Chapter 1

Introduction and Literature Review

1.1 Nitrate contamination sources

Nitrate is the second most commonly found pollutant in surface and groundwater (Back et al., 2018). More than 50 % of water pollution in streams, rivers and groundwater is caused by agricultural activities such as the use of pesticides and fertilizers, which drip out from their places into water bodies (Ahada and Suthar, 2018; Balamurugan et al., 2020; Liu et al., 2021).The explosives used in open mines to extract minerals are also responsible for nitrate contamination in mining effluents (Dutta et al., 2018). Leakage of nitrate from the sewer system, seepage and explosives from mining processes are washed off and disintegrate in rainwater, which eventually seeps into nearby streams and rivers, resulting in elevated nitrate concentrations in groundwater (Brindha et al., 2017; Feng et al., 2020b). Atmospheric deposition of nitrogenous compounds through industrial emissions, combustion of fossil fuels and vehicular exhausts etc. can also be converted to nitrate during weathering and penetrates into the aquatic layers and pollutes groundwater (Ayub et al., 2019).

1.2 Health risks of nitrate contaminated water

Water contaminated with nitrate has no taste, odour or colour, making it dangerous because it cannot be recognized visually or through taste and smell (Avisar et al., 2008). In India, 118 million people drink water with nitrate levels ranging from 45 to 100 mg L^{-1} and more than 108 million people consume water with more than 100 mg L^{-1} of nitrate (Karunanidhi et al., 2020). According to the Indian scenario, the Bureau of Indian Standards (BIS) recommends 45 mg L^{-1} as an acceptable level of nitrate concentration in drinking water (Adimalla and Qian, 2021). Nitrate can enter in the human body by both exogenous and endogenous mechanisms and when reduced to nitrite, it becomes fatal. Nitroso compounds in the diet can also have detrimental health effects (Ward et al., 2018). The World Health Organization (WHO) has recognized a Maximum Contaminant Level (MCL) of nitrate in drinking water at 50 mg L^{-1}. Water consumption above 45 mg L^{-1} can cause methemoglobinemia (bluebaby syndrome), in which red blood cells reduce their oxygen carrying capacity. It also causes birth disorders, cancer, spontaneous abortions, thyroid disorders, teratogenesis and mutagenesis (WHO, 2017; Ward et al., 2018; Wu et al., 2018; Tian et al., 2020) High level of nitrate in water bodies causes

problems such as deteriorating water quality, creating eutrophication and producing toxic algal blooms (Briki et al., 2017). In east china risk of non-carcinogenic was infants > children > females > males Gao et al., (2020b). Table 1.1 shows various nitrate contamination reports in India.

Table 1.1 Ground water contamination due to nitrate reported from India

Region	Nitrate Level (mg L^{-1})	References
Gautam Budh Nagar, Uttar Pradesh	> 45	(Agarwal et al., 2019)
Hanumangutta, Andhra Pradesh	760.12	(Suvarna et al., 2020)
Pratapgarh, Uttar Pradesh	557.80	(Maurya et al., 2020)
Nagpur, Maharashtra	432	(Marghade, 2020)
Lucknow, Uttar Pradesh	212.6	(Singh et al., 2020)
Central Telangana	212	(Adimalla and Qian, 2020)
Yamuna River Basin, Palwal, Haryana	164.1	(Ahmad et al., 2020)
Ganga River, Raebareli, Uttar Pradesh	139.76	(Shukla and Saxena, 2020)
Panipat, Haryana	113	(Rishi et al., 2020)
Vattamalaikarai River Basin, Tamil Nadu	100	(Arya et al., 2020)
Yercaud, Tamil Nadu	94	(Panneerselvam et al., 2021)
Ganga River Basin, Kanpur, Uttar Pradesh	70	(Santy et al., 2020)
Kadava River Basin, Nashik, Maharashtra	68.62	(Wagh et al., 2020)
Hindon River, Ghaziabad, Uttar Pradesh	40	(Sharma et al., 2021)
Tiruppur, Tamil Nadu	290	(Karunanidhi et al., 2020)
Wardha River Basin, Maharashtra	480	(Nawale et al., 2021)
Coimbatore and Tirupur districts	415	(Jayarajan and Kuriachan, 2021)
Salem, Tamilnadu	46.45	(Ramalingam et al., 2022)
Palani, South India	34.16	(Panneerselvam et al., 2022)
South India	86.1	(Suvarna et al., 2022)

1.3 Chemical, Physical and Biological methods for nitrate removal

Several conventional technologies have been established for the remediation of nitrate from wastewater which include adsorption (Ouardi et al., 2015), ion exchange process (Samatya et al., 2006), electrochemical methods (Zhang et al., 2016b; Martinez et al., 2017), reverse osmosis (Epsztein et al., 2015), biological methods (Kodera et al., 2017)

and chemical methods. However, these techniques have several limitations such as in conventional adsorption techniques, adsorption efficiency, reusability and disposal of the adsorbents are major issues (Bhatnagar and Sillanpaa, 2011). Ion exchange techniques are sensitive to different contaminants and it requires post treatment (Kapoor and Viraraghavan, 1997). Reverse osmosis is susceptible to biofouling and is sensitive to contaminants other than nitrate. Uses of chemical methods are limited due to their toxicity and expensive installation. To address the aforementioned operational issues, the biological nitrate remediation strategy discussed below could be ecologically acceptable solution for mitigating nitrate contamination and recovering most industrial wastewaters.

1.4 Biological denitrification

Biological denitrification is one of the most preferred, efficient, cost-effective and environmentally-friendly process (Lim et al., 2017). This process is carried out by denitrifying bacteria that reduce nitrate to non-toxic nitrogen gas under anaerobic or low oxygen environment (Costa et al., 2018). They use nitrate as terminal electron acceptor and organic substances as electron donors and energy sources to sustain their growth (Ghafari et al., 2008).The biological denitrification process is of two types: Autotrophic denitrification and Heterotrophic denitrification. Autotrophic denitrifiers derive their energy from hydrogen, iron or sulfur compounds and their carbon from inorganic carbon compounds like carbon dioxide and bicarbonate (Karanasios et al., 2010). Heterotrophic denitrifiers are the most common denitrifiers in nature which use organic carbon compounds as a carbon source (Van Rijn et al., 2006; Zhao et al., 2011). In the microbial mediated heterotrophic denitrification process, nitrate acts as a terminal electron acceptor and different carbon sources such as methanol, acetate and sucrose act as electron donors (Bill et al., 2009; Rajmohan et al., 2018). Heterotrophic biological denitrification is considered to be more cost-effective and practically used on large scale (Schipper et al., 2010). Table 1.2 shows various denitrifying bacteria used for nitrate and nitrogen removal processes. Enzymes involved in the denitrification process are nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase sequentially (Ji et al., 2015).These reductases are sensitive to oxygen. They carry out the following steps in the biological denitrification process.

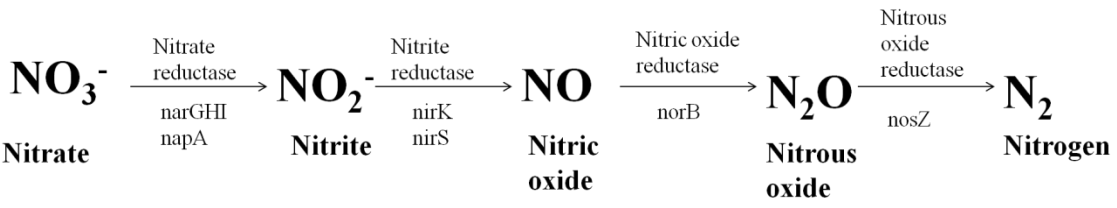

Table 1.2 Denitrifying bacteria used for nitrate and nitrogen removal process

Denitrifying bacteria	References
Alcaligenes defragrans B21	(Flores et al., 2007)
Halomonas campisalis	(Guo et al., 2013)
Enterobacter cloacae	(Guo et al., 2016)
Cupriavidus sp. S1	(Sun et al., 2016)
Janthinobacterium sp. M-11	(Yang et al., 2018)
Enterobacter sp. FL	(Wang et al., 2018)
Bacillius salmalaya	(Dadrasnia et al., 2017)
Paracoccus sp. strain YF1	(Liu et al., 2012)
*Acinetobacte*r sp. H36	(Su et al., 2017b),
Simplicispira hankyongi	(Siddiqi et al., 2020)
Shewanella oneidensis MR-1	(Jiang et al., 2020)
Corynebacterium pollutisoli SPH6	(Liu et al., 2018)
Pannonibacter phragmitetus	(Bai et al., 2019)
Pseudomonas mendocina	(Zhang et al., 2021)
Acinetobacter sp. YS2	(Lang et al., 2020)
Paracoccus denitrificans Z195	(Chakravarthy et al., 2011; Zhang et al., 2020a)
Pseudomonas balearica RAD-17	(Ruan et al., 2020)

1.5 Bioaugmentation

The use of a microbial consortium for bioremediation instead of a pure culture is more advantageous because it provides the metabolic diversity and robustness required for field applications (Rahman et al., 2002a, b). Specific functional consortium has been found to provide possibilities for Total Nitrogen (TN) removal, such as *Brodadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis* for anaerobic ammonium oxidation (Zekker et al., 2012).

Addition of specific contaminant degrading bacteria to the site contaminated with pollutants is known as bioaugmentation process (Quan et al., 2005). This approach is highly suitable for the sites that lack sufficient microbial cells or the native bacterial population does not possess the metabolic pathways required for pollutant degradation

(Liu et al., 2018).This approach has been also used in denitrifying reactors to increase nitrate removal because lack of favorable organisms results in poor reactor performance due to reduction in total biomass amount (Eldyasti et al., 2013).

Table 1.3 Use of consortia in the treatment of various wastewaters

Microorganisms	References
Ralstonia pickettii K50 and *Actinomycetes Streptomyces griseus* in artificial wastewater	(Takaki et al., 2008)
Treatment of tannery effluent with *Bacillus* sp. SFC 500-1E and *Acinetobacter guillouiae* SFC 500-1	(Ontanon et al., 2015; Fernandez et al., 2019)
Treatment of piggery effluent with *Pseudomonas stutzeri* TR2	(Ikeda et al., 2013)
Treatment of Coke making wastewaters with *Paracoccus* sp. BW001, *Shinella zoogloeoides* BC026 and *Pseudomonas* sp. BC001	(Bai et al., 2010)
Alkaline phenol wastewater treatment with *Pseudomonas* sp. JY-2	(Qu et al., 2011)
Tannery effluent treatment with *Brachymonas denitrificans*	(Leta et al., 2005)
Treatment of paper industry effluent with *Pseudomonas aeruginosa* (DSMZ 03505), *Pseudomonas aeruginosa* (DSMZ 03504) and *Bacillus megaterium* (MTCC 6544)	(Tiku et al., 2010)
Treatment of paper industry effluent with *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus* and *Bacillus* sp.	(Chandra et al., 2007)
Achromobacter xylosoxidans MCM1/1 (78%), *Enterobacter cloacae* MCM2/1 (50%), and *Ochrobactrum anthropi* MCM5/1 (52%) and the fungus *Exophiala dermatitidis* MCM3/4 (14%) for methyl tert-butyl ether (MTBE) degradation	(Barbera et al., 2011).

Bioaugmentation process is not only used for denitrifying bacteria but it has been also used for treatment of various wastewaters. It comprises mixed microbial cultures which are important for efficient operation of biotreatment processes. Unlike microbially mediated production processes, microbial mediated environmental protection and restoration processes involve microbial cultures comprising multiple microbial consortia. Microbial consortia encompass consortia performance, rather than individual strain performance. Biotreatment processes involve multiple substrates (pollutants) which can be degraded by highly complex mixed microbial cultures. Use of microbial consortia is

more suitable for the efficient operation of biotreatment processes (Hamer, 1997). Table 1.3 shows list of various consortia used for the treatment of various wastewaters.

1.6 Biological wastewater treatment

Biological treatment using microorganisms is the most cost-effective and efficient way of treating complex organic carbon compounds and removing inorganic nutrients like nitrates from wastewater. Biological treatment can be of two main types, based on whether the functional microbial communities are maintained as planktonic assemblages (floc-based systems) or as surface-associated biofilms (attached growth systems). In floc-based systems the microbial rich slurry in the sewage treatment plants containing bacteria and other microfauna and flora, is known as Activated Sludge (AS). The sludge, also known as the mixed liquor, is a flocculant suspension of various organisms. First step in activated sludge process is aeration process in which oxygen is provided for the respiration of aerobic bacteria. It keeps the microbial flocs in an agitated suspension and ensuring maximum contact between the flocs surface and the wastewater. The flocculated biomass settles swiftly out of suspension to form sludge in the second stage and the cleared effluent is discharged as particle-free final effluent (Hreiz et al., 2015). In biofilm reactors, microbial biomass is attached to the surface of support materials, which results in an increase in the biological removal rate and makes them more resistant to overloading and toxic compounds (Lee et al., 2006). Biofilm reactors are generally categorized into two groups: Fixed-bed and Expanded-bed reactors. In fixed bed reactors, biofilm develop on static media and it can be divided into submerged beds, trickling filters, rotating biological contactors and membrane biofilm reactors (1) In submerged beds the biofilm particles are completely immersed in the liquid (Cheng et al., 2009; Cheng et al., 2010); (2) Trickling filters in which biofilm is formed on the fixed bed (Howell and Atkinson, 1976); (3) Rotating Biological Contactor (RBC) in which the biofilm forms on the surface of a partially submerged vertical disc that rotates within the liquid. (Bungay and Serafica, 1999) (4) Membrane Biofilm Reactors (MBR) in which the microbial biofilm forms on the surface of porous gas-permeable membrane (Yashino et al., 1996). On the other hand, in expanded bed reactors, biofilm is formed on continuously moving media which are further divided into two categories: (1) Fluidized

beds in which particles move up and down within the expanded bed in the well defined zone of the reactor and (2) Moving beds in which the whole expanded bed/media circulates throughout the reactors. Fig. 1.2 shows schematic diagram of various biofilm based reactors. The biofilm systems have distinct advantages over AS systems. They are more compact, incur low cost, stable, have no sludge recycling retain highly concentrated biomass and have reduced hydraulic retention time. They develop more diverse microbial communities than AS, which allow degradation of a wide range of organic pollutants (Wilderer and McSwain, 2004; Bassin and Dezotti, 2018). Table 1.4 shows bioreactors used for denitrification process. Among different biofilm reactors many drawbacks are seen for e.g. trickling filters are not volume-effective, rotating biological contactors often have more mechanical failures, granular media biofilters require backwashing and fluidized bed reactors are unstable.

1.7 Moving Bed Biofilm Reactor

To overcome operational problems faced by biofilm reactors, Moving Bed Biofilm Reactor (MBBR) was developed. In MBBR the biofilm grows on specifically engineered carriers that move freely in the tank, making it highly effective in its performance (Rusten et al., 2006). MBBR development was initiated in the 80s in Norway (Odegaard 2006). This technology was patented as Kaldnes Moving Bed TM Biofilm process. (Eur. Pat. No. 0575314, US pat. No.5458779) (Rodgers and Zhan, 2003). It combines the best features of suspended and biofilm-based processes. Fig. 1e represents schematic diagram of MBBR. In MBBR biofilm is attached to the carriers that are suspended in the reactor, moving freely by aeration (in oxic reactors) and mixing (in anoxic reactors). The carriers in MBBR ‘‘carry’’ the microorganisms in the reactor as the microbes are adhered to the surfaces of the carriers in the form of biofilms (Leiknes and Odegaard, 2006). Biofilm thickness is also important parameter because substrates need to diffuse into the biofilm (Odegaard, 2006).Transportation of substrates into the biofilms is increased by increasing turbulence in the reactor which prevents the biofilm from getting too thick due to shear forces. Thickness of the biofilm can be regulated by sloughing off from the carriers, erosion, predator grazing and abrasion; thin and evenly distributed biofilm is the salient feature of efficient MBBR (Rusten et al., 2006).

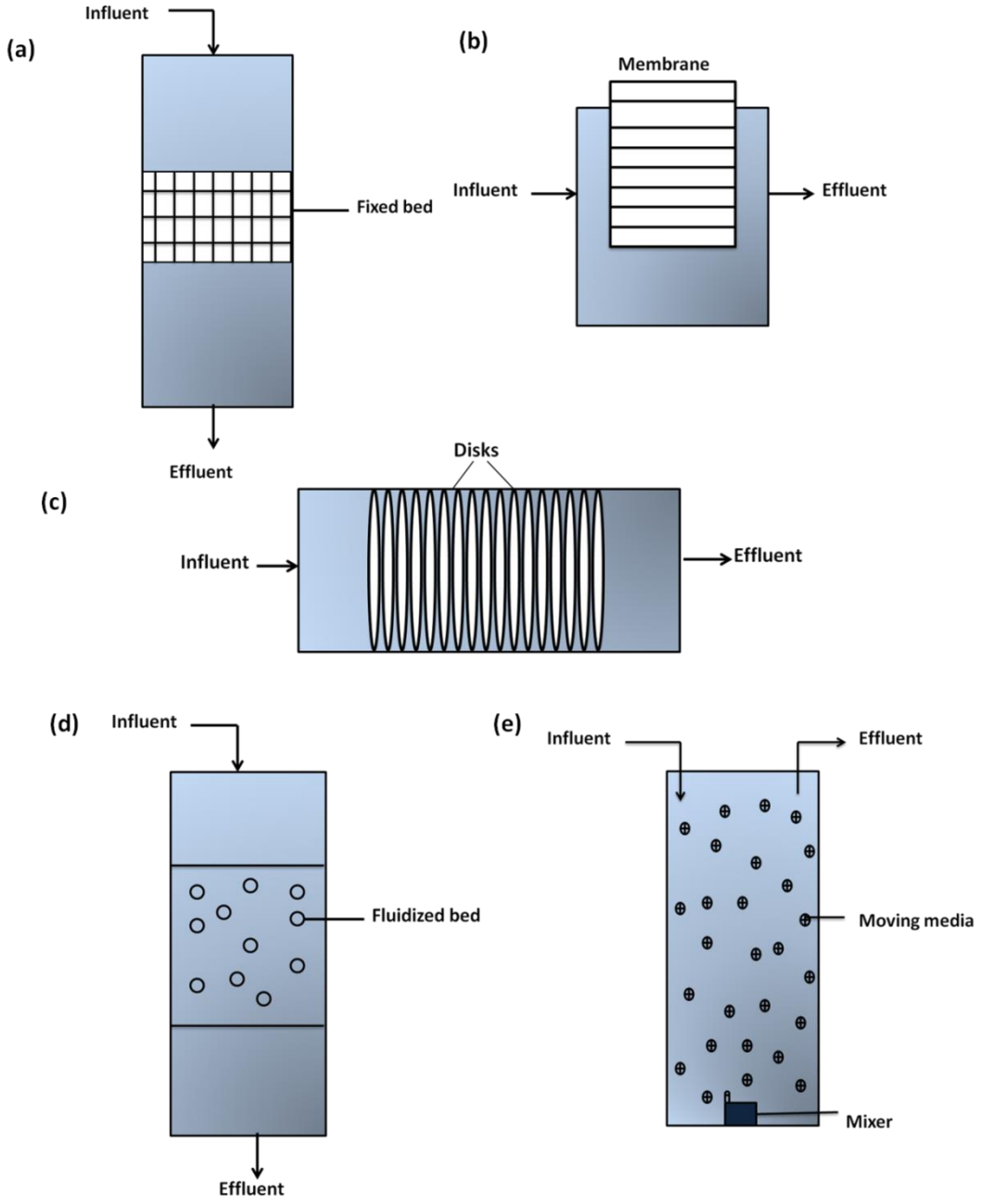

Figure 1.1 Schematic diagram of biofilm reactors (a) Trickling filter (b) Membrane Biofilm Reactor (MBR) (c) Rotating Biological Contactor (RBC) (d) Fluidized Bed Reactor(FBR) (e) Moving Bed Biofilm Reactor(MBBR)

Table 1.4 Bioreactors used for denitrification process

Denitrifying biofilm bioreactor	Process	References
Up-flow solid-phase denitrification biofilm reactor	Simultaneous nitrate and dissolved organic matter removal	(Gao et al., 2020a)
MBBR	Simultaneous nitrification and denitrification	(Bhattacharya and Mazumder, 2021)
Denitrifying MBBR	Denitrification	(Bill et al., 2009)
Denitrification bioreactor – aerobic biofilm reactor	Denitrification	(Dong et al., 2019)
Biological denitrification biofilm reactors	Denitrification	(Chen et al., 2000)
MBBR	Sulfide-oxidizing autotrophic denitrification	(Cui et al., 2019)
Anoxic sequencing batch biofilm reactor	Denitrification	(Ding et al., 2019)
Solid-phase denitrification	Denitrification	(Feng et al., 2020a)
Upflow sludge bed denitrifying reactors	Denitrification	(Franco et al., 2006)
MBBR	Partial nitritation-anammox process	(Gu et al., 2020)
Solid-phase denitrification biofilm reactor	Denitrification	(Han et al., 2018)
Microaerobic MBBR	Simultaneous nitrification denitrification and phosphorus removal	(Iannacone et al., 2019)
A continuous-flow moving bed biofilm reactor	Simultaneous nitrification and denitrification coupled to phosphorus removal (SNDPR)	(Iannacone et al., 2020)
MBBR	Simultaneous partial nitrification and denitrification (SPND) to biological phosphorus removal	(Iannacone et al., 2021)
MBBR	Simultaneous Nitrification and Denitrification	(Jia et al., 2020)
Hydrogenotrophic denitrification reactor	Denitrification	(Keisar et al., 2021)

Denitrifying automatic circulate reactor	Denitrification	(Li et al., 2014)
MBBR	Nitrification and denitrification	(Ooi et al., 2018)
Solid-phase denitrifying reactors	Denitrification	(Qi et al., 2020)
Anoxic MBBR	Denitrification	(Song et al., 2021)
Denitrification reactors	Denitrification	(Saliling et al., 2007)
Denitrifying MBBR	Denitrification	(Stavrakidis et al., 2019)
Sequencing batch biofilm reactors	Denitrification	(Kłodowska et al., 2018)
Membrane biofilm reactor	Anammox with denitrifying anaerobic metse oxidation in a membrane	(Xie et al., 2018)

Biofilm formed on the carriers and in the internal structures of carriers can degrade various dissolved pollutants and remove nitrate from the wastewater. MBBR has been proven to be very suitable for the removal of nitrogen and treatment of industrial effluents generated from poultry processing, pulp and paper industry, refinery and slaughterhouse, landfill leachate and various types of industrial wastewaters (Jahren et al., 2002; Chen et al., 2007; Leyva-Diaz et al., 2017; Jiang et al., 2018; Johnson et al., 2018). Various denitrifying bacteria were used in the MBBR for bioaugmentation purposes, like bioaugmentation of *Pseudomonas* sp. SZF15 efficiently removed nitrate in the MBBR (Su et al., 2019). *Corynebacterium pollutisoli* SPH6 in the MBBR system showed potential for nitrogen removal (Liu et al., 2018). *Pseudomonas mendocina* IHB602 is also used for nitrate removal (Hong et al., 2020). *Acinetobacter* sp. CN86 showed promising approach for simultaneous removal of nitrate, Cd^{2+} and Ca^{2+} and was the main contributor to the effective removal of pollutants during the MBBR process (Su et al., 2019). Table 1.5 shows application of MBBR for the treatment of different wastewater.

Table 1.5 Application of MBBR for the treatment of different wastewaters

Application	References
Antibiotic (Tetracycline)removal	(Chen et al., 2018)
Dairy wastewater	(Zkeri et al., 2021)
Drinking wastewater	(Doederer et al., 2019; Tak et al., 2020)
Pesticide wastewater	(Chen et al., 2007)
Hospital wastewater	(Casas et al., 2015)
Laundry wastewater	(Bering et al., 2018)
Palm oil mill wastewater	(Abu Bakar et al., 2018)
Pesticide industry wastewater	(Bachmann et al., 2018)
Phosphorous wastewater	(Iannacone et al., 2019)
Pulping industry wastewater	(Jahren et al., 2002)
Recalcitrant wastewater	(Hapeshi et al., 2013)
Micropollutant wastewater	(Torresi et al., 2019; Kora et al., 2020)
Treatment of kraft mill effluent	(Chamorro et al., 2016)
Treatment of pulping whitewater	(Jahren et al., 2002)
Treatment of municipal wastewater	(Gustavsson et al., 2020)
Treatment of phenolic wastewater	(Hosseini and Borghei, 2005)
Tannery wastewater treatment	(Sodhi et al., 2021)
Coal pyrolysis wastewater	(Zheng et al., 2019)
Hospital wastewater effluents	(Khan et al., 2020)
Laundry wastewater treatment	(Bering et al., 2018)
Pharmaceutical wastewater	(Tang et al., 2017)
Treatment of mature landfill leachate	(Xiong et al., 2018)
Detergent industries wastewater	(Taghavi et al., 2017)

1.8 Operational parameters influencing the performance of MBBR

Various studies have proven that the nitrogen removal performance of MBBR could be enhanced by optimizing the operating conditions (Aygun et al., 2008; Daija et al., 2015; Wang et al., 2016; Jaafari et al., 2017). Biofilm formed on the carriers is influenced by various process parameters such as carrier type, filling ratio of carriers, the physicochemical surface of carriers, Hydraulic Retention Time (HRT) and hydrodynamics inside MBBR. Optimization of these parameters increased nitrate removal efficiencies in the reactor by directly affecting microorganisms and the speed of biofilm development inside MBBR (Abzazou et al., 2016).

1.8.1 Carrier selection

The carrier media used in denitrifying MBBR is a key element that influences its performance. In MBBR, carriers with high suspendability, surface roughness and high mechanical strength showed high performance (Yuan et al., 2015). Carrier shape, structure and surface properties protect the microbial community developed on the carriers. Biofilm developed inside carriers are thicker whereas microbial growth developed on the abrasion-exposed surface area forms thin biofilms (Comett et al., 2004; Mahendran et al., 2012; Zhang et al., 2017b). Carriers made up of polyethylene materials reported as the best carrier for denitrification. High denitrification efficiency can be achieved with polyethylene (PE), polypropylene (PP), polyurethane foam (PUF) and haydite carriers (Yuan et al., 2015).Suspended ceramic products, activated carbon, diatomaceous earth, polymeric nano-fibrous material and bioplastic based material have been used as carriers for nitrogen removal in MBBR systems (Dong et al., 2011). Other carrier media, such as cellulose, woodchips, plastic media, non-woven carriers and modified carriers, were also used for MBBR (Deng et al., 2016; Young et al., 2016; Peng et al., 2018). Carriers with the high surface area can provide a unique, cost-effective wastewater treatment technology as microbial adhesion is made easier (Massoompour et al., 2020). Depending upon strength of given wastewater the filling ratio of carrier in the reactor can be decided.

1.8.2 Filling ratio

The filling ratio of carriers is the volume of carriers added to the reactor. It should be below 70 % to move the carriers freely in suspension. Zhang et al., (2016a) found the highest nitrogen removal at 20 % filling ratio. As the filling ratio of carriers increased the biofilm thickness on each carrier reduced (Wang et al., 2005). High filling ratio causes more carrier collision, which leads to the selection of bacteria that can grow on the carrier under specific reactor conditions, making the system more efficient. But too high filling ratio reduces the anoxic zone due to the thinner biofilm which increases aerobic microorganisms leading to decrease in denitrification efficiency.

1.8.3 Modified carriers for MBBR

Modification of the carrier surface area and carrier is also an important feature to achieve appropriate biofilm and initial attachment of cells to the surface and is a promising option for wastewater treatment (Sarjit et al., 2015; Sonwani et al., 2019). To enhance biofilm homogeneity, thickness, density and shear strength of nitrifiers community, amino-functional group (-NH2) was introduced on polyethylene (PE) and polypropylene (PP) and plastic carriers (Lackner et al., 2009). Polymer blending by using toluene diisocyanate, polyether polyol and foam stabilizer also increased positive charges and hydrophobicity of the carriers (Chu et al., 2014). MBBR with sponge-modified biocarriers enhanced nutrient removal compared to conventional MBBR carriers (Deng et al., 2016). MBBR developed with zeolite powder-based polyurethane sponges showed higher performance than sponges as biocarriers (Song et al., 2019).

1.8.4 Hydrodynamics/ Shear stress in MBBR

High shear stress caused by turbulence on MBBR carriers is called the hydrodynamic boundary layer. Due to hydrodynamic control, carriers in MBBR did not clog and thin biofilm was maintained which could easily denitrify seawater (Dupla et al., 2006). At lower shear forces, thinner biofilms were formed and biofilm surface roughness was high (Liu and Tay, 2001; Wang et al., 2005; Odegaard, 2006) whereas higher shear stress in MBBR supported stable and stronger biofilms with increased EPS production (Liu and Tay, 2001; Bassin and Dezotti, 2018). Proper hydrodynamics inside MBBR should be maintained to develop a stronger biofilm which ultimately increases the performance of denitrifying MBBR.

1.8.5 Hydraulic retention time (HRT)

HRT is the contact time of the influent wastewater with microbial biomass inside the reactor (Ji et al., 2016). An appropriate HRT improves nitrate reduction (Wang et al., 2009). If HRT is too long, it may waste treatment capacity and consume high energy and if HRT is too short it will reduce contact time between microbial biomass and pollutant (Wang et al., 2009). Different HRTs have been optimized in literature, which is important for denitrification in the reactor.

1.9 Environmental and nutritional factors important for the efficient denitrification process in MBBR

1.9.1 Effect of carbon source

In the heterotrophic denitrification process, acetate, methanol, glucose and ethanol have been more effective carbon sources. These carbon sources are simple sources with high nitrate reduction efficiency because they are easy to utilize and provide an adequate amount of electrons for denitrification (Mohan et al., 2016). Due to its metabolic properties, acetate is more suitable for denitrification than other carbon sources like glucose or methanol (Onnis-hayden and Gu, 2008). The use of other carbon sources like glycerol and ethanol enhanced denitrification compared to methanol (Bill et al., 2009). In the denitrifying MBBR, raw Arundo donax pieces (perennial grass with hollow stems) also showed efficient denitrification performance (Li et al., 2019). Carbon sources are also known to influence the community and biofilm structure of bacteria, according to Srinandan et al., (2012) and Li et al., (2016).

1.9.2 Effect of C/N

The C/N ratio is crucial parameter for determining whether sewage is suitable for biological denitrification (Meng et al., 2019). Balance between electron donor and electron acceptor plays an important role in biological denitrification. Low C/N ratio limits denitrification efficiency by accumulating denitrification intermediates such as NO_2^-, NO and N_2O by limiting the electron supply. Alternatively, high C/N ratio increases the COD of effluents.

1.9.3 Effect of nitrate loading

Nitrate concentration varies in different wastewaters. High nitrate concentration strongly affects bacterial denitrification by affecting bacterial activity (Dhamole et al., 2007; Banihani et al., 2009). As a result, bacterial denitrification with nitrate concentrations of more than 100 mM has been studied in some investigations (Dhamole et al., 2007; Miao et al., 2015). Nitrite buildup is common when nitrate concentrations are high, which is

also an inhibitor of bacterial activity due to its toxicity (Cua and Stein, 2011; Albina et al., 2019).

1.9.4 Effect of metal ions

Certain nutritional factors are known to influence biofilm formation. Mainly cations have a role in biofilm development through physio-chemical interactions, gene regulation, signal transmission and protein component function (Wang et al., 2019b). This plays positive role in biofilm structural stability because of the interaction between divalent cations and negatively charged functional groups of EPS (Mangwani et al., 2014). Cations have an impact on the mechanical properties of biofilms by acting as cross linkers (Korstgens et al., 2001). High ionic strength slows down cell deposition rate in biofilm due to cell aggregation in the bulk. High concentrations of succinate, Mg^{2+}, Ca^{2+} and Mn^{2+} induced biofilm formation (Srinandan et al., 2012).

1.9.5 Effect of Dissolved Oxygen (DO), temperature and pH

Temperature, pH and DO also play a significant role in influencing denitrifier growth, metabolism, denitrification gene expression and denitrification rate.

pH has a significant impact on nitrate removal as well as bacterial growth and metabolism and is one of the most important factors influencing bacterial denitrification ability (Zhang et al., 2012b). Bacterial surface charge can be influenced by pH. It was noted that neutral pH enhanced denitrification rate (Cai et al., 2015). The optimal pH for denitrification was found to be 7-8, which is also the optimal pH for most environmental denitrifying bacteria (Glass and Silverstein, 1998). Parkin et al., (1985) showed that when the pH was reduced from 6 to 4, soil denitrification rate and denitrification was decreased 2-3 times.

DO is a very important parameter for the efficient denitrification process in denitrifying MBBR. The presence of oxygen in high-strength denitrification reactors may induce nitrite build-up, according to Glass and Silverstein, (1998). In biological treatment systems, DO levels have a substantial impact on the microbial community of biofilm (Feng et al., 2012). At high DO concentration denitrification rate was reduced due to

shortening of the anoxic zone in MBBR (Pochana and Keller, 1999). The denitrification process was inhibited by DO concentrations greater than 10 mg L^{-1} (Luo et al., 2016).

Temperature is another important environmental factor for bacterial growth, their survival and the denitrification process (He et al., 2018). Low temperature affects the activities of microorganisms and the composition of the biofilm community (Gilbert et al., 2015; Young et al., 2017). Optimum temperature for the denitrification process ranges from 25 °C to 37 °C, which is generally the optimum temperature for bacterial activity (Ji et al., 2015). At low temperature, biofilm formation was found to be higher compared high temperature (Morimatsu et al., 2012). At high temperature, the biofilm of *Pseudomonas putida* can be led to detach not only through a decrease in the viscosity of exopolysaccharides but also through an increase in the rate of bacterial growth (Morimatsu et al., 2012).

1.10 Biofilms as a potential strategy to improve bioaugmentation in wastewater treatment system

Denitrification process can be promoted by cell immobilization (Kunapongkiti et al., 2019). Immobilization (entrapment or encapsulation) of living microorganisms in a semi-permeable gel or carrier materials, leading to several advantages over the free cell bioaugmentation: it can protect microorganisms against protozoa grazing, bacteriophage infections, variations of temperature, pH and various other abiotic stresses such as the inhibitory effect of toxic compounds or heavy metals as well as the increase of shear stress (Jain et al., 2013). Overall, encapsulation is associated with high biomass concentration and enhanced cell survival.

Microorganisms in biofilm form require less space, have a higher concentration of relevant organisms and do not require biomass return which are important parameters for the performance of wastewater treatment plants (Odegaard, 2006). Cells in biofilms are bound together by extracellular polymeric substances (EPS) attached to a surface by cell to cell and cell to surface interactions (O'Toole et al., 2000). Biofilms are known as "city of microbes " (Watnick and Kolter, 2000) and the EPS matrix as "house of the biofilm cells" (Flemming and Wingender, 2010). The EPS produced by microbes accounts for 90 % of the biofilm content and it is composed of proteins, polysaccharides, lipids and

extracellular DNA (eDNA)(Flemming and Wingender, 2010). Table 1.6 shows various techniques used to observe biofilm inside MBBR.

1.11 Development of biofilm

Biofilm formation is a cyclic process. It comprises four distinct stages (Fig. 1.2). (1) Initial attachment, (2) Microcolony formation, (3) Biofilm maturation and (4) Dispersion. Detached cells go back to the planktonic mode of growth, thus closing the developmental life cycle of biofilm (Stoodley et al., 2002). Fig. 1.2 shows biofilm development on the solid substratum.

1.11.1 Stage 1- Initial attachment

Initial biofilm formation on the carrier material is triggered by the adsorption of molecules like proteins, polysaccharides, nucleic acids, humic acids and lipids on the surface. Absorbed molecules form conditioning films, which can lead to various effects like alteration of the surface physicochemical characteristics, a source of nutrient for microbes, release of toxic surface metal ions, detoxification of the bulk etc. Once the surface is prepared, cells begin to attach (Lewandowski, 2010). Adhesion of bacteria includes two conditions: One is adhesion and aggregation of bacteria to carriers mediated by high-affinity adhesion factors (membrane transport protein, viscous polysaccharide, extracellular DNA) or accessory structures (i.e., flagellum, pili) of the bacterial surface. The other condition for adhesion is triggered by the recognition of specific glycoprotein and glycolipid adhesion proteins on the surface of bacteria to surface receptors, which is also known as specific adhesion (Jefferson, 2004; Verstraeten et al., 2008).

1.11.2 Stage 2 and 3: Microcolony formation and Biofilm maturation

Microcolony formation is marked by production of EPS by the bacterial cells. The major components of EPS are proteins, carbohydrates, lipids and eDNA. These EPS components can vary from organisms to organisms. The formed microcolonies can further develop into mature biofilms during maturation process. Matured biofilms consists of bacterial cell clusters, interstitial voids and conduit channels (Davies et al., 1998). The biomass in mature biofilms can persist for several years (Biswas et al., 2014). The EPS of biofilm, cells in biofilms and thickness of biofilm causes diffusional barriers

which results in concentration gradient of different metabolites and nutrients, which leads to physiological heterogeneity. Zhu et al., (2015) reported that Actinobacteria and filamentous bacteria were dominant in mature biofilms and resisted sloughing. They also claim that bacterial adhesion force is related to the amount of EPS on MBBR carriers (Zhu et al., 2015). Components affecting biofilm characteristics and microbial attachment /detachment interactions become more prominent in mature biofilm EPS.

1.11.3 Stage 4: Biofilm dispersion

Due to the collision of carriers biomass is detached from MBBR carriers (Bassin and Dezotti, 2018). Dispersion is an active event in which sessile, matrix-encased biofilm cells return to the planktonic mode of growth, which is apparent from single cells actively escaping from the biofilm. This process is driven by the release of chemical concentration gradients of nutrient resources, oxygen and waste products from biofilm development (Rumbaugh and Sauer, 2020). Reactor operational parameters such as hydrodynamics, shear stress, HRT and organic loading rates, environmental parameters of the wastewater, microbial physiology and metabolism, microbial interactions and microbial cell properties are all major factors affecting biofilm development, structure and composition in MBBR (Flemming et al., 2016). Biomass surface charge is a complicated interaction among diverse ionic functional groups of proteins, humic substances, polysaccharides, uronic acids, extracellular DNA and cations in MBBR (Wilén et al., 2003; Mahendran et al., 2012; Sarjit et al., 2015). The rapid formation of biofilms on carriers is caused by the combination of microbial layers and bacterial metabolic activity (Sonwani et al., 2019). Contribution of several factors thus is essential for the efficient functioning of MBBR. Table 1.6 shows various techniques used to observe biofilm formation in MBBR.

1.12 Metagenomics in wastewater treatment

The application of Molecular Biology methods in wastewater treatment plants in the 1990s was a revolution. However, use of culture-based techniques to characterize the microbial community present in wastewater was insufficient because only 0.1 to 10 % of microbial cultures could be cultured in the laboratory. Recent advances in Molecular Biology have enabled the development of new molecular tools, such as Next Generation Sequencing (NGS), to characterize the total microbial diversity in wastewater treatment

processes (Shomar et al., 2020). In wastewater treatment, biological reactors are considered as "black boxes'' in which microorganisms play an important role. NGS technologies have made it possible to decode the genetic composition of multiple communities present in these black boxes without relying on cloning-based approaches (Kapley and Purohit, 2009; Shah et al., 2013; Saunders et al., 2016). It not only resolves the complex community but also provides insights into the overall functional potential of the population (Albertsen et al., 2012; Mason et al., 2012; Yuan et al., 2015; Derilus et al., 2019).

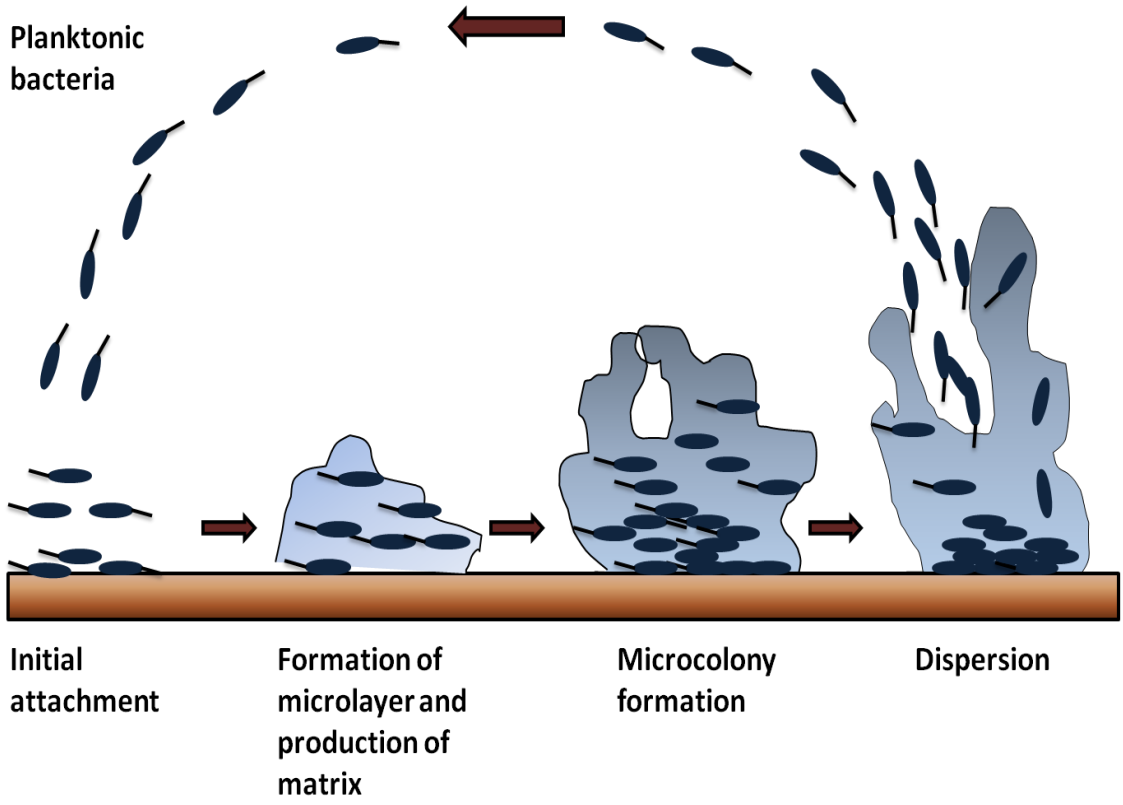

Figure 1.2 Steps involved in biofilm formation

Table 1.6 Techniques used to observe biofilm inside MBBR

Methods		Description of method	References
Microscopy	Confocal laser scanning microscopy	A useful tool for observing the 3D structure of biofilm	(Hoang et al., 2014)
	Scanning electron microscopy (SEM)	Characterization of the biomass from carriers	(Bassin et al., 2012)
	Variable pressure electron scanning microscope	Used for direct imaging of biofilm specimens without pre-treatment; thus eliminating the destructive effects of SEM pre-treatment	(Bassin et al., 2012)
Spectroscopy	Fourier transform infrared (FTIR)	Provide detailed information on EPS composition and structure	(Li et al., 2019)
Biotechnology and molecular biology method	Fluorescence in situ hybridization (FISH)	Characterize biofilm structure	(Persson et al., 2014)
	Quantitative polymerase chain reaction (qPCR)	Used to analyze particular genes and an abundance of microorganisms	(Zekker et al., 2017; Zhao et al., 2019)
	Denaturing gradient gel electrophoresis (DGGE)	To detect the diversity of the most abundant organisms from the biofilm.	(Zekker et al., 2017)
Real-time monitoring method	MRI(Magnetic Resonance Imaging)	Biofilm's physical structure and mass transport behavior of biofilm.	(Ranzinger et al., 2016)
	X-Ray diffraction (XRD)	Analyze components of biofilm.	(Su et al., 2019)

Metagenomic sequencing has evolved from Sanger technology to high throughput sequencing over time and decrease in sequencing costs encourages the development of novel technologies (Gilbert et al., 2008; Gilbert et al., 2010). Metagenome sequencing concerning the bioremediation process provides potential insights into the diversity of

detoxifying microbes, detoxification mechanisms and identification of the genes with novel functions (Ju et al., 2019). It has been widely used to study the microbial communities present in grassland soil (Delmont et al., 2012), human gut (Qin et al., 2010), marine water (Mason et al., 2012), conventional activated sludge (CAS) (Albertsen et al., 2012; Ye et al., 2012; Yu and Zhang, 2012) and wastewaters of various industrial, municipal and hospital (Yadav et al., 2015; Shu et al., 2016; Manoharan et al., 2021). Metagenomics studies in various denitrifying bioreactors also revealed that bioaugmentation of denitrifying bacteria improved nitrogen removal and increased microbial community in the reactors (Liu et al., 2018; Zhao et al., 2019). Table 1.7 shows NGS analysis carried out in various denitrifying bioreactors. This information related to community structure and function in the bioreactors or in wastewater treatment plant can be used in designing efficient bioremediation strategies for a wide range of environmental pollution, including the Common Effluent Treatment Plant (CETP) ecosystem (Guo et al., 2013; Krishnamoorthy et al., 2021). 454-pyrosequencing (Roche) and Illumina are the most widely used commercial platforms for high-throughput DNA sequencing (Illumina Inc.). Despite the higher read lengths obtained with the 454 pyrosequencing platform, Illumina replaced pyrosequencing due to its high capacity, lower price and lower error rate (Sanz and Kochling, 2019). Other massive-sequencing technologies such as HeliScope, SOLiD technology, single-molecule real-time (SMRT) and Ion Torrent DNA sequencing are rarely used in Wastewater treatment (Sanz and Kochling, 2019).

Table 1.7 NGS analysis in denitrifying bioreactors

Denitrifying reactors	References
Upflow Anaerobic Sludge Blanket Reactor (UASB)	(Zhao et al., 2019)
Sequencing Batch Biofilm Reactors (SBBR)	(Yue et al., 2018)
Moving Bed Biofilm Reactor (MBBR)	(Liu et al., 2018)
Sequential batch biological reactors (SBRs)	(Bucci et al., 2020)
Granular Sequencing Batch Reactor	(Bucci et al., 2021)
Rotating Biological Contactor (RBC)	(Ito et al., 2019)
Up-flow anaerobic sludge bed (UASB)	(Wang et al., 2017)
Up-flow cylindrical anammox reactor	(Park et al., 2021)
Pressurized hydrogenotrophic denitrification reactor	(Keisar et al., 2021)

1.13 *Thauera* as a potential denitrifying organism for the treatment of wastewater

In 1993, Macy et al., reported *Thauera* as a novel genus via polyphasic taxonomy. Genus *Thauera*, named after Prof. Rudolf K. Thauer, is a Gram-negative bacterium, having the ability to denitrify nitrogenous oxides under anoxic conditions (Macy et al., 1993). Their biochemical pathways are similar to *Achromobacter, Acidovorax, Alcaligenes, Bordetella, Burkholderia, Comamonas, Pseudomonas, Ralstonia* and *Zoogloea* (Heider and Fuchs, 2015). *Thauera* spp. is mainly found in wet soil, polluted freshwater and activated sludge (Takahashi et al., 1980; Qiao et al., 2018). They use oxygen, oxides of nitrate, selenate etc. as the terminal electron acceptor. They are chemoorganotrophs and can utilize various organic acids, amino acids, as well as aromatic and aliphatic compounds. Table 1.8 shows reported species of *Thauera* and their functional roles in biodegradation. They are most commonly found in biological wastewater treatment systems, where they are involved in the mineralization and detoxification of xenobiotic contaminants. Table 1.9 shows recent reports of *Thauera* dominated denitrification reactors. Their ability to degrade a wide range of recalcitrant compounds, including aromatic and haloaromatic compounds makes them a potential organism for the treatment of various wastewaters (Heider and Fuchs, 2015).

Table 1.8 *Thauera* species and their role in biodegradation

***Thauera* species**	**Role**	**References**
T. aromatica	Denitrification and degradation of several aromatic and haloaromatic compounds	(Heider and Fuchs, 2015)
T. butanivorans	Alkane degradation capability	(Dubbels et al ., 2007)
T. chlorobenzoica	Halobenzoate-degrading bacterium	(Louie et al., 2021)
T. humireducens	Denitrification, ability to reduce humus	(Yang et al., 2013)
T. linaloolentis	Model organisms for anaerobic monoterpene utilization	(Marmulla et al., 2016)
T. mechernichensis	Denitrification	(Chang et al., 2011)

T. phenylacetica	Denitrification	(Mechichi et al., 2002)
T. selenatis	Reduction of selenate	(Dridge et al., 2007
T. sinica	Degradation of m-cresol, 2,4-dimethylphenol, 3,4-dimethylphenol, 2,5-dimethylphenol, o-cresol, 3-ethyphenol, 2,6-dimethylphenol, p-cresol, 2,3-dimethylphenol and 3,5-dimethylphenol	(Qiao et al., 2018)

Table 1.9 Recent reports of *Thauera* dominated denitrification reactors

Reactors	**References**
Mixotrophic Denitrification in Sequencing Batch Reactor	(Liang et al., 2020)
Combined Heterotrophic and Autotrophic Denitrification Reactors	(Xu et al., 2020)
MBBR inoculated with Heterotrophic Nitrifiers and Aerobic Denitrifiers	(Zhang et al., 2020b)
Denitrification in Artificially Constructed Wetlands	(Fu et al., 2020)
Hypoxic Quinoline-Denitrifying Sequencing Batch Reactor (Major Contributor: *T. aminoaromatica*)	(Wu et al., 2020)
Aerobic Nitrifying-Denitrifying Membrane Bioreactor (Major Contributor: *T. mechernichensis*)	(Chang et al., 2011)
Denitrification Under Antibiotic Stress in Expanded Granular Sludge Bed Digestion Reactor	(Li et al., 2021)
Microbial Fuel Cell - Granular Sludge Coupling System	(Deng et al., 2020)
Simultaneous Nitrification And Denitrification by *Thauera sp.* SND5 (Pure culture)	(Wang and He, 2020)
Pyrite-Based Denitrification Bioretention system	(Chen et al., 2020)

Rationale

Nitrate pollution occurs in a variety of ecosystems as a result of various sources and processes. The widespread use of fertilizers, metal processing, the dye manufacturing industry, animal and human waste and other factors contribute to nitrate contamination in wastewater. When nitrate-contaminated wastewater is consumed, it causes severe diseases such as methemoglobinemia (bluebaby syndrome), abortions, birth disorders, thyroid disorders, cancer, etc., and when it is accumulated in the environment it leads to dire ecological problems; therefore it has to be removed by the wastewater treatment plants. Biological denitrification carried out by bacteria is the most widely employed and, cost effective process for nitrate removal from wastewater. In this process, denitrifying bacteria play an important role and have been used in the traditional biological treatment systems that use anoxic processes of suspended growth of bacteria. Biofilm-based technology for effluent treatment like Moving Bed Biofilm Reactor (MBBR) has benefits afforded by both attached and suspended growth systems. It can be applied to wide range of wastewaters and thus is effective in removing carbon as well as nitrogen but negligible studies are focused on the biofilm developed on the carrier media. Studies are particularly lacking on the bacterial biofilm development and its structure in MBBR. MBBR developed with special bacterial seed, therefore would be expected to give better performance. Also, sludge recycling, loss of biomass and requirement of large footprints are the major drawbacks of the suspended growth process, which leads to poor treatability of the wastewater. In contrast to this, MBBR is superior because of its simplicity, minimal space requirements, high biomass concentration (in the form of biofilm), minimal loss of biomass, no sludge separation, no sludge recycling, moreover existing suspended growth plants can be converted to MBBR with less expense. In spite of its advantages, MBBR has been facing the challenges of slow start-up and poor treatment performance. Bioaugmentation of specific microorganisms would provide a simple and cost effective way of improving MBBR performance. However, bioaugmentation studies have been paid less attention as can be perceived from literature. Therefore, in the studies undertaken in this thesis, the aim was to develop denitrifying MBBR (abbreviated as dMBBR) with a special bacterial seed consortium of biofilm forming denitrifying bacteria as a model system for a biofilm reactor and evaluate the

influence of important factors affecting nitrate removal; study the universality of the developed consortium; characterize the biofilm formed on the carriers in dMBBR bioaugmented with the specially developed seed consortium and finally characterize the applicative potential of the most potent, persistent and dominant bacterium found in the reactor. With this perspective, the following objectives were coined.

Objectives

1. Selection of special biofilm forming denitrifying bacteria from activated sludge.

2. Performance of bench scale Moving Bed Biofilm Reactor (MBBR) with the consortium of the selected biofilm forming denitrifying bacteria and evaluation of its performance.

3. Characterization of the biofilm produced by the selected bacterial isolates and its potential in treatment of different effluents.

4. Studies on most persistent and dominant denitrifying bacterium in continuously operated MBBR.