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Identification of Bacterial Strains and GIS-Based Buffer Analysis of Canoli Canal, Kozhikode, Kerala, India.

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ABSTRACT

One of the greatest concerns of the water consumers with respect to the quality of drinking water is the contamination of pathogenic microorganisms. The present work was done to identify the potential bacterial contaminants in Canoli canal a man made urban water way and the surrounding open wells of Kozhikode district, Kerala State, India, using three methods for taxonomical identification of bacterial strains. The methods used were: morphological, physiological and biochemical tests, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and the sequencing of 16S rRNA gene. The MALDI-TOF MS indicated the presence of eight different bacterial species, among which 100% of the isolates were used to identify the species level with a high score ≥2.Two of the isolates were identified using the 16S rRNA gene sequencing method which also proved the existence of the same taxonomical units. Buffer zone of bacterial contamination within 1 km radius of canal was accomplished using Geographical Information System (GIS) tool. From this study, it can be concluded that canal water is contaminated with pathogenic bacteria with varied health risks and this contamination is playing a role in the transmission of potentially harmful organisms into the open wells. This work reports the use of MALDI-TOF MS as an efficient tool for reliable identification and rapid discrimination of bacterial strains isolated from canal water and well water sources with minimal time demand. Also the results of buffer mapping can be used to raise public concern in drinking well water within 1km radius of the canal which can be hazardous to health of the residents.

Keywords: bacteria, buffer zones, MALDI-TOF MS, canoli canal, open wells

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INTRODUCTION

Research has shown the complexity of microbial populations in environmental samples (Uhlik *et al.,* 2009) [24]. Sewage waste matter contains a wide range of pathogenic microorganisms which may cause a health hazard to human population when they are discharged into the recreational water (Juan *et al.,* 2010) [1] and the health hazard might be high in a heavily colonized country such as India. Over burden of the population pressure, unplanned urbanization, unrestricted exploration and dumping of the polluted water at inappropriate place enhance the infiltration of harmful compounds to the groundwater.

Wells are a common groundwater source readily explored to meet community water requirement or make up the short fall (Adekunle, 2008) [2]. Kerala a southern State of India is strongly dependent upon groundwater wells and has considerable value both for its economic and social uses. Open dug wells are important groundwater extraction structures in the coastal belt of Kerala and groundwater is the most common source of drinking water in these areas. Of late, these precious resources are getting contaminated by various contaminants and anthropogenic activities. Open wells of Kerala have the problem of bacteriological contamination and a study conducted by CWRDM indicates that 70% of the drinking water wells have fecal contamination (Harikumar, 2009) [3].

Canoli Canal, which is an artificially constructed canal, is flowing through the heart of Kozhikode city and is heavily polluted with the untreated sewage discharging into the water body. The source of pollution includes waste from hospitals, hotels, garages, timber industries, slaughter houses as well as residential areas. Many drainage outlets are connected to the canal and the water body receives the storm water, household grey water and sewage. All these activities contribute to the poor condition of the canal water.

Until now, bacterial identification has always been a major challenge in all microbiological fields including water microbiology. The identification of bacterial strains isolated from sewage water is mainly based on the morphological, physiological and biochemical tests. Altogether, these tests allow the identification of most bacterial isolates with great accuracy, but they are costly and time-consuming. The matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) method was first used in microbiology for the identification of small molecules released from cells (Anhalt and Fenselau, 1975) [6]. Over time, this method has become an important tool for bacterial identification. It can be used to generate protein fingerprint signatures from whole bacterial cells (Fenselau and Demirev, 2001) [7] and it allows identification of both Gram-positive (Smole et al., 2002 [25]; Friedrichs et al., 2007 [22]) and Gram-negative bacteria (Conway et al., 2001[8]; Mellmann et al., 2008 [10]) to the species level in a few minutes by measuring the molecular masses of proteins and other bacterial components obtained from whole bacterial extracts. By comparing these fingerprints to a database of reference spectra by the use of various algorithms, bacteria can be rapidly identified (Fenselau and Demirev, 2001) [7]. The advantage of this technique include a fast sample preparation time, which allows the researcher to perform the analysis in approximately 10 min (Krishnamurthy and Ross, 1996), rapid results, and very low reagent costs. Although upcoming spectral evaluation does not have to be so explicit, MALDI-TOF MS has a great potential for massive identification screening in clinical microbiology as well as environmental microbiology (Ruelle et al., 2004) [11].

Geographic information systems (GIS) provide ideal platforms for converging disease specific information and their analyses in relation to population settlements, and natural and constructed environments (Zeilhofer *et al..,* 2007) [19]. GIS helps generate thematic maps that depict the intensity of a disease or vector (carrier of a disease). It can create buffer zones around selected features and combine this information with disease incidence data to determine how many cases fall within the buffer (Pallavi, 2007) [23]. Various case studies demonstrate the application of GIS and spatial analysis in health risk assessment. Njemanze *et al*., 1999 [4] conducted a spatial risk analysis of diseases associated with diarrhea in Nigeria. They identified an increase in health risk where populations have easy access to untreated surface water and live near human and industrial activities causing environmental hazards. In Brazil, Barcellos *et al.,* 1998 [5] evaluated geographical aspects of the relationship between health risk and water supply in a case study in the city of Rio de Janeiro.

Ideally, drinking water should not contain any microorganisms known to be pathogenic or any bacteria indicative of fecal pollution. For the effective maintenance of microbial quality of water, one needs to continuously monitor the water sources. To the best of our knowledge, no specific work has been reported so

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far to isolate the pathogenic bacteria from this area. Considering these facts, this study was conducted to isolate and identify various bacteria in Canoli canal and the surrounding open wells, thereby to create a buffer analysis map to understand the extent of bacterial contamination. We also tested the suitability of MALDI-TOF MS as an efficient tool for water microbiology research, especially since it has become a routine identification method in clinical laboratories.

MATERIALS AND METHODS

Study area

The study area is located along the Canoli Canal in the heart of Kozhikode City, Kozhikode District, Kerala, India. The canal is a man-made water way and constructed in 1848, connects the Korapuzha River in the north and the Kallai River in the south. The canal has a length of 11.4 km and the width ranges from 6 to 20 m. The water depth in the peak monsoon season varies from 0.5 to 2 m. The banks of the canal are highly urbanized except certain patches in the northern sector.

We have selected 11 sampling stations for monitoring the water quality.They are Mooriyad (CC1), Puthiyapalam (CC2), Kaluthankadavu Bridge (CC3), Arayidathupalam (CC4), Swapnanagari (CC5), Eranjipalam (CC6), Karaparamba (CC7), Kakkuzhipalam (CC8), Edakkade (CC9), Kaipurathupalam (CC10) and Eranhikkal (CC11). The details of the stations are given in table 1. A number of representative wells within 1 km radius of the canal were also sampled along with the canal water.

Table 1: Sampling details of Canoli canal

Sample code	Name of Station	Location	
		Latitude	Longitude
CC1	Mooriyad	N11˚19'25.45"	E75˚45' 50.25"
CC2	Puthiyapalam	N11˚14'39.32"	E75˚47' 38.30"
CC3	Kaluthankadavu bridge	N11˚15'07.83"	E75˚47' 41.63"
CC4	Arayidathupalam	N11˚15'33.53"	E75˚47' 35.22"
CC5	Swapnanagari	N11˚16'22.06"	E75˚47' 15.46"
CC6	Erajipalam	N11˚16'58.93"	E75˚47' 01.02"
CC7	Karaparamba	N11˚17'14.53"	E75˚46' 54.30"
CC8	Kakkuzhipalam	N11˚17'55.52"	E75˚46' 41.06"
CC9	Edakkade	N11˚18'22.59"	E75˚46' 26.87"
CC10	Kaipurathupalam	N11˚18'47.42"	E75˚46' 15.98"
CC11	Eranhikkal	N11˚19'25.45"	E75˚45' 50.25"

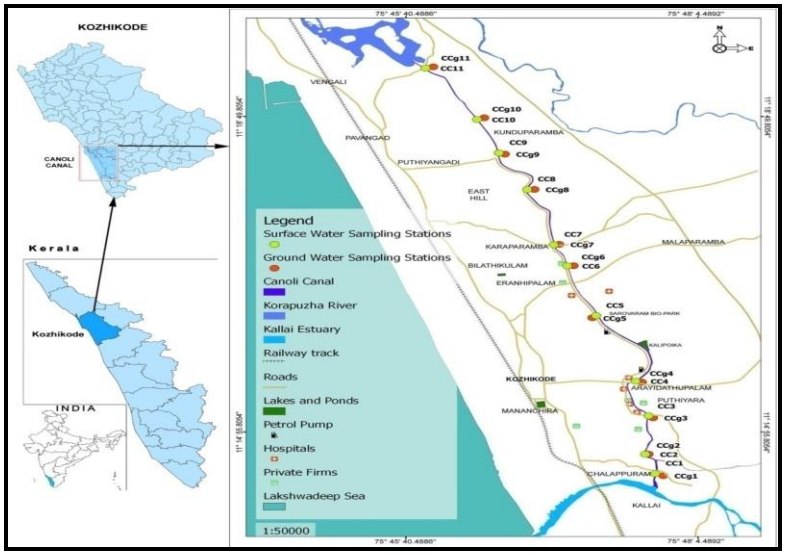

Figure 1: Detail map of the study area

Sample collection

A total of 33 water samples, 11 surface and 22 well water samples, during pre-monsoon (February-May) were collected during the year 2013 and analyzed for the presence of various bacterial pathogens. The exact sampling locations were fixed by using Global Positioning System (GPS). All the water samples were collected in the morning hours between 7:00 A.M-11:00 A.M, during which necessary precautions were taken to collect undisturbed water samples from the canal. Samples were collected aseptically and transferred to 10 ml sterile bottles and properly labelled. Water samples were then kept in the icebox and transported to laboratory for further analysis and were preserved at 4°C before isolation and identification in the laboratory.

Bacterial isolation and presumptive identification

The experiments were carried out immediately after collection .Bacteria isolated from the sampling sites (described above), were grown on nutrient agar at 37°C for 18 to 24 hours. Each colony was the subject of the following tests: morphological, physiological and biochemical tests. Then our results were compared to the known characteristics of bacteria in Bergey's Manual of Systematic Bacteriology (Holt and Williams, 1989) [14].

Whole-cell MALDI-TOF mass spectrometry analysis

The MALDI-TOF mass spectrometry protein analysis was carried-out as previously described (Seng et al., 2009 [17]; Bizzini et al., 2010 [12]). The identification of the isolates by MALDI-TOF MS was performed on a Microflex LT instrument with FlexControl (version 3.0) software (Bruker Daltonics) for the automatic acquisition of mass spectra in the linear positive mode within a range of 2 to 20 kDa, according to the instructions of the manufacturer. Automated analysis of the raw spectral data was performed by the MALDI BioTyper automation (version 2.0) software (Bruker Daltonics); it makes use of a large database containing reference spectra for more than 3200 reference strains (Nagy et al., 2009) [16].

The score value is defined by three components, the matches of the unknown spectrum against the main spectrum, the matches of the main spectrum peaks against the unknown spectrum, and the correlation of intensities of the matched peaks. This leads to a first score, from 0 (no match) to 1,000 (perfect identity), which is converted into a log score from 0 to 3. When the score is greater than 2.0 is considered to indicate good species-level identification and scores above 2.3 correspond to excellent species-level identification. Values between 1.7 and 2.0 correspond to reliable genus-level identification and values below 1.7 indicate no identification (no significant similarity) (Lartigue et al., 2009 [15]; Cherkaoui et al., 2010 [13]).

PCR amplification of the 16S rRNA gene and sequencing

Genomic DNA was isolated from the bacteria isolated from water sample using Sigma Aldrich DNA extraction Kit. Further 16S rDNA was amplified by PCR from the above isolated genomic DNA. A single discrete band was observed when resolved on Agarose Gel. The PCR amplicon was purified by column purification in order to remove contaminants. DNA sequencing was carried out with PCR amplicon. The 16S rDNA sequence was used to carry out BLAST with the nrs database of NCBI genbank database.

Polymerase Chain Reaction

16S rDNA region was amplified by PCR from fungal genomic DNA using PCR universal pimers: 16S Forward Primer : 5'-AGAGTTTGATCMTGG -3' 16S Reverase Primer: 5'-ACCTTGTTACGACTT-3' PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. 3µl of PCR product was loaded with 3µl bromophenol blue (Loading Dye) in 1.5% agarose gel. The gel was ran at constant voltage of 100 V and current of 45 A for a period of 30 min till the bromophenol blue has travelled 6 cms from the wells. Further the gel was viewed on Gel documentation system.

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Purification & DNA Sequencing of Samples

Amplified PCR product was purified using column purification as per manufacturer's guidelines, and further used for sequencing reaction. The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on Genetic Analyzer.

Sequence Analysis of 16S rDNA

Each nucleic acid sequence was edited manually to correct falsely identified bases and trimmed to remove unreadable sequence at the 3 'and 5' ends (considering peak and Quality Values for each base) using the sequence analysis tools. The edited sequences were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) program in the NCBI GenBank which is a DNA database for identifying the bacterial strains.

Buffer analysis mapping

A GIS based buffer analysis was used to estimate the critical zones of bacterial contamination in and around the canoli canal. In this study the buffer zone is defined as a specified distance around a selected map feature. The software used to create the Buffer analysis map is Arc Map 10.1, in which the buffer tool in the geoprocessing menu creates buffer zones around the sampling points to a specific distance. A radius of 1 km was assigned to the buffer zones, created around the surface water sampling sites in canoli canal. Buffers are used not only in analysis of distances and areas around point, line and area features, they are also used in mapping to achieve a number of cartographic effects, such as proximity analysis. Buffer zone maps were created in such a way that distances which are less or equal to the buffer distance were considered high risk zones, whereas distances greater than the buffer distance were considered as low risk zone.

RESULT AND DISCUSSION

A total of 33 water samples were collected from different areas of Canoli canal, of which 11 were surface water samples and 22 open well samples. Quality of both the surface and groundwater in the study area showed bacterial population.

Table 2: Morphological, physiological and biochemical characteristics of the different bacterial strains

Strains	Identification	Tests													
		Morphological		Physiological			Biochemical								
		Gram	Mob[a]	ONPG[b]	OX[c]	Cat[d]	Lac[e]	Saccharose	Mannose	H_2S	Urease	Ind[f]	Cit[g]	MR[h]	VP[i]
S1	*Escherichia coli*	-	+	-	+	+	+	-	+	-	-	+	-	+	-
S2	*Klebsiella sp*	-	-	+	-	+	+	+	+	-	+	-	+	-	+
S3	*Bacillus sp*	+	+	+	-	+	+	-	-	-	+	+		-	+
S4	*Staphylococcus sp*	+	-	-	-	+	-	-	-	-	+	+		+	+
S5	*Aeromonas sp*	-	+	+	+	+	+	+	+	-	-	+	+	+	-
S6	*Klebsiella sp*	-	-	+	-	+	+	+	+	-	+	-	+	-	+
S7	*Citrobacter sp*	-	+	+	-	+	+			+	+	-	+	+	-
S8	*Pseudomonas sp*	-	+	-	+	+	+	+	-	-	-	-	+	-	-

(+): Strains positive; (-): Strains negative.
(a): Mobility; (b): Ortho-Nitrophenyl-β-galactoside; (c): Oxidase; (d): Catalase; (e): Lactose; (f): Indole production; (g): Citrate;
(h): Methyl red; (i): Voges-Proskauer

We obtained eight bacterial isolates from water samples; these strains were designated as S1- S8 and were characterized by their morphological, physiological and biochemical profiles (table 2). The morphological, physiological and biochemical tests represent one of the oldest methods used for bacterial identification and characterization in bacteriological studies. But as seen in table 2, all the eight bacterial strains were identified

to genus level only, which is not sufficient for a complete and accurate identification of our strains. Their important advantages include low cost and easy performance.

In our experiment, we used the software MALDI Biotyper (Bruker Daltonics) to compare the collected spectra of our eight bacterial strains with the reference database and to generate a numerical value (score) based on the similarities between the observed and stored datasets. As shown in table 3, all the nine spectra aligned with the MALDI Bio Typer database were correctly identified to the species level (scores ≥2). The 16S rRNA gene sequencing supported the results from the mass spectrometry; we obtained the same results for the selected bacterial species. The entire identification process is summarized in table 4.

The identification studies has clearly indicated that, the bacteria isolated from the collected samples were known to be present in all sorts of environment of human involvement, majority of them are human as well as animal pathogen (table 5).

Table 3: Identification of the eight bacterial strains by MALDI-TOF MS

Strains	Identification according to MALDI Biotyper	Scores
S1	*Escherichia coli*	2.301
S2	*Klebsiella pneumoniae*	2.442
S3	*Bacillus cereus*	2.139
S4	*Staphylococcus aureus*	2.374
S5	*Aeromonas jandaei*	2.219
S6	*Klebsiella variicola*	2.090
S7	*Citrobacter braakii*	2.181
S8	*Pseudomonas aeruginosa*	2.101

Table 4: Identification of bacterial strains by 16S rRNA gene sequencing.

Strains	Identification	Accession Numbers
S1	*Escherichia coli*	KP005067
S8	*Pseudomonas aeruginosa*	KP005068

Table 5: Probable sources of the bacterial species and their pathogenicity reported by Public health agency of Canada, 2011 [28]

Sl no	Organism	Sources / specimens	Pathogenicity/toxicity
1	*Escherichia coli*	Stools and fecally contaminated material	Acute, profuse, watery diarrhea, which rarely becomes persistent.
2	*Klebsiella pneumoniae*	all sites within the human body, RT and UT most common	Leading cause of community-acquired and nosocomial pneumonia and lung abscesses.
3	*Bacillus cereus*	Human stool, Food specimen and soil	Self-limiting (24-48 hours) food-poisoning syndromes (a diarrheal type and an emetic type), opportunistic infections and clinical infections such as endophthalmitis and other ocular infections.
4	*Staphylococcus aureus*	CSF, joint aspirates, blood, abscesses, aerosols, feces, and urine	Leading cause of food poisoning and a variety of self-limiting to life-threatening diseases in humans.
5	*Aeromonas jandaei*	Soil, hospital water supplies, dialysis fluids, meat and dairy products.	Gastroenteritis
6	*Klebsiella variicola*	blood, banana plants, rice, sugar cane, and maize	Pathogenic to humans
7	*Citrobacter braakii*	Human feces , brain abscesses, cerebral fluids, laboratory mice, eye, urine, intestines, umbilicus, skins pustules, hands, environmental sources (soil, water)	Cause urinary tract infections, blood stream infections, intra abdominal sepsis, brain abscesses, and pneumonia and other neonatal infections, such as meningitis, neonatal sepsis, joint infection or general bacteremia.
8	*Pseudomonas aeruginosa*	Blood cultures, urine, skin, sputum, soft tissue samples, lower respiratory tract secretions, wound exudates, contaminated water samples	Otitis externa, open burn wounds, causing infections, abscesses, and sepsis, with edema and/or discoloration of unburned skin at wound margins and green pigment in subcutaneous fat.

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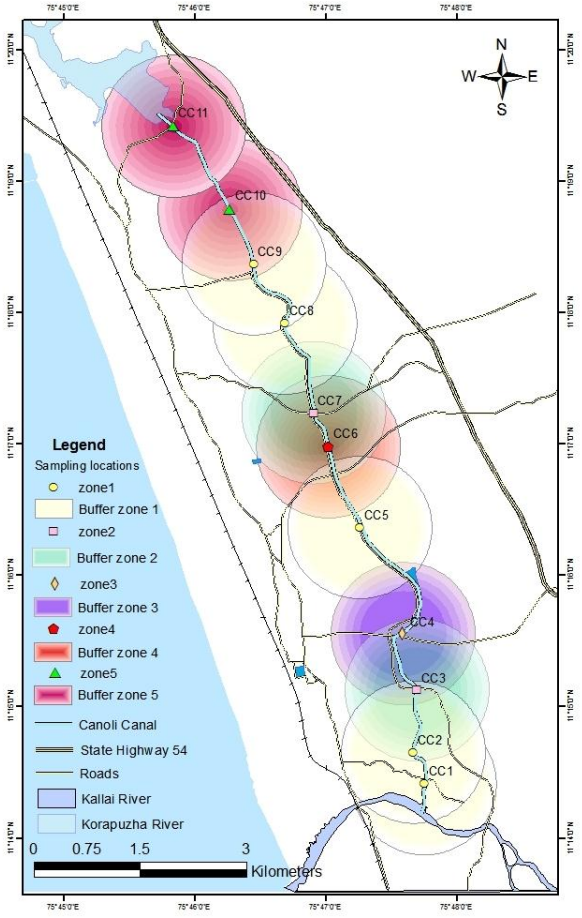

Figure 2: Bacterial contamination zones in and around Canoli canal

The bacterial species identified from various stations of the Canoli canal were represented in the GIS buffer zone map (figure 2). The bacterial contamination was highly concentrated at all sites. Each buffer zone represents a set of bacterial species. There are five different buffer zones represented in different colors and symbols. Zone 1 consists of a set of 4 bacteria (*Escherichia coli, Bacillus cereus, Klebsiella variicola, Citrobacter braakii)* which were isolated from the surface and groundwater wells of Mooriyad, Puthiyapalam, Swapnanagari, Kakkuzhipalam and Edakkade. Similarly zone 2 consists a set of 5 bacteria (*Escherichia coli, Bacillus cereus, Klebsiella variicola, Citrobacter braakii, Aeromonas jandaei*) which were islolated from water samples collected from Kaluthankadavu Bridge and Karaparamba. Zone 3, 4 & 5 consists of a set of 6 bacteria (*Escherichia coli, Aeromonas jandaei, Klebsiella pneumonia, Staphylococcus aureus, Bacillus cereus, Citrobacter braakii*) from Arayidathupalam, a set of 6 bacteria (*Escherichia coli, Bacillus cereus, Klebsiella variicola, Citrobacter braakii, Aeromonas jandaei, Klebsiella pneumonia*) from Erajipalam and a set of 5 bacteria (*Escherichia coli, Bacillus cereus, Klebsiella variicola, Citrobacter braakii, Pseudomonas aeruginosa*) from Kaipurathupalam and Eranhikkal respectively.

Zone 3 & 4 were reported with more pathogenic bacteria, the reason can be attributed to the presence of hospitals and slaughter houses. The canal is receiving the waste water from those establishments. The data also revealed that, the highest proportion of Enterobacter species were noticed in the samples collected from Kaluthankadavu Bridge and Karaparamba sites (zone 2). When these sites were surveyed, it was found that sewage disposal practices in that area are very poor. Also Kaluthankadavu bridge site is a slum area were municipal drainage facilities are not provided. Low bacterial counts were observed in the samples collected from Kaipuarhupalam and Erajikkal area (zone 5) where well established municipal drainage system was present. Majority of the sampling stations came under zone 3 which were observed to have coliform bacteria than other pathogenic strains. This may be due to the controlled release of municipal waste water.

The wells situated within 1 km radius from the Canoli canal were also contaminated with various bacteria. Proximity of contaminating surfaces and interaction with surface water are some of the factors, likely to control the presence and transport of bacteria in groundwater (Chitanand *et al.,* 2006) [26]. According to BIS guidelines (IS 10500:2012) [27] Total coliform, Fecal coliform and *E.coli* counts should be zero in the water which is used for drinking purpose. In such circumstances, the present results indicate gross pollution of wells near the study area. All the samples from all the sampled sites, tested positive for fecal coliforms and other pathogenic bacteria. Higher densities of indicator organisms during the study period can be attributed to the percolation, as well as seepage of domestic sewage through the soil.

The results of this study will provide invaluable information to public and health officials to develop effective control measures in dumping untreated waste directly into the canal. This study has investigated only the influence of Canoli canal in the contamination of open wells.

CONCLUSION

Presence of pathogenic bacteria was detected in surface and 22 open well samples collected from the areas in and around the canal. The study also concluded that, canal water is contaminated with pathogenic microbes and this contamination may be playing a role in the transmission of potentially harmful organisms into the open wells. The introduction of GIS has automated the data handling and computation process, and has provided an effective cartographic presentation of the results. With these flexibilities, the GIS methodology has been potentially used in creating a buffer analysis map to identify the bacterial contamination zones in and around the canal.

Consistent and periodical examination of water samples and disinfection process should be carried out in the Canoli canal area and in the adjacent well water sources to prevent the spread of pathogenic microbes. Public attention should be also raised to discharge only treated municipal waste water to the Canoli canal.

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