Chapter 17
High Levels of Nitrate in Well Waters of Saipem Ward, Candolim, Goa

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Abstract Water is essential for life, but many people across the world do not have access to clean and safe drinking water. Saipem ward in Candolim is located below a plateau onto which the Pilerne Industrial Estate is situated and locals have been complaining of industrial waste and sewage polluting the wells since the last several years. We analysed water samples from five different households to determine water quality as part of our community outreach programme. We examined dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), nitrate, total phosphorus, total dissolved solids (TDS), total suspended solids (TSS) and most probable number (MPN) test to detect coliform in well water samples. With the exception of TSS and phosphorus, the DO, BOD and COD were low while TDS was higher than the permissible limit indicating the presence of organic or inorganic elements. The nitrate content was greater than 45 mg/l (maximum acceptable limit). The coliform index was also very high and variable from 33 to greater than 2400 coliforms/100 ml, thus indicating water unfit for drinking purposes. We carried out subsequent confirmatory tests using differential media and functional characterization of coliform bacteria. These results indicate that the well water samples are not potable due to high coliform load, low dissolved oxygen and high nitrate levels. Moreover, the anaerobic coliform bacteria have the ability to convert nitrate to nitrite to support their metabolic needs. There was no presence of *E. coli* (common faecal coliform indictor) in the well water samples. Further studies are warranted to determine the likely source of nitrate contamination as no faecal contamination or nearby agricultural activity is reported.

Keywords Groundwater contamination · nitrate · multiple fermentation tests · coliform · industrial waste · phosphorus

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17.1 Introduction

Water is a vital component for sustenance of life on Earth and is required for various domestic, agricultural and industrial purposes. The rapid growth in population, industrialization, improper waste disposal and excessive use of chemical pesticides and fertilizers have led to substantial reduction in groundwater table and groundwater pollution. Groundwater reserves are rapidly depleting across many agricultural regions across the world, especially in India, which is the world's largest consumer of groundwater for irrigation purposes [1]. Moreover, water pollution is of serious concern in India as 70% of surface water resources are contaminated by biological, toxic, organic and inorganic pollutants [2, 3]. Groundwater contamination due to raw sewage, improper human and animal faecal waste disposal and farmland runoff contribute to disease outbreak and mortality, with 600,000 children under 5 years old dying each year in India [4]. Faecal coliforms such as *Escherichia coli*, which are commonly found in gastrointestinal tracts of animals and humans, are classical bacteria indicating water unfit for domestic purposes and are used globally for water quality monitoring as per standards set by the World Health Organization (WHO) [5]. Furthermore, physiochemical properties of water such as pH, dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) are important parameters for assessing water quality [6]. Water pollution due to organic matter or phosphorus and nitrate elements causes increased microbial activity due to rapid consumption of dissolved oxygen further affecting the water ecology and potability [7, 8]. Excessive levels of nitrate and phosphates in water bodies promote algal growth leading to eutrophication. Moreover, higher than permissible limits of nitrate in potable water are known to cause adverse health effects such as methaemoglobinaemia or blue baby syndrome in infants [9] and associated with higher risk of stomach cancer in adults [10].

Saipem ward at Candolim is situated below the plateau on which the Pilerne Industrial Estate (15°31′55.0″N 73°47′39.6″E) is located. Locals have been complaining about their well water pollution due to the industrial effluent discharge [11]. In the current study, we analysed well water samples from five different households in Saipem ward, Candolim, Goa for their potability and to identify possible sources of contamination. The results of our study indicate high coliform bacterial load and low dissolved oxygen in all well water samples. There was no total organic carbon and phosphorus; however, higher than permissible limits of nitrate were detected, indicating water unfit for consumption. The anaerobic coliform bacteria were found to utilize their nitrate reduction properties for metabolism in the absence of sufficient dissolved oxygen. The absence of *E. coli* and no reported agricultural activity in the vicinity rule out faecal and fertilizer run-off-induced contamination, respectively.

17.2 Materials and Methods

17.2.1 Selection and Collection of Water Samples

With reference to groundwater contamination issue raised by the villagers of Saipem ward, five different well water samples were collected randomly which were up to 100 m distance apart from each other. The pH was measured using a calibrated standard pH meter, and water quality analysis was carried out.

17.2.2 Multiple-Tube Fermentation Technique

This is a three-stage (presumptive stage, confirmed stage and completed test) process in which the results are statistically expressed in terms of the most probable number (MPN). Three sets of five test tubes each containing sterile single- and double-strength 2% brilliant green bile (BGB) broth (HiMedia laboratories Pvt. Ltd., Mumbai, India) were prepared for the study. BGB broth is widely used for detection of faecal coliforms in water as it inhibits the growth of lactose-fermenting gram-positive bacteria and allows selective growth of lactose-fermenting gram-negative bacteria. Inverted Durham's tubes were placed in all the tubes to detect the release of carbon dioxide gas in the form of trapped gas bubbles. Each set was inoculated with three different sample volumes (10 ml, 1 ml, 0.1 ml) of water sample and incubated for 24–48 h at *37* °C. Growth of lactose-fermenting bacteria results in fermentation of lactose present in the broth to form lactic acid, and carbon dioxide is released. Gas production in Durham's tube was recorded as positive tube, and coliforms were counted based on the reference MPN index [12]. The presence of coliform was confirmed by streaking sample from positive tubes onto eosin methylene blue (EMB) and Endo Agar and incubated for 24 h at *37* °C. Finally, gram staining of colonies was carried out as part of test completion.

17.2.3 Dissolved Oxygen (DO) Levels and Biological Oxygen Demand (BOD) of Water Samples

DO and BOD of water samples were estimated using Winkler's method as stipulated in IS: 3025 Part 44 [13]. Clean and sterilized airtight amber-coloured bottles were taken to the study site and filled with well water sample to the brim, and 2 ml each of manganese sulphate and alkaline iodide-azide solution was added to fix dissolved oxygen (DO), mixed well, and acid precipitate was allowed to settle. The acid precipitate was then titrated with neutralizing compound to determine the end point, which coincides with DO content in water. BOD was estimated by Winkler's method by calculating the difference in DO at day zero and day five.

17.2.4   Chemical Oxygen Demand (COD)

COD of water sample was determined using the potassium dichromate method. A known fixed amount of potassium dichromate as an oxidizing agent was added to the water sample to oxidize the organic matter present in the water sample. Excess of oxygen was allowed to react with potassium iodide to liberate iodine in amounts equal to the excess oxygen, which was titrated with sodium thiosulphate using starch as an indicator [14].

17.2.5   Total Dissolved Solids (TDS)

Clean and dried empty porcelain dishes were placed in an oven at *100* °C for 1 h in order to remove all the organic and inorganic impurities and then cooled in a desiccator and weighed. Subsequently, water samples (100 ml each) were filtered using Whatman paper (#40), and 10 ml of filtered sample was placed in the porcelain dish and kept in an oven again at *100* °C for 1–2 h or until liquid evaporates off. Finally, the dish was allowed to cool again in the desiccator and final weight noted to calculate the TDS.

17.2.6   Total Suspended Solids (TSS)

Whatman filter paper (#40) was washed with distilled water and dried in an oven at *100* °C for 1 h, cooled in desiccator and weighed. Later, it was used to filter 100 ml each water sample and filter paper allowed to dry again and final weight noted to calculate TSS. The protocol and stipulations for TDS and TSS analysis were as per IS: 3025, Part 16 and Part 17, respectively [15, 16].

17.2.7   Isolation of Xenobiotic-Degrading Bacteria by Selective Enrichment

The bacterial cultures or well water samples were inoculated in minimal salts (M9 media) containing various ingredients and either one of the carbon sources (1% sodium benzoate/lactic acid (HiMedia)) or emapunil (Cayman chemicals, Michigan, USA) and inoculated at *37*°C on an orbital shaker at 50–100 rpm for seven days. An aliquot of this sample was inoculated in fresh M9 media and incubated in similar conditions for two times, and subsequently M9 agar containing either of the carbon sources was incubated at *37*°C for 24–48 h [17].

17.2.8   Estimation of Total Phosphorus

Phosphates in test water samples react with ammonium molybdate to form molyb-dophosphoric acid, which upon reaction with stannous chloride gives blue-coloured complex which was spectrophotometrically estimated at 690 nm. The stipulation for phosphorous estimation was as per IS 6092-3-2 [18].

17.2.9   IMViC Tests

'IMViC' stands for indole (I), methyl red (M), Voges-Proskauer (V) and citrate (C) and were employed for identification and differentiation of members of family Enterobacteriaceae [19]. Briefly, the cultures were inoculated in tryptone broth (indole test) and methyl red-Voges-Proskauer broth (MR-VP broth) streaked on citrate agar and allowed to grow for 24–48 hrs at *37* °C.

17.2.10   Nitrate Estimation Using Cadmium Reduction Method

Different concentrations of standard sodium nitrate solution (2–10 mg/ml) in increasing order followed by equal volume of well water samples were passed through a column filled with cadmium pellets which reduces nitrates to nitrites. Subsequently, reduced nitrites were detected by adding colour reagent (sulfanilic acid and alpha-naphthylamine), incubated for 30 mins, and absorbance was measured at 543 nm. The stipulation for nitrate estimation was as per IS:3025, Part 34 [20].

17.2.11   Microbial Nitrate Reduction Test

Briefly, the microbial cultures were inoculated in test tubes with nitrate broth and Durham's tube and incubated for 24 h at *37* °C. The microorganisms having presence of nitrate reductase enzyme reduce the nitrate present in the broth to nitrite which was detected by the presence of red colour precipitate on addition of colour reagent (sulfanilic acid and alpha-naphthylamine). The procedure was described in *Bailey & Scott's Diagnostic Microbiology*, *13th Edition* [21].

17.2.12 UV Spectrometric Analysis for Total Organic Carbon and Nitrate

Well water samples were analysed with ultraviolet (UV) spectrometry for absorbance at 254 nm for total organic carbon (TOC) on Shimadzu UV-2600 spectrometer as there is high correlation coefficient between A_{254} nm and TOC [22]. The nitrate levels were estimated similarly at absorbance of 200–220 nm [23].

All the analyses were carried out in duplicate or triplicate for each well water sample.

17.3 Results and Discussion

17.3.1 High Coliform Load in Well Waters

The pH of the well water samples were 7.16 + 0.2. There was no objectionable smell in the well water samples. The presumptive MPN test result for well water samples was variable with 33 to more than 2400 coliforms per 100 ml indicated by the presence of gas bubbles inside Durham's tube (Fig. 17.1). The confirmatory and completed test revealed the presence of purple-black to colourless colonies on EMB agar (Fig. 17.1) and pink mucoid colonies on Endo agar plates which indicated the presence of vigorous lactose fermenters, and the gram staining indicated the presence of gram-negative cocci or rods. However, no colonies with green metallic sheen were observed on the EMB agar plate. Thus, it could be inferred that faecal
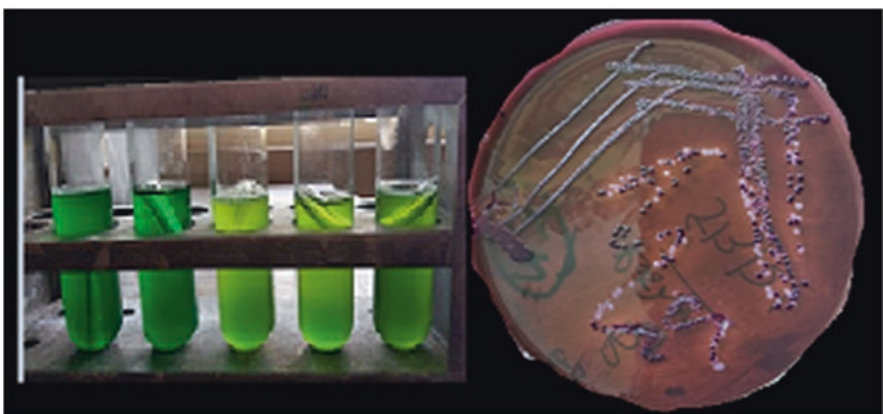

Fig. 17.1 Vigorous lactose fermenters observed in brilliant green bile broth indicated by gas bubbles in Durham's tube causing them to float (left); purple-black to colourless colonies on eosin methylene blue agar plates (right)

coliform *E. coli* was absent in the well water samples. As per the Bureau of Indian Standards (BIS) [24], no coliform should be detected in 100 ml of drinking water sample. Thus, the well water samples with such a high load of coliforms were unfit for drinking purpose.

17.3.2   DO, BOD, COD, TDS and TSS of Well Water Samples

The DO levels of the well water samples under the present study were found to be between 1.88 and 6.05 mg/L (Table 17.1). With reference to the Water Quality Standards in India (Source IS 2296:1992), the best quality water should have minimum 6 mg/L of DO. As per the results obtained, it can be observed that the DO levels of water are comparatively low which can be probably due to high organic/inorganic matter and microbial load. The BOD of water samples was found to be between 0.5 and 1.9 mg/L (Table 17.1). No specific permitted BOD levels are prescribed by WHO or BIS for drinking water. Ideally, BOD for drinking water is considered to be zero which is practically impossible in the untreated open source of water, such as well water sample. Hence, as per the Water Quality Standards in India (Source IS 2296:1992), the best water sample should have maximum BOD level 2 mg/L. Thus, the BOD obtained from the results indicate that the well water sample is clean.

The COD of the water samples determines the amount of oxygen required for the oxidation of organic matter present in the water. The COD obtained from the analysed samples ranged between 8 and 32 mg/L, respectively, indicating the presence of organic matter (Table 17.1). Four out of five water samples were found to have TDS more than 500 mg/L, which is above the permissible limit set by BIS for drinking water. However, the TSS of all water samples was well below the desired 500 mg/L limit (Table 17.1).

Table 17.1   Coliform count per 100 ml and concentrations of DO, BOD, COD, TSS, TDS and nitrate (NO3-) in mg/L in well water samples. Maximum acceptable limit for TDS (1200 mg/L), TSS (500 mg/L) and nitrate (45 mg/L) for drinking water per IS 10500: 2012

Sample	MPNIndex	Day1 DO	Day5 DO	BOD	COD	TSS	TDS	Nitrate
	per 100 ml	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
B1	33	6.0	4.2	1.9	NE	108	490	8612
C1	240	3.0	1.9	1.1	NE	61	1050	5484
M1	>2400	4.2	3.5	0.7	8	69	2020	3579
N1	>2400	1.9	1.3	0.5	13.9	68	270	1857
G1	130	5.4	4.2	1.2	32	46	890	5302

NE not examined

17.3.3 Isolation and Characterization of Xenobiotic-Degrading Bacteria

We did not observe any colonies on M9 agar media plates with the three different carbon sources indicating that the coliforms do not utilize these carbon sources for their metabolic requirements. The IMViC test results were not conclusive in identifying the organism in the coliform (Enterobacteriaceae) group (Table 17.2).

However, interestingly, the coliform bacteria in all five well water samples have nitrate reductase enzyme which converts nitrate to nitrite indicated by the presence of red-coloured precipitate upon addition of colour reagent (sulfanilic acid and α-naphthylamine). In the absence of sufficient oxygen, the anaerobic bacteria utilize denitrification process by converting nitrate to nitrite for metabolic needs, which occurs at neutral pH (7.0), and this process has been utilized for wastewater treatment [25]. Similarly, in our own work, we have observed low dissolved oxygen and neutral pH, which allow anaerobic bacteria to thrive and carry out denitrification process.

17.3.4 Phosphorus and Nitrate in Well Water Samples

The total phosphorus estimated was below 0.01 mg/ml for all the five well water samples. Recent studies have also shown that water with no phosphorus has low DO and COD [8] akin to our own observations. Nitrate estimation using cadmium reduction method revealed high nitrate concentration (maximum acceptable limit is 45 mg/L as per IS 10500: 2012) as the major contaminating factor in well waters of all the five different households (Table 17.1). Natural deposits of nitrate and decomposition of soil organic matter are also known to contribute to nitrate contamination in excess of 1000 mg/L [26–28]. The soil type in our study is laterite, and previous studies have observed differential adsorption of nitrate to laterite soil [29]. The major source of nitrate contamination is fertilizer run-off from agricultural fields and faecal contamination, though both these sources for nitrate contamination are ruled out based on our data. Although this is a small study, higher than usual values of nitrate indicate local geological or even industrial pollution sources which would

Table 17.2 B1, C1, M1, N1 and G1 are the five well water samples

Colony	B1	C1	M1	N1	G1
Indole red test	+	+	+	+	+
Methyl red test	+	+	+	+	−
Voges-Proskauer test	−	+	−	+	+
Simmon"s citrate test	+	+	+	+	+
Catalase test	+	+	+	+	+

The + sign indicates a positive reaction while − sign indicates a negative reaction in the IMViC test
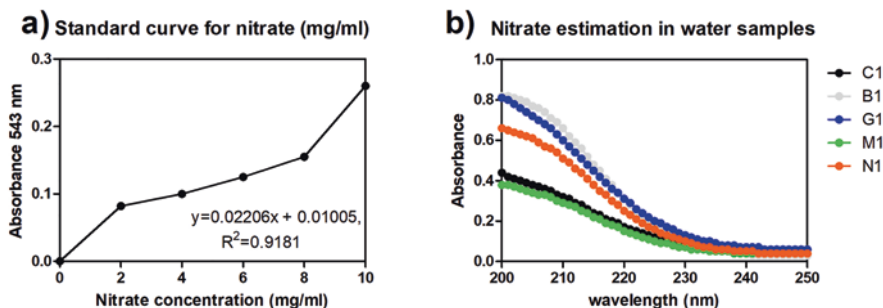
Fig. 17.2 (**a**) Standard curve for nitrate concentration (0–10 mg/ml) with absorbance at 543 nm. (**b**) Ultraviolet absorbance spectra for nitrate with strong absorption at 200–220 nm with well water samples

warrant further research and would be potentially valuable information for understanding similar phenomena in other locations.

17.3.5 Ultraviolet Spectrometric Analysis of TOC and Nitrates

UV absorbance at 254 nm did not indicate the presence of TOC in well water samples, while the presence of nitrate level was further confirmed by UV light absorbance in the wavelength range of 200–220 nm (Fig. 17.2).

17.4 Conclusion

In all the well water samples, the dissolved oxygen content was up to 6 mg/L, which likely favours growth of anaerobic coliform bacteria, which uses nitrate (in the absence of sufficient oxygen) as the final electron acceptor for respiration. The absence of *E. coli* coliform confirms that the contamination is not of faecal origin. There are no reported agricultural activities in the vicinity that could contribute to nitrate contamination. Further studies are warranted to identify sources of nitrate contamination which could be of industrial or geogenic origin. There is significant potential to bio-augment with selective bacteria that can further convert nitrite to nitrous oxide and dinitrogen gas liberating it into the atmosphere and leading to bioremediation. The use of microorganisms would be a good alternative for nitrate bioremediation as it is environmentally friendly and a low-cost approach. The major source of nitrate contamination is agricultural run-off and faecal contamination. Although this is a small sample size study, higher than usual values of nitrate indicate local geological or even industrial pollution sources which warrant further research and would be potentially valuable information for understanding similar phenomena in other locations.

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