




# Simultaneous nitrification–denitrification by phosphate accumulating microorganisms

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Received: 15 April 2020 / Accepted: 5 September 2020 / Published online: 14 September 2020  
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## Abstract

Nitrogen and phosphorous are important inorganic water pollutants that pose a major threat to the environment and health of both humans and animals. The physical and chemical ways to remove these pollutants from water and soil are expensive and harsh, so biological removal becomes the method of choice to alleviate the problem without any side effects. The identification of microorganisms capable of simultaneous heterotrophic nitrification and aerobic denitrification has greatly simplified the sequestration of nitrogen from ammonium ( $\text{NH}_4^+$ ) into dinitrogen ( $\text{N}_2$ ). Further, the discovery of phosphorous accumulating organisms offers greater economic benefits because these organisms can favourably and simultaneously remove both nitrogen and phosphorous from wastewaters hence reducing the nutrient burden. The stability of the system and removal efficiency of inorganic pollutants can be enhanced by the use of immobilized organisms. However, limited work has been done so far in this direction and there is a need to further the efforts towards refining process efficiency by testing low-cost substrates and diverse microbial populations for the total eradication of these contaminants from wastewaters.

**Keywords** Nitrification · Denitrification · Simultaneous heterotrophic nitrification-aerobic denitrification · Phosphorus uptake · Phosphorus accumulating organisms · Wastewater

## Introduction

Increasing population and subsequent industrialization over the past decades has created new challenges for the environment. Every day, an enormous amount of waste from industries, agricultural reforms, mining activities, chemical fertilizers, pharmaceutical production plants, hospitals, landfills, and communal individuals is being disposed of into rivers and oceans (Khalaf 2016). This has increased the level of unwanted organic and inorganic material in the waters, causing pollution to exorbitant levels. As a consequence of this unjustified pollution, the crucial biogeochemical cycles of nitrogen and phosphorus have been extremely disturbed (Ahlström and Cornell 2018). Nitrogen mainly in the form of nitrate ( $\text{NO}_3^-$ ) and phosphorous in the form of phosphate ( $\text{PO}_4^{3-}$ ) has become the most common inorganic pollutants that have entered the water bodies.

The impact of these pollutants is profound and may prolong for many years (Ahlström and Cornell 2018). High levels of nitrate pose extensive health hazards such as methemoglobinemia, thyroid problems, respiratory problems, tumours, etc. to humans and animals (Rajta et al. 2019). Nitrate and phosphate amalgamation in the aquatic ecosystems leads to eutrophication causing an increase in biomass in the form of algal blooms, phytoplankton, and macrophytes. This adulteration changes the color of rivers, lakes, and the marine environment (Yang et al. 2016), reduces penetration of oxygen and light, and results in the deaths of aquatic life. This completely upsets the ecosystem balance and deteriorates the quality of water.

Nitrate is naturally available due to the processes of nitrification and mineralization of organic matter in the soil (Rajta et al. 2019). It is usually present in optimal concentrations (Lockhart et al. 2013), however, being very soluble and mobile, its excessive amounts can easily seep from the surface into groundwater and accumulate for decades causing adulteration of drinking water (Guadie et al. 2013). Excessive nitrate originates from agricultural sources such as nitrogen-based fertilizers, waste tips, manure collected from the farming industry, landfills as well as non-agricultural

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sources such as combustion of fossil fuels, domestic waste and industrial discharge (Savci 2012). Phosphorous is a non-renewable resource but its agricultural utilization is inefficient. Large amounts of dissolved phosphorous (orthophosphate form) are lost to the soil and water bodies even though restricted inflow in water bodies is necessary to maintain high biodiversity. Non-point, anthropogenic sources such as natural decomposition of rocks and minerals, erosions, agricultural runoff, and point sources such as household contributions including human waste, laundry cleaners, household cleaning products and industrial waste (Parsons and Smith 2008; Elser 2012) are responsible for phosphorus overload in surface and ground waters beyond ecological relevance.

Removal of nitrate and phosphate is undoubtedly a priority concern in wastewater treatment to retain the quality of open and ground waters (Fabro et al. 2015). Microorganisms mediated simultaneous remediation of both is an environment-friendly and efficient strategy. The process is self-sustaining and has economical benefits as more than one pollutant can be targeted by the same microorganism. However, not much has been achieved so far in this direction and the process is still in its nascent stage of development.

This review describes the process of simultaneous nitrification–denitrification and phosphorous removal (SNDPR) by aerobic microorganisms. Diverse microbial types that can perform different enzyme-mediated conversion reactions of nitrogen and phosphorous and the influencing factors are also discussed. Finally, a model for the process of SNDPR has been proposed. Previous reviews (Nancharaiah et al. 2016; Winkler and Straka 2019) have mainly focussed on nutrient removal from wastewaters by the combined application of biological and bio-electrochemical treatment systems to minimize energy requirements. Their large scale implementation is still awaited. This review enables a better understanding of the microbial process per se and describes the progress and lacunas in the simultaneous removal of nitrogen and phosphorous from contaminated waters by biological means.

## Treatment technologies for environmental pollutants

Untreated waters coming from various sources may contain high doses of contaminants such as calcium, iron, lead, chloride, magnesium, fluoride, phosphate, and nitrate (Sharma and Bhattacharya 2017). These wastewaters can be treated by different physical, chemical, and biological methods. Physical methods involve the application of physical forces. Clarifying the effluents by filtration, flocculation, floatation, and mixing (Topare et al. 2011) are some of the physical methods. Chemical methods involve the addition of chemicals. Chemical precipitation, chemical oxidation,

and advanced oxidation are some of the chemical-based methods (Topare et al. 2011; De-Bashan and Bashan 2004). Biological treatment involves the breakdown of organic and inorganic wastes by several anaerobic and aerobic microorganisms such as nitrifiers and denitrifiers (He et al. 2016b), converting them into gaseous forms. The chemical method is most appropriate for dealing with toxic inorganic compounds. But, in turn, it further increases the chemical load of the waters. Moreover, these processes do not completely remove nitrogen and phosphorus from surface and groundwater. Their success rate depends upon the type of operation and other impurities found in water (Carolyn et al. 2017). As such, the biological approach is most acceptable for the complete removal of nitrogen and phosphorous compounds. A description of the various treatments and their advantages and disadvantages are summarized in Table 1.

## Microbial proficiency for nitrogen removal

Microorganisms are the main biological components that help to remove nitrogenous pollutants from wastewater. Nitrification and denitrification are the two most important processes involved in its biological treatment (Rajta et al. 2019). Conventionally, treatment methods were based on autotrophic nitrification and anaerobic denitrification. These were time-consuming and energy-intensive methods (Khardenavis et al. 2007) and involved treatment with different microorganisms having differing requirements for oxygen and electrons (Wan et al. 2017). In recent times, bacteria with a vast potential for treatment and removal of nitrogen under a variety of environmental conditions have been identified. These include diverse nitrifiers, denitrifiers, and those that can participate in simultaneous nitrification and denitrification (SND) (Bai et al. 2019). Identification of these microorganisms has revolutionized the wastewater treatment technology making it faster and more proficient than the conventional methods.

Nitrification implies the biological oxidation of ammonium ( $\text{NH}_4$ ) to nitrate ( $\text{NO}_3$ ) under strict aerobic conditions (Thakur and Medhi 2019). Till the identification of complete ammonia oxidizers (Cotto et al. 2020), it was considered to be a two-stage process performed by different groups of organisms. The first stage of ammonia oxidation can be carried out by the autotrophic ammonia-oxidizing bacteria (AOB) and mixotrophic archaea and the second stage is performed by the nitrite-oxidizing bacteria (NOB). Ammonia oxidizing archaea (AOA) predominate over the AOB in many environments, especially in extreme conditions, possess a higher affinity for oxygen and ammonia (Winkler and Straka 2019), and account for vigorous ammonia oxidation (Hou et al. 2013). AOA mainly belong to phylum *Thaumarchaeota* with *Nitrosopumilus maritimus* and

**Table 1** Treatment technologies for removal of environmental pollutants from wastewater

Method	Advantages	Disadvantages	References
<b>Physical methods</b>			
Membrane filtration	Simplest method of separation	Membrane filtration design could differ perceptively	Crini and Lichtfouse (2019)
Nanofiltration (NF)	Produces quality effluent	Investment cost very high for a start-up for small industries	
Ultrafiltration (UF)	No chemicals requirement	High energy requirement	
	Eradicates every single type of salts, mineral by-products, and dyes		
	Low solid waste generation		
Irradiation	Simplest and efficient method	Difficult recovery on a large scale	Anjaneyulu et al. (2005)
Electron beam and radiation	Reduces the colour of sewage plant	High cost of maintenance and operation	
Adsorption	Economically feasible process	Post-treatment disposal	Ahmed et al. (2017)
	Produces a quality product	Requirement of large quantity	
	Adsorbents can be made from low-cost waste materials		
Coagulation	High removal efficiency		
	Ability to inactivate bacteria	Increased volume of sludge	Bratby (2016)
	Accessibility of diversity in viable chemicals	High cost	
	Good capital cost	Low exclusion of arsenic	
<b>Chemical methods</b>			
Chemical precipitation	Economical	Futile removal of metal ions in low concentrations	De-Bashan and Bashan (2004); Crini and Lichtfouse (2019)
	Effective at removing metals and fluorides	Generation of a large amount of sludge treatment	
	Lack of selectivity of the metal greatly reduces the demand for Chemical oxygen	Disposal problems	
Chemical oxidation	Simple, efficient and speedy process.	Unidentified intermediates formation	Bratby (2016)
	No sludge production	Requirement of chemicals	
	Oxidation helps to eliminate colour and odour	Chemical oxidation releases some volatile compounds	
Advanced oxidation processes (AOP)	Rapid degradation	Expensive	Parsons (2004)
	Effective for dyes, drugs, etc.	Not used at small level industries	
	No sludge generation	By-product formation	

Table 1 (continued)

Method	Advantages	Disadvantages	References
Electrochemical technologies	High removal efficiency Cost-effective	Requires chemicals for flocculation and coagulation High cost of yearly upkeep	Chen (2004)
Electro-coagulation (EC)	Compact facility for treatment Anodic oxidation doesn't require a large number of chemicals	Deposition of sludge can slow down electrolytic properties in continuous operation Efficiency of separation depends on bubble size in EF	
Electro-oxidation (EO) Electro-flotation (EF)			
Biological methods			
Constructed wetland systems	Cost-effective approaches and simple techniques are involved to design it High constancy in wildlife habitats as well as changes in environmental situations. Long life	High energy demands Excessive production of sludge	De-Bashan and Bashan (2004); Lee et al. (2009); Zhang et al. (2015)
EBPR by activated sludge	Cheap, effective and acceptable way to eliminate phosphorus	Insufficient carbon source issues	Cai et al. (2019)
Immobilization of biologicals	High Alteration rate due to cell stacking densities. increased testability resistance of microbes to the outer environment	Chemically stable organic adsorbents whereas inorganic adsorbents are soluble in alkaline solution. High leakage rate	De-Bashan and Bashan et al. (2010); Abdel-Raouf et al. (2012); Bouabidi et al. (2019)
Microalgae treatment	Biomass is used as a fertilizer	Seasonal variation in efficiency	Matamoros et al. (2015)

*Nitrososphaera viennensis* as the dominant species (Limpiyakorn et al. 2011). Ammonia oxidizing bacteria (AOB) carry out the oxidation of ammonia to hydroxylamine by the enzyme ammonia monooxygenase (AMO) and hydroxylamine is further oxidized by hydroxylamine oxidoreductase (HAO) to nitrite. Nitrite is converted to nitrate by the nitrite oxidase enzyme of the nitrite oxidizers (NOB) (Lei et al. 2016). Ammonia oxidizing archaea possess the AMO genes but lack the HAO genes which catalyze the hydroxylamine oxidation process. Heterotrophic nitrification involves the oxidation of both inorganic and organic forms of nitrogen to nitrates by various heterotrophic bacteria (Stein 2011). In some of these microorganisms, the mechanism of ammonia oxidation is the same as in autotrophic ammonium oxidizing bacteria (AOB). This is supported by the observation of structural and functional similarity in the ammonia monooxygenase and hydroxylamine oxidoreductase enzymes of heterotrophic nitrifiers, most particularly *Paracoccus pantotrophus* GB17, with that of ammonia-oxidizing bacteria (AOB) (Yokoyama et al. 2012). Other heterotrophic organisms may carry out the oxidation of hydroxylamine by the cytochrome P460 (Zahn et al. 1994). The final step involving oxidation of nitrite to nitrate is catalyzed by the catalase enzymes (Stein 2011).

Comammox or the complete ammonia oxidizers are autotrophic organisms that catalyze the complete conversion of ammonia to nitrate by employing ammonia and nitrite as electron donors (Cotto et al. 2020). These organisms are found in both terrestrial and aquatic habitats and involve similar enzyme-catalyzed reactions as the AOB/AOA and NOB. However, the ammonia monooxygenase enzyme is phylogenetically distinct from that found in AOB and AOA (Daims et al. 2015). Sublineage II of genus *Nitrospira* is the distinctive branch responsible for complete nitrification. Comammox bacteria have a high affinity for ammonia and oxygen but a slower growth rate and offer great advantages in energy-saving and nitrogen polishing (Lawson and Lucker 2018; Ren et al. 2020).

Anammox is an anaerobic process in which anaerobic ammonia-oxidizing bacteria use nitrite or nitrate as an electron acceptor to oxidize ammonium nitrogen to molecular nitrogen (Abbassi et al. 2014). These bacteria belong to the phylum *Planctomycetes* and include five genera identified to date that play a vital role in the nitrogen cycle. These include *Candidatus Brocadia*, *Candidatus Kuenenia*, *Candidatus Jettenia*, *Candidatus Scalindua*, and *Candidatus Anammoxoglobus* (Nancharaiah et al. 2016; Ren et al. 2020). Initially, half of the available ammonium is oxidized to nitrite by oxygen. In anammox, this nitrite then acts as an electron acceptor and converts ammonium to nitrogen via the formation of two intermediates, nitric oxide (NO) and hydrazine (N<sub>2</sub>H<sub>4</sub>) (van Niftrik and Jetten 2012; Nancharaiah et al. 2016). The key enzymatic players

in anammox are the nitrite reductase (NIR), hydrazine synthase (HZS) and the hydrazine dehydrogenase (HDH). It is mainly an autotrophic process with a high affinity for ammonium and less release of greenhouse gases (Ren et al. 2020) making it more effective than both nitrification and denitrification.

Aerobic denitrification, on the other hand, converts nitrate into gaseous nitrogen in a series of enzyme guided steps, nitrate reductase being the first enzyme of the pathway (Rajta et al. 2019). Here, co-respiration is an important mechanism where oxygen and nitrate are simultaneously used as electron acceptors (Chen and Strous 2013). In some aerobic microorganisms mechanism of heterotrophic nitrification is clubbed with aerobic denitrification (Fig. 1). Heterotrophic genera capable of SND include *Alcaligenes* (Joo et al. 2005), *Bacillus* (Kim et al. 2005), *Diaphorobacter* (Khardenavis et al. 2007), *Providencia* (Zhao et al. 2010), *Achromobacter*, *Comamonas*, *Agrobacterium* (Chen and Ni 2011), *Pseudomonas* (Qiu et al. 2012; He et al. 2018; Zhang et al. 2019; Su et al. 2019), *Serratia* (Huang et al. 2017), *Enterobacter* (Wan et al. 2017), *Rhodococcus*, *Klebsiella* (Su et al. 2019), *Acinetobacter* (Xia et al. 2020). These microorganisms have several advantages such as a high growth rate and their individual ability to convert ammonium (NH<sub>4</sub><sup>+</sup>) into nitrogen (N<sub>2</sub>). Identification of these microorganisms has made nitrogen removal from wastewaters simpler as nitrification and denitrification can be performed at the same time under the same set of conditions. While the comammox organisms can potentially shorten the process of simultaneous nitrification–denitrification, their activity may be limited by the presence of organic pollutants in wastewaters. Ammonia oxidizing archaea, on the other hand, can contribute only to ammonium oxidation during SND (Wang et al. 2017). Anammox organisms can be very useful for the complete removal of nitrogenous substances with limited requirements. However, their potential is restricted by their extremely slow growth rate with a doubling time of 11 days or even more (Lan et al. 2011) making process development quite difficult.

As such, simultaneous heterotrophic nitrification-aerobic denitrification becomes the process of choice for the treatment of wastewaters with a considerable organic load. However, there are differences in the biochemical routes followed by different bacteria capable of SND and this makes it very tough to draw generalizations. For example, although denitrifying enzymes are found in *Thiosphaera pantotropha*, nitrate reductase is absent in *Alcaligenes faecalis* (Chen and Ni 2011) even though it performs SND. Therefore, it is important to identify a wider range of organisms with potentially enhanced abilities and pathways for SND (Zhao et al. 2010).

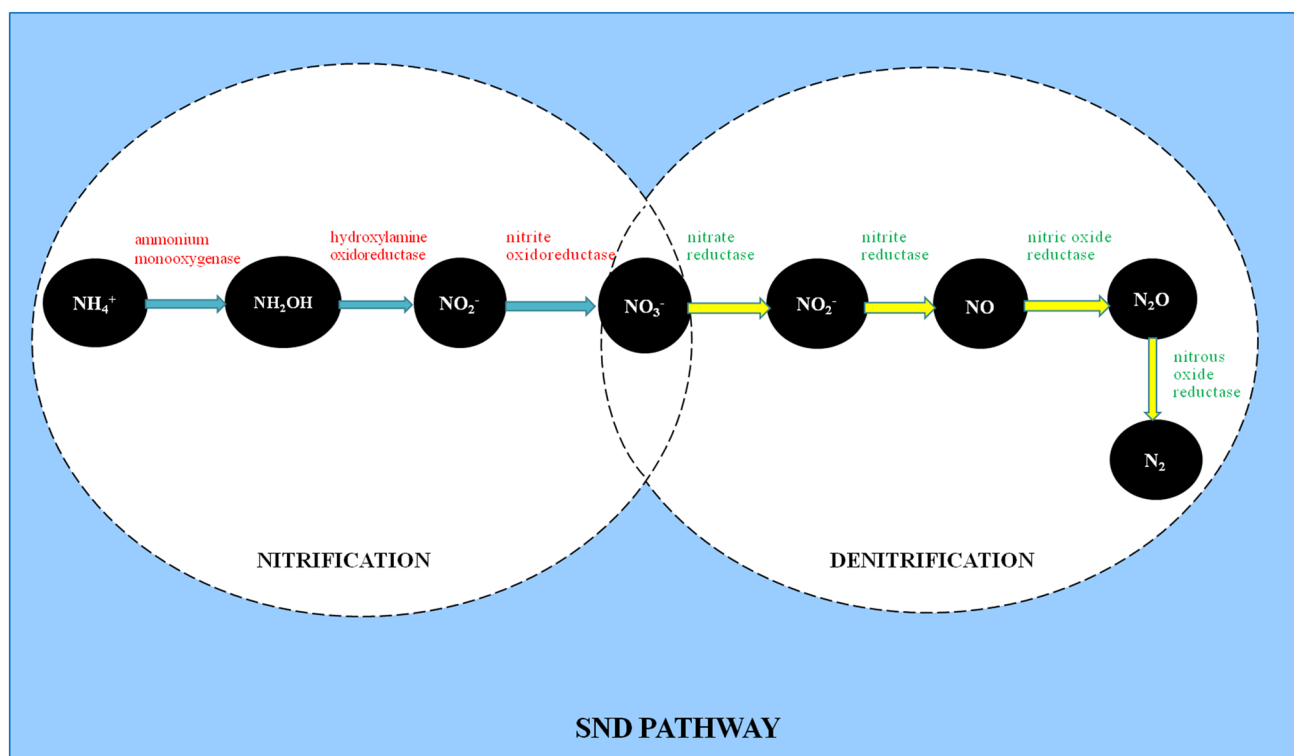


Fig. 1 Simultaneous nitrification–denitrification pathway in bacteria

## Factors Affecting Simultaneous Nitrification–Denitrification

The activity of different organisms involved in nitrogen removal is mainly dictated by environmental conditions. Type of carbon source, C/N ratio, temperature, pH, and levels of dissolved oxygen have a significant influence on nitrogen removal by SND bacteria. Accordingly, the effect of these parameters on simultaneous nitrification–denitrification has been widely investigated.

On the way to accomplish simultaneous heterotrophic nitrification and aerobic denitrification, several carbon sources such as glucose, succinate, acetate, sodium citrate, mannitol, trehalose, and glycerol have been reported for different bacteria including *Pseudomonas* (Yang et al. 2016), *Enterobacter cloacae* (Guo et al. 2016), *Anoxybacillus contaminans* (Chen et al. 2015b), *Paracoccus denitrificans* (Medhi and Thakur 2018), *Bacillus cereus* (Barman et al. 2018), *Pseudomonas putida* (Zhang et al. 2019), *Acinetobacter* sp. (Xia et al. 2020). Wan et al. (2017) used six different carbon sources (glucose, sucrose, mannitol, formic acid, trehalose, and sodium acetate) on *Enterobacter cloacae* HW-15 for SND activity and observed sodium acetate to be the most efficient carbon source. Similarly, Rout et al. (2017) reported 94%  $\text{NH}_4\text{-N}$ , 97%  $\text{NO}_3\text{-N}$ , and 84%  $\text{NO}_2\text{-N}$  removal in 36 h by *Bacillus cereus* GS-5 using sodium acetate as a

carbon source. Glucose was judged as the best carbon source for *Paracoccus denitrificans* ISTOD1 and *Bacillus cereus* (Medhi et al. 2017; Barman et al. 2018). Appropriate carbon source helps to attain a high rate of growth and nitrogen removal in simultaneous heterotrophic nitrification–denitrification. Besides this, the concentration of carbon directs the flow of electrons in the process of nitrogen removal. Therefore, SND is also influenced by an optimum C/N ratio. Extremely low or high C/N ratios can adversely affect the process efficiency by inhibiting the growth of bacteria (Chiou et al. 2007). So far, a C/N ratio between 8 and 10 has been reported for efficient SND (Xia et al. 2020).

Many findings have confirmed that when the level of dissolved oxygen (DO) exceeds its critical value the process of nitrogen removal by aerobic bacteria gets blocked. For example, Hocaoglu et al. (2011) investigated the effects of dissolved oxygen on simultaneous nitrification and denitrification in membrane bioreactors and found that best nitrogen removal could be achieved between dissolved oxygen levels of 0.15 to 0.35  $\text{mgL}^{-1}$ . Further increasing the oxygen concentration significantly reduced the effectiveness of nitrate removal. Jin et al. (2015) have reported DO value between 0.3 and 0.8  $\text{mgL}^{-1}$  as most effective for SND whereas, Li et al. (2018a) carried out simultaneous nitrification and denitrification at 1.2  $\text{mgL}^{-1}$  DO. It is important to maintain DO levels according to

specific strain requirements (Barman et al. 2018). Lei et al. (2019) and Xia et al. (2020) reported  $6.08 \text{ mgL}^{-1}$  to be the best dissolved oxygen concentration for *Ochrobactrum anthropic* LJ81 and *Acinetobacter* sp. ND7, respectively. Therefore the level of dissolved oxygen is critical for simultaneous heterotrophic nitrification-aerobic denitrification process. Temperature is another important factor that impacts the metabolic motion of the organism and improves the reaction rate in simultaneous heterotrophic nitrification-aerobic denitrification (Guo et al. 2013). Most SND bacteria are sensitive to variations in temperature and perform best within a temperature range of  $25\text{--}37 \text{ }^\circ\text{C}$  (Rout et al. 2017; Zhang et al. 2019; Xia et al. 2020). A longer lag phase and interruption in gene expression were observed in SND capable *Pseudomonas mandelli* when grown at  $10 \text{ }^\circ\text{C}$  (Lakha et al. 2009).

One of the drawbacks of the conventional method of nitrogen removal was the need for maintenance of specific pH conditions. While mild alkaline conditions (7–8) are appropriate for nitrification, a weakly acidic environment is preferred by denitrifiers (Lei et al. 2019). pH values closer to either 5 or 10 resulted in no change or increase in nitrogen removal due to the unsuitability of the strongly acidic or alkaline environment for the survival of bacteria (Zhang et al. 2019). However, simultaneous nitrification–denitrification creates, in a cyclic manner, pH conditions appropriate for the process as a whole. The acidity generated during nitrification is suitable for the denitrification process which in turn creates alkaline conditions appropriate for nitrification (Li et al. 2018b). Kim et al. (2005) reported that a pH value of 7 has a positive effect on simultaneous nitrification and denitrification ability of *Bacillus* strain. There are also available strains like *Pseudomonas putida* ZN1 (Zhang et al. 2019) which prefer pH 7 as optimum for processes aerobic denitrification, heterotrophic nitrification, and simultaneous nitrification- denitrification.

The use of heterotrophic nitrification-aerobic denitrification technology for real wastewater treatment is still in its early stages. This is mainly due to the reduced processing efficiency of potential strains. Fluctuations in salinity and nitrogen concentrations in real effluents also have a significant impact on the performance of these strains (Zhang et al. 2012; Gui et al. 2017). Therefore, it is urgent to identify strains capable of performing SND in hostile environments such as those with high concentrations of nitrogen and salt. He et al. (2019) isolated and identified *Pseudomonas mendocina* TJPU04 and examined its cell growth and yield. Results expressed that strain TJPU04 is effective in nitrogen removal from effluent under non-sterile conditions of high salt content and variable nitrogen concentrations. Chlorate is another inhibitor of cell growth and denitrification (Zhou et al. 2014). However, studies performed by Lei et al. (2019) using the strain *Ochrobactrum anthropic* LJ81 confirmed

that the addition of chlorate only affects denitrification and not the nitrification process.

Different studies have employed the use of different organisms and successfully achieved simultaneous nitrification and denitrification. Lee et al. (2001) used a mixed methanotrophic culture in a batch reactor and reported complete nitrate removal within 10 h but ammonia removal at a slower rate. Hibiya et al. (2003) successfully and completely removed nitrogen and carbon components from domestic wastewater by simultaneous nitrification–denitrification in a membrane -aerated biofilm reactor using different bacteria distributed horizontally and vertically in biofilms fixed on hollow fiber membrane in a single reactor. Similarly, Qi et al. (2003) also reported an efficient SND process using aerobic granular sludge and controlling the amount of carbon in a sequencing batch reactor. Khardenaviet et al. (2007) demonstrated the ability of *Diaphorobacter* for simultaneous nitrification and denitrification under aerobic conditions with 85–93% COD removal and 92–96% ammonia removal and suggested their application in the treatment of high-nitrogen-containing wastewaters. *Providencia rettgeri* exhibited the ability to heterotrophically nitrify and aerobically denitrify ammonium within 12–48 h under conditions of C/N 10,  $30 \text{ }^\circ\text{C}$ , 120 rpm (Taylor et al. 2009). Compact suspended carrier biofilm reactor system was operated by Xia et al. (2010) at different C/N ratios (10:1, 5:1, and 3:1) and the whole process was able to achieve 83.3% effective nitrification–denitrification at C/N ratio 3:1.

Chen and Ni (2011) isolated three strains, *Achromobacter* sp. GAD3, *Comamonas* sp. GAD4 and *Agrobacterium* sp. LAD9, from landfill leachate system. Out of these, GAD4 was able to achieve the highest aerobic nitrification–denitrification rate of  $0.381 \text{ mmol L}^{-1} \text{ h}^{-1}$ , followed by LAD9 ( $0.374 \text{ mmol L}^{-1} \text{ h}^{-1}$ ) and GAD3 ( $0.346 \text{ mmol L}^{-1} \text{ h}^{-1}$ ). Qiu et al. (2012) screened *Pseudomonas* sp. as an aerobic nitrifying-denitrifying bacterium from activated sludge with the capability to remove 94% of  $70 \text{ mgL}^{-1} \text{ NH}_4^+\text{-N}$  and 90% of  $50 \text{ mgL}^{-1} \text{ NO}_3^-\text{-N}$ . *Alcaligenes faecalis* C16 identified by Liu et al. (2015) was found to have the ability to heterotrophically nitrify and aerobically denitrify in the presence of both nitrate and ammonium. High ammonium tolerance of the organism was associated with the use of citrate and acetate as carbon sources with a C/N ratio of 7 for acetate and 14 for citrate. Lei et al. (2016) identified heterotrophic *Zobellella taiwanensis* DN-7 for the conversion of nitrite, nitrate, and ammonium to  $\text{N}_2$  as the primary end product and advocated it as a promising candidate for high-strength ammonium wastewater treatments.

Flat-panel air-cathode microbial fuel cell (FA-MFC) system using *Nitrosomonas* and *Nitratireductor* sp. was developed by Park et al. (2017) for treating domestic wastewater and complete removal of total nitrogen was achieved at a rate of  $0.62 \text{ kg-Nm}^{-3} \text{ d}^{-1}$ . Zhang et al. (2019) performed nutrient

removal by a *Pseudomonas putida* strain and accomplished 97.47% ammonium, 86.08% nitrate, and 71.57% nitrite removal by heterotrophic nitrification-aerobic denitrification. *Ochrobactrum anthropic* was found to remove sole and mixed nitrogen sources without accumulation of nitrite during the SND process wherein more than 80% of initial nitrogen was converted into gaseous nitrogen (Lei et al. 2019).

Disproportionate levels of nitrogenous pollutants in different ecological niches with different environmental conditions highlight the need for metabolic diversity and robustness among the bacterial community to achieve successful nitrogen removal from wastewaters. So far, the identification and performance of SND capable microorganisms are quite promising, especially in the most prevalent conditions of variable COD levels. However, it is necessary to further evaluate and establish their performance under in situ stress conditions of heavy metals, salt, and the presence of other microflora.

## Phosphorous accumulating microorganisms

Excessive discharge of phosphorous into water bodies leads to eutrophication and other perilous effects associated with it. Enhanced biological phosphorus removal (EBPR) is the most effective and sustainable method to get

rid of phosphorus from wastewaters (Nielsen et al. 2019). It employs the phosphate accumulating organisms (PAO) which follow an alternative aerobic-anaerobic cycle (Liu et al. 2018). During the anaerobic phase, PAOs hydrolyze the intracellular phosphate and glycogen reserves for energy generation. This energy is utilized for the uptake and storage of volatile fatty acids in the form of polyhydroxyalkanoates (PHAs). PHA synthesis is accompanied by the extracellular release of phosphate ions. Uptake of organic matter gives a competitive advantage to PAOs. During the aerobic phase, these organisms degrade the accumulated PHAs as a source of energy and use a part of this energy to accumulate more phosphorous into their cells than that released during the anaerobic phase and store it as polyphosphate, thus removing phosphorus from wastewaters (Shen and Zhou 2016). Diagrammatically the process is depicted in Fig. 2. Microbial genera capable of EBPR include *Microcylunatus*, *Tessaracoccus* (Stokholm-Bjerregaard et al. 2017), *Tetrasphaera* (Marques et al. 2018), *Candidatus Accumulibacter* (Rubio-Rincon et al. 2019) and *Dechloromonas* (Anuar et al. 2020) along with *Pseudomonas stutzeri* (Li et al. 2015), *Enterobacter cloacae* (Wan et al. 2017), *Arthrobacter* sp. (Zhang et al. 2020). *Candidatus Accumulibacter phosphatis* is considered important for phosphorous removal since it has been consistently found in wastewaters (Marques et al. 2018; Nielsen et al. 2019). It belongs to the family *Rhodocyclaceae*

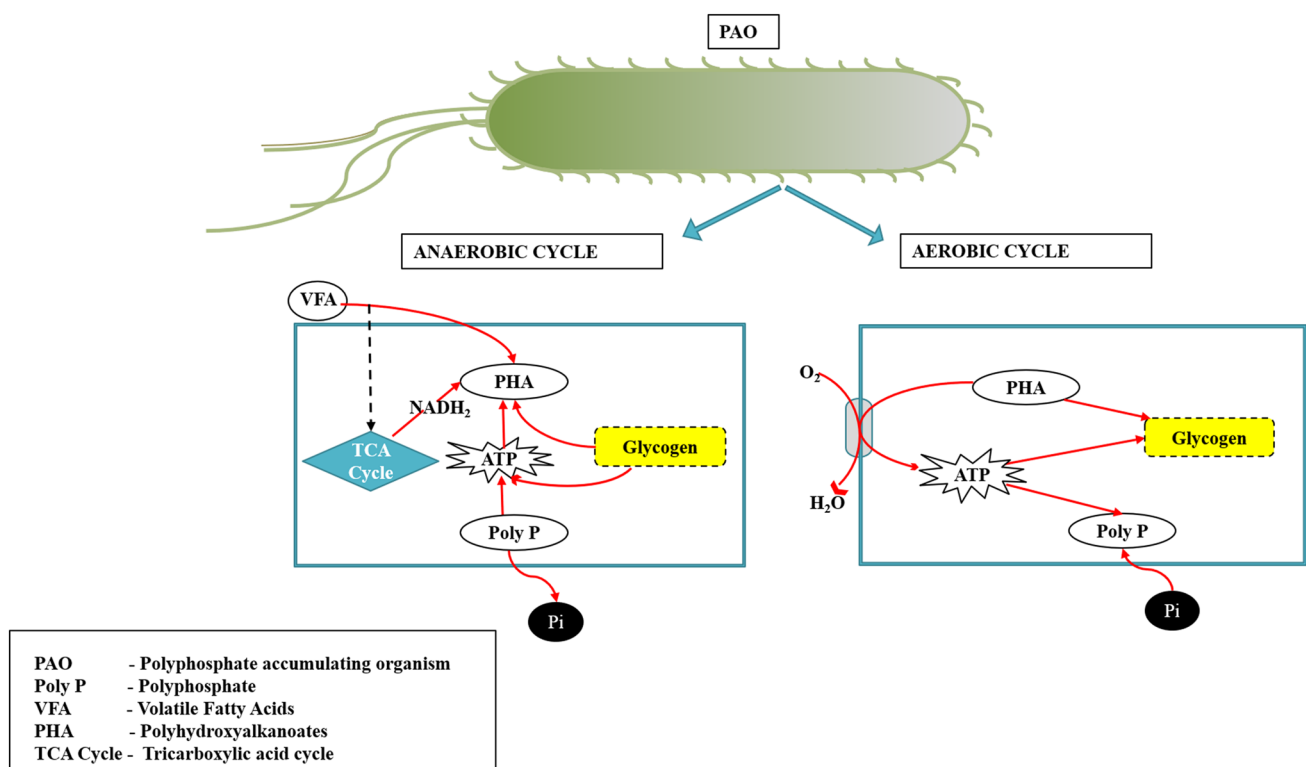


Fig. 2 Biological route of phosphorous removal



of class *Beta Proteobacteria* (Dorofeev et al. 2019). Activated sludge from wastewater treatment plants is the most well-known habitat of *Candidatus Accumulibacter phosphatis* besides sediments of estuaries and freshwater bodies (Dorofeev et al. 2020).

Process of phosphorous uptake is catalyzed by the activity of the enzyme polyphosphate-adenosine diphosphate phosphotransferase, also known as polyphosphate kinase (ppk), encoded by the *ppk* gene (Li et al. 2015). It helps in the elongation of the poly-P chain (Achbergerova and Nahalka 2011). PAOs chiefly prefer volatile fatty acids (acetate and propionate) for the synthesis of intracellular polyhydroxyalkanoates (PHAs). Their activity gets augmented at a pH between 6.5–7.5 and temperatures between 15–30 °C (Nancharaiah et al. 2016). Recently, Zhang et al. (2020) tested different conditions of pH and temperature and achieved above 99% phosphorus removal by *Arthrobacter* sp. HHEP5 at temperatures between 18–28 °C and pH 5.5–8.5.

PAOs have a higher capacity for intracellular phosphorus accumulation than the non-phosphorous accumulating heterotrophic bacteria (Dorofeev et al. 2020). They may be used in laboratory-scale reactors and full-scale wastewater treatment plants forming a dominant part of the bacterial community involved in the removal of phosphorus (Nancharaiah et al. 2016). However, low phosphorus removal efficiencies have been reported in PAOs identified so far. Therefore it is important to focus more on the isolation and identification of strains of PAOs with higher efficiencies since most species of known PAOs are significantly rare.

DNPAOs or the denitrifying phosphate-accumulating organisms are a subgroup of PAOs. These microorganisms are metabolically similar to PAOs (Bassin et al. 2012). Most significant DNPAOs belong to class *Beta Proteobacteria* and are *Dechloromonas* and *Zoogloea* (Kondo et al. 2009). Whereas PAOs can only use oxygen as an electron acceptor for respiration, DNPAOs can also use nitrite or nitrate (Yuan and Oleszkiewicz 2010) to absorb phosphates by degrading PHAs (Zhang et al. 2010). However, the affinity of PAOs towards oxygen is higher and phosphorous uptake faster than the DNPAOs (Salehi et al. 2019). Even then, the chemical oxidation demand is a limiting factor for PAOs and is overcome by the process of simultaneous nitrification–denitrification and phosphorous removal (SNDPR) that can achieve the removal of both nitrogen and phosphorus at low carbon levels in wastewaters (Bassin et al. 2012).

## Simultaneous Removal Of Nitrogen And Phosphorus

The discovery of microorganisms capable of performing both simultaneous nitrification–denitrification and phosphorous removal (SNDPR) makes single-stage removal

of nitrogen and phosphorous an attractive strategy (He et al. 2016a). So far, only a few microbial species with this potential have been cited (Table 2). These are mostly found in aquaculture environments and employ the same set of enzymes and genes as those involved in the individual processes of EBPR and SND (Zhang et al. 2020). A model for the mechanism of SNDPR is illustrated in Fig. 3. Under heterotrophic aerobic conditions, organisms capable of SNDPR are concomitantly able to convert nitrogen compounds (mainly ammonium and nitrate) and uptake phosphorous intracellularly. While phosphorous gets accumulated in the form of polyphosphate, ammonium and nitrate undergo a series of enzyme-catalyzed steps resulting in the formation of dinitrogen. Part of this nitrogen is used up by the organism for its growth and the rest escapes into the environment.

SNDPR strains perform stable nitrogen and phosphorus removal at a low C/N ratio (Chen et al. 2020). The effective temperature is within the mesophilic range and pH between 7 and 8 (Wang et al. 2018; Salehi et al. 2019). Major advantages associated with the SNDPR process are lower COD demand, lesser sludge production, and low aeration cost (Wang et al. 2016). The potential of SNDPR organisms promises to solve the problem of bacterial changes to remove both nitrogen and phosphorus from wastewaters (Wan et al. 2017). Also, their success rate ensures a cost-effective and sustainable strategy for wastewater treatment.

## Role of SNDPR organisms in bioremediation

The identification of novel bacteria with a unified potential of SND and EBPR has opened ways for the simultaneous treatment of accumulated nutrients in industrial and domestic wastewaters, eutrophic lakes, and aquaculture systems. However, the numbers and efficiency of these bacteria known so far are extremely low and their applicability is still very limited. Yang et al. (2010) performed simultaneous removal of nitrogen and phosphorus in a sequencing batch moving bed membrane bioreactor. COD/N ratio was kept between 5.6–12.9 and 93.5% carbon and 82.6% nitrogen removal was achieved. Also, an increase in time duration by 2 h resulted in 84.1% total phosphorus removal. It was also observed that dissolved oxygen (3 mg L<sup>-1</sup>) in the aerobic phase was a major factor contributing to the simultaneous removal of nitrogen and phosphorus.

Over 13 months, synthetic wastewater was treated under alternating anaerobic and aerobic conditions in a lab-scale sequencing batch reactor sown with granular sludge. The loading rates of organic carbon, nitrogen, and phosphorus were 2.7 g CODL<sup>-1</sup> d<sup>-1</sup>, 0.43 g NL<sup>-1</sup> d<sup>-1</sup>, and 0.06 g PL<sup>-1</sup> d<sup>-1</sup> respectively and removal efficiency of 68% total COD, 86% total nitrogen, and 74% total phosphorous were obtained indicating that granular sludge facilitated the

**Table 2** Microbial spp involveld in simultaneous removal of nitrogen and phosphorus

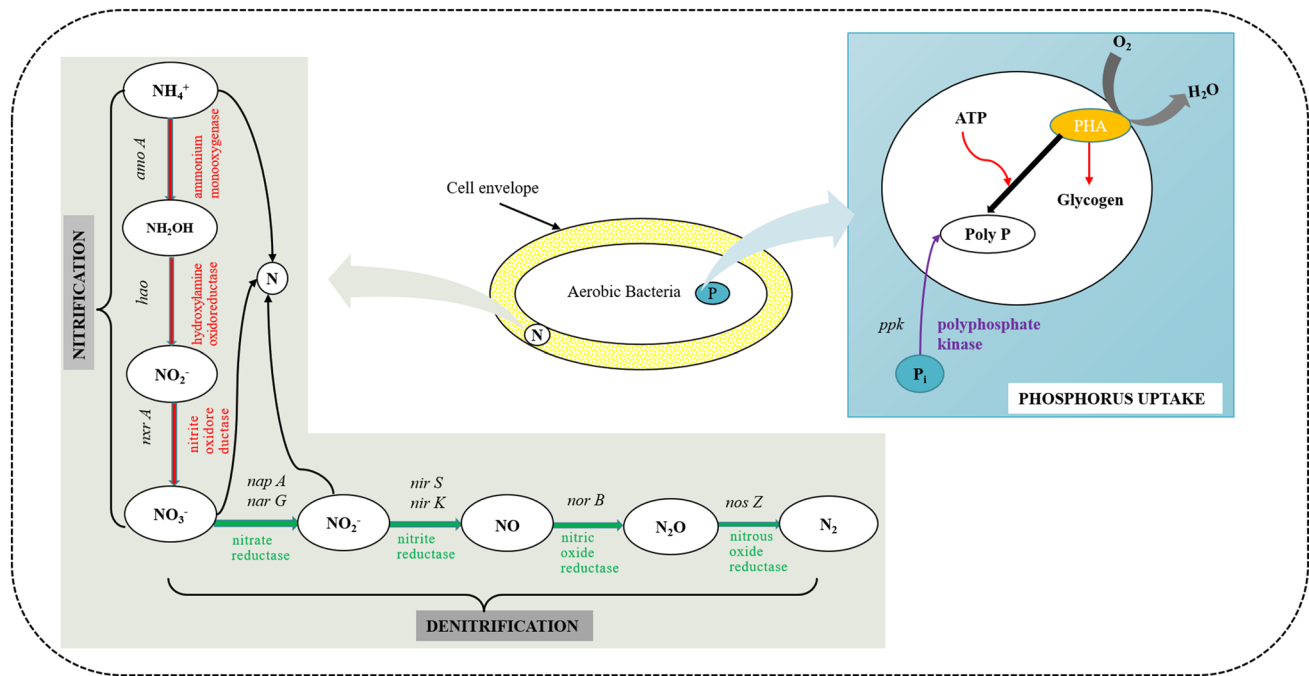
Bacterial strain	Reactor	Isolation site	Country	Type of waste-water	Nitrogen removal efficiency (%)	Phosphorus removal efficiency (%)	References
<i>Auxenochlorella protothecoides</i> UMN280	–	Saint Paul, Minnesota	United States	Municipal wastewater	59%	81%	Zhou et al. (2012)
<i>Pseudomonas stutzeri</i> YG-24	–	Beijing	China	Domestic wastewater	80%	51%	Li et al. (2015)
<i>Bacillus cereus</i> GS-5	–	Bhubaneswar, Odisha	India	Domestic wastewater	–	–	Rout et al. (2017)
<i>Enterobacter cloacae</i> HW-15	–	Beijing	China	Domestic wastewater	96.16	73%	Wan et al. (2017)
<i>Pseudomonas denitrificans</i> ISTOD1	–	JNU, New Delhi	India	Domestic wastewater	71%	93%	Medhi and Thakur (2018)
<i>Accumulibacter</i> sp	Sequencing batch reactor (SBR)	Queensland	Australia	Abattoir wastewater	93%	89%	Yilmaz et al. (2008)
<i>Bacillus origin</i> MCC0008, MCC2059, MCC2071	Glass and Scaled up Bioreactor	Keshtopur, Kolkata	India	Domestic wastewater	94%	68%	Saha et al. (2018)
<i>Cadidatus Competibacter</i> , <i>Cadidatus Accumlibacter</i> , <i>Tetrasphaera</i>	Partial nitrification endogenous denitrification and phosphorus removal (PNEDPR)	Beijing	China	Municipal wastewater	86.8%	90.9%	Zhao et al. (2019)
<i>Pseudorhodobacter</i>	Sequencing batch biofilm reactor (N-SBBR)	Tianjin	China	Domestic wastewater	75.47%	65.87%	Chao et al. (2020)
<i>Arthrobacter sp.</i> HHEP5	–	–	–	Domestic wastewater	95%	99%	Zhang et al. (2020)

SNDPR process (Yilmaz et al. 2008). Similarly, Kishida et al. (2009), Lochmatter et al. (2013), and Wei et al. (2014) also successfully used aerobic granular sludge systems for simultaneously removing nitrogen and phosphorus from wastewaters.

*Auxenochlorella protothecoides*, a microalgal strain was examined for nutrient removal and showed 59% removal of total nitrogen and 81% total phosphorus removal from municipal wastewater in six days (Zhou et al. 2012). Rasoul-Amini et al. (2014) used batch cultures of five microalgae strains for removal of  $\text{NO}_3\text{-N}$  and orthophosphate ( $\text{PO}_4^{3-}\text{-P}$ ) from wastewater. It was observed that *Chlorella* sp. (YG01) achieved a higher N removal (84.11%) whereas strains *Chlamydomonas* sp. (YG05) and *Chlamydomonas* sp. (YG04) achieved higher P uptake (100%) from wastewater. Similarly, a microalgae-bacteria consortium was prepared by Delgadillo-Mirquez et al. (2011) for the removal of nutrients (nitrogen and phosphorus) and achieved an average of 72–83% nitrogen and 100% phosphorus removal, besides

converting these nutrients into biomass. Therefore, even microalgae have been found helpful in the removal of nutrients from wastewater.

Phosphate accumulating bacteria *Pseudomonas stutzeri* YG-24 (Li et al. 2015) and *Enterobacter cloacae* HW-15 (Wan et al. 2017) have presented a great ability of nitrogen and phosphorus removal from real wastewaters. *Achromobacter* and *Agrobacterium* also possess a versatile genomic potential for concurrent removal of nitrogen and phosphorus in a single unit system during the treatment of wastewater (Liu et al. 2018). Rout et al. (2017) used a *Bacillus cereus* strain and reported efficient simultaneous removal of ammonium, nitrate, nitrite, and phosphate from domestic wastewater with average rates of 2.62, 2.69, 1.16 and 0.42  $\text{mg L}^{-1} \text{h}^{-1}$  respectively under aerobic conditions. Zhang et al. (2020) isolated *Arthrobacter* sp. HHEP5 as a novel PAO from a mariculture environment and demonstrated its ability in mariculture and domestic wastewater treatment with 99% phosphorus and 95% nitrogen removal



**Fig. 3** A model for the simultaneous nitrification–denitrification and phosphorous removal (SNDPR) in aerobic microorganisms

in both the environments. They also reported that varying the environmental factors (pH, temperature, C/N ratio, P/N ratio, salinity, and rpm) improved the removal efficiencies of HHEP5.

The application of a microbial consortium is one of the fastest and energy-efficient technologies to date. Three different strains of *Bacillus* (MCC 0008, MCC 2059, MCC 2071) were employed as a consortium at ambient temperature in a biofilm reactor and showed simultaneous sequestration of 94% nitrate, 68% phosphorus, 93% COD, 97% BOD and 73% total organic carbon (Saha et al. 2018). Zhao et al. (2019) appraised a novel system of nitrogen and phosphorus removal that could aid the removal of nitrogen deprived of carbon wherein *Candidatus* Competibacter ensured 86.8% nitrogen removal and *Candidatus* Accumulibacter phosphatis and *Tetrasphaera* achieved 90.9% phosphorus removal. Rout et al. (2018) accomplished highly effective removal of nitrogen and phosphorus at the same time by immobilizing *Bacillus cereus* GS-5 strain in an inventive single unit multi-layered packed bed bioreactor which also included some packing parts containing a mixture of solid organic wastes and dolochar. The reactor was operated for 70 days using both synthetic and domestic wastewater and was able to achieve 87–93% removal of  $\text{NH}_4^+\text{-N}$ , 69–88%  $\text{NO}_3\text{-N}$  removal, 84–100%  $\text{PO}_4^{3-}\text{-P}$  removal and also 69.8–92% reduction of COD.

The bioremediation of nitrogen and phosphorous pollutants is an economical and sustainable approach in comparison to other physical and chemical methods. However,

the focus of most studies until now has been the microbial analysis of this system, and its adaptability and efficacy are quite unsatisfactory. The overall process requirements and time management become a lot simpler but performance would depend on the ability of microorganisms to increase removal efficiencies and overcome natural environmental conditions which bring in the need to explore newer strains with enhanced abilities for simultaneous removal of nitrogen and phosphorous under aerobic conditions using cost-effective substrates.

### Immobilization of biological components for removal of inorganic contaminants from water

Immobilization technology is receiving a lot of attention in treating wastewaters. Immobilization involves the restricted movement of cells by natural or artificial means. Some of the most common immobilization techniques include covalent coupling, affinity immobilization, capture behind the semi-permeable membrane, entrapment in polymers, and adsorption (Xie et al. 2020). Basic requirements for immobilized systems such as retention of viability, high cell density, and low leakage of cells from a matrix must be fulfilled for successful water treatment. Apart from these, an ideal matrix for immobilization should be chosen which is resistant to disruption, non-toxic, and able to retain biomass. Studies have stated that immobilized cells can increase the removal

efficiency of nutrients by more than 60% (Bouabidi et al. 2019). Compared to free cells, immobilization of cells offers several great advantages in bioremediation such as increased conversion rates due to higher cell loading densities, continuous supply of nutrients without any competition with other organisms (De-Bashan and Bashan 2004), protection against environmental stress, no need for cell suppression (De-Bashan and Bashan 2010) and elimination of washout possibilities even at high dilution rates hence allowing a continuous process.

Different studies report the immobilization and co-immobilization of organisms performing specific functions for the treatment of waters containing high concentrations of nitrogen and phosphorous (Shen et al. 2017). For example, co-immobilization of nitrifiers and denitrifiers allows the two separate processes of nitrification and denitrification to be conducted as if single staged. In this direction, Santos et al. (1996) prepared a simultaneous or one reactor system using a bubble column reactor in which *Nitrosomonas europaea* and *Pseudomonas denitrificans* were co-immobilized in a double layer of (25% v/v) alginate carrageenan beads and successfully achieved a high nitrogen removal rate ( $5.1 \text{ mmol N m}^{-1} \text{ gel s}^{-1}$ ). Similarly, Hill and Khan (2008) conducted a bench-scale study using two reactor systems to remove ammonia from sludge digester. One system comprised of immobilized nitrifiers and the other consisted of both co-immobilized denitrifiers and nitrifiers. It was seen that the co-immobilized cell reactor removed 8.5% more total nitrogen than the cell reactor with individually immobilized nitrifiers. Large voids caused due to  $\text{N}_2$  gas in several co-immobilized beads samples provided evidence of denitrifying activity.

Immobilization of microorganisms involved in nitrogen and phosphorous removal has also been carried out using different organic and inorganic carrier materials with a high success rate. *Rhodobacter sphaeroids* was immobilized on a porous ceramic plate for denitrification of sewage water and a high nitrate removal rate of  $5.04 \text{ kg m}^{-3} \text{ d}^{-1}$  was obtained (Nagadomi et al. 2000). Phosphate accumulating strain *Pseudomonas stutzeri* YG-24 was isolated from Taihu Lake, China, and immobilized in polyvinyl alcohol -sodium alginate beads. Even though both the free and immobilized cells were able to remove 97% to 100% phosphorus from synthetic wastewater, the removal process was faster using immobilized cells (8 h) than the free cells (12 h) (Li et al. 2012). Similarly, Wang et al. (2013) isolated and immobilized denitrifying *Pseudomonas* using polyvinyl-alcohol and reported that immobilized pellets had a significantly higher nitrate removal rate than the non-immobilized bacteria. Complete nitrate removal was achieved when pellets were reused in 12 h.

Ma et al. (2015) used three different supports, sodium alginate beads, mycelial pellets, and polyurethane foam

cubes for immobilization of *Pseudomonas stutzeri* and achieved excellent nitrogen removal. Immobilization in sodium alginate beads had 57.25% nitrogen removal as compared to 29.7% by free cells. However, Tang et al. (2020) used gel beads of sodium alginate, graphene oxide, and polyvinyl-alcohol as a carrier material for *Pseudomonas fluorescens* Z03 to improve nitrogen removal efficiency at very low temperatures of 6–8 °C and found graphene oxide beads to be most effective with a removal efficiency of 96.38–97.24% and 98.82–99.12% for  $\text{NH}_4^+-\text{N}$  and  $\text{NO}_3^--\text{N}$  respectively.

Chen et al. (2015a) incorporated biomass entrapped in bio-plates having cellulose triacetate structure into a reactor basin for carrying out simultaneous nitrification and denitrification and observed that bio-carriers were able to achieve 74% total nitrogen removal in domestic effluent without any supplemental carbon or alkalinity. This demonstrated the immobilization of biomass to be a very efficient mechanism for wastewater treatment plants. Simultaneous removal of nitrogen and phosphorus was achieved by Yin et al. (2015) using a sequencing batch reactor-biofilm system with an average removal of 95% COD, 94% total nitrogen, and 97% total phosphorus over 3 months. Li et al. (2016) used a bio-trickling filter packed with biochar from porous palm residues and inoculated with microbial consortia for treatment of nitrogen and phosphorus-rich wastewater and achieved 80% ammonium and 68% phosphorus removal. *Acinetobacter* sp. TX5 was immobilized on the spent *Hypsizygyus marmoreus* substrate for simultaneous nitrification and denitrification of raw piggery wastewater (Yang et al. 2018). In a batch system, the immobilized *Acinetobacter* sp. TX5 achieved 89%  $\text{NH}_4^+-\text{N}$  removal in 8 h; and in a continuous system it achieved 94–95%  $\text{NH}_4^+-\text{N}$ , 73–93% total nitrogen and 54–82% COD removal in 96 h.

Katam and Bhattacharyya (2019) compared the use of immobilized microalgae and suspended activated sludge to suspended co-culture for nitrogen and phosphorous removal and concluded that immobilization of algae in alginate beads was synergistically better than suspended co-culture. Nancharaiah and Sarvajith (2019) and He et al. (2020) have promoted the use of aerobic granular sludge composed of self-immobilized microbial groups to achieve simultaneous nitrogen and phosphorus removal (SNDPR) from industrial and domestic wastewaters. Therefore the performance of microorganisms involved in nutrient removal from wastewaters has been enhanced using different immobilization matrices. However, the nature of the immobilization material can further influence the removal process which may also depend on the type of microbial species, wastewater, and environmental conditions.

## Conclusion

The bioremediation of polluted waters is imperative to maintain water quality. Among the diverse range of organisms that can participate in the biological removal of inorganic pollutants from wastewaters, the potential of bacteria with the ability for simultaneous heterotrophic nitrification aerobic denitrification is far-reaching. Faster growth, simpler process requirements, conversion from ammonium to nitrogen, and the ability to perform under a variety of environmental conditions, places them ahead of other organisms with similar functions. Further, identification of novel phosphate accumulating bacteria that can remove both phosphorous and nitrogen has opened ways for the simultaneous treatment of accumulated nutrients. The success of SNDPR organisms ensures an economical and sustainable strategy and promises to overcome problems encountered in wastewater treatment such as the need for a shift in the microbial community due to different process requirements. However, studies about their adaptability and applicability are still very limited. Performance would depend on the ability of these microorganisms to increase removal efficiencies and overcome natural environmental conditions. The use of immobilized microorganisms over free cells can further enhance the removal rates. Therefore, it is urgent to explore newer strains with enhanced abilities for simultaneous removal of nitrogen and phosphorous using cost-effective and environmentally responsive substrates for the total eradication of contaminants from wastewater.

## Future prospects

The dangers of nutrient pollution need to be addressed to find long-term, economical solutions. Although the discovery of aerobic heterotrophic bacteria that can simultaneously remediate both nitrogen and phosphorous in a single unit is a boon to a great extent, there are still deficiencies that need to be overcome to achieve complete removal. There is a persistent requirement of extensively researched efficient strains that can withstand different environmental conditions to treat real wastewaters. The role of various genes and variability in metabolic pathways followed in aerobic organisms also need further understanding. Knowledge of the ecological interactions of these organisms with other microbiota can assist in the development of efficient consortia which can be applied to treat several other contaminants as well. There is an increasing need to focus on improving the performance and applicability of these organisms and the use of various

natural carbon sources to reduce process costs. Thus, till the time these gaps are filled, the process of simultaneous nitrification–denitrification by phosphate accumulating microorganisms remains a partially fulfilled task in the treatment of contaminated waters.

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