Isolation of Aerobic Heterotrophic and Anaerobic Sulphate Reducing Bacteria from Model Water System by Filtration Method

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Abstract

The purpose of this study was to compare the efficacy of the filtration method with direct method (without filtration) for detection and enumeration of either aerobic heterotrophic bacteria or anaerobic sulphate reducing bacteria (SRB). In addition we compared the results of colony morphology types of heterotrophic bacteria performed by filtration-spread plate experiments with spread plate experiments (i.e. plating on R2A agar plates). We detected that there was no statistically significant difference in the mean of SRB and heterotrophic bacteria counts from unfiltered and filtered samples. The results of the experiments comparing the cultivation and enumeration success of the filtered samples with unfiltered samples clearly demonstrate that filtration method enables the cultivation and enumeration of SRB and heterotrophic bacteria. In addition, the numbers of distinct colony types recovered from filtered samples were usually higher than unfiltered samples.

Keywords: Aerobic heterotrophic bacteria, sulphate reducing bacteria, filtration, spread plate method, cooling tower.
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Introduction

Cooling towers are heat rejection systems and provide suitable conditions for microorganisms such as *Legionella pneumophila* that is the agent responsible for Legionnaires' disease and SRB considered as the major bacterial group involved in microbiologically influenced corrosion (MIC) (Türetgen et al. 2005; Hamilton 1985, Choudhary 1998). For metabolic activities, SRB utilize sulfate ions as an electron acceptor and produce H_2S that is agent of corrosion (Hamilton 1985; Widdel and Pfennig 1984). The distribution of SRB in cooling towers and their capability of generating H_2S could thus be an indicator for possible bacterial corrosion. Ilhan-Sungur et al. (2005) showed that SRB were present at 46.6% (14 of 30) of the cooling towers which belonged to the 6 hotels and 8 business centers.

In our previous studies (Ilhan-Sungur et al. 2005; Ilhan-Sungur et al. 2007), samples were serially diluted to 10^{-1}-10^{-10} and SRB counts were determined by the most-probable-number (MPN) technique using Postgate's medium B that contained an iron indicator.

In this study we used a filtration method, which is used for isolation of *Legionella pneumophila* bacteria (British Standard 1998). The method consists of filtration, resuspension and spread plate steps. However, for SRB detection, it was modified. The purpose of this study was to compare the efficacy of the filtration method with direct method (without filtration) for detection and enumeration of either aerobic heterotrophic bacteria or anaerobic sulphate reducing bacteria. In addition we compared the results of colony morphology types of heterotrophic bacteria

performed filtration-spread plate experiments with direct spread plate experiments (i.e. plating on R2A agar plates).

Material and Methods

The laboratory model system

The present study was performed using an open recirculating flow model system to simulate the environment of an industrial cooling water system (Figure 1). Model system was made from polypropylene (height 70 cm, diameter 44 cm) and operated with 80 l potable water. Water was recirculated with a recirculation pump (Standard Pump, model JET M) and heated to keep constant at 27°C by a heater (ATMAN, AT-100, 100W). Also it was removed and replaced daily with potable water.

Four water samples having 1 l volume were taken from model recirculating water system in sterile containers monthly during 13 months. While two of the samples were exposed to filtration process, the other water samples were used directly without filtration.

Membrane filtration procedure

Water samples were concentrated by filtration through a 0.22 μm pore size polyamide filter (Sartolon, Sartorius AG, Goettingen, Germany), and then membrane filters were resuspended in 50 ml sterile tap water by stomacher (IUL Instruments) for 1 minute (British Standard 1998). The suspensions were used as inoculum for isolation of SRB and heterotrophic bacteria.

Isolation and enumeration of SRB

Unfiltered water samples and the resulting suspensions of filtered water samples were serially diluted to 10^{-1}-10^{-10}. SRB counts were determined by the MPN technique using Postgate's medium B. Postgate's medium B consists of $C_3H_5O_3Na$ (3.5 g/l), KH_2PO_4 (0.5 g/l), NH_4Cl (1.0 g/l), Ca_2SO_4 (1 g/l), $MgSO_4 \times 7H_2O$ (2 g/l), yeast extract (1.0 g/l), $C_6H_7O_6Na$ (0.1 g), $C_2H_3O_2SNa$, (0.1 g), $FeSO_4 \times 7H_2O$ (0.5 g/l), $C_6H_5O_7Na_3$ (0.3 g/l). pH adjusted to 7.2 (Postgate 1984).

MPN tubes were incubated in the dark at 30°C for 3 months (The Institute of Petroleum 1995). In each inoculated tube, growth of sulphate reducers was indicated by the formation of a black FeS precipitate and by turbidity.

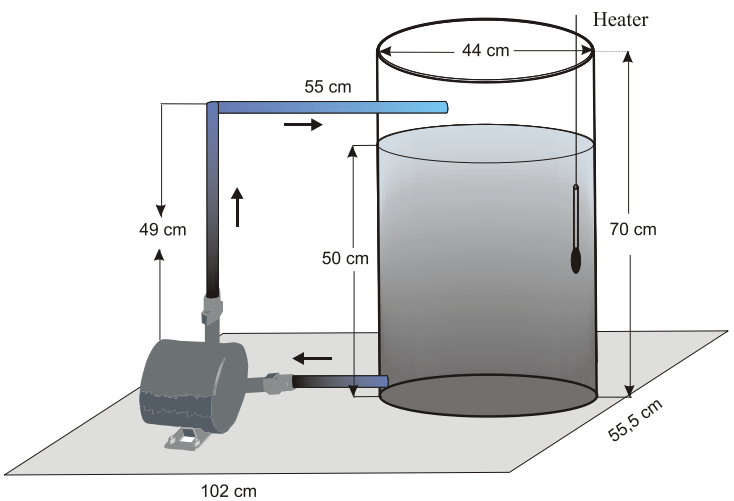

Figure 1. Schematic diagram of model recirculating water system, arrows indicate the flow direction.

Recovery of Heterotrophic plate count (HPC)

Samples of 100 µl from dilution series of unfiltered and filtered water were directly plated out in triplicate on R2A (Oxoid) and incubated for 7 days at 27°C (Regsoner and Geldrich 1985). After the incubation time, the plates were observed under a colony counter (WTW, model BZG 30) and the number of visually different colonies was recorded.

Statistical analysis

SRB and heterotrophic bacteria counts were $\log_{10}$ transformed and standard deviations of the means were calculated. Mann-Whitney U test was employed to detect statistically significant changes in the bacteria counts. Pearson product moment correlation coefficient test was used to examine the relationship between bacterial counts measured with both of the methods (filtration and without filtration).

Results

The results for the HPC determined on R2A medium by different two methods were shown in Table 1 and Fig. 2. The maximum HPCs recovered from unfiltered and filtered samples were detected as 2820000 ± 43589 cfu/ml in May and 938333 ± 16073 in June respectively (Table 1). A Mann-Whitney U analysis revealed that there was no statistically significant difference in the mean of R2A plate counts from unfiltered and filtered sample ($Z = -0.026$, $P > 0.05$). In addition, Pearson's correlation analysis demonstrated a significant positive relationship between HPC obtained from unfiltered and filtered water samples (correlation coefficient = 0.592, $P < 0.01$) (Fig. 2).

In order to investigate the numbers of different heterotrophic bacteria, the counts of distinct colony types detected on the R2A plates were recorded according to size, color and morphology (Fig. 3). Yellow, orange, pink, white and cream colonies were viewed on R2A medium plated by both of the different methods. However green fluorescence colonies were seen only on R2A medium that filtered samples were plated. In addition, the numbers of distinct colony types recovered from filtered samples were usually higher than unfiltered samples (Fig. 3).

Table 1. Heterotrophic and sulphate reducing bacteria counts obtained from unfiltered and filtered samples.

	HPC ± SD (cfu/ml)		SRB ± SD (cell/ml)	
Months	**Unfiltered sample**	**Filtered sample**	**Unfiltered sample**	**Filtered sample**
March	275 ± 35	53 ± 3	*	1750 ± 707
April	801297 ± 170236	896667 ± 48589	8250 ± 1061	4625 ± 177
May	2820000 ± 43589	618333 ± 27538	115000 ± 35355	200000 ± 0
June	2747500 ± 206135	938333 ± 16073	2500000 ± 0	2000000 ± 0
July	66667 ± 15275	12500 ± 2887	350000 ± 70711	2000000 ± 0
August	71333 ± 2082	15875 ± 1652	925000 ± 35355	143750 ± 37500
September	107333 ± 1528	10183 ± 161	925000 ± 35355	450000 ± 0
October	1000 ± 0	13000 ± 707	675000 ± 318198	212500 ± 17678
November	10533 ± 416	6225 ± 233	825000 ± 176777	400000 ± 70711
December	20050 ± 1485	33500 ± 614	575000 ± 459619	343750 ± 137500
January	3133 ± 551	39333 ± 1607	2450000 ± 2192031	1625000 ± 530330
February	987 ± 415	12500 ± 3211	600000 ± 424264	600000 ± 212132
March	430 ± 96	1615 ± 185	275000 ± 176777	266667 ± 72169

*: not carried out

The values of SRB count determined by different two methods were shown in Fig. 4. The maximum SRB counts recovered from unfiltered and filtered samples were detected as 2500000 ± 0 cell/ml in June and 2000000 ± 0 both in May and June respectively (Table 1). A Mann-Whitney U analysis showed that there was no statistically significant difference in the mean of SRB counts from unfiltered and filtered sample ($Z = -1.011$, $P > 0.05$). Also we detected a significant positive relationship between SRB counts obtained from unfiltered and filtered water samples (correlation coefficient = 0.592, $P < 0.05$). In addition it was determined that standard deviations according to means of SRB count obtained by filtered sample were lower than unfiltered sample.

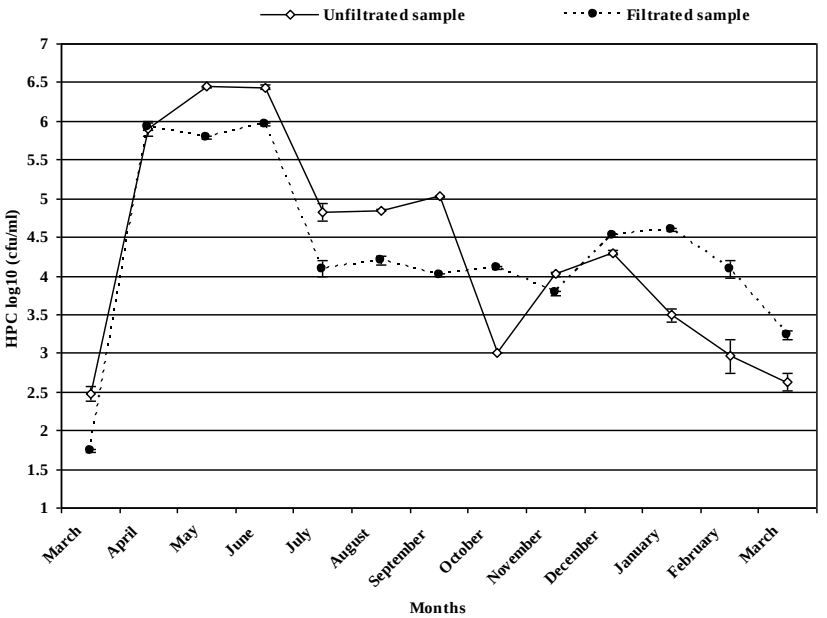

Figure 2. HPC values recovered from unfiltered and filtered samples. The plotted values represent $\log_{10}$ transformed, viable bacterial counts and arithmetic means of triplicates. The error bars represent the standard deviation of the mean.

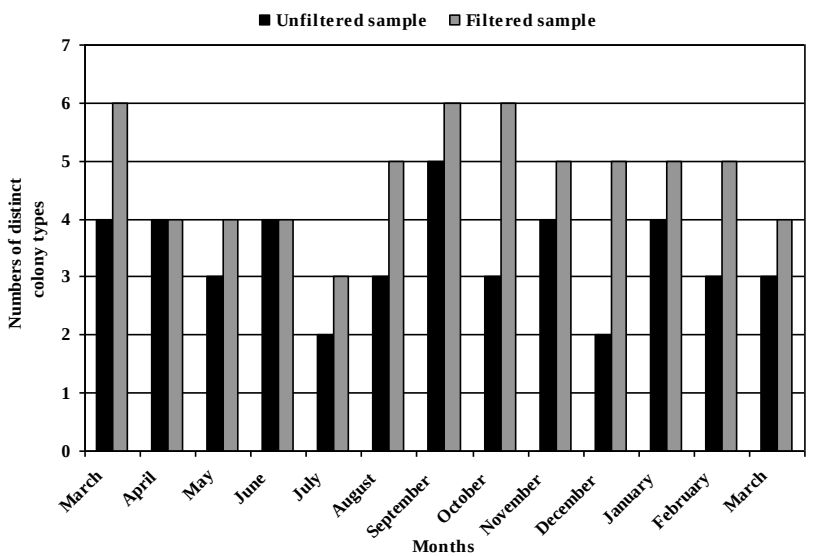

Figure 3. The numbers of visually distinct colony types recovered on R2A medium.

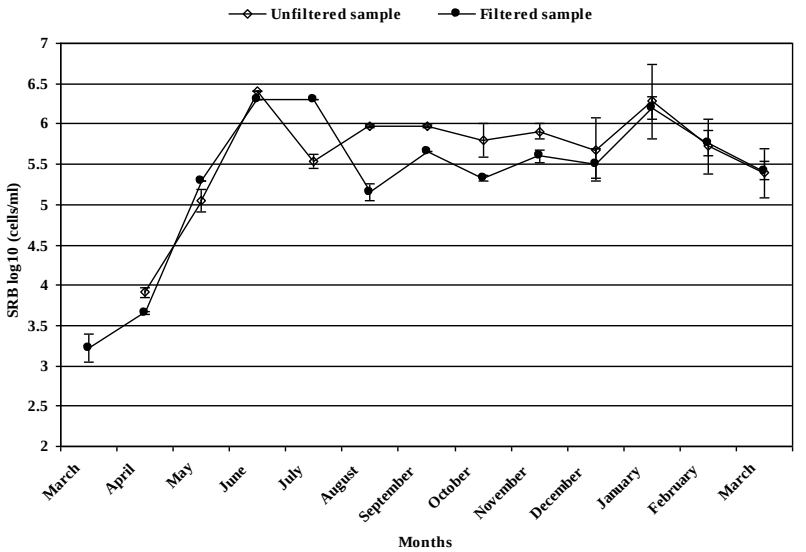

Figure 4. Growth curves of SRB recovered from unfiltered and filtered samples. The plotted values represent $\log_{10}$ transformed, viable bacterial counts and arithmetic means of duplicates. The error bars represent the standard deviation of the mean.

Discussion

Membrane filtration technique is used for a wide variety of bacterial assessments as well as *L. pneumophila* (Türetgen et al. 2005). The membrane filtration method allows the analysis of the more volume of water, depending on the water quality. Thus, it provides detection of bacteria found at very low concentrations in water sample (APHA 1981). SRB are anaerobe and so, they can be found at low values in water sample (Widdel and Phenning 1984). Therefore we applied filtration procedure in this study.

The counting area on the media of the membrane filtration method is smaller than the spread plate method. Thus, for HPC, we used spread plate method after filtration to provide higher counting area on the media.

We detected that there was no statistically significant difference in the mean of SRB and heterotrophic bacteria counts from unfiltered and filtered sample. However Punakabutra et al. (2004) reported that membrane filtration method gave higher bacterial recovery than the spread plate method. It could be explained by the less CFU values.

While yellow, orange, pink, white and cream colonies were viewed on R2A medium plated by both of the different methods, green fluorescence colonies were seen only on R2A medium that filtered samples were plated. The result shows that filtration-spread method is more suitable for recovery of distinct colony types of heterotrophic bacteria than direct spread plate experiment.

The results of the experiments comparing the cultivation and enumeration success of the filtered samples with unfiltered samples clearly demonstrate that filtration method enables the cultivation and enumeration of SRB and heterotrophic bacteria. In addition we can say that filtration method is more suitable for isolation of SRB, since standard deviations according to means obtained by filtered sample were lower than unfiltered sample.

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