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Chapter 1

Introduction

1.1. Nitrogen cycle

Nitrogen constitutes 78.08% by volume of earth's atmosphere and nitrogen cycle is one of the most important cycles after the carbon (Fig. 1.1). Nitrogen exists in the oxidation states of +5 (NO_3^-) to -3 (NH_4^+) and has importance in biological systems as it is a constituent of one of the major organic molecules proteins, in the cell. However, nitrogen is very valuable for organisms because all the amino acids contain nitrogen which is important in formation of polypeptide chains. Nitrogen approximately makes up 12% of microbial cell dry weight whose scarcity can control the biomass growth. The major microbial processes involved in cycling the nitrogen are the nitrogen fixation, nitrification and denitrification. Through nitrogen fixation, the atmospheric nitrogen is made available to the biosphere as ammonia by some symbiotic as well as free living bacteria. Nitrification occurs by a two-step process where the ammonia is oxidized into nitrite by the ammonia oxidizing bacteria (AOB) and the nitrite is oxidized into nitrate by nitrite oxidizing bacteria (NOB). The nitrate is reduced by both assimilatory and dissimilatory processes, with dissimilatory denitrification being the prominent one. Dissimilatory nitrate reduction to ammonium (DNRA) which is also called as nitrate ammonification is another dissimilatory nitrate reducing process and the organisms capable of this process compete with denitrifiers in anoxic environments (Tiedje et al. 1982).

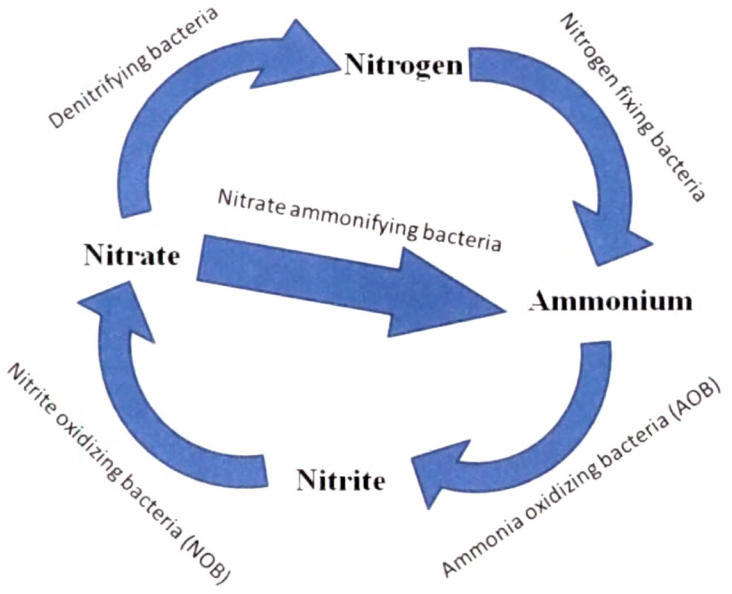

Fig.1.1. Nitrogen cycle

1.2. Nitrate as a pollutant

Wastewaters of certain industries producing chemicals, fertilizers, explosives, etc. contain very high amounts of nitrate with more than 1000 mg L^{-1} (Constantin & Fick 1997; Glass & Silverstein 1999; Zala et al. 1999; Fernandez-Nava et al. 2008), which when released into the environment such as lakes or rivers causes eutrophication and also contaminates the drinking water. The WHO guideline value for nitrate and nitrite in drinking water is 50 mg L^{-1} and 3 mg L^{-1} respectively, because consumption of high nitrate and nitrite concentrations are known to cause methemoglobinemia in infants and other health hazards (WHO 1998).

Biological denitrification is a widely used process in removal of high nitrates from the wastewaters (Mateju et al. 1992). Activated sludge, which is a suspended-growth process, is commonly used for treating wastewaters by industries. The objectives of a biological wastewater treatment are to coagulate and remove the nonsettleable colloidal solids and to stabilize the organic matter (Metcalf & Eddy 1991). The different wastewater treatment processes come under five major kinds: aerobic, anoxic processes, anaerobic processes, combined aerobic, anoxic and anaerobic processes, and pond processes (Metcalf & Eddy 1991). However, the usage of attached-growth or biofilm reactors is increasing due to its conferred advantages as compared to the suspended-growth systems like low space requirement, operational flexibility, reduced hydraulic retention time, resilience to changes in the environment, increased biomass residence time and reduced growth rate leading to lower sludge production (Nicolella et al. 2000; Qureshi et al. 2005; Andersson 2009).

1.3. Denitrification

Denitrification is defined as a bacterial respiratory process that couples electron transport phosphorylation to sequential reduction of nitrogenous oxides (Zumft 1992).The pathway of denitrification is as follows:

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$$

The denitrification pathway is carried on by the nitrate, nitrite, nitric oxide and nitrous oxide reductase enzymes which is encoded by the *nar*, *nir*, *nor* and *nos* genes respectively. The theoretical energy yields of denitrification are given in the Table 1.1.

Table 1. 1. The theoretical yield from each step of denitrification (Adapted from Tiedje 1994)

Reaction	No. of electrons accepted/N	G°′ (kJ/mol)
$NO_3^- + H_2 \longrightarrow NO_2^- + H_2O$	2	-161
$NO_2^- + 1/2H_2 + H^+ \longrightarrow NO + H_2O$	1	-76.2
$2NO + H_2 \longrightarrow N_2O + H_2O$	1	-306
$N_2O + H_2 \longrightarrow N_2 + H_2O$	1	-340
Total Process $2NO_3^- + 5H_2 + 2H^+ \longrightarrow N_2 + 6H_2O$	5	-1121

1.3.1. Enzymes and genes involved in denitrification

Nitrate reductase is the first enzyme involved in the denitrification process which reduces nitrate to nitrite. Three different nitrate reductases are found in bacteria, with assimilatory type (Nas) present in the cytoplasm and two dissimilatory types, membrane-bound respiratory type (Nar) and the other periplasmic dissimilatory type (Nap) (Moreno-vivian et al. 1999). More than one type of enzyme is present in an organism, but all the three enzymes are also reported to coexist in *Paracoccus denitrificans* and *Alcaligenes eutrophus* (Warnecke-Eberz & Friedrich 1993; Sears et al. 1997). However, all the nitrate reductases contain molybdenum as a cofactor in its active site (Moreno-vivian et al. 1999). The respiratory nitrate reductase is encoded by the genes *nar*GHIJ (Zumft 1997).

Nitrite reduction is the fate determining step for denitrification because it converts nitrite into nitric oxide, while the other nitrate reducing bacteria convert it into ammonia. There are two kinds of nitrite reductases found in denitrifiers, with either of them present in bacteria but not both. One type contains the cytochrome cd_1 as its cofactor and the other contains copper (Zumft 1997). Nitrite reductases are periplasmic enzymes. The

cytochrome cd1 nitrite reductase is encoded by the *nir*S gene and *nir*K codes for the copper containing enzyme.

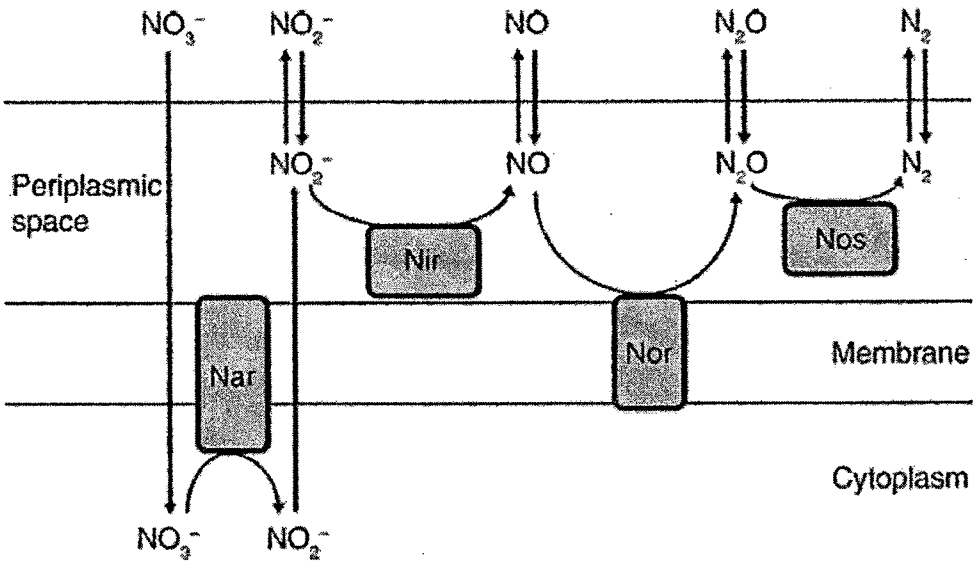

Fig. 1.2. Topology of the denitrification system (Wallenstein et al. 2006)

Nitric oxide is a toxic intermediate and the nitric oxide reductase enzyme converts it into nitrous oxide. The bacterial nitric oxide reductase enzyme is membrane-bound (Averill 1996) and is encoded by the *nor*CB genes. The *nir* and *nor* genes are closely linked and harbors the structural and some functional information (Zumft 1997).The last step of denitrification is the reduction of nitrous oxide to dinitrogen by the nitrous oxide reductase enzyme which is periplasmic and has copper as a cofactor (Zumft 1997). The nitrous oxide reductase is coded by the *nos*Z gene.

1.3.2. Regulation of denitrification in bacteria

The topology of the denitrification apparatus is shown in Fig. 1.2. The catalytic site of the nitrate reductase faces towards the cytoplasm due to which nitrate has to be imported into the cell to reduce it and the nitrite has to be exported because the nitrite reductase is periplasmic in nature (John 1977; Kristjansson & Hollocher 1979). Nitrate and nitrous oxide reductase are independently regulated; whereas nitrite and nitric oxide reduction are controlled inter-dependently (Zumft 1997). Denitrification can proceed either sequentially or coordinately in an organism. Denitrifying bacteria are basically aerobes, but low partial pressure of oxygen induces the denitrification enzymes, therefore

denitrification is best studied in static conditions in the laboratory (Tiedje 1994). Presence of nitrogenous oxides, and also the metal ions like copper, iron and molybdenum is important in the biosynthesis of denitrifying enzymes (Zumft 1997). FNR-like factors, which are oxygen labile molecules, are mainly associated in the regulation of anaerobic respiration including denitrification in many organisms (Spiro 1994). However, there are also a few reports of aerobic denitrification (Robertson & Kuenen 1984; Robertson et al. 1989; Chen et al. 2003).

1.3.3. Environmental factors affecting denitrification

Different environmental factors are known to affect the denitrification, important among them being the oxygen. Partial pressure of oxygen is reported to determine the synthesis of reductases differentially and also nitrate is known to affect nitrite reduction in *P. stutzeri* (Korner & Zumft 1989). Almeida et al. (1995b) observed the dependency of nitrite reduction on the nitrate concentrations in *Pseudomonas fluorescens*. Lower reduction of nitrite than nitrate causes nitrite accumulation, which limits denitrification and growth (Almeida et al. 1995a). Almeida et al. (1995b) showed that nitrite concentration above 130 mg N L^{-1} limits growth of *P. fluorescens*. Carbon sources play an important role in denitrification because they are the source of electrons. Different volatile fatty acids (VFA) were reported by Rijn et al. (1996) to have different effects on denitrification of *Pseudomonas stutzeri* with nitrite accumulation in presence of acetate and propionate while no nitrite accumulation was observed when butyrate, valerate and caproate was provided as carbon source. Denitrification is rapid at slightly alkaline pH (Focht 1982). Sequential reduction of the nitrogenous oxides were found at pH 5.5 while pH of 8.5 was shown to accelerate the denitrification process with very little amount of accumulation of the intermediates in *Paracoccus denitrificans* (Thomsen et al. 1994). Temperature is also known to affect the denitrification (Focht 1982).

1.3.4. Distribution of denitrifying bacteria

More than a century has passed after the denitrifying bacteria were first cultivated (Payne 1986). Gamble et al. (1977) had done an exhaustive isolation of denitrifying bacteria from various sources of different parts of the world. Their study showed the *Pseudomonas* and *Alcaligenes* species to be numerically dominant denitrifiers. Heylen et al. (2006) used an evolutionary algorithm to optimize the media parameters most suitable for cultivation of denitrifying bacteria and found Alpha and Betaproteobacteria to

dominate in the sludges. They isolated denitrifiers from Gamma & Epsilonproteobacteria, Firmicutes and also an isolate from Bacteroidetes.

However, cultivation-dependent approaches yield bacteria that are rare in whole community cloning studies (Fuhrman 2009). The unculturable methods used to explore the diversity of denitrifying bacteria target functional genes of denitrification because of the polyphyletic distribution of denitrifiers (Table 1.2). Various molecular techniques used to circumvent the problem of culture-dependent approaches in the study of denitrifying bacterial distribution are cloning and sequencing, RFLP, T-RFLP, DGGE and FISH (Scala & Kerkhof, 1999; Stres et al. 2004; Enwall et al.2005; Ginige et al. 2005; Smith et al. 2007; Henry et al. 2008; Enwall & Hallin 2009). The advances in molecular techniques have widened the horizon and it is now known that the denitrifiers are distributed widely in almost all the groups of bacteria including Archaea (Table 1.2).

The functional genes studied to investigate the diversity of the denitrifying bacteria are the *nar*G, *nir*S, *nir*K, *nor*B and the *nos*Z. The nitrate reductase gene (*nar*) is present in most of the organisms including the ones which does not denitrify, hence it is not widely used for studying the diversity of denitrifiers (Philippot et al. 2002). The nitrite reductase gene (*nir*) was initially targeted for finding the diversity of denitrifiers in the environment (Braker et al. 1998). Nevertheless, both *nir*S and *nir*K have to be studied to get an idea of the denitrifier community because either of the enzymes is present in an organism, but not both. Because of this redundancy in the *nir* gene, it is better to study the *nor*B or *nos*Z genes for understanding the community composition of denitrifiers. There are a few reports targeting the *nor* gene encoding nitric oxide reductase (Braker & Tiedje 2003), however, Heylen et al. (2007) showed incongruence of the *nor*B gene and the organism phylogeny based on the 16S rRNA gene. The diversity of nitrous oxide reductase gene (*nos*Z) is more widely studied and it is one of the best markers to study the abundance and diversity of denitrifiers (Scala & Kerkhof 1999; Stres et al. 2004; Mounier et al 2004; Enwall et al 2005; Henry et al. 2008).

Philippot & Hallin (2005) proposed denitrifying bacteria as a model functional community for understanding the relationship between the community structure to function. Nonetheless, Wallenstein et al. (2006) reviewed the prominent factors affecting the denitrifier community composition and the denitrification activity. The illustration of

Table 1.2. Distribution of denitrifiers among Bacteria and Archaea (Adapted from Zumft 1997)

Archaea	Bacteria (gram-negative)
Organotrophic	Diazotrophic
Halophilic	*Aquaspirillum*
Haloarcula	*Azospirillum*
Halobacterium	*Azoarcus*
Haloferax	*Bacillus*
Hyperthermophilic	*Bradyrhizobium*
Pyrobaculum	*Pseudomonas*
	Rhodobacter
Bacteria (gram-positive)	*Rhodopseudomonas*
Organotrophic	*Sinorhizobium*
Spore forming	Thermophilic
Bacillus	*Aquifex*
Nonspore forming	*Bacillus*
Jonesia	*Thermothrix*
	Psychrophilic
Bacteria (gram-negative)	*Aquaspirillum*
Phototrophic	*Halomonas*
Rhodobacter	Halophilic
Rhodopseudomonas	*Halomonas*
Rhodoplanes	*Bacillus*
Lithotrophic	Pigment-forming
S oxidizing	*Chromobacterium*
Beggiatoa	*Flavobacterium*
Thiobacillus	*Pseudomonas*
Thioploca	Budding
H_2 oxidizing	*Blastobacter*
Ralstonia	*Hyphomicrobium*
Paracoccus	Gliding
Pseudomonas	*Cytophaga*
NO_2^- or NH_4^+ oxidizing	*Flexibacter*
Nitrobacter	Magnetotactic
Nitrosomonas	*Magnetospirillum*
Organotrophic	Pathogenic
Carboxidotrophic	*Achromobacter*
Pseudomonas	*Alcaligenes*
Zavarzinia	*Agrobacterium*
Oligocarbophilic	*Campylobacter*
Aquaspirillum	*Eikenella*
Hyphomicrobium	*Flavobacterium*
Fermentative	*Kingella*
Empedobacter	*Moraxella*
Azospirillum	*Morococcus*
Facultatively anaerobic	*Neisseria*
Alteromonas	*Ochrobactrum*
Pseudomonas	Oligella
Aerobic	*Pseudomonas*
Paracoccus	*Sphingobacterium*
Alcaligenes	*Tsukamurella*

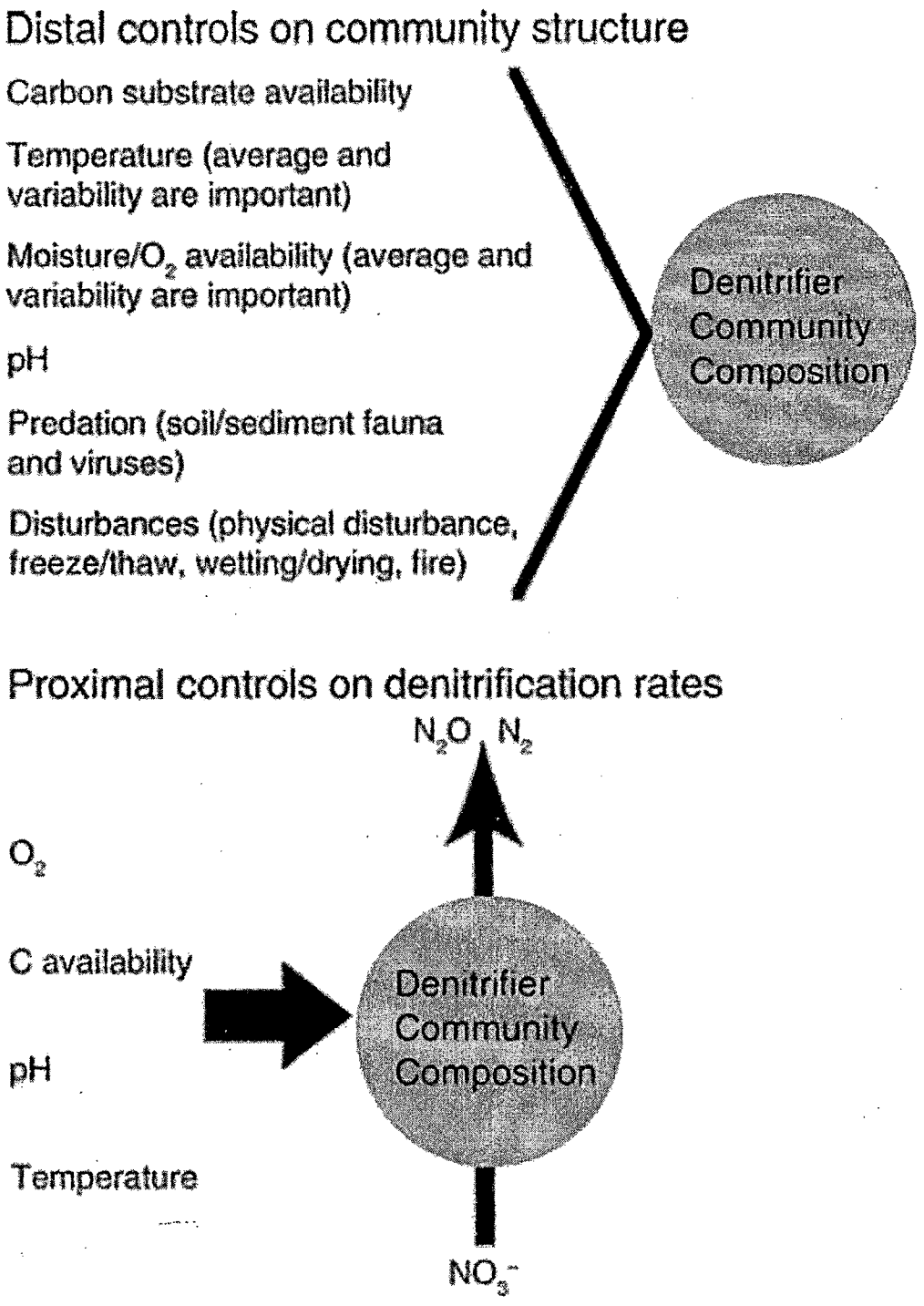

Fig. 1.3. Conceptual illustration of the distal and proximal controls on denitrifying community and denitrification proposed by Wallenstein et al. (2006)

the controlling factors as depicted in Fig. 1.3 shows that the carbon and oxygen availability, temperature, pH and other physical disturbances are the major factors affecting the denitrifier community. However the denitrification rates were shown to be controlled by pH, temperature, oxygen and carbon availability rather than the community in most cases.

1.4. Biofilm

Traditionally microorganisms were viewed as solitary organisms, but this notion has been contradicted now by a vast amount of research showing a variety of social behaviours (Nadell et al. 2009). Multicellularity in bacterial cells was proposed by Shapiro (1998). Quorum sensing and the recent discovery of the cell to cell connecting nanotubes for intercellular communication have refurbished the idea of multicellularity in bacteria (Dubey & Ben-Yehuda 2011). Biofilms are such multicellular structures which are defined by Costerton et al. (1995) as the, "matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces". Parsek & Singh (2003) proposed four criteria for determination of pathogenic biofilm bacteria, which could be modified as follows for general description of biofilms (1) Cells adhere to a surface (2) The bacteria live in clusters or microcolonies encased in an extracellular matrix (3) The bacteria are confined to a particular location, although dissemination occurs as a secondary phenomenon (4) The biofilm cells are difficult to eradicate with antibiotics as compared to their planktonic counterpart.

The biofilm cells are distinctly different from its planktonic lifestyle. Many reports show substantial differences in the gene or protein expression between planktonic and biofilm cells (Tremoulet at al. 2002a; Tremoulet et al. 2002b; Shemesh et al. 2007) important among them being the genes involved in adhesion, quorum sensing, stress response, carbohydrate metabolism, and motility (Jefferson 2004). Trade-off between the growth yield and growth rate also takes place between the planktonic and biofilm cells, with the latter showing a high growth yield (Kreft 2004).

Cooperation is a prominent characteristic of biofilm. As Kreft (2004) pointed out, the biofilm habitat is preferentially favoured to the altruists because of their high growth-yield strategy than higher growth rate. Altruism is successful when it is directed towards other genetically related ones to avoid the development of cheats in the population (West et al. 2006). Another important example of cooperation in biofilm bacteria is the

production of "public goods" including EPS, enzymes, surfactants and signalling molecules (West et al. 2007). It is also argued that biofilm is the default mode of growth in bacteria (Jefferson 2004).

1.4.1. Development of Biofilm

The development of biofilm proceeds through four different apparent stages namely the (a) attachment (b) microcolony formation (c) matured biofilm and (d) dispersal (Fig. 1.4).

Fig. 1.4. Development of biofilm. 1 = reversible attachment, 2 = irreversible attachment, 3 = microcolony development, 4 = matured biofilm, 5 = dispersal. (Stoodley et al. 2002)

1.4.1.1. Attachment – Attachment to a biotic or abiotic surface is the first stage of biofilm development. The cells attach to a surface reversibly first and irreversibly thereafter. The attachment step involves a multitude of factors including surface conditioning, mass transport, hydrophobicity, surface charge and roughness (Palmer et al. 2007). Accumulation of the molecules at the solid-liquid interface because of the bulk flow or release of some surface-active compounds by the organism causes conditioning of the surfaces (Neu 1996; Palmer et al. 2007). Conditioning layer includes small and polymeric compounds such as lipids, proteins, complex polysaccharides and humic substances which affect the surface free energy, hydrophobicity and electrostatic charges (Neu 1996). Attachment to the surface is mediated by van der Waal's forces, electrostatic

or hydrophobic interactions (Palmer et al. 2007). Bacteria can be transported to the surfaces by Brownian motion, sedimentation or convective mass transport. Active transport towards the surface is mediated by the flagellar motility in different organisms (deWeger et al. 1987; Pratt & Kolter 1998; O'Toole & Kolter 1998a; Lemon et al. 2007). Type IV pili is implicated in the initial stages of biofilm formation in *Peudomonas aeruginosa* (O'Toole & Kolter 1998a). *P. aeruginosa* uses type IV pili to finding a suitable place on the surface and moves by the mechanism of twitching motility (Mattick 2002). Bacteria form a monolayer after the initial attachment to the surface (Moorthy & Watnick 2004).

1.4.1.2. Microcolony formation – After adhering irreversibly to a surface, the bacteria forms a microcolony with few aggregations of the cells. The primary colonizers of the surface can either promote or preclude the succession of the secondary colonizers (Costerton et al. 1994). Klausen et al. 2003 showed that the cells formed in the microcolony are clonal in nature, suggesting kin selection to take place. The microcolony is also marked by the production of exopolymeric substances (EPS), which is metaphorically called as the "House of Biofilm Cells" (Flemming et al. 2007). The EPS consists of sugars, lipids, proteins and also DNA. Composition of the EPS differs in different organisms with *E.coli* producing colanic acid (Prigent-Combaret et al. 1999), alginate is produced by *P. aeruginosa* (Davies & Geesey 1995) and *Vibrio cholera* EPS consists of glucose and galactose with N-acetyl glucosamine, mannose and xylose as its constituents (Yildiz & Visick 2009). Proteins are shown to mediate adhesion in *Pseudomonas fluorescens* WCS365 (O'Toole & Kolter 1998b) and presence of protein component in the extracellular matrix is also shown in *Bacillus subtilis* (Branda et al. 2006). Extracellular DNA (eDNA) is shown to be important in the initial stages of biofilm formation in *P. aeruginosa* (Whitchurch et al. 2002). The eDNA has also been reported to be present in the biofilm matrix of other species (Steinberger & Holden 2005; Qin et al. 2007; Vilain et al. 2009; Harmsen et al. 2010). The EPS has various important functions in the biofilm and is highlighted in reviews by Sutherland (2001) and Flemming et al. (2007) (Table 1.3).

Quorum sensing is another important phenomenon in the development of biofilm. Davies et al. (1998) showed involvement of cell to cell signalling in the biofilm development of *P. aeruginosa*. *Streptococcus mutans* also require quorum sensing for biofilm formation (Li et al. 2002). The release of extracellular DNA is mediated in many organisms by

quorum sensing (Spoering & Gilmore 2006). Cell-cell signalling is important for cooperation among the biofilm bacteria and Parsek & Greenberg (2005) have extensively reviewed the connection of quorum sensing and biofilm formation. However, the strategies of quorum sensing and biofilm formation differs among species with most of the organisms, for example, *P. aeruginosa* start producing EPS for biofilm formation after a quorum is reached (Davies et al. 1998; Sakuragi & Kolter 2007), while *Vibrio cholerae* stops EPS production after a quorum is reached (Hammer & Bassler 2003). The biofilm forming fitness of these contrasting strategies have been evaluated by Nadell et al. (2008).

Table 1.3. Functional importance of EPS in biofilms (Flemming et al. 2007)

Effect of EPS component	Nature of EPS component	Role in biofilm
Constructive	Neutral polysaccharides Amyloids	Structural component Structural component
Sorptive	Charged or hydrophobic polysaccharides	Ion exchange, sorption
Active	Extracellular enzymes	Polymer degradation
Surface-active	Amphiphilic Membrane vesicles	Interface interactions Export from cell, sorption
Informative	Lectins Nucleic acids	Specificity, recognition Genetic information, structure
Redox active	Bacterial refractory polymers	Electron donor or acceptor?
Nutritive	Various polymers	Source of C, N, P

1.4.1.3. Matured biofilm – The microcolonies are developed into a matured biofilm subsequently and during the maturation, the cells undergo differentiation. Klausen et al. (2003) showed non-motile and a migrating subpopulation of *P.aeruginosa* which formed stalks while the motile subpopulation formed mushroom-shaped caps on these stalk cells by migrating with the help of type IV pili. Boles et al. (2004) reported functional diversification of *P. aeruginosa* cells in the biofilm, with a subpopulation forming a wrinkly colony on the agar surface while another forming a mini colony. The wrinkly

colony subtype had an increased biofilm forming capability, whereas the mini subtype showed high ability to disseminate. Boles & Singh (2008) found that the production of endogenous oxidative stress during biofilm formation was the reason for the diversification and this process confers insurance effects (Boles et al. 2004). Chai et al. (2008) observed only a subpopulation of *B. subtilis* biofilm cells to produce the EPS matrix. The matrix production genes in *B. subtilis* are under control of spo0A whose stochastic expression causes a subpopulation of cells to trigger EPS production. Programmed cell death (PCD) by a subpopulation of cells in biofilm is also reported (Bayles 2007). It could thus be inferred from these reports that the biofilm possibly has division of labour (Kearns 2008).

The matured biofilm is characterized by a complex architecture (Wood et al. 2000; Davey et al. 2003; Bridier et al. 2011). Watnick & Kolter (2000) called biofilm metaphorically as the "City of Microbes". A typical biofilm architecture consists of cell clusters, interstitial voids and conduit channels (Fig. 1.5) (deBeer et al. 1994; Costerton et al. 1995). The architecture of biofilm could emerge as a consequence of the bacteria adapting to its microenvironment (Nadell et al. 2008). However, the EPS, thickness and clusters of cells in the biofilm causes diffusional barriers resulting in concentration gradients of the nutrients and metabolites, due to which physiological heterogeneity can be seen. Both vertical as well as horizontal oxygen concentration gradients were observed by deBeer et al. (1994). The patterns of chemical heterogeneity in biofilms was shown by Stewart & Franklin (2008), where they proposed three kinds of chemical heterogeneity with respect to the metabolic substrate, metabolic product and the metabolic intermediate (Fig. 1.6)

1.4.1.4. Dispersal – The last step in the development of biofilm is the dispersal of the cells. The cells detach from the biofilm possibly for colonizing other surfaces or in search of nutrients. The dispersal of the biofilm bacteria could be active or passive as influenced by the environment (Fig. 1.7). Three types of dispersal strategies are found in biofilm (Hall-Stoodley et al. 2004) (1) Swarming/Seeding dispersal, where the individual cells are released from the microcolony into the bulk fluid (2) Clumping dispersal, in which aggregates of cell are shed as clumps and (3) Surface dispersal, where the biofilm structures move across surfaces.

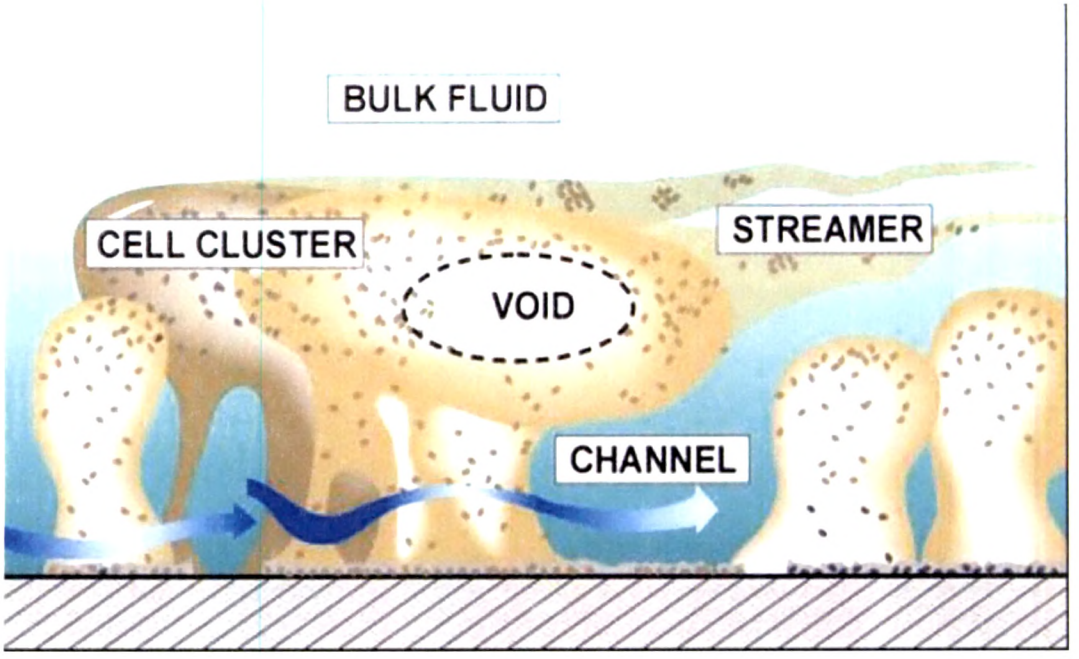

Fig. 1.5. A typical biofilm structure (Image courtesy: Centre for Biofilm Engineering, Montana State University)

Starvation conditions are shown to induce dispersion in *Pseudomonas* species (Hunt et al. 2004; Gjermansen et al. 2005), but bacteriophage could also be implicated in detaching the biofilm cells by reducing the viscosity of the EPS (Hanlon et al. 2001). Nitric oxide which is an intermediate of denitrification is also shown to cause dispersal in *P. aeruginosa* (Barraud et al. 2006). Enzymatic release of cells by degrading the EPS is reported in a number of organisms (Lee et al. 1996; Kaplan et al. 2004; Kaplan 2010). Surfactants, quorum sensing and other environmental factors including shear stress are also reported to be implicated in biofilm dispersal (Kaplan 2010).

1.4.2. Environmental cues for biofilm formation

Cyclic-di-GMP signalling has emerged as an important cue to induce the sessile lifestyle in bacteria (Jenal & Malone 2006). The cyclic-di-GMP is synthesized by a class of enzymes containing GGDEF domains and hydrolysed by another family containing EAL domains (Jenal & Malone 2006). Various environmental factors are shown to either induce or inhibit biofilm formation; nevertheless, Klausen et al. (2006) proposed that the biofilm bacteria optimize colonization in response to environmental conditions.

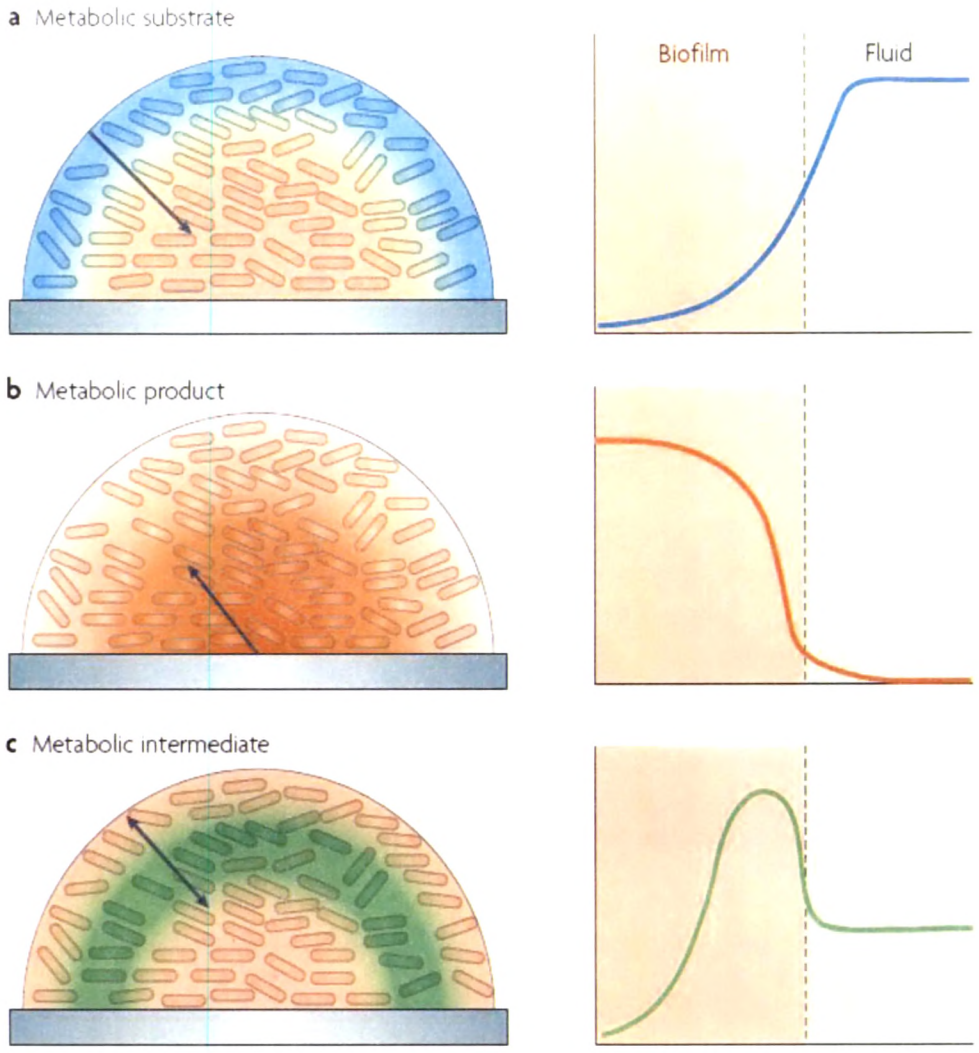

Fig. 1.6. Chemical heterogeneity in biofilms. (Stewart & Franklin 2008)

Different physical factors which prominently influence biofilm formation are the kind of substratum surface (Palmer et al. 2007) and hydrodynamics (Purevdorj et al. 2002). Various chemical factors known to influence biofilm formation are pH (Dunne & Bird 1992; Palmer et al. 2007), phosphate concentration (Monds et al. 2007) and various divalent cations (Turakhia et al. 1983; Dunne & Bird 1992; Kierek & Watnick 2003; Banin et al. 2005; Song & Leff 2006).

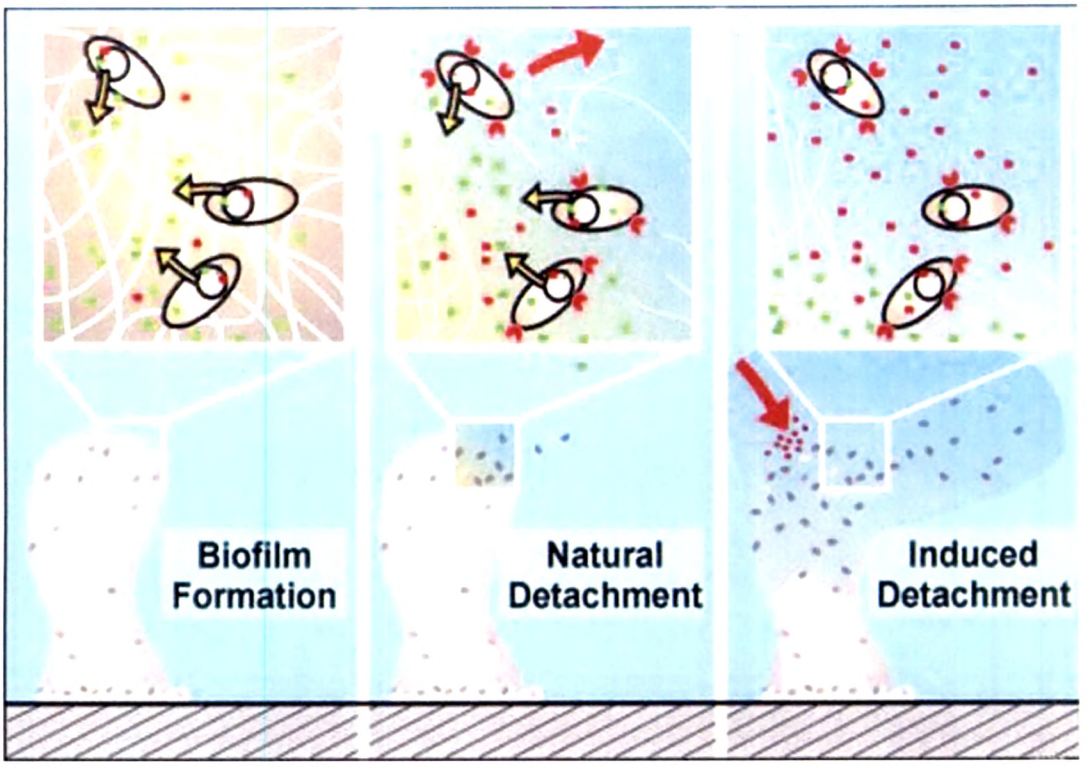

Fig. 1.7. Active and passive forms of biofilm detachment (Image courtesy: Centre for Biofilm Engineering, Montana State University)

1.4.3. Significance of biofilms

Biofilms are viewed as microbial landscapes (Battin et al. 2007), where the different ecological theories like the neutral and dispersal ecologies are applied. However, the evidence for biofilms also appears in the fossil records (Hall-Stoodley et al. 2004). Biofilms, both harmful and beneficial are formed on the tissue surfaces of plants (Ramey et al. 2004). Nevertheless, the most important aspect of the bacterial biofilms is the resistance to anti-bacterial compounds. A very high level of resistance to a dose of more than 1000 times than planktonic forms towards antibiotics has been reported by the biofilm bacteria (Stewart & Costerton 2001). The possible mechanisms for anti-bacterial resistance of the biofilm include (Mah & O'Toole 2001) (a) failure of the antibacterial to penetrate due to production of EPS (b) slow or no growth and (c) heterogeneity of biofilm cells. Numerous infections including nosocomial infections spread especially due to the biofilm formation on medical devices like catheters, difficult to treat with antibiotics are caused by these resilient biofilm bacteria (Parsek & Singh 2003; Hall-Stoodley et al. 2004). Biofouling of industrial pipelines, which cause decrease in the flow rate and also biofouling of ship hulls, which causes corrosion yielding huge economic

losses. Biofilm based reactors are also used in wastewater treatment processes (Qureshi et al. 2005).

1.4.4. Methods used to study biofilms

Though various different methods are employed to study biofilms, the most widely used techniques are the microtitre plate assay, flow cells and the CDC biofilm reactor (Fig. 1.8). The 96 well microtiter plate is extensively used to screen biofilm formation, where the biofilm formed on the surfaces of the wells are quantified using a simple stain, usually crystal violet, which measures the biomass adhered to the surface of the wells (O'Toole & Kolter 1998b; Djordjevic et al. 2002; Meritt et al. 2005). Flow-cells or the drip flow reactors are used to grow biofilms on slide surfaces in continuous flow of nutrients, which tries to mimic the natural conditions and the biofilm formed on slides, are further used for microscopic analysis. The flow cells are widely used for many kinds of analysis including dispersal of the biofilm cells (Heydorn et al. 2000a; Hunt et al. 2004; Gjermansen et al. 2005; Pamp et al. 2009). The CDC biofilm reactors have higher volumes than that of the flow cells, and the biofilms are grown on coupons mounted to polypropylene rods. A baffled stir bar in the reactor is rotated with the help of magnetic stirrer to support proper mixing of the nutrients. CDC reactors are mostly used for testing the antibiotic susceptibility of biofilm (Goeres et al. 2005; Honraeta et al. 2005; Buckingham-Meyer et al. 2007; Kim et al. 2008)

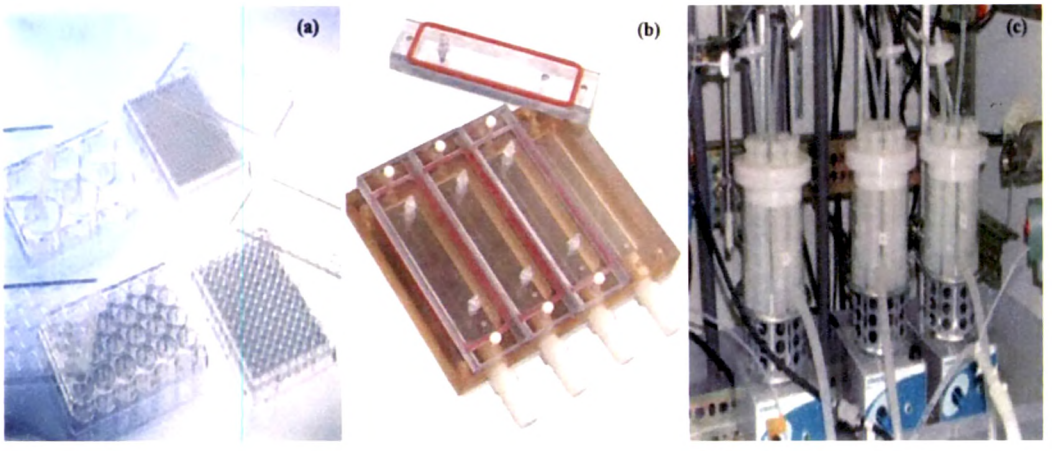

Fig. 1.8. Some most widely used tools to study biofilms in the laboratory (a) Microtitre plates (b) Flow cell (c) CDC biofilm reactor.

Biofilm architecture is analysed by either visualization or quantification of the biofilm structures from the microscopic images. Confocal microscope is one of the best methods to acquire biofilm images because of its non-invasiveness and ability to acquire images vertically through slices, which can be combined to get the 3-D image by the use of several softwares (Pamp et al. 2009). The biofilm forms a complex architecture which can be dissected into different structural parameters like the thickness, roughness, diffusion distance, fractal dimension, aerial porosity, etc. (Hermanowicz et al. 1996; Heydorn et al. 2000b; Beyenal et al.2004). Different softwares are developed to quantify these biofilm structural parameters from the confocal images like the widely used COMSTAT, Image Structure Analyzer (ISA) and PHLIP (Heydorn et al. 2000b; Beyenal et al.2004; Mueller et al. 2006). Scanning electron microscopes are also used in biofilm studies to get high resolution images (Costerton et al. 2003; Tomaras et al. 2003; Hunter & Beveridge 2005; Priester et al. 2007), however, though not yet widely used, the 3-D images of the biofilms are also analysed by atomic force microscope (Kolari et al. 2002; Ahimou et al. 2007; Schmid et al. 2008). The number of viable cells in the biofilm is studied by either staining them with live/dead stains like the SYTO9 and propidium iodide and then measuring the ratio of the stained bacteria by image analysis or scraping off the biofilm cells by using rubber policeman and plating them to enumerate the CFU.

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