African Journal of Microbiology Research. Vol.(2) pp. 224-228, August, 2008
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2008 Academic Journals

Full Length Research Paper

Detection and identification of groundwater bacteria in Sebha City, Libya

Salem, A. Mahfouz[1] F. M. Shaieb[2] and A.E.K Elzen[1]

[1]Botany Department, Faculty of Science, Sebha University, Libya.
[2]Botany Department, Faculty of Science, Omar Almukhtar University, Libya.

Accepted 8 August , 2008

Drinking water systems supplied by untreated groundwater were examined to determine whether coliform or heterotrophic plate count bacteria are capable of growing. Filterable bacteria were present in 42% of the 46 groundwater sources examined by using nonselective media (R2A and full strength m-HPC agars). Pseudomonads were the most frequently identified group of filterable bacteria detected. *Flavobacterium*, *Alcaligenes*, *Acinetobacter* and *Achromobacter* isolates were also identified. Total coliforms were recovered only from six samples taken from wells near the wastewater lagoon, following filtration through 0.45-µm-pore-size membrane filters by using selective M-Endo LES agar or mT7 agar. In addition, none of the isolates identified from nonselective media were coliforms. Similarly, neither total coliforms nor specifically *Escherichia coli* were detected in these filtrates when Colilert P/A medium was used.

Key words: ground water, contamination, *Flavobacterium, Alcaligenes, Acinetobacter, Achromobacter* and *E. coli*

INTRODUCTION

Groundwater contamination is nearly always the result of human activities. Groundwater is especially vulnerable in areas where population density is high and human use of the land is extensive. Virtually any activity whereby chemicals or wastes may be released to the environment, either intentionally or accidentally, has the potential to pollute groundwater. Groundwater is often used as a source of drinking water and human consumption of water containing intestinal pathogens may spread disease (Entry and Farmer, 2001). When groundwater becomes contaminated, it is difficult and expensive to purify. Liquid waste discharged onto soil initiates solute and microbe movement that follows natural groundwater drainage patterns and may contaminate groundwater.

Groundwater provides almost 99% of water for drinking, domestic use or irrigation in Sebha city, serving an estimated 120 000 people. Current membrane filtration methodologies may not recover all bacteria from groundwater, in part because miniaturized or filterable bacterial cells may pass through standard porosity (0.45-µm-pore-size) membrane filters. Filterable bacteria have been isolated from seawater (MacDonnell and Hood, 1982; Tabor et al., 1981) and are generally regarded as those bacteria capable of traversing a 0.45 µm-pore-size membrane filter (Anderson and Heffernan, 1965). Anderson and Heffernan (1965), using a double-filtration method, reported detection of small marine bacteria that passed through a 0.45-µm-pore-size filter but were retained on a 0.22-µm-pore-size filter. Selected isolates were subsequently identified as *Spirillum, Leucothrix, Flavobacterium*, *Cytophaga*, and *Vibrio* spp. Miniaturization of bacterial cells may reflect the lack of one or more critical energy-yielding substrates in the surrounding environment for a specific bacterium. Miniaturization reportedly increases the cell's surface-to-volume ratio; hence, the organism's capacity to scavenge available energy-yielding substrates will be increased (Morita, 1982). Failure to detect miniaturized cells on standard 0.45 µm-pore-size membrane filters could result in a substantial underestimation of the sanitary quality of a water supply. Accordingly, the goals of this study were the following: (i) to determine whether groundwater bacteria were capable of escaping detection when standard porosity (0.45-µm-pore-size) membrane filters were used; and (ii) to attempt to identify those bacteria which passed through 0.45 µm-pore-size filters and were

*Corresponding author. E-mail: salmahfouz@yahoo.com.

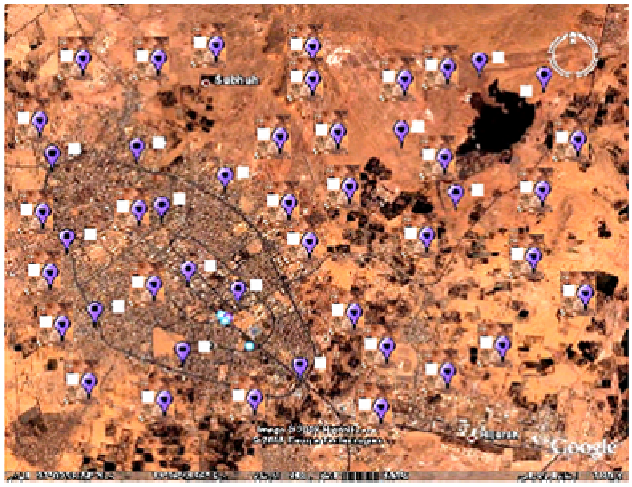

Figure 1. The 46 locations of groundwater wells in Sebhah city.

subsequently entrapped on 0.22 µm-pore-size filters.

MATERIALS AND METHODS

Collection of groundwater samples

Water samples were collected from 46 untreated, groundwater systems supplied from drilled wells. The groundwater supplies were located in Sebhah city, Libyan Jamihiriya in a circle having nearly a 5 kilometers diameter (Figure 1). Samples were obtained in sterile 1-liter polypropylene containers after any screening devices were removed and the cold water tap was flushed for 2 min. Samples were placed on ice immediately after collection. Samples were collected daily and were analyzed within 2 h of collection. Only 10 samples were stored in the dark at 13°C for 70 or 240 days before analysis for filterable bacteria.

Membrane filtration procedure

A membrane filter tower was constructed from two polycarbonate filtration units (Millipore Corporation, Bedford, Mass.) with a type HA 0.45 µm-pore-size membrane filter (Millipore) aseptically placed on the upper filter holder and a type GS 0.22-µm-pore-size membrane filter inserted on the lower filter holder. This design was previously used by Shirey and Bissonnette (1991). Such a design permitted entrapment of miniaturized bacteria on the 0.22 µm-pore-size filter, which had passed the larger 0.45-µm-pore-size membrane and consequently, provide evidence of filterable bacteria. All membrane filters were from the same lot and were sterilized by autoclaving. Sample processing was conducted according to Standard Methods (APHA, 1989). Samples were filtered by the use of a vacuum with a cotton plug trap to prevent back contamination. Nonselective media used to recover heterotrophic plate count (HPC) bacteria included R2A agar (Difco Laboratories, Inc., Detroit, Mich.) and full-strength (lx) and 1/10-strength (0.1x) m-HPC agar (Difco), whereas mT7 agar (Difco), M-Endo LES agar (BBL Microbiology Systems, Cockeysville, Md.) and M-Endo medium (BBL) were used for coliform detection. R2A agar, a modification of Henrici medium (Henrici, 1938), was developed by Reasoner and Geldreich (1985) and has been successfully used to enumerate bacteria from oligotrophic waters such as treated and nontreated potable water (Reasoner et al., 1989). M-Endo medium and 0.1x m-HPC agars were supplemented with 1.5% agar. Media were incubated at 35°C for 18 to 22 h (mT7 agar), 22 to 24 h (M-Endo medium and M-Endo LES agar) or 72 h (R2A, m-HPC, and 0.1x m-HPC agars).

Both the 0.45 and 0.22 µm-pore-size filters were placed on R2A agar and incubated for 72 h at 35°C. No colonies were detected on 0.45 µm-pore-size filters prior to filtration of groundwater samples; whereas two to four colonies were detected on four of the 0.45-µm-pore-size control filters after the groundwater samples had been filtered. Only one 0.22 µm-pore-size control filter contained two colonies, which grew following water sample filtration. The corresponding groundwater sample did not contain any colonies on the 0.22 µm-pore-size filter. In addition to being filtered through a membrane filter tower, 100 ml of some groundwater samples were vacuum filtered through a type HA 0.45-µm-pore-size membrane filter (Millipore) into a sterile 500 ml Erlenmeyer flask containing Colilert P/A medium (Access Analytical Systems, Branford, Conn.) of (Edberg et al., 1990). Another 100 ml of groundwater was added to Colilert P/A medium in a sterile 250 ml Erlenmeyer flask as an unfiltered control. Following 28 h of incubation at 35°C, the medium was examined according to the manufacturer's instructions for turbidity, yellow color formation (indicative of total coliforms) and fluorescence (indicative of presence of *Escherichia coli*). The liquid was then examined for bacterial cells by the acridine orange method (Hobbie et al., 1977).

Isolation and identification of filterable bacteria

Following incubation, membrane filters were examined for growth. Colonies on 0.22-µm-pore-size membrane filters were enumerated for those filters containing <100 colony forming units (CFU) per plate by using a dissecting microscope at a magnification of 15x. Colonies from lx m-HPC, 0.1x m-HPC and R2A media were isolated by streaking onto R2A agar. Isolates were preliminarily examined for oxidative or fermentative metabolism (oxidation-fermentation basal medium with 1% glucose), cytochrome oxidase (Kovac's oxidase), pigmentation, Gram stain reaction and morphology. Gram-negative, nonfermentative isolates were further identified with API Rapid NFT (Analytab Products, Inc., Plainview, N.Y.).

RESULTS

Bacterial cell miniaturization during *in vitro* starvation

Indigenous bacteria in ten different groundwater samples were starved for 70 or 240 days to determine the presence of filterable bacteria by using a membrane filter tower. Natural heterotrophic microfloras were recovered on 0.22 µm-poresize filters from all 10 groundwater sources (A through J) on R2A agar (Table 1).

Only bacteria from sample 4 were recovered on 0.22 µm-pore-size filters on 1x m-HPC agar. All 10 samples contained coliforms, which survived the extended starvetion period, as evidenced by colonies on M-Endo LES and mT7 media. However, coliforms were detected only on the standard 0.45 µm-pore-size filters.

Recovery of filterable bacteria from groundwater

Thirty-six rural, untreated groundwater supplies were examined by using a membrane filter tower to determine whether bacteria were capable of escaping detection on standard 0.45 µm-pore-size membrane filters. Filterable

Table 1. Recovery of filterable bacteria from groundwater samples stored *in vitro*.

Ground water Source [a]	Starvation Period (days)	Presence of colonies on medium on a membrane filter tower [b]							
		R2A		1x m-HPC		M-Endo LES [c]		mT7 [c]	
		0.45 µm	0.22 µm	0.45 µm	0.22 µm	0.45 µm	0.22 µm	0.45 µm	0.22 µm
1	240	+	+	+	-	+	-	+	-
2	240	+	+	+	-	+	-	+	-
3	240	+	+	+	-	+	-	+	-
4	70	+	+	+	+	+	-	+	-
5	70	+	+	+	-	+	-	+	-
6	70	+	+	+	-	+	-	+	-
7	70	+	+	+	-	+	-	+	-
8	70	+	+	+	-	+	-	+	-
9	240	+	+	+	+	+	-	+	-
10	240	+	+	+	-	+	-	+	-

a Sample volume of 100 ml
b (+): ≥ 1 colony; (-) no colonies
c Total coliform

Table 2. Recovery of total coliform on selective media on a membrane filter tower.

Medium	No. of groundwater samples with colonies on membrane filters on two pore sizes [a]	
	0.45 µm	0.22 µm
mT7		
Yellow[b]	27(59%)	4(9%)
Non-yellow	32(70%)	6(13%)
M-End0 LES		
Sheen[b]	16(57%)	0(0%)
Non-sheen	28(61%)	0(0%)

a Sample volume of 100 ml; 46 samples
b Total coliform

bacteria were recovered on 0.22 µm-pore-size filters from 16 samples on 0.1x m-HPC, 10 samples on lx m-HPC, and 10 samples on R2A agar. Ten samples had only one colony on the 0.22 plm-pore-size filter, whereas six samples contained an excess of 100 colonies. All six samples with >100 colonies were obtained on R2A agar.

In one case, most of the approximately 64 colonies recovered on the 0.22-µm-pore-size membrane filter on R2A agar exhibited similar colonial morphology and six isolates were subsequently identified as *Pseudomonas vesicularis*. Three groundwater sources contained 30 to 40 colonies on 0.22 µm-pore-size filters, with corresponding 0.45 µm-pore-size filters containing an estimated 300 to 400 colonies.

An attempt was made to determine whether total coliforms were able to escape entrapment on 0.45 µm-pore-size membrane filters by using membrane filter towers. Approximately 72% of the 46 groundwater samples were positive for total coliforms on the 0.45 µm-pore-size membrane filters, but only one 0.22 µm-pore-size filter on mT7 agar contained a presumptive total coliform (Table 2). However, this isolate was not verified as a coliform since gas was not produced during incubation in lauryl sulfate broth or brilliant green lactose bile broth. In addition, the isolate did not fit any of the API 20E or Rapid NFT biotype descriptions. Three samples contained non-coliform bacteria that were able to form colonies on the 0.22-µm-pore-size filters on selective mT7 agar, whereas no presumptive coliforms or non-coliforms were recovered on M-Endo LES agar.

Recovery of groundwater bacteria by using Colilert P/A medium

A new medium (Edberg et al., 1990) designed for the simultaneous detection, specific identification and verifycation of total coliforms and *E. coli* was used to evaluate filtered groundwater samples for the presence of filterable bacteria. Only six of the 46 filtered (0.45 µm-pore-size) groundwater samples were positive for total coliforms or *E. coli* (Table 3). However, after 28 h of incubation, one-half of the sample filtrates did contain bacteria that escaped entrapment on the 0.45 µm-pore-size membrane filter, corroborating the findings of the membrane filter tower studies using nonselective media.

Identification of miniaturized bacteria

Approximately 72% (23 of 32) of the isolates were capable of growth to the density necessary for identification by API Rapid NFT. None of the isolates recovered from the nonselective media were identified as coliforms. The ma-

Table 3. Recovery of bacteria from filtered and unfiltered groundwater by using colilert P/A medium.

Groundwater sample	Incubation Time (h)	No. of groundwater samples positive by using Colilert P/A [a]		
		Total coliforms	*E. coli*	Bacterial cells[b]
Unfiltered	24	18	8	ND
	28	21	8	36
Filtered [c]	24	0	0	ND
	48	0	0	14

a Sample volume of 100 ml; 46 samples
b determined by acridine orange direct count method
ND, not determined
c filtered through 0.45 µm membrane filter only

jority of bacteria capable of escaping entrapment on 0.45-µm-pore-size membrane filters with subsequent colony formation on nonselective media were *Pseudomonas* spp. Other isolates identified were *Acinetobacter calcoaceticus* and *Flavobacterium, Achromobacter* and *Alcaligenes* spp.

DISCUSSION

Much effort has been directed toward maximizing the detection of coliform bacteria in the environment. Improved methods have been developed to permit recovery of sub-lethally injured coliforms from water, including modification of selective recovery media (Presswood and Strong, 1978), pre-incubation on resuscitation media, and exogenous addition of compounds that degrade or prevent the formation of peroxides. In the present work, the possibility that erroneous conclusions about the sanitary quality of groundwater may be related to miniaturization of coliform bacteria, resulting in non-detection of cells when standard-porosity membrane filters are used for enumeration.

The morphology of bacteria is related to their existence and persistence in the natural habitat and includes adaptations to low-nutrient environments. Large reductions in cell size have been reported as a specific physiological survival response by microorganisms in oligotrophic environments (Lappin-Scott et al., 1988; Lillis and Bissonnette, 2001). *In vitro* studies using long periods of starvation have suggested that one possible survival mechanism is a decrease in cell size from large rods to small cocci. Bakhrouf et al. (1989) reported that prolonged starvation of *Pseudomonas aeruginosa* in sterile, nutrient-free seawater resulted in the development of small cells capable of passing through a 0.45-µm-pore-size membrane. The present findings give no evidence that coliforms inhabiting groundwater decrease in size, since coliforms were not detected on M-Endo LES, mT7, or Colilert P/A media from filtrates passing through a 0.45-µm-pore-size membrane filter (Tables 2 and 3). In addition, coliforms did not shrink in size, despite prolonged starvation in a laboratory microcosm (Table 1). These observations are reassuring, because evaluation of the bacteriological quality of drinking water from rural groundwater supplies are apparently not compromised because of a failure to detect miniaturized coliform bacteria. Similar results were reported by Shirey and Bissonnette (1991) who used the same techniques.

On the other hand, further investigation is warranted since lack of detection of coliforms on the lower 0.22 µm-pore-size membrane filter may be related to inadequacy of the selective recovery medium. If coliform bacteria did pass through the upper 0.45-µm-pore-size filter of the membrane filter tower, it is possible that these cells would be physiologically debilitated and difficult to culture on 0.22 µm-pore-size membrane filters on selective media. In this regard, application of recently developed immunofluorescence and polymerase chain reaction techniques on filtrates from 0.45 µm-pore-size filters may provide increased sensitivity for detection of cells which may be in a viable but non-culturable state. Historically, the evaluation of the sanitary quality of drinking water has focused on enteric microorganisms associated with fecal pollution. More recently, efforts have been directed toward isolation and identification of non-enteric bacteria. The possible public health significance of HPC bacteria in drinking water warrants such attention. In particular, the presence of excessive background HPC bacteria could potentially mask the presence of total coliforms, thereby compromising the evaluation of the water's safety. In addition to their importance as coliform antagonists, some non-coliform heterotrophic bacteria include opportunistic pathogens such as *Aeromonas, Acinetobacter* and *Pseudomonas* spp. (Lamka et al., 1980). For these reasons, support has been given to the necessity of concurrent enumeration of HPC bacteria with coliforms to correctly evaluate the sanitary quality of drinking water. Our observations indicate that heterotrophic bacteria other than coliforms were able to escape entrapment on 0.45-µm-pore-size membrane filters (Tables 1 and 3). Of the 24 groundwater sources examined, three samples in which approximately 10% of the HPC bacteria were capable of escaping detection on standard-porosity 0.45-p.m-pore-size filters, were identified. Further investigation is required to determine the quantitative significance of

miniaturized bacteria in groundwater. A limited number of colonies appearing on the 0.22-µm-pore-size membrane filters were isolated and identified. Some of these isolates may be opportunistic pathogens, further adding to the potential significance of filterable cells. Interest in and use of the HPC for enumeration of bacteria in potable water has grown in recent years. The presence of filterable HPC bacteria in rural drinking water supplies could be of public health significance in evaluating the use of the Standard Methods (APHA, 1989) membrane filtration technique with 0.45-µm-pore-size filters for the enumeration of these bacteria in groundwater.

REFERENCES

American Public Health Association (APHA) 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.

Anderson JIW, Heffernan WP (1965). Isolation and characterization of filterable marine bacteria. J. Bacteriol. 90: 1713-1718.

Bakhrouf AM, Jeddi A, Bouddabous, Gauthier MJ (1989). Evolution of *Pseudomonas aeruginosa* cells towards a filterable stage in seawater. FEMS Microbiol. Lett. 59:187-189.

Edberg SC, Allen MJ, Smith DB, Kriz JJ (1990). Enumeration of total coliforms and Escherichia coli from source water by the defined substrate technology. Appl. Environ. Microbiol. 56:366-369.

Entry JA, Farmer N (2001). Movement of coliform bacteria and nutrients in ground water flowing through basalt and sand aquifers. J. Environ. Quality. 30:1533-1539.

Henrici AT (1938). Studies of freshwater bacteria. IV. Seasonal fluctuations of lake bacteria in relation to plankton production. J. Bacteriol. 35:129-139.

Hobbie JE, Daley RJ Jasper S (1977). Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Lamka KG, LeChevallier MW, Seidler RJ (1980). Bacterial contamination of drinking water supplies in a modern rural neighborhood. Appl. Environ. Microbiol. 39:734-738.

Lappin-Scott HM, Cusack F MacLeod A, Costerton JW (1988). Starvation and nutrient resuscitation of *Klebsiella pneumoniae* isolated from oil well waters. J. Appl. Bacteriol. 64:541-549.

Lillis TO, Bissonnette GK (2001). Detection and characterization of filterable heterotrophic bacteria from rural groundwater supplies. Letters in Appl. Microbiol., 32: 268-272.

MacDonnell MT, Hood MA (1982). Isolation and characterization of ultramicrobacteria from a Gulf Coast estuary. Appl. Environ. Microbiol. 43:566-571.

Morita RY (1982). Starvation-survival of heterotrophs in the marine environment.Adv. Microbiol. Ecol. 6:171-198.

Presswood WG, Strong DK (1978). Modification of M-FC medium by eliminating rosolic acid. Appl. Environ. Microbiol. 36:90-94.

Reasoner DJ, Blannon JC, Geldreich EE, Barnick J (1989). Nonphotosynthetic pigmented bacteria in a potable water treatment and distribution system. Appl. Environ. Microbiol. 55:912-921.

Reasoner DJ, Geldreich EE (1985). A new medium for the enumeration andsubculture of bacteria from potable water. Appl. Environ. Microbiol. 49:1-7.

Shirey JJ, Bissonnette GK (1991). Detection of groundwater bacteria capable of escaping entrapment on 0.45 –pore-size membrane filters. Appl. Envion. Microbiol., 57(8): 2251-2254.

Tabor PS, Ohwada K, Colwell RR (1981). Filterable marine bacteria foundin the deep sea: distribution, taxonomy, and response to starvation. Microbiol. Ecol. 7:67-83.