NITRATES AND PHOSPHATES REMOVAL FROM GROUNDWATER:

A REVIEW OF METHODS OF BIOREMEDIATION

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APRIL 2020

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A DISSERTATION REPORT

Submitted in Partial Fulfilment

of

The Degree of M.SC. (Biochemistry)

By

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To the

School of Chemical Sciences

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APRIL 2020

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Abstract

Keywords: Nitrates, phosphates, eutrophication, groundwater contamination, methemoglobinemia, bioremediation, phytoremediation.

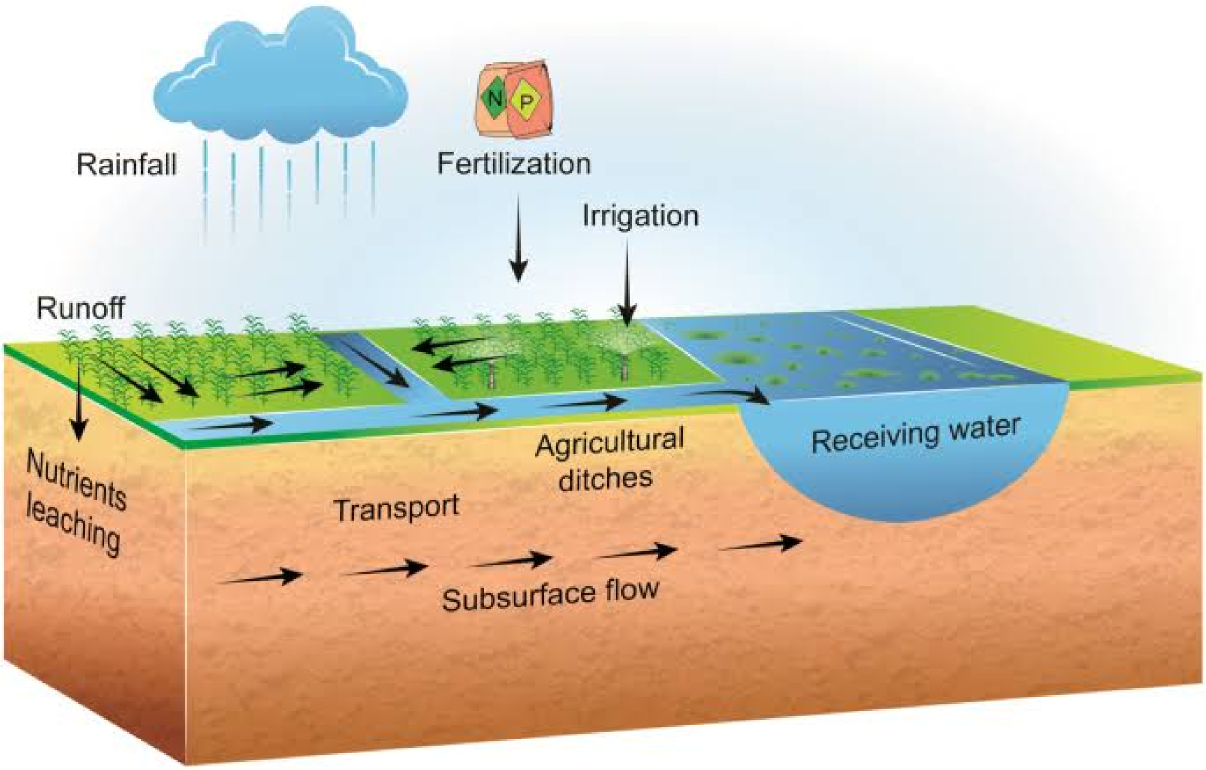
1.Introduction

Water is of fundamental importance for life on earth. Water plays an important role in maintaining plant and animal life. Water makes up 60 – 75% of human body. Plants require water for transfer of nutrients and photosynthesis. Water governs and supports all life forms. About 70% of the earth's crust is covered with water, out of the 70% only 2.5 – 3% is freshwater rest is saline water. 2.5 - 3% of freshwater, 69% is glacier and snow cover, 30% is groundwater and remaining is lakes and rivers. Groundwater is the largest source of unfrozen freshwater. Groundwater is a major source of freshwater used of agriculture, industrial and domestic use. Due to increase in population, industrialization and development there has been over exploitation of groundwater resource. Groundwater supplies more than half of all the freshwater used for drinking in the world. Worldwide there are concerns of groundwater contamination with pollutants. The groundwater contaminants may have natural or anthropogenic sources. Some natural sources may include seawater, poor quality surface water, minerals, bacteria and algae. Major anthropogenic sources of groundwater contamination are industrial discharge, agricultural runoff, septic leakage etc.

In waterbodies nitrogen and phosphorus exist in the form of nitrates and phosphates. Nitrates in the water are used by algae and fishes to synthesize amino acids and proteins. Phosphorus is essential for cellular growth and reproduction as it is a vital nutrient for converting sunlight unto usable energy. Excessive nitrates can cause severe illness in infants and domestic animals. (Li et al. 2022)

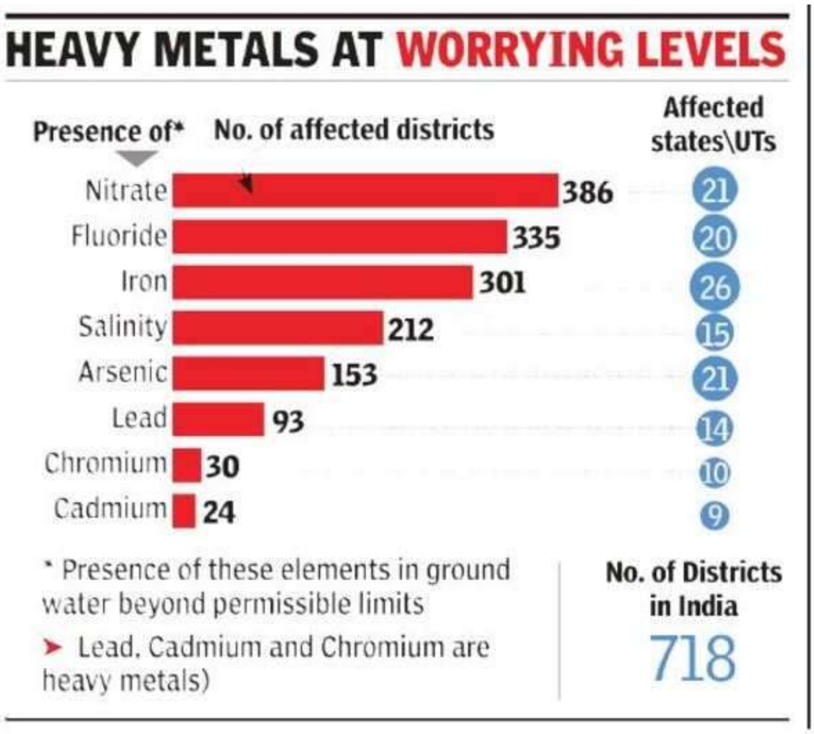
The fertilizers used for the enhancement of the crops contribute to major groundwater contaminants. Fertilizers are used so as to provide nutrients to the crop and increase the yield. Nitrogen, phosphorus and potassium are the 3 primary nutrients in commercial fertilizers. Each of these nutrients have a key role in plant nutrition. NPK fertilizers contribute to major groundwater contaminants Nitrates (NO_3) and Phosphates (PO_4). Nitrate is one the main groundwater pollutant. The large quantities of fertilizers applied to the crops contribute to nitrate and phosphate contamination of groundwater. The applied fertilizers diffuse into the groundwater. The applied fertilizer diffuses into the groundwater due to irrigation and rainfall. Groundwater nitrate and phosphate contamination has been reported from regions from all over the world. Nitrate and phosphate contamination exceeding the permissible limit makes the groundwater unfit for consumption.

A figure explaining leaching of NPK fertilizers into groundwater.



SOURCE: Xia, Y., Zhang, M., Tsang, D.C.W. et al. Recent advances in control technologies for non-point source pollution with nitrogen and phosphorus from agricultural runoff: current practices and future prospects. Appl Bio Chem 63, 8 (2020).

Over the years nitrate contamination in groundwater has increased in many countries. Current nitrate contamination scenario in Goa is of major concern as the nitrate levels in groundwater are high. Major toxins in groundwater of India.



SOURCE - Across India, high levels of toxins in groundwater. https://m.timesofindia.com/india/govt-body-find-high-levels-of-groundwater-contamination-across-india/articleshow/65204273.cms. Accessed on 28/03/22

1.1 Effects of Nitrate and Phosphate pollution in groundwater

Nitrate contamination is a global problem. Nitrates and phosphates containing compounds when released in water without treatment pose a serious problem to human health.

1.1.1 Effect on Human health

Nitrate is a stable and chemically unreactive species of nitrogen and is the most oxidised form of nitrogen (Huno et al. 2018). Nitrate contamination in groundwater is life threatening. High levels of nitrate and phosphates in drinking water can have negative health effects. In infants nitrate poisoning is a major concern. Infants consuming water contaminated with high nitrate level can cause methemoglobinemia, also referred as “blue baby syndrome”. The drinking water standard for nitrate set by the WHO is 50mg/L.

1.1.1a Methemoglobinemia

Characterized by blue skin colour caused by red blood cells unable to carry oxygen from the lungs to the rest of the body. The standard for nitrate in drinking water is set at 10mg/L for babies under age of 3 months. Infants under the age of 3 months are sensitive to nitrate toxicity. The bacteria present in the digestive tract of such infants readily convert nitrate to toxic nitrite. Nitrite converts haemoglobin to methaemoglobin. This methaemoglobin is more stable than the oxygen haemoglobin complex that facilitates the oxygen transport. Methaemoglobin prevents the transport of oxygen. As the concentration of methaemoglobin exceeds more than 5% in blood the first symptoms are noticeable and when concentration exceeds 50% it leads to anoxia. As the babies grow the acid increases in the stomach and bacteria in the digestive tract also change and hence nitrate is not that readily converted to nitrite (Adelana, 2005).

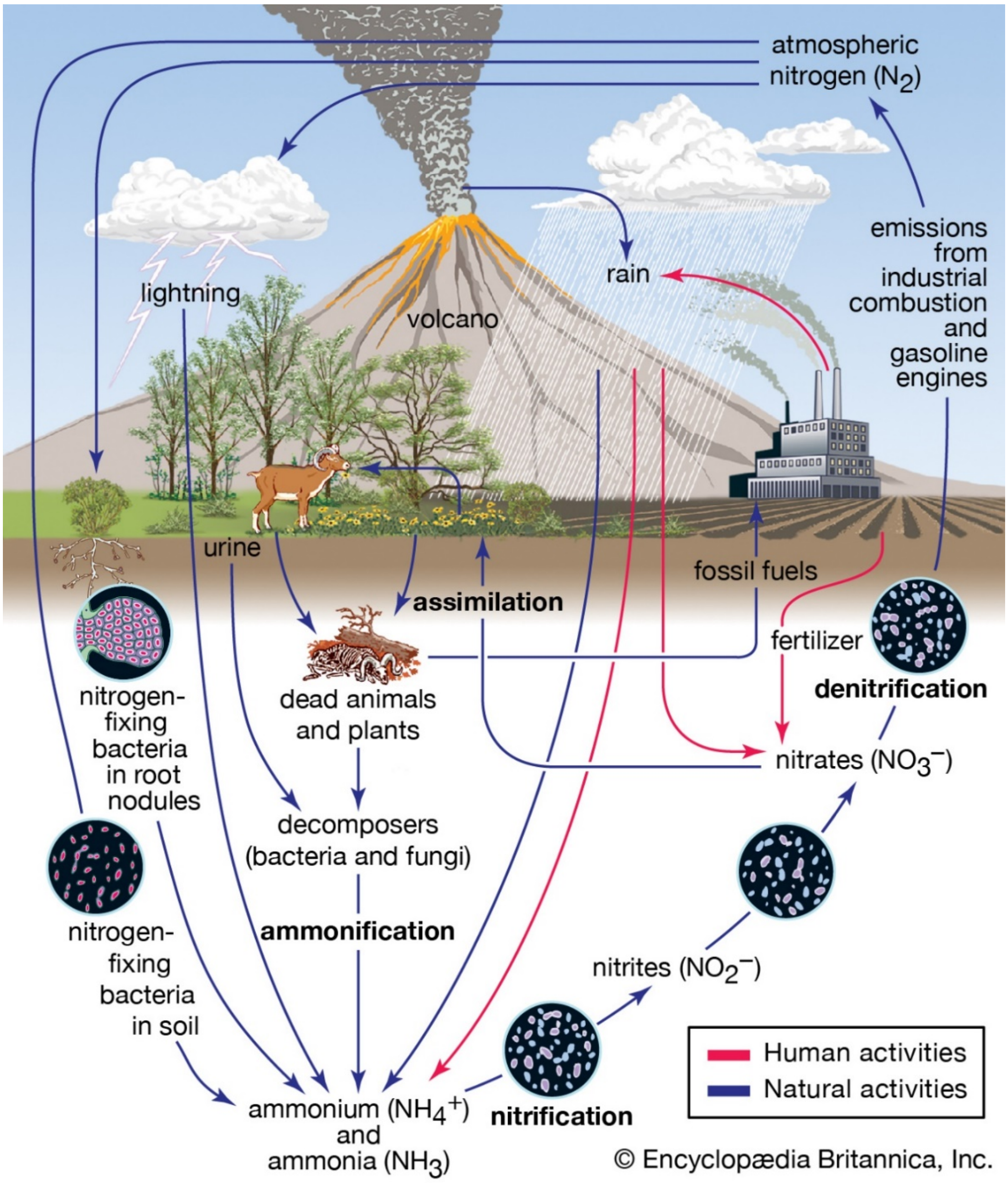
High nitrate concentration is known to cause gastric cancer and other health effects in adults (Majumdar and Gupta, 2000).

High levels of phosphates in drinking water can cause digestive problems. Phosphates are not toxic to the human health unless it is present in high concentrations. Beyond permissible limits it causes kidney damage and osteoporosis (Kumar and Puri 2012; Adelana, 2005).

1.1.2 Effect on Environment

Industrialization has caused major concerns of environmental pollution. It has led to pollution of soil, air and water. Caused due to discharge of untreated chemicals and effluents into the environment. As nitrates and phosphates concentration increases in water body like lakes it enhances the growth of aquatic plants and algae. High levels of nitrates and phosphates in waterbodies like lakes stimulates the growth of algae and aquatic plants. Excessive growth of aquatic plants and algal blooms diminishes light penetration and the water becomes depleted in oxygen hence decreases the aquatic life(fish) affecting the entire ecosystem. Creating negative effect on water quality such as bad odour, decreased oxygen levels eventually suffocating the aquatic life(fish) and eventually resulting in eutrophication. The waterbodies like lakes over the years of time are converted into small forest covers.

1.2 NITROGEN CYCLE



SOURCE: https://www.britannica.com

Nitrogen gas (N_2) makes up 80% of the Earth's atmosphere. The nitrogen in the gaseous form cannot be used by the plants and animals. The nitrogen gas needs to be converted into a form that can be used by the living beings. The nitrogen cycle describes how the nitrogen moves through the environment.

STEP 1: Nitrogen Fixation

Nitrogen is a part of all amino acids which make proteins that are an important part of the cell. Nitrogen gas cannot be used by plants so the first step converts the nitrogen gas into a form that can be used by the plants. The process of conversion of nitrogen gas into ammonia (NH_3) is called nitrogen fixation

$$\mathbf{N_2 + H^+ \rightarrow NH_3 + H_2}$$

Nitrogen fixation is carried out by nitrogen fixing bacteria in the soil and bacteria in the roots of certain plants like legumes (e.g., Peas, clover, soybeans). Rhizobium species are mostly associated with root nodules and carry out nitrogen fixation. The nitrogen fixation is facilitated by enzyme complex called nitrogenase that catalyse the reduction of N_2 to NH_3. Nitrogen fixation also occurs when lightning strikes the air. Plants metabolize ammonia and use it.

STEP 2: Nitrification

Nitrification is the process in which ammonia is converted to nitrite (NO_2^-) and further nitrite is converted to nitrate (NO_3^-). Not all ammonia produced from nitrogen fixation is taken up by plants excess ammonia is converted to nitrates. The nitrification steps are exclusively carried out by prokaryotes. The first step in nitrification is oxidation of ammonia to nitrite. This step is carried out by microorganisms called as ammonia oxidizers. Ammonia oxidation is carried by only a few types of bacteria from the genera Nitrosomonas, Nitrosospira and Nitrosococcus.

1. $\mathbf{NH_3 + 0_2 \rightarrow NO_2^- + H^+}$

2. $\mathbf{2NO_2^- + 0_2 \rightarrow 2NO_3^-}$

The second step in nitrification is oxidation of nitrite to nitrate. This step carried out by bacteria known as nitrite-oxidizing bacteria some of the genera are Nitrobacter, Nitrococcus, Nitrospira and Nitrospina. Nitrification helps to maintain healthy waterbody/aquaria by removing toxic ammonium.

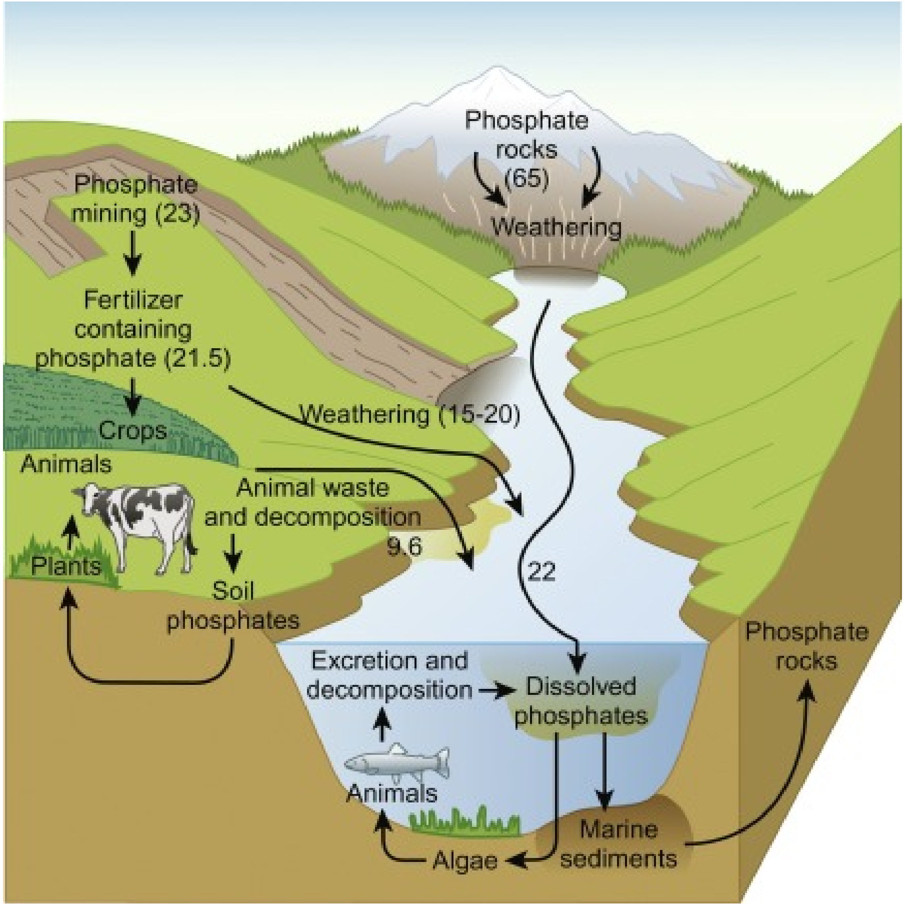
STEP 3: Denitrification

The conversion of nitrate to nitrogen gas is called as denitrification. The reaction removes the bioavailable nitrogen and returns it to the environment in form of dinitrogen gas(N_2). Denitrification is an important as it removes nitrate from the ecosystem and returns to the atmosphere in inert form. Denitrification removes unwanted or excess nitrates from waterbodies and reducing the chances of growth of algal blooms and further eutrophication.

1. $\mathbf{NO_3^- \rightarrow NO_2^- \rightarrow NO + N_20 \rightarrow N_2}$

Denitrifying bacteria include species from the genera Bacillus, Paracoccus and Pseudomonas.

1.3 PHOSPHORUS CYCLE



SOURCE: https://www.sciencedirect.com

STEP1: Weathering

Phosphorus is found in abundance in rocks. Due to weathering the phosphate salts are broken down from rocks and are washed into the ground or waterbody and further mix with the soil.

STEP2: Absorption by plants

The phosphate salts are absorbed by the plants from the soil. The amount of phosphorus in soil is very less. NPK fertilizers are applied to the crops by the farmers which enhances the yield of crops and provides the crop with essential nutrients.

STEP3: Absorption by animals

Animals absorb phosphorus by consuming plants, pant products and plant-eating animals.

STEP4: Return of phosphorus back to environment

Microorganisms decompose decaying plant and animal which converts organic phosphate into inorganic form which is recycled back to soil and water and further end up in sediments and rocks.

1.4 Sources of contamination

Sources of nitrate and phosphate contamination/pollution can be divided into two groups nonpoint sources (diffuse sources) and point sources.

Nonpoint or Diffuse sources

"Diffuse sources pollution is mainly caused by the extensive use of synthetic and organic nitrogen fertilizers." (Zhou, 2015). The extensive use of fertilizers does produce higher yields also on the other hand contribute to groundwater contamination of nitrates and phosphates. The crops do not take up or utilize all the nitrate and phosphate in the fertilizer, it accumulates in the soil and due to irrigation is leached out into the groundwater. Agricultural runoff is the largest nonpoint source of contamination affection groundwater. (Zhou, 2015)

Point sources

Point sources are single and identifiable source of contamination. Some of the point sources include spills of nitrogen rich compounds, leaky septic, leaky sewer system etc. Points sources can be identified and treated to stop further contamination.

1.5 Preventive measures

Nitrate and phosphate contamination in the groundwater is a global problem and yet there is no accurate/effective treatment process the best we can do is prevent further contamination of the groundwater.

1.5.1 Agriculture management/Crop management

The most important step in prevention of groundwater contamination of nitrates is to reduce the nitrate leaching. Nitrate leaching can be reduced by limiting the amount of nitrogen applied in the area or agricultural land. Slow-release nitrogen sources applied can reduce the nitrate contamination. Over irrigation of the agricultural land should be avoided as it increases the chance of nitrate leaching (Zhou., 2015).

Crops do not use more than 50% of the applied nitrogen, application of fertilizer management strategies can reduce the nitrate leaching. Application of optimum fertilizer rates can prove effective in reduce nitrate leaching. Time of fertilizer application to mirror nitrogen uptake of crops can lead to reduced nitrate leaching. Crop rotation strategies show promise of reducing leaching loss of fertilizer nitrogen. By increasing awareness among the farmers regarding the fertilizer overuse and environmental costs also help controlling fertilizer related nitrogen pollution of groundwater. (Singh and Craswell, 2021)

The Punjab Scenario: Wheat and rice are grown in Punjab in annual rotation. Negligible losses of nitrate beyond 2 metre depth have been recorded when fertilizer was applied during the wheat season. Nitrates leached beyond the rooting zone during the wetland rice season as it receives more than 150cm of irrigation with addition of 33cm average rainfall. Nitrate contamination in the groundwater in Punjab has been increasing since 1975, the Central Ground Water Board of India categorized Punjab as the high-risk zone with respect to nitrate pollution of groundwater (Singh and Craswell, 2021).

1.5.2 Sewage system management

Wastewater before discharging into waterbodies need to be treated. Wastewater treatment is necessary to minimize environmental pollution. Wastewater contains harmful chemicals, organic matter and nutrients which pollute the water body. These substances need to be treated before discharging into the waterbody. The aim of wastewater treatment is to purify the effluent by removal of harmful and hazardous substances and plant nutrients. Methods of nitrate and phosphate removal from wastewater include biological denitrification process, ion-exchange process, reverse osmosis, electrodialysis and chemical precipitation (Kumar et al. 2017).

1.5.3 Environmental Protection Policies

The Water (Prevention and Control of Pollution) Act was enacted in 1974 for prevention and control of water pollution and maintaining and restoring of wholesomeness of water in the country. The Act was amended in 1988.

The Environmental (Protection) Act was enacted in 1986 with the objective of providing the protection and improvement. It helps in preventing environmental pollution in all its forms and to tackle specific environmental problems that are peculiar to different parts of the country. The Act of last amended in 1991.

SOURCE: Central Pollution Control Board (CPCB)

1.6 Drinking water standards

The drinking water standard for nitrates is 50mg/L (WHO, 2004). The drinking water standard for phosphates is 5mg/L (EPA).

2. Materials and Methods/Sample Analysis

2.1 NITRATE

A standard of sodium nitrate solution of increasing concentration (2-10mg/ml) followed by the water sample of equal volume were passed through column filled with cadmium pellets which reduce nitrate to nitrite by addition of colouring reagent sulfanilic acid and alpha-naphthylamine and incubated for 30 min and OD measured at 543nm.

UV spectrophotometric analysis – Concentration of nitrates can be estimated at absorbance of 200-220nm.

2.2 PHOSPHATES

100ml collected water sample add one drop phenolphthalein indicator, 2 drops 2M sulphuric acid, 4ml molybdate reagent and 10 drops stannous chloride reagent with mixing. Samples allowed to cool and analysed using spectrophotometer at 690nm. This method is according to American Public Health Association (APHA), American Water Works Association (AWWA), Water Environmental Federation (WEF). (Isiuku and Enyoh 2020)

3. METHODS OF BIOREMEDIATION

3.1 Bioremediation of Nitrates

3.1.1 Biological denitrification

Biological denitrification removes nitrates from water. Nitrate is reduced to nitrogen gas. Biological denitrification targets only removal of nitrates. It is cost effective technique for removal of nitrates from contaminated water. Ex-situ nitrate removal involves extraction of contaminated groundwater, treatment and discharging the water back into the aquifer. Nitrate treatment may not be achieved within a single cycle as there will be re-mixing of the treated water with untreated water. Recontamination is one of the limitations and ex-situ method is also cost intensive. In-situ nitrate removal involves treatment of contaminated water in the aquifer.

3.1.1a Heterotrophic denitrification of groundwater

Heterotrophs are a group of microorganisms that utilize complex carbon compounds as substrates for cell synthesis and energy production. In groundwater the dissolved organic carbon may be used for the process of denitrification however the dissolved organic carbon is insufficient and limits the rate on denitrification. Which leads to high levels of nitrate in groundwater as there is limited denitrification. The process of denitrification can be facilitated in-situ by addition of external carbon compounds. To facilitate nitrate removal by denitrification external carbon compounds like acetate, glucose, sucrose, ethanol, methanol, starch, molasses etc can be used. If the external carbon source is methanol then both nitrate and methanol participate in cellular respiration as well as anabolism reactions. Nitrate is reduced to nitrogen gas and methanol is converted to water and carbon dioxide.

Total respiratory reaction

$$6NO_3^- + 5CH_3OH \rightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^-$$

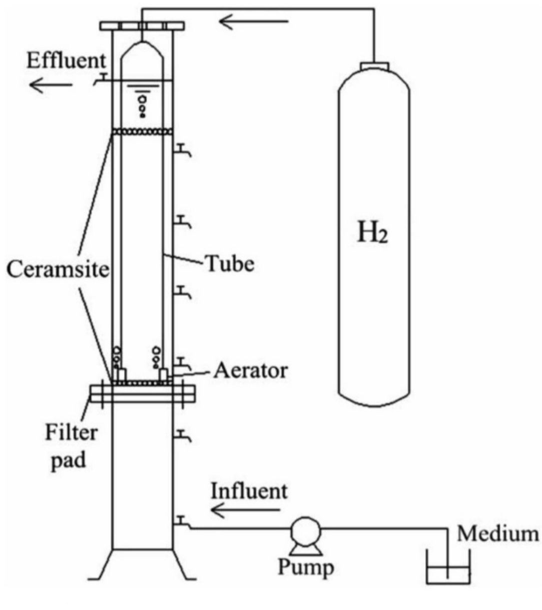
Methanol and ethanol used as substrate gave the nitrate removal efficiencies in the range of 95-97% and 88-92% respectively. Some common heterotrophic denitrifies include species of *Pseudomonas, Bacillus, Acinetobacter* (Huno et al. 2014; Rezvani et al. 2017; Shrimali and Singh 2000).

Calderer et al. 2010 conducted a study to develop strategy for *in-situ* denitrification of groundwater contaminated with nitrate. Glucose and acetate were the two substrates used as the carbon source. The denitrification was carried out by the indigenous microorganisms of the aquifer. The aquifer was partially aerobic so the assays were run under both aerobic and anaerobic condition at a temperature of 17^0C. High nitrate removal were obtained with lower denitrification rate under aerobic condition. Under anaerobic condition the denitrification was complete and faster. Glucose and acetate yielded similar nitrate removal under initial aerobic condition. Glucose was preferred over acetate as acetate led to accumulation of nitrite in high levels. The study demonstrates that glucose can be used a substrate for *in-situ* bioremediation of groundwater. The adaptability of this process for a particular site is not known, laboratory analysis is to be done before implementing this process at a particular site (Calderer et al. 2010).

3.1.1b Autotrophic denitrification of groundwater

Autotrophic organisms oxidize inorganic matter and produce energy for their metabolism and growth by delivering released electron to an electron acceptor. In case of denitrifiers, nitrate serves as the electron acceptor. Carbon dioxide and bicarbonate ($C0_2$ and HCO_3^-) is used as the carbon source for cell synthesis. Bacteria species of genera *Ferrobacillus, Gallionella, Leptothrix* carry out autotrophic denitrification using ferrous iron as the electron donor. Reduced sulphur compounds can be utilized as energy source for denitrification by bacteria of species *Paracoccus, Thiobacillus, Thiosphaera.* *Thiobacillus denitrificans* effectively removes nitrate from water by utilizing a reduced sulphur compound like sulphide or elemental sulphur. *Micrococcus denitrificans* and *Paracoccus denitrificans* remove nitrate by oxidizing H_2 and using nitrate as electron acceptor (Huno et al. 2014; Rezvani et al. 2017; Shrimali and Singh 2000).

Autohydrogenotrophic denitrification is excellent method for denitrification as it uses H_2 which is clean, does not persist in treated water, does not create biological instability and has a low biomass yield. “Autohydrogenotrophic denitrification can be carried out using a H_2-fed bio-ceramsite reactor. Bacteria use the ceramsite as carrier and forms a biofilm on the surface of ceramsite in order to treat nitrate contaminated groundwater. The highest denitrification rate (7.41 mg NO_3^-N/L/h) was observed at nitrate loading of 130 mg NO_3^-N/L however nitrate removal was inhibited when loading was increased. C/N ratio was not a significant factor. Optimum temperature for the reactor was 25 – 35^{0}C and optimum pH was 7-8. H_2-fed bio-ceramsite reactor was effective for treatment of nitrate from groundwater (Chen et al. 2014).



Source: Chen et al.2014

Factors affecting groundwater denitrification

1. DO – Bio-denitrification is favoured where DO concentration is below 0.5mg/L. The effect of DO depends on the type of bacteria and strain.

2. Temperature – Denitrification typically occurs in optimal temperature of 25 - 30°C.

3. pH – Denitrification by heterotrophic bacteria mostly occur in the pH range of 5.5 – 8.0. Denitrification is inhibited in acidic environments.

4. Toxic inorganic compounds – Presence of toxic inorganic compounds in groundwater inhibit the denitrification process. Heavy metals concentrations have inhibitory effect on aerobic denitrification. (Huno et al. 2014)

5. C/N (carbon: nitrogen) ratio of 1.5 is optimum for efficient denitrification process.

Disadvantages of Heterotrophic Denitrification

1. An external carbon source is required to be added. Pumping of the carbon source into the groundwater can increase the operation cost.

2. Nitrous oxide (N_2O) is generated as by-product of incomplete heterotrophic denitrification. Also, it releases greenhouse gasses like carbon dioxide.

3. It generates high levels of biomass.

Autotrophic denitrification is considered better method for removal of nitrates from groundwater.

3.1.1c Microalgae-based water treatment

Microalgae with their ability to remove nutrients like inorganic nitrogen and phosphorus, heavy metals and toxic organic pollutants can be used to remove nitrates and phosphates. Photosynthetic microorganisms like microalgae and cyanobacteria require nitrogen light and inorganic carbon for growth. *Chlorella* is one of the most common species of microalgae. *Chlorella vulgaris* and *Chlorella sorokiniana* are used because of their ability to grow in mixotrophic conditions. The use photosynthetic microorganisms for nitrate removal is an area of research as there are some requirements for development of bioreactors, operation parameters, and microbiological characteristics to start large scale groundwater treatment (Rezvani et al. 2017).

3.2 Bioremediation of Phosphates

Acinetobacter sp. is the is one of the most common bacteria for removal of phosphates. Bacteria accumulate phosphate inside their cell in polyphosphate bodies. *Acinetobacter iwoffi* under aerobic condition and in presence of sodium acetate as energy source can remove 75 – 80% phosphate.

There is inhibition of phosphate uptake in the presence of carbon and phosphate sources at the same time. Phosphate uptake will occur after the consumption and exhaustion of carbon sources. In *Pseudomonas sp.* phosphate removal is enhanced in presence of glucose as carbon source. 68% of phosphate removal is achieved by *Pseudomonas sp.* in the presence of glucose as carbon source. A bacterial consortium (*Bacillus sp., Pseudomonas sp. and Enterobacter sp.)* can reduce the phosphate in presence of lactose as carbon source within seventy-two hours (Singh, A. L .2016).

The *Cyanobacterium* and *Phormidium bohneri* is able to simultaneously remove both nitrates and phosphates (Velusamy et al. 2021).

3.3 Phytoremediation of Nitrates and Phosphates

Three potential plant species were studied for nitrate and phosphate removal from groundwater water lettuce (*Pistia stratiotes), water hyacinth (Eichhornia crassipes)* and water spinach (*Ipooea aquatica)*. All the plants were grown in open bottles of 1.5L or 25L using groundwater. The water and the plants were tested weekly for level of nitrates and phosphates. *Pistia stratiotes* was tested to remove 61 - 92% of nitrate during a period of six weeks. Highest nitrate removal was observed during first week. It also recorded 90 – 99% of phosphate removal. Phosphate was efficiently removed than nitrates. *Eichhornia crassipes* recorded 40 – 63% of nitrate removal. Phosphate (75 – 97%) was more efficiently removed than nitrates. *Ipooea aquatica* showed significant removal of nitrates in the range of 29 – 75%. The nitrate in the tissues of the water spinach was found to be 26.3mg/kg while the concentration of nitrate in which it was growing was 54mg/L. *Ipooea aquatica* which is used as vegetable is an excellent choice for treating nitrate contaminated water. The other two plants are also used as animal feed (Sundaralingam and Gnanavelrajah 2013).

Ceschin et al. 2020 studied the potential of duckweeds *Lemma minuta* and *Lemma minora* for phytoremediation of nutrients (nitrates and phosphates). *Lemma minuta* was found to be more efficient in removing nitrates and phosphates than *Lemma minora*. *Lemma minuta* is the ideal choice for phytoremediation between the two. Use of *Lemma minuta* for phytoremediation is to be carried out under strict controlled conditions as it is regarded as an invasive species. It can invade vast areas (water bodies) and affect the aquatic ecosystem which then can be difficult to control and manage (Ceschin et al. 2020).

3.4 Transgenic approach

Paddy cultivation requires lot of irrigation and excessive irrigation is one of the major causes of groundwater contamination with nitrates and phosphates. Rice (*Oryza sativa*) can be genetically modified in such a way that it does not require much irrigation for growth.

Drought-tolerant transgenic plants are in development. Transgenic rice plants show enhanced tolerance towards drought conditions These drought-tolerant rice plants are able to grow in small amounts of water. Genetically engineered plants have been developed using gene encoding proteins that control drought regulatory networks. The drought-tolerant rice exhibit decreased plant height. Further more research is required in this area of agricultural biotechnology to develop transgenic rice plants which can use less irrigation and yield good crop (Todaka et al. 2015).

DISCUSSION

CONCLUSION

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