to 78 % of POC and 34 to 79 % of TPN during the pre-monsoon season. These results showed the importance of bacteria in cycling of carbon and nitrogen in tropical estuary.

❖In chapter 5B, the fate of the organic matter and the bacterial contribution to the sediment of Bay of Bengal was assessed using the L-and D-amino acids. For this purpose six sediment cores from various water depths and locations in the Bay of Bengal during fall inter-monsoon season were analyzed for TOC, TN, L- and D-amino acid concentration and composition.

The quality and quantity of organic matter from the Bay of Bengal sediment was influenced by the physical, chemical and biological factors such river discharge, nutrients, eddies and the primary production occurring in the overlying water column and the bacterial community existing in the sediment core. The degree of organic matter decomposition in Bay of Bengal sediment was identified from the various biogeochemical indicators such as C/N ratios, amino acid yields, amino acids composition, degradation index (DI), and D-amino acids. The C/N ratio, amino acid composition and yields, DI, and D-amino acids indicate that shelf station (18a) contained relatively fresher surface organic matter as compared to deep-water stations (20 and 24). Moreover, these biological indicators suggest that the organic matter was relatively degraded at greater sediment core depths. The deviation observed for degradation index values for station 24 was due to higher Mole % glycine in the surface compared to greater sediment depths. The positive DI values, and higher D-amino acids concentration and contribution at slope stations 14 and 16 was a result of higher benthic bacterial biomass and not due to degradation processes. The observed D-amino acid, Mol % D-amino acids and % AApep/L-AA indicate that bacterial cell wall components contributed substantially at greater depths to the pool of OM. This was supported by the observed bacterial–C of 1.1 to 14.5 % of OC and 3.8 to 45.4 % of ON contribution to the sediment core.

Chapter7

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List of Published Papers

List of Publications

1) **Loreta Fernandes***. Origin and biochemical cycling of particulate nitrogen in the Mandovi estuary. **Estuarine, Coastal and Shelf Science, 94 (2011)**, 291-298, **IF: 2.247**.

2) **Loreta Fernandes***, Narayan B. Bhosle, S.G. Prabhu Matondkar, Ravi Bhushan. Seasonal and spatial distribution of particulate organic matter in the Bay of Bengal. **Journal of Marine Systems**, 77 (2009), 137 – 147, **IF: 2.126**.

3) Vishwas B. Khodse, **Loreta Fernandes***, Narayan B. Bhosle and Sugandha Sardessai. Carbohydrates, uronic acids and alkali extractable carbohydrates in contrasting marine and estuarine sediments: Distribution, size fractionation and partial chemical characterization. **Organic Geochemistry, 39 (2008)**, 265-283, **IF= 2.785**.

4) **L. Fernandes**, F. D'Souza and N. B. Bhosle. Carbohydrates in size fractionated dissolved organic matter in a station of the Bay of Bengal. **Indian Journal of Marine Science, 36 (2007)**, 193 – 198, **IF= 0.422**.

5) Vishwas B. Khodse, **Loreta Fernandes**, V.V. Gopalkrishna, Narayan B. Bhosle, Veronica Fernandes, S. G. Prabhu Matondkar, Ravi Bushan. Distribution and seasonal variation of concentrations of particulate carbohydrates and uronic acids in the the northern Indian Ocean. **Marine Chemistry, 103 (2007)**, 327–346, **IF= 3.074**.

6) Loreta Fernandes, Fraddry D'Souza, S.P.G. Matondkar, Narayan B. Bhosle, Amino sugars in suspended particulate matter from the Bay of Bengal during summer monsoon of 2001. **Journal of Earth System Science, 11 (2006)**, 363 – 370, **IF= 0.820**.

7) Narayan B.Bhosle, Anita Garg, Loreta Fernandes, and Pierre Citon., Dynamics of amino acids in the conditioning film developed on glass panels

immersed in the surface seawater of Dona Paula Bay. **Biofouling, 21 (2005)**, 99 –107, **IF= 4.429**.