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ASTM Symposium on the Recovery and Enumeration of Mycobacteria from the Metalworking Fluid Environment

ABSTRACT: Since 1994, when the first documented hypersensitivity pneumonitis cluster was reported at a metalworking plant, there have been approximately 200 diagnosed cases of hypersensitivity pneumonitis within the metalworking industry. Although there are a variety of bacterial and fungal etiologic agents associated with hypersensitivity pneumonitis, metalworking industry stakeholders have focused their attention on a hypothesis that links *Mycobacterium immunogenum* exposure to the disease.

A number of barriers confound attempts to test this hypothesis. Today's symposium opens a dialogue on two significant barriers. There is no consensus practice for sampling and recovering *Mycobacterium immunogenum* from either bulk metalworking fluids or metalworking fluid aerosols. There is no consensus method for quantifying mycobacteria that may be present in either bulk fluid or aerosol samples.

This paper provides a context for the symposium's other presentations. After offering a brief overview of the history of hypersensitivity pneumonitis in the metalworking environment, the author will address the current state of knowledge regarding *Mycobacterium immunogenum* distribution in metalworking fluids. Finally, the author will summarize the three primary strategies for enumerating mycobacteria: microscopic examination of acid-fast stained preparations, viable counts and non-conventional methods.

KEYWORDS: hypersensitivity, pneumonitis, Mycobacteria, metalworking fluids, health, immunogenum, disease, immunology, microbiology.

Introduction

Historical Context

Hypersensitivity pneumonitis (HP) is a respiratory disease caused by a variety of biological and chemical agents. Its etiology is not well understood. The disease may be the result of the irritant effect of respired particles (biological or non-biological) on alveoli [1]. Certain inhaled particles may be more toxic than others [2; 3]. Alternatively, HP may be an allergic response [4].

Passman and Rossmore recently reviewed the current state of understanding of HP in the metalworking fluid (MWF) environment [5]. Since the sentinel outbreak at a Midwestern automotive parts manufacturing plant in 1992 [6] there have been approximately 200 cases of HP reported amongst manufacturing industry employees chronically exposed to MWF and MWF aerosols [7; 8]. The typical incidence of HP for the general population is 2 cases/100,000 person years (py) [9]. An estimated one-million metalworking industry employees are exposed to MWF routinely. This translates to approximately 1.4 cases/100,000 py; essentially indistinguishable from the background population HP incidence rate. However, HP has consistently occurred in clusters at metalworking facilities [7]. In a recent outbreak, 3% of the workers at a plant were diagnosed with HP [8].

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Linking cause and effect between specific etiologic agents and HP in the MWF environment is complicated by several critical variables. As demonstrated in Table 1, at least a half-dozen bacterial and fungal species routinely recovered from MWF are known etiological agents of HP [3, 4, 6, 10 11 and 12]. A combination of microbiological, aerosol, clinical and immunological testing has been attempted after several of the MWF industry disease outbreaks. More often, one or more of these parameters was not included in the post-outbreak investigations. Bernstein *et al.* [10] suggested a relationship between *Pseudomonas fluorescens* recovery, high antibody titer and HP incidence at one plant. Zacharisen *et al.* [12] linked *Acinetobacter lwoffii* serum precipitins (antibodies), viable *A. lwoffii* recoveries and HP at an automotive plant. Shelton *et al.* [13] suggested that *Mycobacterium chelonae* antigen (subsequently characterized as *M. immunogenum* [14]) was either the most likely cause or an important adjuvant for HP amongst metalworking industry workers. Falkinham [15] noted that several mycobacteria cell wall constituents, including mycolic-acid containing glycolipids, can induce immune responses in test animals. Moreover, Mycobacteria produce a variety of extracellular metabolites known to induce immune system responses. Significantly, Shelton asserted that plants with no HP are consistently free of measurable *M. immunogenum*. As we shall hear during this symposium, the growing database may not substantiate Shelton's hypothesis.

Symposium Focus

A debate regarding the importance of *M. immunogenum* relative to other known HP-causing MWF microbes is beyond the purview of this symposium. There is currently no consensus method for quantifying *M. immunogenum* in MWF or MWF aerosols. A critical step in identifying relationships amongst variables is figuring out acceptable means for quantifying them. Today's focus is on methodology. Specifically, the authors who will share their research with us will be describing three principal approaches: direct counts, viable counts and quantification by polymerase chain reaction (PCR). Without stealing the thunder of any of our other speakers, my objective in this paper is to provide symposium participants with an overview of each of these the strategies. As part of this introduction to today's symposium, I'll also address the issue of sampling as it affects *Mycobacterium* recovery.

Sampling and Recovery

The Babylonian Talmud, compiled during the 4th century C.E., teaches that the answers we get depend on the questions that we ask. This remains as true today as it was 16 centuries ago, and is particularly relevant to today's topic. How, when and where we sample affects what we recover from fluid systems and MWF aerosols. How we handle samples between collection and analysis also affects our analytical results. The recurrent theme of today's presentations will be the effect of test methodology on our perceptions about both the distribution and abundance of *M. immunogenum* in MWF and MWF aerosols.

Sample Source

Recirculating MWF systems, particularly large [$> 38 \text{ m}^3$ (10,000 gal)] central systems, have considerable surface areas. A typical system with 1,000 m (3,000 ft) of piping and a 38m^3 sump has approximately 128 m^2 ($1,400 \text{ ft}^2$) of surface area. The typical population density of a biofilm community is 10^7 to 10^9 cells/cm². This means that the system just describe is likely to be home to 10^{13} to 10^{14} biofilm-embedded cells. The significance of this number relates to our understanding of the relative efficacy of microbicides against planktonic (free-floating) and

epiphytic (attached) microbes. Biofilm chemistry and community interactions render microbes within biofilms substantially more difficult than planktonic microbes to kill. Yet most commonly, we measure only the planktonic population's response to microbicide treatment. This sampling strategy creates several problems. First, it underestimates total system bioburden by at least six orders of magnitude. Second, it results in apparent knock-downs of microbial viable counts, followed by rapid population recoveries. This occurs because biofilm communities are in dynamic equilibrium. Biomass flocs are released into the recirculating community frequently; reintroducing planktonic cells into the fluid as soon as the concentration of microbicide active ingredient falls below the minimum lethal dose. Third, it biases data towards that fraction of the population that is present in the bulk fluid. Obligate anaerobes and other species that depend on biofilm consortia interactions typically won't be recovered from bulk fluid samples.

To better understand how the microbial population is responding to contamination control measures, it's critical to include surface samples in condition monitoring programs. This may be accomplished either by identifying sampling sites or using coupons. The advantage to using pre-selected surfaces within the system is that no artificial surfaces are being introduced to new, undefined sources of variation. The disadvantage to using system surfaces is that uniform sample collection may be hindered by access limitations and variability of the dimensions of the swab area. Coupons facilitate quantitative recovery from well defined surface areas. Replicate coupons can be used to determine data variability, compare data from different parameters, or both. If flow dynamics across the coupons aren't the same as those across system surfaces, the biofilm ecology may be non-representative. Similarly, if the material from which the coupons are made is different from that of the system, the biofilm community on the coupons may not be representative of the MWF system biofilm community. If coupons are used to monitor biofilm control, validation tests should be run to ensure that actions based on coupon biofilm data reflect impact on system biofilms.

Sample Perishability

Both bulk fluid and surface samples are extremely perishable. The microorganisms in the samples remain active between the time of sampling and time of analysis. During this lag time population succession may occur. Microbes predominant in the system may die off as minor members of the system community may increase in relative and actual abundance. To minimize the effects of post-sampling population dynamics, testing should be performed as soon as possible after sampling. Some tests (for example: gross observations, odor detection, dissolved oxygen and adenosine triphosphate – ATP) may be performed tankside. Others (for example: viable counts by dip-slide, oxygen demand, and catalase activity) may be performed at the plant with hours after sample collection. More specialized tests (for example: endotoxin concentration, mycotoxin analysis, *M. immunogenum* and other species specific enumeration) require instrumentation and technical expertise not found at metalworking facilities routinely. Samples must be sent to laboratories qualified to perform the desired analyses. Samples destined for analysis by outside laboratories should be kept refrigerated between the time of collection and analysis. Analysis should be started within 24h after collection. Data from refrigerated samples that have aged longer than 24h or unrefrigerated samples that have aged longer than 8h are suspect. The relationship between such data and conditions in the system from which the sample was collected are tenuous at best.

Test Methods

Direct Count Methods

The common element of all direct count methods is that microbes are applied to a substrate that can be placed onto a microscope stage and counted. The simplest direct count method entails placing a drop of fluid sample into the chamber of a counting device such as a Petroff-Hauser counter. The chamber of the counter is reticulated (scored) to facilitate cell counting (figure 1). These devices are calibrated so that cells/ml can be computed from the average number of cells/reticule square. Alternatively, a known volume of fluid (typically 10 μL) may be placed onto a microscope slide, heat fixed (gently heated to evaporate the fluid), stained (more on this below) and counted. For this protocol an ocular objective (eye piece) with a reticulated lens is used. Cell per mL are computed from the average number of cells per field the area visible per field and sample volume. A third strategy is to filter a sample through a 0.45 μm pore-size membrane, stain the cells and then determine cells/mL from the average number of cells per field, surface area/field and sample volume.

Direct counting is labor intensive. Moreover, in fluids with substantial numbers non-microbial particles that are in the same size range as microbes, it may be difficult to differentiate between microbes and inert matter. Moreover, it is impossible to differentiate between viable and non-viable microbes unless special stains, known as vital stains are used.

Staining

Some of the limitations of the direct count method are overcome using stains. The most commonly used stain was first developed by Christian Gram in 1884 [16]. In this protocol, a head-fixed smear is first treated with an iodine solution [16]. It's then rinsed and decolorized with ethanol and counter stained with Safranin (a red dye). The carbohydrate envelope of Gram positive bacteria retains the iodine stain, making these cells appear blue to violet when viewed through a light microscope. Gram negative bacteria appear pink to red.

Of particular relevance to this symposium is the acid fast stain. The high lipid-content of mycobacteria and actinomycetes caused these microbes to bind carbolfuschin stain, so that when viewed under a light microscope, they will appear red. In this staining procedure, cells are first stained with carbolfuschin, then decolorized using acid alcohol (95% ethanol + hydrochloric acid), and finally counterstained with a methylene blue solution [17].

Acridine orange epifluorescent microscopy has been used for enumerating environmental bacteria since 1973 [18]. By the early 1980's investigators were coupling various vital stains and antibody tags onto fluorescent dyes in order to differentiate between living and dead cells and to detect targeted species [19-21]. Fluorescence acid-fast microscopy has been used recently to study mycobacteria ecology [22].

Direct count methods are the theme of two of today's presentations and are included in a third presentation.

Viable Count Methods

All viable count methods depend on the capture of viable microbes from a sample, and their subsequent proliferation in or on a nutrient medium [23]. When liquid nutrient media are used, population densities are estimated either by time lapsed between inoculation and observable change in the medium, visible growth through a single series of ten-fold dilutions (extinction dilution) or visible growth in an array of replicate series of extinction dilutions (most probable number – MPN). Observable change may be color, electrochemistry (for example impedance) or turbidity.

Colony counts may be obtained by one of four methods. Spread plates are prepared by spreading dilutions of the sample onto the surface of a solid medium. Pour plates are prepared by suspending dilutions of the sample into molten agar, pouring that agar into Petri dishes and allowing the agar to solidify. Sloppy agar (nominally nutrient media with half the agar concentration of that used in pour plates or spread plates) vials are inoculated with dilutions of the sample, and then shaken to distribute the sample. The membrane filter method is used for samples expected to have < 1 CFU/mL. A known volume of sample is filtered through a sterile 0.45µm membrane which is then placed upon with a solid nutrient medium or a broth-saturated absorbent pad.

After inoculation, the media are incubated at a specified temperature for a pre-designated time period. Colonies become visible when they contain approximately 2×10^9 cells. For fast growing species, colonies may be visible with 24 to 32h. Colonies of slow growing species, such as *M. immunogenum* may require > 1 week² incubation before they are visible.

Roszak and Colwell [24] reviewed the limitations of viable count methods in 1987. Only a small percentage of microbes living in the sampled environment are likely to elaborate into colonies on any given growth medium. The complex interaction effects of cell physiological state, inter-species competition, nutrient requirements, optimal growth temperatures and oxygen tension requirements affect both total colony counts and species recoveries. Early anecdotal evidence of acid-fast bacteria in MWF came from the examination of colonies that appeared on dip-slides after approximately two-week's incubation. Routinely, dip-slides are observed and discarded within 72h after inoculation. When colonies appear within 24 to 36 h, growth may become confluent (individual colonies grow large enough to touch one another – covering the agar surface completely) within 4 to 5 days. Colonies of slower growing microbes will not be observed, even if the slower growing species are more abundant in the original sample. Only when fast-growing species are suppressed (either through microbicide treatment in the system or use of selective growth media) can the slower growing microbes form detectable colonies. Early reports of *M. immunogenum* selection in MWF being caused by the use of microbicides known to be effective against Gram negative bacteria may have reflected the effect on viable count data rather than *in situ* population ecology. This and related issues will surface during today's proceedings.

² Typically, *M. immunogenum* colonies are visible at 10 to 14 days. Some sulfate reducing bacteria may require 3 to 4 weeks incubation before their colonies are visible.

Non-conventional Methods

Chemical methods used to quantify microbial populations are classified as non-conventional methods [ASTM Guide for Evaluating Nonconventional Tests Used for Enumerating Bacteria, E1326]. Some chemical methods - such as endotoxin [25], protein [26], deoxyribonuclease (DNA) [27] and ATP [28] concentrations – depend on the extraction and measurement of a particular cell constituent. Others measure the activity of general metabolism (two-hour oxygen demand) or specific enzyme systems (for example NAD-NADH reductase activity [29], catalase activity [30]). Recently, a number of methods have been used with some success to quantify both species abundance and taxonomic diversity. Fatty acid methyl ester (FAME) profiles are species specific [31] and can be used to determine population diversity profiles that include non-culturable species. One current limitation of FAME analysis is that the existing databases against which unknown chromatograph spectra are compared are comprised mainly of clinically important microbes. Non-clinical species may be misidentified by so called best fit chromatograph profile comparison programs. A recent FAME survey of MWF yielded profiles that had no species in common with those previously reported in MWF [32]. More research is needed to determine why the FAME derived taxonomic profiles were unique. Polymerase chain reaction (PCR) testing has been used in a variety of environmental studies. At 2003 microbially influence corrosion (MIC) investigation applied PCR methodology to characterize a population of *Archaea* species responsible for the corrosion [33]. Ecological surveys using PCR are changing our understanding of microbial diversity in environmental [34] and industrial [35] systems.

Specific PCR methods will be detailed in two of today's presentations. Briefly, the method relies on the activity of the enzyme polymerase.

Fluorescence antibody staining was described earlier under **Direct Count Methods**. A recent fluorescence technology development uses photomultiplier technology to amplify and quantify the light emitted by the target-bound dye-antibody complex. An *M. immunogenum* specific variation of this method will be presented this afternoon [Thomas, unpublished] this afternoon.

The Current Situation

Routine *M. immunogenum* surveillance has increased over the past decade. However, there is no consensus about a particular strategy or method for quantifying *M. immunogenum* in MWF system samples – surface, bulk fluid or aerosol samples. Although each lab has a high level of confidence in their data, a recent round robin suggests that this self-confidence may be unfounded [D'Arcy, unpublished]. Direct counts, viable counts and PCR methods are being used, but there is no consensus on a referee standard against which to evaluate the precision and bias of any given method. Despite the absence of a consensus quantification method, ASTM subcommittee E35.15 on antimicrobial pesticides has been approached to develop a method for evaluating MWF microbicide performance against acid-fast bacteria. Major corporations are making microbial contamination control decisions based on data generated by non-consensus methods, the precision and bias of which have yet to be determined. Industrial hygienists and industry stakeholders are posing hypothesis regarding the relationship between *M. immunogenum* exposure and HP, and proceeding as if those hypotheses have already been proven, even though there are data suggesting that other etiologic agents present in MWF also

cause HP. At present, *M. immunogenum* surveillance is not being complemented with equally aggressing endotoxin, mycotoxin or total bioaerosol surveillance. Given that the ratio of HP cases to total worker population is so small, epidemiological treatment of the data remains severely challenged.

The Path Ahead

The papers presented during this symposium demonstrate that additional work must be completed before we have a consensus protocol for quantifying mycobacteria in MWF. Viable counts, direct counts and quantification by qPCR all seem to have potential value, but each method also has limitations. Before data from any of these methods can be used reasonably to test the relationship between mycobacterial recovery and disease conditions such as HP a number of issues must be resolved.

First, we need a clear understanding of the recovery efficiencies and detection limits of each method. We also need to understand data variability. What is the anticipated data range for multiple tests performed by a single analyst on portions of a given sample? What's the data range amongst several analysts at the same testing facility? What's the range amongst different laboratories asked to analyze splits of the same sample? How does fluid chemistry and condition affect recovery? Do different MWF classes (emulsifiable oil, semisynthetic and synthetic) affect *Mycobacteria* survival (actual presence of *Mycobacteria* sp. in the tested fluid), impact recoveries (bias caused my test method) or some combination of the two? How do test results from the three different types of quantification methods covary? Until these questions have been answered, mycobacteria abundance and prevalence data are suspect. To address these precision and bias questions, ASTM Subcommittee E.34.50 has chartered three task forces – one to evaluate direct counts, viable counts and qPCR respectively.

Once we understand the variables affecting test precision and bias, the next step will be to complete MWF system surveys. Unless mycobacterial data are collected as part of a multivariate survey, hypotheses regarding the relationship between the presence of mycobacteria and HP will remain speculative. Other microbiological parameters such as heterotrophic bacteria plate counts, fungal plate counts, mycotoxin, endotoxin and total mist concentrations should be integrated with immunological testing and plant engineering/industrial hygiene surveys. The fact that *M. immunogenum* has been recovered from numerous systems at plants where HP has not been reported suggests that any potential relationship between mycobacteria exposure is complex. Eradicating *M. immunogenum* is likely to be insufficient for educing the HP risk in the MWF environment.

References

[1] Merck, "Hypersensitivity Diseases of the Lungs," in *The Merck Manual of Diagnosis and Therapy*, 2001, Merck & Co., Inc. Whitehouse Station, NJ.,

<http://www.merck.com/pubs/mmanual/section6/chapter76/76b.htm>.

[2] Rose C. "Hypersensitivity Pneumonitis," in *Occupational and Environmental Respiratory Diseases*, Harber P., Schenker, M.B. and Balmes, J. R. eds. Mosby, St. Louis, pp 201-215. 1996.

- [3] Cormier, Y. "Hypersensitivity Pneumonitis," in *Environmental & Occupational Medicine*, 3rd Ed. Rom, W.N. ed, Lippincott-Raven, Philadelphia, pp 457-465. 1998.
- [4] Schuyler, M., "Lesson 6, Volume 14 – Hypersensitivity Pneumonitis," 2001, <http://www.chestnet.org/education/pccu/vol14/lesson06.html>.
- [5] Passman, F. J. and Rossmoore, H.W., "Reassessing the Health Risks Associated with Employee Exposure to Metalworking Fluid Microbes," *Lubrication Engineering*, July 2002, **58**(7):30-38.
- [6] Muilenberg, M. L., Burge, H. A. and Sweet, B. S., "Hypersensitivity Pneumonitis and Exposure to Acid-Fast Bacilli in Coolant Aerosols," *Allergy Clin. Immunol.*, 1993, **91**(part 2), p. 311.
- [7] Kreiss, K. and Cox-Ganser, J., "Metalworking Fluid-associated Hypersensitivity Pneumonitis: a Workshop Summary," *Am. J. Ind. Med.*, **32** (4), pp 423-432. 1997.
- [8] Anon., "Respiratory Illness in Workers Exposed to Metalworking Fluid Contaminated with Nontuberculosis Mycobacteria – Ohio, 2001. MMWR, 2002, **51**(16): 349-352.
- [9] **Cases/yr citation**
- [10] Bernstein, D.I., Lummus, Z.L., Santilli, G., Siskosky, J. and Bernstein, I.L. (1995), "Machine Operator's Lung. A Hypersensitivity Pneumonitis Disorder Associated with Exposure to Metalworking Fluid Aerosols," *Chest*, 1995, **108**, pp 593-594.
- [11] Dutkiewicz, J., Kus, L., Dutkiewicz, E., and Warren, C. P. W., "Hypersensitivity Pneumonitis in Grain Farmers due to Sensitization to *Erwinia herbicola*," *Annal. Allergy*, 1985, **54**(1), pp 65-68.
- [12] Zacharisen, M.C., Kadambi, A.R., Schluter, D.P., Krup, V.P., Shack, J.B., Fox, J.L. Anderson, H.A., and Fink, J.N., "The Spectrum of Respiratory Disease Associated with Exposure to Metal Working Fluids," *J. Occup. Environ. Med.*, 1998, **40** (7), pp 640-647.
- [13] Shelton, B.G., Flanders, W.D. and Morris, G.K., "*Mycobacterium* sp. as a Possible Cause of Hypersensitivity Pneumonitis in Machine Workers," *Emerg. Infect. Dis.(serial online)*, 1999, **5** (2), <http://www.cdc.gov.ncidod/eid/vol5no2/shelton.htm>.
- [14] Wilson, R.W., Steinggrube, V.A., Bottger, E. C., Springer, B, Brown-Elliott, B.A., Vincent, V, Jost, K.C. Jr., Zahang, Y., Garcia, M. J., Chiu, S.H., Onyi, G.O., Rossmoore, H., Nash, D.R., Wallace, R.J. Jr., "*Mycobacterium immunogenum* sp. nov. a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy." *Int. J. Syst. Evol. Microbiol.* September 2001, **51**(Pt 5): 1751-64.
- [15] Falkinham, J.O. III, "Mycobacteria Aerosols and Respiratory Disease." *Emerg. Infect. Dis.*, July 2003, **9**(7): 763-767.
- [16] Gram, C., "Ueber die isolirte Farbung der Schizomyceten in SchnittÄund Trockenpreparaten." *Fortschritte der Medicin*, 1884, **2**:185-189.
- [17] Doetsch, R.N., "Determinative Methods of Light Microscopy." *In*, Gerhardt, P. *et al.* Eds. "Manual of Methods for General Microbiology," 1981, American Society for Microbiology. Pp: 21-33.
- [18] Francisco DE, Mah RA, Rabin AC, "Acridine orange-epifluorescence technique for counting bacteria in natural waters. *Trans Am Microsc. Soc.* 1973 Jul; **92**(3):416-21
- [19] Betts, R.P., Bankes, P. and Banks, J.G., "Rapid Enumeration of Viable Micro-organisms by Staining and Direct Microscopy." *Appl. Microbiol.*, 1989, **9**:199-202.

[20] Amann, R.I., Krumholz, L. and Stahl, D.A., "Fluorescent-oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic, and Environmental Studies in Microbiology." *J. Bacteriol.*, 1990, **172**: 762-770.

[21] Hodson, R.E., Dustman, W.A., Garg, R.P. and Moran, M.A., "In Situ PCR for Visualization of Microscale Distribution of Specific Genes and Gene Products in Prokaryotic Communities." *Appl. Environ. Microbiol.*, 1995, **61**: 4074-4082.

[22] Strahl E.D. Gillaspy, G.E. and Falkinham, J.O., III, "Fluorescent Acid-Fast Microscopy for Measuring Phagocytosis of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* by *Tetrahymena pyriformis* and Their Intracellular Growth." *Appl. Environ. Microbiol.*, October 2001, 67(10): 4432-4439.

[23] Anonymous. "9215 Heterotrophic Plate Count." In: Greenberg, A.E., Clesceri, L.S. and Eaton, A.D. Eds., *Standard Methods for the Examination of Water and Wastewater*. 1992. American Public Health Association, Washington, DC. pp: 9-32 to 9-39.

[24] Roszack, D. B and Colwell, R. R. "Survival Strategies of bacteria in the Natural Environment." *Microbiological Rev.* 1987, **51**(3): 365-379.

[26] Endotoxin

[27] Lowery, O. H., Rosebrough, N. J. Farr, A. L. and Randall, R. J. "Protein measurement with the Folin-Phenol reagents." *J. Biol. Chem.* 1951. **193**: 265-275.

[28] Kehrmeier, S. R., Appelgate, B. M., Pinkart, H. C., Hedrick, D. B. White, D. C., and Sayler, G. S. "Combined Lipid/DNA Extraction Method for Environmental Samples."

[29] Karl, D. M. Total Microbial Biomass Estimation Derived from Measurement of Particulate Adenosine-5'-triphosphate." In: Kemp, P.F., Sherr, B. F, Sherr, E. B. and Cole, J. J. Eds., *Handbook of Methods in Aquatic Microbial Ecology*. 1993. Lewis Publishers, Boca Raton, Fl. Pp: 359 – 368.

[30] NAD-NADH Reductase

[31] Gannon, J. E. and Bennett, E. O. "A New Rapid Technique for Determining Microbial Loads in Metalworking Fluids." *Tribol. Internat.* 1981. **14**: 7-9.

[32] Guckert, J. B., Antworth, C. B., Nichols, P. D., and White, D. C. "Phospholipid, Ester-linked Fatty Acid Profiles as Reproducible Assays for Changes in Prokaryotic Community Structure of Estuarine Sediments." *FEMS Microbiol. Ecol.* 1985, **31**:147-158.

[33] van der Gast C. J., Whiteley* A. S. , Lilley, A. K., Knowles, C. J. and Ian P. Thompson, I. P. "Bacterial Community Structure and Function in a Metal-working Fluid." *Environ. Microbiol.* 2003, **5**(6):453-461.

[33] Gibbon, D. "DNA Sequencing Pinpoints Corrosion-Causing Bacteria." *Materials Perform.* 2004, **43**(3): 42-43.

[35] PCR ecology

[36] PCR industrial syst

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TABLE 1—Results of tests. **{Journal Caption Centered}**

Specimen ^a	Length, m	Width, m
1	10	5.5
2	8	0.2
3	6	0.5
4	...	0.4

^aRefrigerate at 20°C for three days.

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