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*Full Length Research Paper*

# Application of fluorescence *in situ* hybridization (FISH) to the analysis of sulfate reducing bacterial community in an oily bench scale reactor

**Abd El-Latif Hesham[1, 2]* and Saad A. Alamri[1]**

[1]Biological Department, Faculty of Science, King Khalid University, Abha, Saudi Arabia.
[2]Genetics Department, Faculty of Agriculture, Assiut University, Assiut, Egypt.

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**Advances in the field of genomics and meta-genomics have led to rapid and accurate strategies for the monitoring of microbial biodiversity and have revealed its potential for biotechnological applications. In this study, fluorescent *in situ* hybridization (FISH) as a culture-independent molecular approach using specific CY3-labelled oligonucleotide probes was used to study the dynamics of the sulfate reduction bacterial community (SRB) of the activated sludge from an oily wastewater treatment system. The relative abundance of members of the dominant bacteria in the oily water reactor was determined by FISH for 16S rRNA using EUB338 probes, for detecting general eubacteria, and SRB385 for targeting SRBs and major species of delta-*proteobacteria* sulfate reducers. The percentage of cells hybridizing with probe EUB338 for the dominant bacteria decreased from 25.85 to 6.25%, while with the SRB385 probe for SRB bacteria, it increased from 7.21 to 10.20% of total cells during the reactor process. These data show that SRB bacteria dominated the active microbial community in the system. It is interesting that delta-proteobacterial SRBs occupied a high percentage and took place in an oily biological system under aerobic conditions.**

**Key words:** Sulfate reducing bacteria, fluorescence *in situ* hybridization, 16S rRNA oligonucleotide probes, microbial community, dynamics.

## INTRODUCTION

Oily wastewater, generated by various industries and subsequently discharged into the natural environment, creates a major ecological problem throughout the world. Such wastewater must be treated before being released into the environment; otherwise its high mineral and organic content may severely pollute coastal waters, estuaries, rivers, the seashore, soil, and even the air (Wang et al., 2007) Sulphate-reducing bacteria (SRB) play a significant role in the mineralisation of organic matter in anaerobic environments and in the biogeochemical cycling of sulphur. SRB group are characterized by specific features: their cell walls do not contain peptidoglycan, the cell membrane has a specific composition of lipids, and ribosomes are characterized by a specific sequence of nucleotides in the RNA chain (Alcamo, 2001).

Fluorescently labeled rRNA-targeted oligonucleotides are used to specifically sain different members of microbial communities. The specificity of the probes ranges from the phylotype to the kingdom, depending on the targeted region on the rRNA (Amann et al., 1995). The

*Corresponding author. E-mail: hesham_egypt5@yahoo.com , hesham.egypt5@yahoo.co , hesham_egypt5@hotmail.com. Fax: +2088-2331384.

**Abbreviations: FISH,** Fluorescent *in situ* hybridization; **SRB,** sulfate reduction bacterial community.

signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. Presently, only the cyanide dye Cy3 provides sufficient signal intensity to serve as fluorescent label for the direct microscopic visualization of microbial assemblages in the marine environment by FISH with monolabeled oligonucleotide probes (Eilers et al., 2000; Pernthaler et al., 2002; Loy et al., 2003).

The detection, identification and confirmation of micro-organisms such as SRB and other bacteria from environmental samples of water from oil fields representing the population of its habitat can be achieved with greater confidence using FISH than with other techniques (Santos et al., 2010). This molecular tech-nique using 16S rRNA specific oligonucleotide probes is widely used in microbial ecology (Bryukhanov et al., 2011). The rapid and sensitive FISH technique provides information on the qualitative and quantitative composition of microbial communities, which is especially important for the determination of the phylogenetic position of uncultured microorganisms (DeLong et al., 1999). FISH probes could be designed with rRNA sequence specificity for nearly any microbial phylotype or taxon.

The objective of this study was to use highly specific oligonucleotide labelled probes for identifying SRB and eubacteria. Such a probe can be used for *in situ* hybridization. Through the application of these probes, the relative abundance and dynamics of eubacteria and SRB in activated sludge were studied.

## MATERIALS AND METHODS

### Bench scale reactor set up

The experimental system employed in this study consisted of a bench scale reactor with a working volume of 1.5 L. The biological treatment system was inoculated with activated sludge. Air bubbles for aeration were supplied through the reactor bottom. Oily wastewater from oil field was used as the influent. The bioreactor was operated, and the hydraulic retention time (HRT) was about 24 h.

### Samples

The bioreactor was operated using a fill-and-draw mode, and every day, 1 L of oily wastewater was exchanged after 90 min of sedimentation. Weekly sludge samples were taken from the reactor system for the FISH experiment and water for the determination of the chemical oxygen demand (COD).

### COD analysis

10 ml of the discharged water samples were taken from the bioreactor and centrifuged at 5,000 rpm to prepare the cell free supernatant at 4°C. This supernatant was used for COD determination. The COD in the supernatant was measured using a Spectralab COD Digester (2015 M) and a COD Titrator (CT-15) using a platinum combined electrode (Singh et al., 2011).

### Cell counts and fluorescence *in situ* hybridization

Cell counts and FISH were performed on samples collected during the time period from the oily bench scale reactor. Total cell counts were conducted by using 4,6-diamidino-2-phenylindole (DAPI) staining (Zarda et al., 1997).

### Hybridization with oligonucleotide probes

For *in situ* hybridization, we used indocarbocyanine (Cy3)-labeled 16S rRNA oligonucleotide probes EUB338 to target eubacteria and SRB385 for targeting sulfate reducing bacteria (Amann et al., 1990). The specific nucleotide sequence for each probe is summarized in Table 1. Samples for the DAPI and FISH counts were processed within a few hours of sampling by centrifugation at 2,500 g for 5 min. The debris or cell pellets were then re-suspended in 1 ml of 4% paraformaldehyde in phosphate-buffered saline (130 mM NaCl, 7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$). Samples were further processed as described by Zarda et al. (1997); 20 µl from each fixed and dispersed sample was spotted onto ethanol-washed slides. Drying, hybridizations with oligonucleotide probes, DAPI staining and washing were performed under standard conditions (Zarda et al., 1997). The formamide concentrations used in the hybridization mix were 30% for probe EUB338 and 20% for SRB385. Sodium chloride concentrations in the wash buffer were 112 mM for probe EUB338 and 250 mM for SRB385 (Zarda et al., 1997).

### Microscopic evaluation

The slides were visualized using an Olympus BX51 microscope with an Olympus Q-Color camera for image capture. Image analysis was carried out using Image Pro-Plus version 5.1 software. Cells stained with DAPI, and Cy3-labeled probes were visualized using two sets of filters - U-MWU2 - 330/420nm and U-MSWG2 - 480/590nm, respectively.

## RESULTS AND DISCUSSION

### Reactor performance

COD in influent wastewater was determined and the results show that the COD declined from 555 ± 12 $mgL^{-1}$ to 312 ± 33 $mgL^{-1}$. The results indicate that the capacities of decomposing COD and toxic compounds were enhanced as a result of further adapted bacteria.

### SRB community analysis by fluorescence *in situ* hybridization

The DAPI stain includes both inactive and dead cells, so it may overestimate the total number of living bacteria. The FISH technique can, to some extent, solve this

**Table 1.** Cyanine 3-labeled 16S rRNA-specific oligonucleotide probes used in this work.

| Probe | Phylogenetic specificity | Probe sequence (5'-3') | 16S rRNA target fragment | FA (%) | Tm (°C) | Reference |
|---|---|---|---|---|---|---|
| EUB 338 | Most bacteria | GCTGCCTCCCGTAGGAGT | 16S rRNA | 30 | 55 | Amann et al.,1990 |
| SRB385 | Sulfate reducing bacteria | CGGCGTCGCTGCGTCAGG | 16S rRNA | 20 | 59 | Amann et al.,1990 |

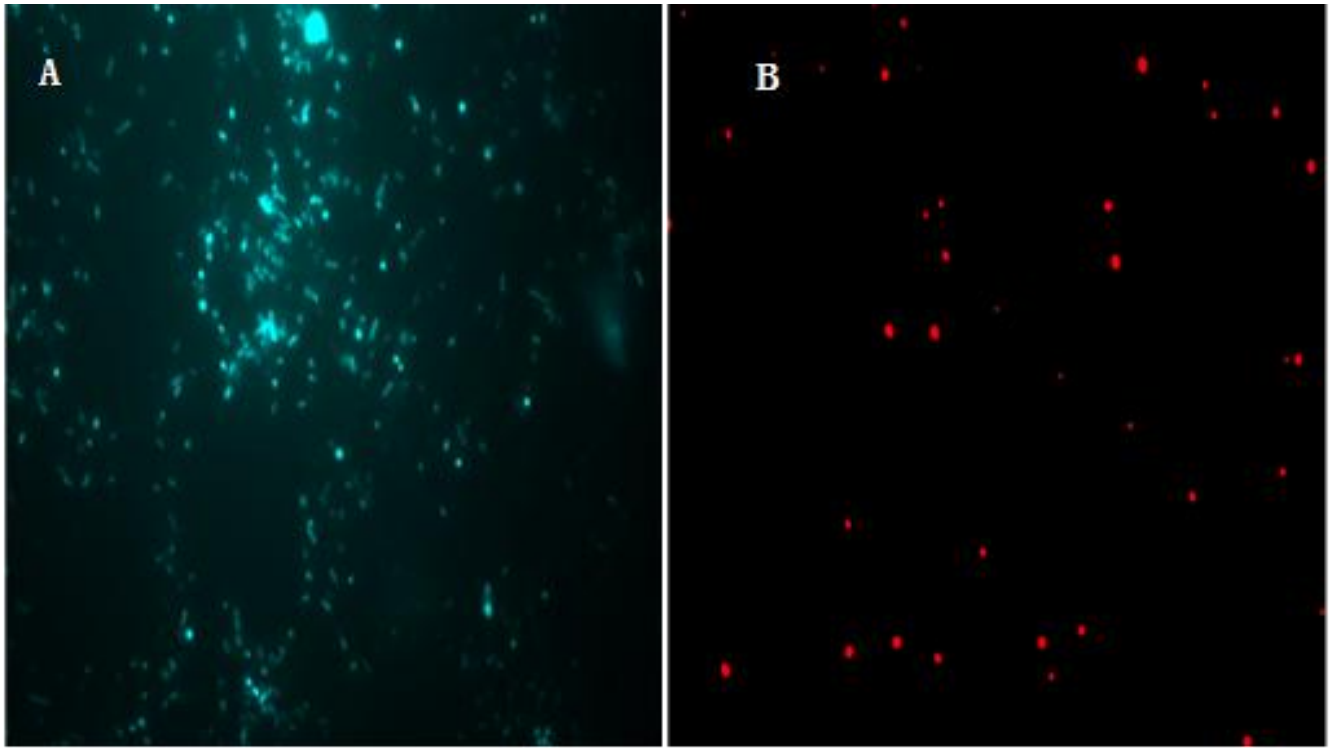


**Figure 1.** FISH image of sludge samples from oily reactor. The detected total bacteria shows by DAPI (a), and active bacteria detecetd by Cy3-labeled probe EUB 338 (red) (b) . FISH, Fluorescent in situ hybridization; DAPI, 4,6-diamidino-2-phenylindole.

problem. Since FISH labels only bacteria with a certain content of ribosomal RNA, therefore only active cells or cells that have recently been active will be enumerated (Bouvier and Giorgio, 2003).bacteria detected with probe EUB338 and of SRB detected with probe SRB385 in the reactor system as determined by the FISH technique are shown in Figures 1 and 2. The percentages of cells hybridizing with probe EUB338 ranged from 6.25 to 25.85%, and with probe SRB385 from 7.21 to 10.20% of the total (DAPI-stained) cells. These numbers underestimate the fraction of living, active cells, as they relate to DAPI counts. As shown in Figure 3, the ratio of the cells hybridizing with probes EUB338 to the number of DAPI-stained cells decreased significantly from 25.85 to 6.25% during the course of the reactor process. This was probably due to the biological reactor containing many bacterial species with a low ability to degrade oil pollutants. The highest number of suspended cells associated with the domain bacteria (EUB338, 25.85%) detected in the reactor, were quite similar to the bacteria numbers determined in a previous study using the same method in different environments (Bolliger et al., 2000; Kleikemper et al., 2002 ).

On the other hand, the number of active SRB revealed by FISH significantly increased from 7.21 to 10.20% of the total (DAPI-stained) cells. These data show that SRB bacteria dominated the active microbial community in the system, and indicated that it has ability to degrade the oil pollutant and to take place in biological systems under aerobic conditions.

As shown in Figure 3, the highest numbers of SRB detected in this study (SRB385, 7.21 to 10.20%) were higher than those detected with activated sludge by other authors using the same method (7 to 7.9% of the total bacteria) from aeration tanks (Manz et al., 1998).

However, they were lower than those detected by

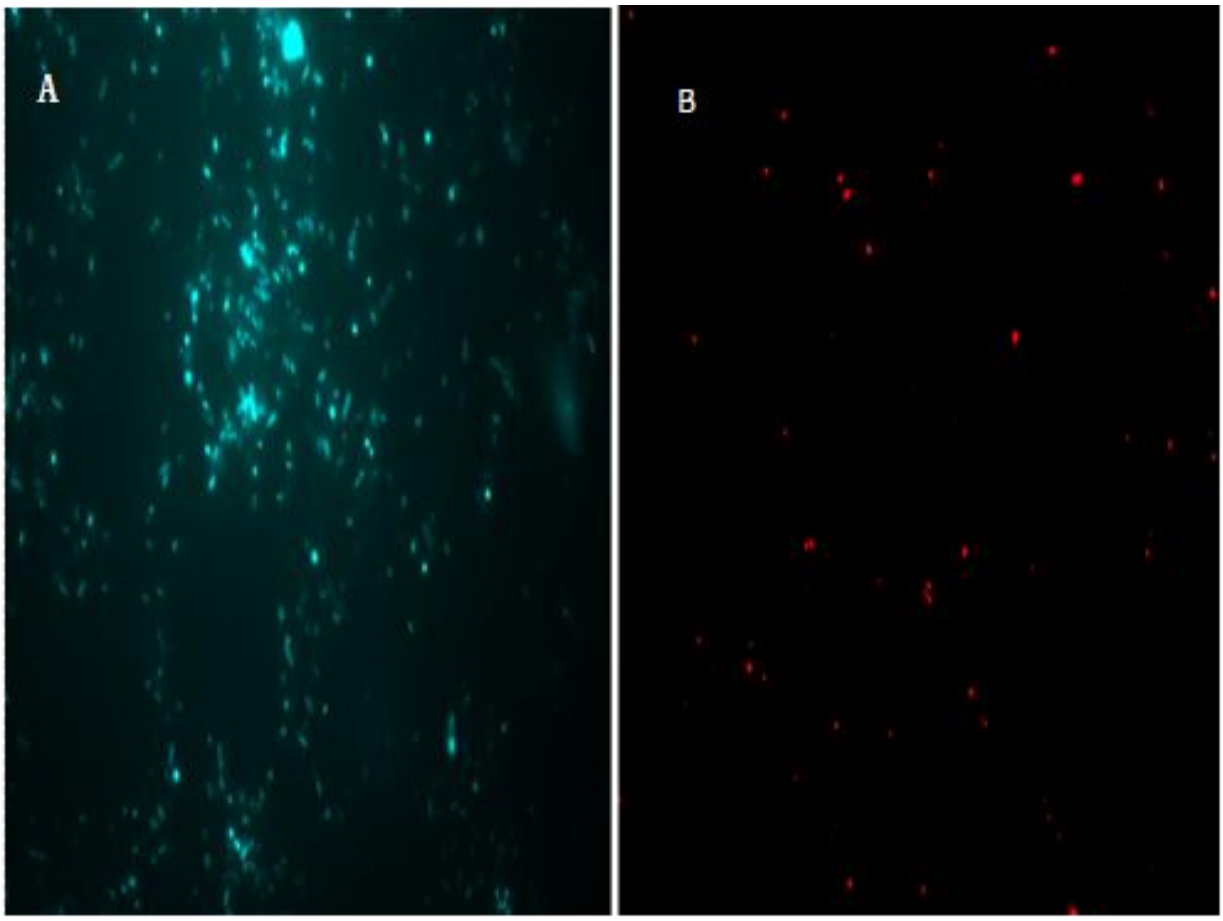


**Figure 2.** FISH image of sludge samples from oily system. The detected total bacteria shows by DAPI (a), and active SRB bacteria detected by Cy3-labeled probe SRB385 (red) (b). FISH, Fluorescent in situ hybridization; DAPI, 4,6-diamidino-2-phenylindole; SRB, sulfate reduction bacterial community.

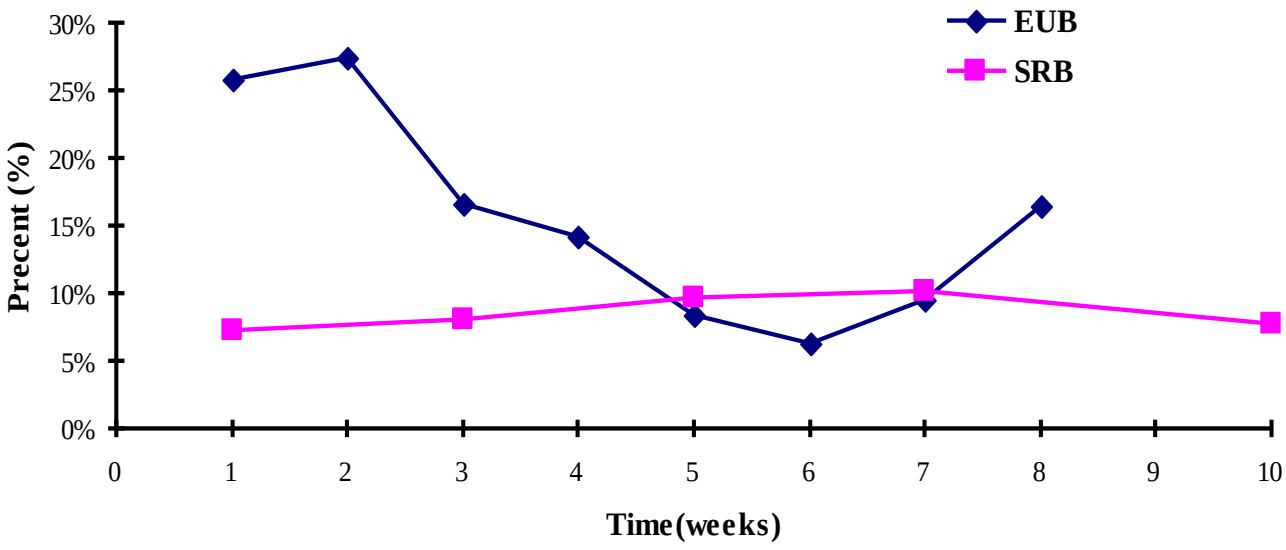


**Figure 3.** Percentages of cells hybridizing with probe EUB338 and probe SRB385 of total (DAPI-stained) cells. DAPI, 4,6-Diamidino-2-phenylindole.

Kleikemper et al. (2002) in aquifer samples (11 to 24%). The reasons for this difference may include the environment that we investigated, and our choice of probe (SRB385), which may have resulted in higher detection rates. Although SRB bacteria are traditionally considered strict anaerobes, Bryukhanov et al. (2011) detected some species of sulfate reducers in aerobic waters and in the oxic/anoxic transitional zone (chemocline) of the Black Sea; their results were in agreement with our findings in terms of the aerobic reactor.

Diagnostic rRNA directed oligonucleotides have been designed for specific groups of SRB (Devereux et al., 1992; Amann et al., 1990; Rabus et al., 1996) and have been demonstrated to represent valuable tools for group- and species-specific hybridization studies of SRB populations in complex communities such as biofilms (Amann et al., 1992; Ramsing et al., 1993), marine sediments (Devereux and Mundfrom, 1994), microbial mats (Ramsing et al., 1993; Risatti et al., 1994), anaerobic bioreactors (Dar et al., 2008) aquifers (Morozova et al., 2011), and sea water (Bryukhanov et al., 2011) without prior isolation of the target organisms.

The conclusion that the bench top wastewater reactor selected for sulfate reducing delta-proteobacteria that were able to degrade crude oil or polycyclic aromatic hydrocarbons seems to be justified and it is in line with other findings (Suarez- Suarez et al., 2011).

## Conclusion

The culture-independent molecular approach using specific fluorescein-labelled oligonucleotide probes was implemented on sludge samples from the oily biological system, in order to identify and quantify the dominant microbial groups. Our results show that FISH analyses have the potential to offer a significant step forward in the process of undertaking reliable quantification of specific bacteria in oil field sludge samples. The study demonstrated that SRBs, delta-proteobacterial SRBs, increased during the wastewater treatment process, suggesting that delta-proteobacterial SRBs played a great role in the removal of oily pollutants. Finally, our findings reveal that SRB bacteria could take place in biological systems under aerobic conditions.

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