IN SITU IDENTIFICATION AND QUANTIFICATION OF BIOAUGMENTATION PRODUCTS IN WASTEWATER TREATMENT FACILITIES

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Abstract

Bioaugmentation of wastewater treatment processes has gained greater industrial support recently. Until recently, there has been no way to quantify the presence and activity of the exogenous organisms added to the complex wastewater treatment communities. Current *in situ* microbial identification methods are focused on 16S rDNA-based methods and include fluorescence *in situ* hybridization (FISH) which offers insight into microbial community dynamics. However, this cannot be reliably used to distinguish between closely related organisms and lacks sensitivity. While end-point PCR is a sensitive alternative, it lacks the specificity needed to accurately identify individual microbial strains such as those added in bioaugmentation schemes. In order to identify and quantify a specific exogenous strain in complex communities, a combination of microbial strain-specific quantitative PCR (qPCR) and a recent advancement in FISH technologies, Recognition of INdividual Gene-FISH (RING-FISH), has been employed. The combination of techniques is highly specific, sensitive and quantitative and can be used to both monitor the presence and integration of bioaugmentation products in wastewater treatment.

Introduction

Recent advances in wastewater treatment research have emphasized the importance of the microbial populations inherent to the processes used (Wagner et al., 2002). The total microbial community structure is responsible for both efficient processing and removal of undesirable wastewater constituents such as COD, phosphorus, nitrate, etc, and for the maintenance of stable plant conditions. The active consortia in these systems are strongly influenced by the influent type, treatment scheme and operational conditions (Manz et al., 1994), and their importance is reflected in the intentional design of wastewater treatment plants specifically for the enrichment of some microbial constituents (i.e nitrifiers) over those with detrimental effects on treatment, such as filamentous bacteria responsible causing bulking problems.

The process of adding microbial members to these complex consortia for beneficial effects is known as bioaugmentation and has been practiced for years, Recent findings provide considerable support for bioaugmentation methods (Bai et al., 2010) however the need for tools to assess whether it is appropriate to a given situation and to monitor the subsequent effects are paramount in its broader acceptance (El Fantroussi and Agathos, 2005).

Bioaugmentation has considerable potential for improving aspects of wastewater treatment. To realize this potential, it is increasingly important that the effects of these treatments be closely monitored and that they are linked to the activity and persistence of the bioaugmentation organisms. Here we present a combination of molecular and microscopic methods aimed at validating and monitoring the effects and presence of bioaugmentation strains. In order to

maintain high specificity for a strain of interest in a microscopic technique, we have employed the Recognition of INdividual Gene Fluorescence *In Situ* Hybridization (RING-FISH) method (Zwirglmaier et al., 2004) based on a known, unique, genomic sequence in a bioaugmentation strain. This method has been successfully applied to visualize the bioaugmentation strain in a variety of wastewater samples from field applications against the native microbial community. The same sequence was used to perform qPCR on treated field samples in order to monitor the abundance of the specific strain of interest.

Methods and Findings

The complete genome of a bioaugmentation strain, NZ-86, was sequenced and a search was performed for unique regions appropriate for qPCR. A qPCR standard curve was created by spiking in known cell numbers of NZ-86 into untreated wastewater from a petroleum refinery, extracting total nucleic acids and plotting cell number against the calculated fluorescence threshold cycle (C_t) value. The selected genomic region was also used to amplify Cy3 fluorescently-labeled RING-FISH polynucleotide probes for microscopic visualization according to the method of (Zimmermann et al., 2001).

Sample #	Industry	Bioaugmentation?	ng/uL DNA	End-point PCR	C_t	Log NZ-86 cells/ml
1	Food Additives	Y	26.8	+	23.68	6.85576
2	Petroleum	Y	28.2	+	26.73	5.88586
3	Poultry	N	178	-	ND	ND
4	Paper	Y	158.5	+	26.95	5.8159
5*	Paper	Y	200.5	+	24.38	6.63316
6	Paper	Y	85.1	+	25.52	6.27064
7	Muni	N	308	-	ND	ND
8	Muni	Y	190.3	+	24.54	6.58228
9*	Petroleum	Y	92.4	+	24.49	6.59818
10	Petroleum	N	265.6	-	ND	ND
11	Paper	Y	288	+	23.83	6.80806
12	Paper	Y	93.2	+	24.15	6.7063
13	Paper	N	36.4	-	ND	ND
14	Paper	Y	47.6	+	25.68	6.21976
15	Paper	N	22.2	-	ND	ND
16	Petroleum	Y	11.5	-	ND	ND

Table 1. Summary of samples used for end-point and quantitative PCR analyses. An example of end-point PCR results can be seen in Figure 1. ND = Not detected * = sample used for RING-FISH analysis

Wastewater, sludge, and mixed liquor samples were collected from 16 treatment plants from several industrial sectors and municipal sites (Table 1). Of these, 11 sites were treated and 5 sites were untreated controls. The samples were split and a subsample of each was immediately fixed for RING-FISH visualization in 4% paraformaldehyde. DNA was then extracted from a 5 ml portion of the remainder for PCR and qPCR. Extracted DNA was quantified on a NanoDrop

spectrophotometer and end-point PCR was run with a total of 300 ng DNA each. Products were analyzed on a 1.2% agarose gel stained with ethidium bromide (Figure 1). qPCR was carried out on a SmartCycler (Cepheid, Sunnyvale, CA) using a FAM/BHQ3 strain-specific probe under standard operating conditions, again using 300 ng of template DNA (Figure 2). qPCR was capable of the specific quantification of strain NZ-86 in complex communities. All but one of the treated sites showed persistence of the strain after treatment and the sites that had not been treated did not result in positive end-point or qPCR, suggesting high specificity for this primer/probe set.

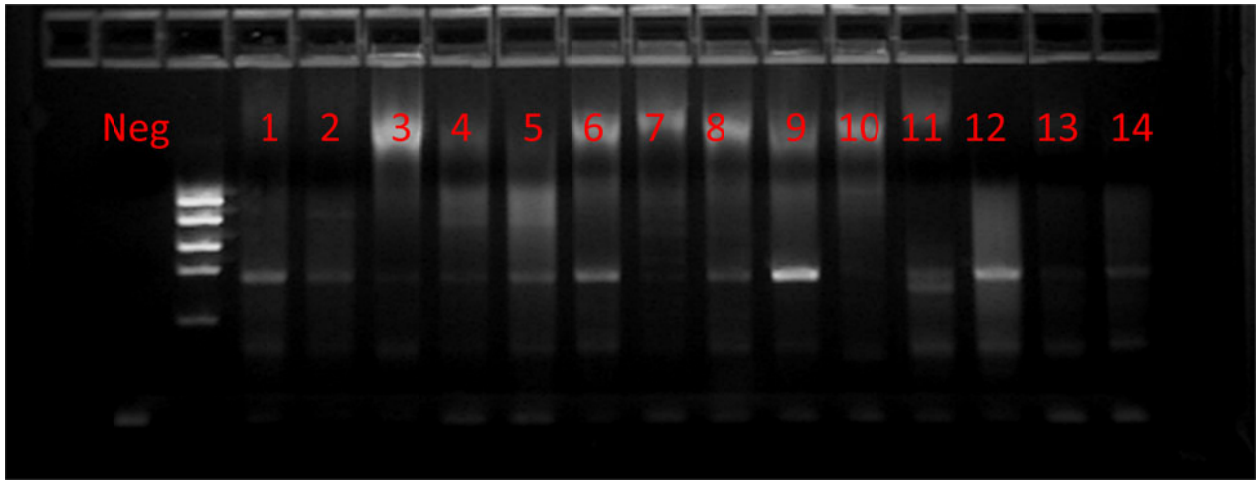

Figure 1. Example of end-point PCR results using primer pair specifically designed for a unique genomic region of strain NZ-86. Sample numbers correlate with those in Table 1.

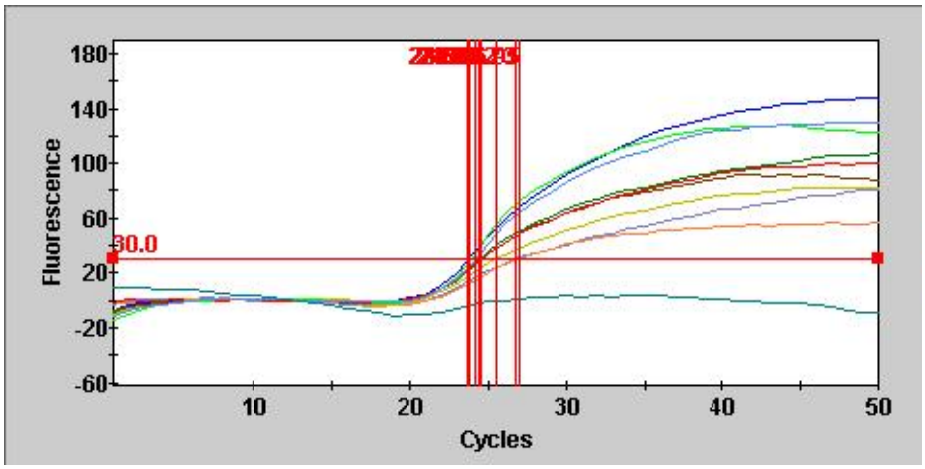

Figure 2. Example of qPCR results using primer/probe set specifically designed for a unique genomic region of strain NZ-86. The threshold value was set to 30 normalized fluorescence units.

Paraformaldehyde-fixed samples were prepared for RING-FISH as previously described (Nielsen et al., 2009; Zwirglmaier et al., 2004). Briefly, the strain specific probe described above was hybridized to the sample then washed off to avoid background fluorescence. After hybridization and washing, the samples were treated with VectaShield® mounting medium

(Vector Laboratories, Burlingame, CA) to protect against photodegredation of the Cy3 labeled probes during visualization on an Olympus BX50 epifluorescent microscope. A pure culture of NZ-86 grown in synthetic medium was first visualized to ensure the specificity and efficacy of the probing procedure (Figure 3). The technique was then applied to a municipal wastewater sample with the addition of NZ-86 just prior to sample preparation in order to assess the feasibility of the technique in a mixed population (Figure 4).

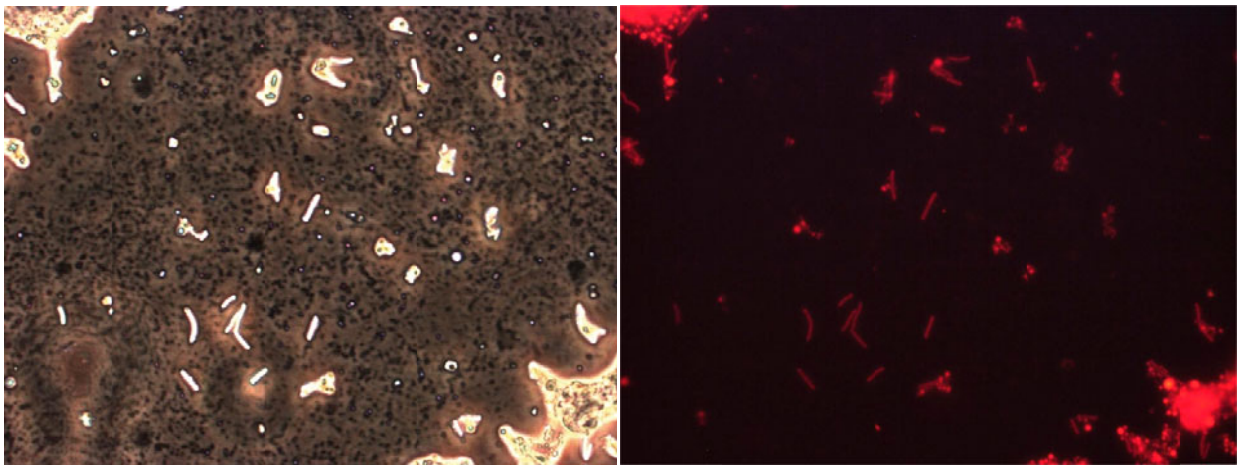

Figure 3. RING-FISH method applied to a pure culture of bioaugmentation strain NZ-86. Left: phase contrast microscopy of the cell culture. Right: epifluorescent image of the same field of view. Target cells appear red.

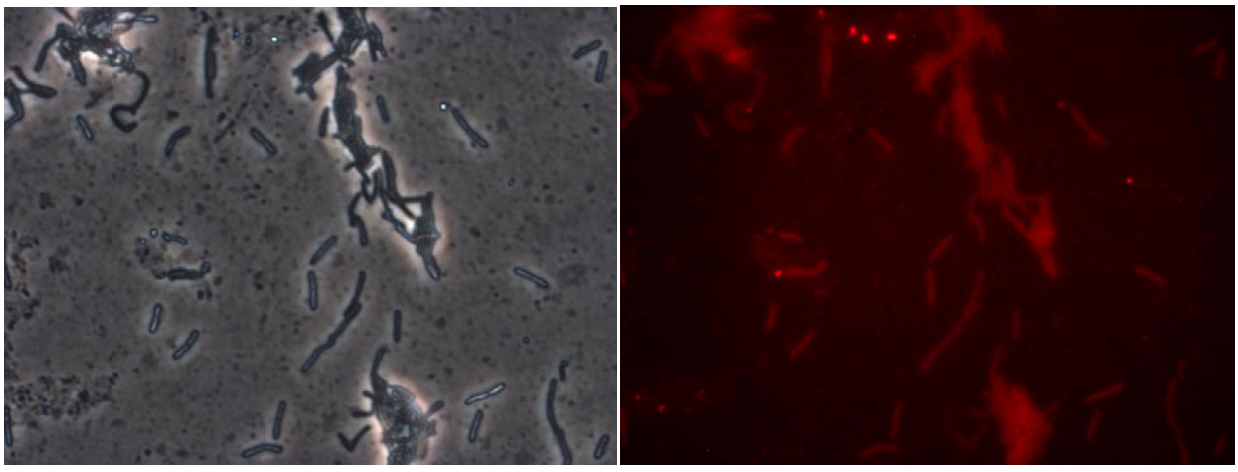

Figure 4. RING-FISH method applied to municipal wastewater sample with a pure culture of NZ-86 added prior to sample processing. Left: phase contrast microscopy of the cell culture. Right: epifluorescent image of the same field of view. Target cells appear red.

Field samples from pulp and paper processing (Figure 5**)** and offshore produced water (Figure 6) treatment facilities that had been treated with bioaugmentation products containing strain NZ-86 were then analyzed without the addition of a pure culture prior to fixation in order to visualize the NZ-86 persisting in the samples following treatment.

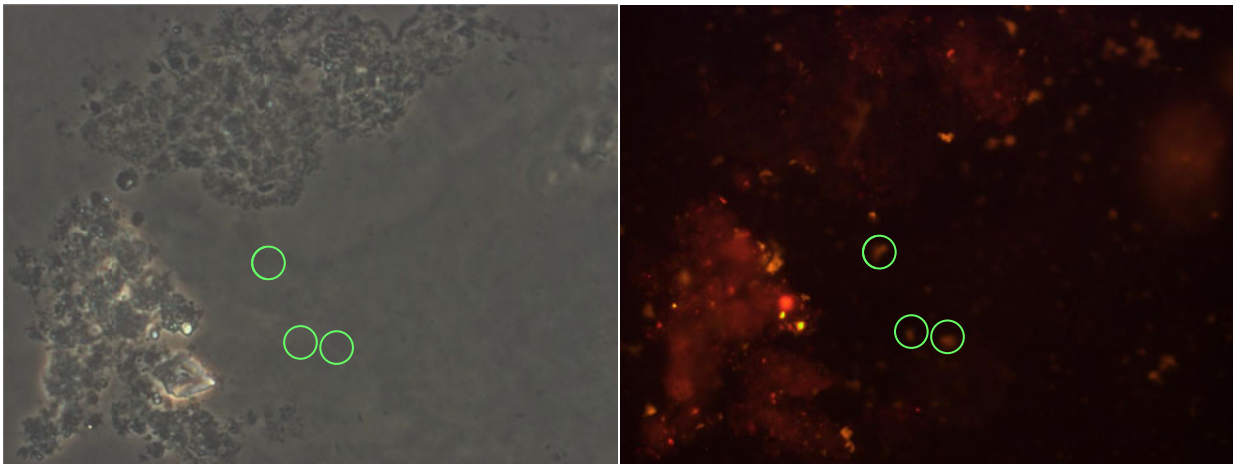

Figure 5. RING-FISH method applied to a pulp and paper processing plant wastewater sample that had been previously treated with a bioaugmentation product containing strain NZ-86. Left: phase contrast microscopy of the cell culture. Right: epifluorescent image of the same field of view. Target strain NZ-86 has been circled for ease of identification.

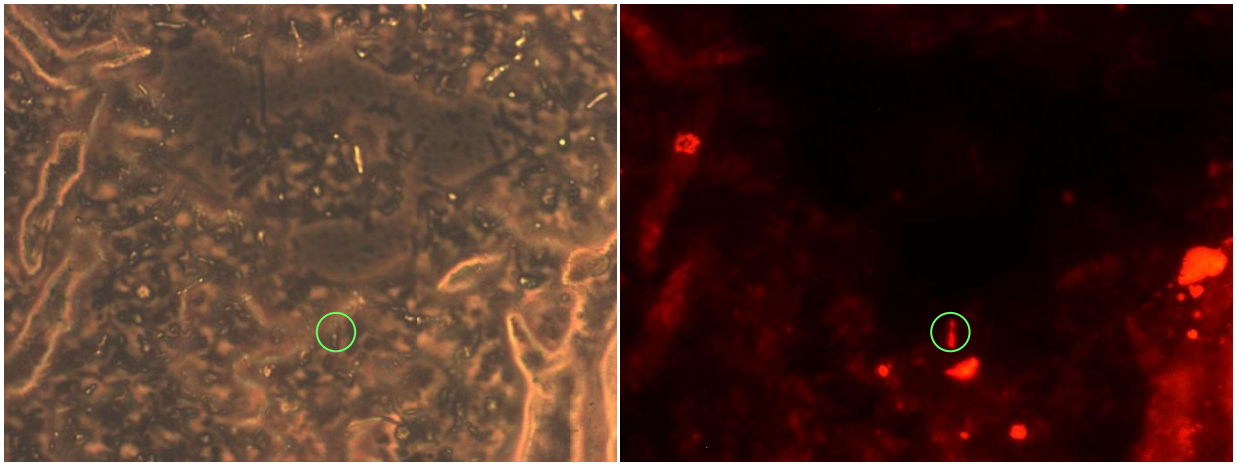

Figure 6. RING-FISH method applied to a petroleum refinery wastewater sample that had been previously treated with a bioaugmentation product containing strain NZ-86. Left: phase contrast microscopy of the cell culture. Right: epifluorescent image of the same field of view. Target strain NZ-86 has been circled for ease of identification.

Conclusions

New tools to identify and quantify bioaugmentation products have the potential to further improve this technology. Molecular methods, both visual and quantitative, can be applied to better understand the changes in complex microbial communities such as those in wastewater treatment facilities with the addition of one or several exogenous strains. Given the complex nature of wastewater treatment environments, methods employed to track the fate and effect of bioaugmentation strains must meet two primary criteria; 1) specificity and 2) adaptability. 16S rDNA based probes have been used to gain visual and quantitative information from complex communities (Addison et al., 2011) however these lack the specificity and resolution to analyze the presence and persistence of a specific strain in a complex community. The methods

employed here are based on a unique genomic sequence resulting in highly specific probing and quantification of the strain of interest despite a phylogenetically broad background. The methods are also adaptable to a range of common wastewater types. qPCR requires the efficient extraction of DNA from samples which can be accomplished through the use of commercially available kits and also allows for normalization of samples based on DNA concentration allowing for its use on samples with widely varying biomass. The RING-FISH method can be applied to samples of varied composition with relatively minor changes to the preparation, hybridization, and washing protocols though the extent of this plasticity remains to be seen.

The strength of these techniques lies in their combination. While qPCR alone can provide quantitative analysis of a unique strain in a system, the RING-FISH method is particularly well-suited to the localization and identification of individual cells of interest *in situ*, for example to answer the question of whether cells are being incorporated into floc particles or whether they remain dispersed. RING-FISH, when combined with qPCR, provides an invaluable tool for the monitoring of bioaugmentation products in a wastewater system. Future studies will be directed at correlating the data obtained through molecular techniques with operational performance from treated and untreated plants. This data could then be used to enhance bioaugmentation application techniques.

References

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