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Genotoxicity of ground water due to nitrate pollution and removal of nitrate by fungal consortium

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ABSTRACT

In this study, a total of 20 individual exposed to contaminated water bodies were investigated for DNA damage. The water samples from pallipalayam were found to be mutagenic when analyzed by comet assay. The nitrate level in the water samples analyzed was higher than the permissible limit. The values of physioco-chemical parameters like TDS, BOD and nitrate of the water samples from pallipalayam were higher than the permissible limit. The higher concentrate of nitrate was removed with the help of organism isolated from ground water. The bio adsorption rate of *Aspergillus niger* adsorbed 94% of the nitrates, *A. flavus* adsorbed 80% of the nitrate, *A. fumigatus* adsorbed 81% of the nitrate and *Penicillium* spp. Adsorbed 75% of the nitrate. Consortium of these cultures adsorbs 95% of the nitrate. In conclusion, ground water at pallipalayam does have genotoxic effect on the consumers and the indigenous microbial consortium can be used in the removal of mutagenic nitrate.

KEYWORDS

Nitrate;
Comet assay;
Microbial consortium;
Genotoxicity.

INTRODUCTION

One of the principal hygienic problems of all time, including the present, is the quality of the water, which should be made available to man in accordance with his physiological needs. No specification that can be applied to water for human consumption, with respect to physiological criteria for its chemical composition, can ignore the contribution made to the regular human intake of these components through food consumption and breathing of the ambient atmosphere. The compositions of water, which will best maintain life in the flora and fauna of the earth is the subject of discussion.

The crucial role ground water plays as a decentralized source of drinking water for millions of rural and urban families cannot be overstated. The national water policy drawn up in 1987 and presently under revision has already accorded the highest allocative priority to drinking water needs of the household sector[3]. Ground water resources feed more than 50% of the total area under irrigation. According to some estimates, it accounts for nearly 80 percent of domestic water needs and 50 percent of the urban needs in India. Ground water is generally less susceptible to contamination and pollution when compared to surface water bodies. Also, the natural impurities in rainwater, which

replenishes ground water system, get removed while infiltrating through soil strata. However, the quality of the ground water available is a concern for its sustainable and effective use. The current pattern of industrial activity alters the natural flow of materials and introduces novel chemicals into the environment[5]. The data at which effluents are discharged into the environment especially water bodies have been on the increase as a result of urbanization. Most of these effluents contain toxic substances especially heavy metals. These heavy metals include arsenic, zinc, copper, nitrite, iron, manganese, mercury, molybdenum, nickel, silver, cobalt, chromium, lead and nitrate. The presence of heavy metals in the environment is of major concern because of their toxicity, bioaccumulating tendency threat to human life and the environment[7,9]. Heavy metals are among the conservative pollutants that are not subject to bacterial attack or other breakdown or degradation process and are permanent addition to the aquatic environment[4]. As a result of this their concentration often exceeds the permissible levels normally found in soil, waterways and sediments. Chemical compounds of toxic potential entering a public water supply may impose an immediate risk on human health[15]. It is known that organisms inhabiting areas influenced by effluent discharges can suffer deleterious somatic effects[12] or genetic damage[2] and that people using polluted water could be at higher risk of similar genotoxic effect and cancer development[14].

Among these toxic chemicals nitrate is the most common chemical contaminant in the world's groundwater aquifers[13]. In most, European countries, nitrate levels in rivers and groundwater have increased gradually over the last decade mainly as a consequence of large-scale agricultural application of soil fertilizer with manure mainly from animal origin. An estimated 42% of the U.S population uses groundwater as their drinking- water supply[8]. In the United States, total nitrogen in stream and nitrate in ground water are highest in agricultural areas, followed by urban areas and areas with mixed land use .The most recent data indicate that about 22% of domestic wells in agricultural areas of the United States exceeded the MCL (U.S. Geological survey, unpublished data). In contrast 3% of public supply wells in major water supplies exceed the MCL (U.S. Geological survey, unpublished data).

Higher nitrate concentration in drinking water has drawn a lot of attention due to its harmful biological effects on health. It has been established that indigestion of water containing higher nitrate concentration causes methemoglobinemia (i.e. infant cyanosis or blue baby syndrome). It also affects the blood in such a way as to reduce its oxygen carrying capacity (OECD, 1988). However, it also has the risk of gastric and intestinal cancer. The functioning of central nervous system and cardiovascular system may also be affected adversely by nitrate rich nitrate rich water[20]. The World Health Organization has recommended the permissible limit of 10mg/l nitrate nitrogen (NO_3-N) or equivalent to 45mg/l of NO_3, which is also accepted by Indian council of medical research.

A wide range of physico chemical process such as ion exchange, reverse osmosis, electro dialysis, chemical denitrification and biological denitrification processes are currently being developed for removal of nitrate from drinking water, essentially for large-scale water treatment plants[10]. These processes may be ineffective or expensive, especially when the heavy metal ions are in solution containing in the order of 1-100mg dissolved heavy metal ions/l[18,19]. Biological methods such as biosorption /bioaccumulation for the removal of heavy metal ions may provide an attractive alternative to physico chemical methods[10].

Microorganisms play a major role in reducing the nitrogen level in the wastewater. The organic nitrogen is converted to ammonia in the first step of the nitrogen cycle. In order to remove nitrogen from wastewater, the ammonia must be oxidized to nitrate (NO_3). This process is commonly referred to as nitrification. An anaerobic environment will promote nitrification. Biological nitrification occurs producing nitrite in an intermediate step and ultimately producing nitrate. Following nitrification, nitrogen gas can be removed from wastewater by reducing the nitrate to nitrogen gas (N_2), which is released to the atmosphere. This process is commonly referred to as denitrification. Denitrification requires anoxic condition as well as organic carbon sources to proceed. Under anoxic condition dissolved oxygen is not available to the microorganism for respiration .Because of this the oxygen is stripped from the nitrate leading to the production of nitrogen gas, carbon dioxide and water are also produced in the pro-

cess, which results from the degradation of BOD(UFWRF, 2003).

Microorganism and microbial material have been used in the recovery or removal of valuable or toxic metals from industrial process streams or effluents and have advantages over conventional methods including ion-exchange resins. The ability of metal uptake by the microorganisms has caught great attention due to its potential to provide an effective and economic means for the remediation of heavy metal polluted waste water[21,22]. Hence there is an increased awareness of the potential role of microorganisms on solving major industrial and environmental problems associated with metals.

Hence as a sequel to the genotoxicity study the present work involves identification of microbes generally present in groundwater and the role of these isolated indigenous micro flora in biosorption and removal of mutagenic nitrates.

MATERIALS AND METHODS

Collection of water samples

Nearly 31 groundwater samples were collected in a sterile conical flask in different location from open well and bore well water at Pallipalayam, Namakkal District in Tamil Nadu. The collected ground water samples were transported to the laboratory within 6 hours in an icebox for further physico-chemical analysis like TDS, BOD and Nitrate (APHA, 1985) and microbial analysis.

Nitrate estimation by ultraviolet spectrophotometric screening methods

The 50ml of known concentration of Potassium nitrate was taken at varying proportion. To this 1N HCL solution was added and read at 220 nm in UV spectrophotometer. A standard graph was plotted with concentration of nitrate in the X-axis and O.D. value on Y-axis. For estimating the nitrate concentration of groundwater samples, the every sample was read at 220 nm in UV spectrophotometer and the O.D. value correlated with nitrate concentration plotted in the standard graph.

Isolation of indigenous fungalflora from groundwater

Pour plate technique was employed for the enumeration of microorganisms such as fungi from the polluted groundwater sample. Serial dilution was made from 10^{-1} to 10^{-5}. The fungal cultures were identified based on their morphological features by performing lacto phenol cotton blue.

Evaluvation of genotoxic effect on human consumers of groundwater by comet assay

1% Normal melting agarose and 0.5% low melting agarose was prepared in the phosphate buffered saline (pH 7.4). The agarose was heated until boiling or agarose was dissolved. The conventional microscopic slides were taken and the slides were dipped in the methanol solution and it was burnt over a blue flame to remove the machine oil and dust. The slides were dipped in the heated 1% normal melting agarose up to one-third the area and slides were gently removed. The slides were air-dried.

The whole blood was used for assay. The 75 microlitre of low melting agarose was added over the normal melting agarose coated slides. The whole blood was diluted with the equal volume of phosphate buffered saline and 5-10 microlitre of blood was added to slides. The cover slip was placed over the slides. After the slides wee harden the cover slip was removed from the slides. After removing the cover slip from the slide the third layer of low melting agarose was added to the slide and the cover slip was placed over the slide, it was removed after the agarose harden.

The slides were placed for at least 2 hours in the lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris Base, 10% DMSO and 1% Triton x-100) at 4°C. The slides were removed from the lysis solution and the slides were placed in the horizontal gel box filled with the electrophoresis buffer (10 N NaOH and 200mM EDTA) for 30 min at 20 volts to allow unwinding of DNA. The slides were taken from the electrophoresis tank and placed in tray for drying. The slides were drop wise coated with the neutralization buffer (0.4M Tris) for 5 min. The Slides were taken and procedure was repeated twice. The slides were stained with ethidium bromide solution for 5 min. 100% Cold ethanol was used for destaining. The slides were observered under 40 x magnifications in the fluorescent microscope.

Nitrate removal by microorganism

Fungi play as a wide role in the removal of nitrate from the polluted groundwater. The stock cultures of *Aspergillus* spp. like A. *niger, A. flavus, A. fumigatus* and *Penicillium* spp. were reconstituted in broth media. Sodium alginate solution prepared in 0.85% of saline water and maintained in 80°C were mixed with broth cultures of the selected organisms individually and in combined form. Alginate broth matrix was dropped into 2% of calcium chloride solution with the help of syringe. Immobilized gel beads containing the organisms were separated and stored in a container.

Immobilized cells of *A. niger, A. fumigatus, A. flavus* and *Penicillium* spp. were subjected for bioadsorption studies. 0.5g of each organism was inoculated into the water sample, which is known to contain nitrate, the concentration that are estimated initially. Sample with microbial consortium and controls were maintained. The media was kept in the shaker for 120rpm for 120 hours. After the sufficient period of incubation the media was allowed for nitrate estimation for checking the removal of nitrate. The sample from experimental flasks were analyzed for their optical density at 220nm and from the OD value adsorption of nitrate was deduced.

RESULTS AND DISCUSSION

Physico-chemical parameters of polluted groundwater

Following the standard method (AHPA, 1985) initially the polluted groundwater were subjected to various physico-chemical parameters like TDS (Total Dissolved Solids), BOD (Biological Oxygen Demand) and Nitrate were estimated. The total dissolved solids of the water sample from the polluted site ranged between 665-7800 mg/l (Figure 1), the biological oxygen demand ranged from the 255-390mg/l (Figure 2) and the nitrate ranged from the 6-32 mg/l (Figure 3). The physico chemical parameters of the groundwater were higher than the permissible limit. As human population is increasing by leaps and bounds, there is need for more production in all spheres. So, more industries are coming up polluting more and more that leaves biosphere foul. As man has become more and more civilized he is

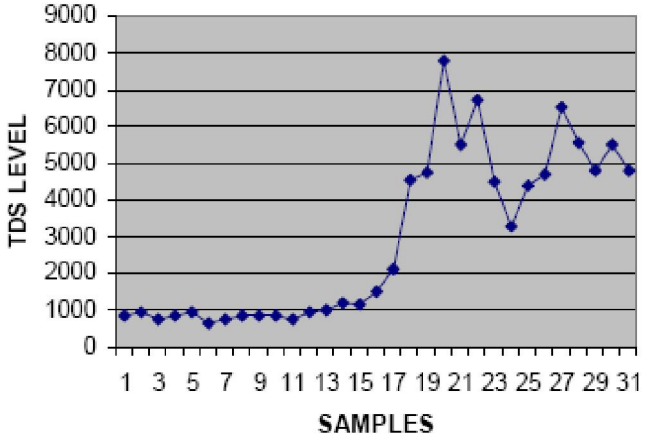

Figure 1: Total dissolved solids levels in polluted groundwater collected from pallipalayam.

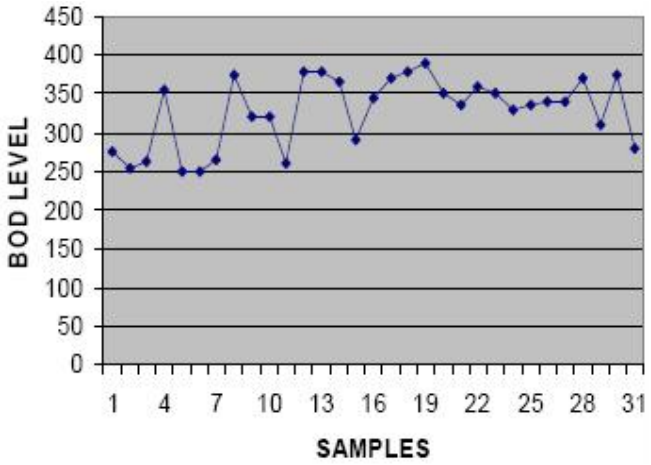

Figure 2: Biological oxygen demand level in polluted ground water

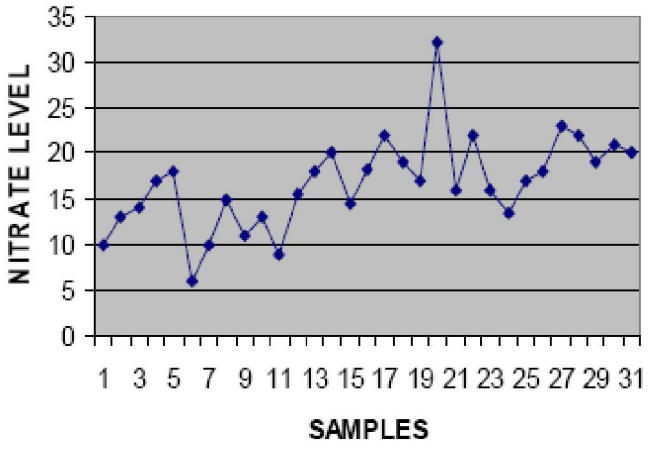

Figure 3 : Nitrate level in polluted ground water collected from pallipalayam

not content with three primary requisites of food, shelter and clothing. Man now has more needs, more entertainment and more comfort, civilization and industrializations associated with the development has polluted

TABLE 1 : Removal of nitrate

S.No	Organisms	Before nitrate level	After nitrate level
1.	*A.niger*	32 mg/l	6 mg/l
2.	*A.flavus*	32 mg/l	7 mg/l
3.	*A.fumigatus*	32 mg/l	7 mg/l
4.	*Penicillium spp*	32 mg/l	5 mg/l
5.	Microbial consortium	32 mg/l	5 mg/l

the Mother Nature considerably. Among this water pollution is taking a heavy toll of human lives. Chemical contamination of groundwater due to unauthorized and uncontrolled release of contaminated effluents from domestic and industrial sector is a growing problem in India. A groundwater source once contaminated tends to remain contaminated for a long period due to slow dispersion and the existence of anaerobic condition, which prevents the oxidation of chemical contaminants. Either chronic or acute, the persons exposed to textile-polluted water bear the major burnt of this hazard. The subjects in this study the peoples exposed to textile effluents contaminated water containing nitrate are reported to be mutagenic.

TABLE 2 : Results for comet assay

Sample no	Gender	Age	Smoking habit	Year of exposure	Comet tail length
1	M	44	-	20	-
2	F	21	-	21	-
3	M	48	-	20	-
4	F	43	-	10	-
5	F	40	-	15	-
6	M	25	-	10	-
7	M	21	-	20	-
8	F	55	-	35	-
9	M	23	+	15	-
10	M	30	+	10	-
11	M	60	+	55	10
12	F	58	-	55	6
13	F	58	-	40	-
14	F	50	-	40	-
15	M	58	+	50	8
Control 1	F	22	-	-	-
Control 2	M	24	-	-	-
Control 3	F	23	-	-	-
Control 4	M	31	-	-	-
Control 5	M	45	-	-	-

Results for comet assay

A total of 20 blood samples were collected from persons consuming the polluted groundwater known to be contaminated with textile discharge were collected to study the incidence of the DNA damage by using the comet assay. Details of their age, smoking habit, duration of exposure to water polluted with textile discharge are presented in the TABLE 2 among 20 blood samples, 3 samples show the positive results. The data on DNA damage in non-smokers and smokers of control group showed an apparent DNA damage. When compared with the control group the exposed group exhibited a linear increase in the DNA damage, with the increase in the age and duration of consumption. Most of the monitoring studies of exposed groups measure mutations or other forms of induced DNA damage involve the evaluation of genotoxic compounds in peripheral blood.

Analysis of DNA damage in exposed cells has been recognized as a biological tool to evaluate genotoxic effects and to monitor the extent of damage. In the present study the DNA damage on peripheral blood of peoples exposed to textile effluent were investigated. Apart from the exposure, the people have the habit of smoking and intoxication. Hence, the observations presenting the investigation are synergistic effects of all these factors. Control group of individuals includes smokers and alcoholics in order to compare the difference between the respective groups.

Analyzed samples were having the exposed period of minimum 10 years to maximum 30 years of exposure of polluted water. The analysis has shown that the frequency of DNA damage is statistically significant in the group of individuals having more than 30 years of exposures. However, the individuals exposed to polluted water below 6 years shows no significance against the control group.

Moreover textile dye effluents have been found to induce DNA damage in aquatic organisms[16], and it is also known that some substances, especially azocompounds discharged from textile dyeing and dye manufacturing units into the environment release monocyclic aromatic amines[11,12]. These amines were some of the first chemicals found to be carcinogenic in humans and in experimental animals[1]. The chemical characterization of the organic residues indicated the presence of such amines in most of the samples.

Bioadsorption of nitrate by indigenous microflora from the polluted groundwater

The bioadsorption of nitrate was done by using the immobilized cells of fungi such as *A. niger, A. flavus, A. fumigatus* and *Penicillium* spp. The samples containing immobilized cells were agitated. After sufficient period of incubation the samples were taken and the percentage of adsorption was measured. The adsorption of nitrate was gradually increased at 120rpm for 120 hours. The combination of fungal cells shows the greatest efficiency of the adsorption. The *A. niger* adsorbed the 94% of the nitrates, *A. flavus* adsorbed 80% of the nitrate, *A. fumigatus* adsorbed 81% of the nitrates and *Penicillium spp* adsorbed 75% of the nitrate. Consortium of these cultures adsorbs the 95% of the nitrate. Moreover textile dye effluents have been found to induce DNA damage in aquatic organisms[16], and it is also known that some substances, especially azocompounds discharged from textile dyeing and dye manufacturing units into the environment release monocylic aromatic amines[11,12]. These amines were some of the first chemicals found to be carcinogenic in humans and in experimental animals[1]. The chemical characterization of the organic residues indicated the presence of such amines in most of the samples.

In order to remediate the water bodies known to be contaminated with nitrate containing polluted groundwater, microorganisms due to their inherent property to degrade and adsorb xenobiotic compounds were employed to remove nitrate. The organisms namely *A. niger, A. flavus, A. fumigatus* and *Penicillium spp* have been isolated from the polluted ground water. Our investigation has revealed that indigenous micro floras isolated from the polluted groundwater have the ability to adsorb nitrate. The *A. niger* adsorb the 94% of the nitrates, *A. flavus* adsorb 80% of the nitrate, *A. fumigatus* adsorb 81% of the nitrates and *Penicillium spp* adsorb 75% of the nitrate. Combination of these cultures adsorbs the 95% of the nitrate. Thanh and Simand[17] reported the same result for the treatment of wastewater by various Yeast species. The study screened 27 yeast strains for their ability to produce a high biomass, while maximizing reduction of phosphate, ammonia and organic matter. Reported phosphate removal ranged from 12% to 100%, total nitrogen removal from 22 to 93%, ammonia nitrogen from 27% to 90%, and COD removal from 0 to 72%. Hiremath et al.[6] performed a similar study except they tested seven fungal species from wastewater stabilization pond. The study reported BOD_5 removal between 53 to 72%, phosphate removal from 34 to 77%, and ammonia nitrogen removal between 49 to 77%.

Water pollution is a global problem and is a major threat at the dawn a of 21st century. Every man must fight against it instead of wringing his hands in despair or joining the carping crowds demanding a halt to our technological advances. Water pollution is woven throughout the fabric of modern life. Man should change his out of sight is out of mind concept and must wholeheartedly participate in the environmental quality improvement programs. The public must be educated to keep their surroundings clean. The environmental engineers and scientist must find out ways and means to convert by product into useful materials and minimize waste to as small an amount as possible. He must copy nature that is he must observe that processes are going on in nature to assimilate foreign material and use the same methods to destroy harmful pollutants arising from the textile industries. If every citizen realizes that it is primary duty to live this world a better place than he had found it, the problems will be automatically solved. But for the damage that has been already done we as a microbiologist should rely on the ability of microorganisms to degrade and adsorb the xenobiotic metals as has been done in the present project. The microbial infallibility may have a solution to the problem of pollution.

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