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Shu-Chi Chang · Tracey I. Anderson
Sarah E. Bahrman · Cyndee L. Gruden
Anna I. Khijniak · Peter Adriaens

Comparing recovering efficiency of immunomagnetic separation and centrifugation of mycobacteria in metalworking fluids

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Abstract The accurate detection and enumeration of *Mycobacterium immunogenum* in metalworking fluids (MWFs) is imperative from an occupational health and industrial fluids management perspective. We report here a comparison of immunomagnetic separation (IMS) coupled to flow-cytometric enumeration, with traditional centrifugation techniques for mycobacteria in a semisynthetic MWF. This immunolabeling involves the coating of laboratory-synthesized nanometer-scale magnetic particles with protein A, to conjugate a primary antibody (Ab), specific to *Mycobacterium* spp. By using magnetic separation and flow-cytometric quantification, this approach enabled much higher recovery efficiency and fluorescent light intensities in comparison to the widely applied centrifugation technique. This IMS technique increased the cell recovery efficiency by one order of magnitude, and improved the fluorescence intensity of the secondary Ab conjugate by 2-fold, as compared with traditional techniques. By employing nanometer-scale magnetic particles, IMS was found to be compatible with flow cytometry (FCM), thereby increasing cell detection and enumeration speed by up to two orders of magnitude over microscopic techniques. Moreover, the use of primary Ab conjugated magnetic nanoparticles showed better correlation between epifluorescent microscopy counts and FCM analysis than that achieved using traditional centrifugation techniques. The results strongly support the applicability of the flow-cytometric IMS for microbial detection in complex matrices.

S.-C. Chang · T. I. Anderson · S. E. Bahrman
A. I. Khijniak · P. Adriaens (✉)
Department of Civil and Environmental Engineering,
University of Michigan, Room 116, EWRE Bldg.,
1351 Beal Avenue, Ann Arbor, MI 48109, USA
E-mail: adriaens@umich.edu
Tel.: +1-734-7638032
Fax: +1-734-7632275

C. L. Gruden
Department of Civil Engineering,
University of Toledo, Toledo, OH, USA

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Introduction

Metalworking fluids (MWFs) are used for cooling and lubrication in a wide range of manufacturing applications. Generally, MWFs are categorized in four classes: straight oil, soluble oil, semisynthetic, and synthetic fluids, of which soluble oil and semisynthetic types are most widely used [10]. The annual United States consumption of MWFs exceeds 2 billion gallons [26]. Semisynthetic MWFs, which exhibit lower oil content and higher heat capacity, have gained increased acceptance among metal fabrication industries. Their formulations are oil-in-water emulsions, which represent prolific substrates for aerobic bacterial growth [3, 21]. Without biocide addition, total cell densities of up to 10^8 or 10^9 cells/mL are common, impacting the chemical properties, cutting efficiency, and subsequent need for MWF replacement [3, 21, 55]. Bacterial aerosolization and the control of microbial growth via biocide addition result in occupational health risks due to human exposure to pathogenic organisms and endotoxins released from lysed Gram-negative bacteria [65].

Historically, bacterial species of concern found in MWFs include *Pseudomonas* sp., *Klebsiella pneumoniae*, *Desulfovibrio* sp., and *Flavobacterium* sp. [3, 36, 55, 62]. Recently, *Mycobacteria* have been isolated from soluble oil, semisynthetic, and synthetic MWFs and demonstrated to be strongly correlated to hypersensitivity pneumonitis (HP) outbreaks. [4, 22, 30, 42, 43, 72]. Consequently, once mycobacteria have been detected in manufacturing operations using MWFs, unscheduled discharges of the whole batch of MWFs to wastewater treatment may be required, hence impacting system downtime. Aggressive formaldehyde condensate biocide-based control of microbial growth resulted in a

change in bacterial flora, favoring proliferation of a non-tuberculous *Mycobacterium* sp. [69]. Several outbreaks in metalworking factories using semisynthetic or synthetic MWF have been linked to *Mycobacterium* spp. [4, 22, 30, 72]. Due to these issues, *Mycobacterium* spp. have become emerging and significant health hazard agents [59, 75], and their accurate detection and quantification is a necessity [48].

Current enumeration methods for bacteria in MWFs include direct and indirect detection technologies (reviewed in [9]). However, for mycobacteria, due to their slow growth rate, fastidious nutrient requirements, and their tendency to form aggregates, these approaches may either underestimate their cell density or are too time-consuming to be effectively used for process control. These limitations necessitate the development of a rapid, sensitive, and accurate quantitative method to effectively screen for mycobacteria in MWF.

In the last few years, flow cytometry (FCM) has been increasingly used for direct optical detection of bacteria in aquatic systems and in environmental samples with complex matrix characteristics [19, 50, 68]. Due to the capability of rapid, simultaneous multi-parametric data acquisition by FCM in combination with newly developed diverse fluorescent nucleic acid dyes, it has become one of the best options for rapid bacterial detection and quantification. FCM technology is based on optical detection of particles that pass through a sheath-fluid-focused flow cell, where incident light is introduced by a laser source. Particles exhibit different light-scattering characteristics due to their size, shape, internal complexity, and granularity. The scattered light is detected and signals are amplified and recorded at the speed of more than tens of thousands of events per second. Recent reviews on FCM applicability in complex environmental matrices can be found elsewhere [19, 67].

Immunomagnetic separation (IMS) has been developed for microbial identification and enumeration in last decade. In the past, two major techniques have been used to immobilize antibody (Ab) onto the surface of magnetite: (1) zero-length linker 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (CDI) [24, 29, 38] and (2) (3-aminopropyl) triethoxysilane (APTES) [61, 70, 71, 78]. Whereas CDI can link the carboxyl group of the primary Ab onto the hydroxyl group on the surface of magnetic nanoparticles (MNP) without fixed orientation [see Fig. 1 for multiple carboxyl groups on an immunoglobulin G (IgG) molecule], APTES is able to achieve oriented conjugation of primary Ab by interlinking with protein A. Due to the high specificity of protein A toward the Fc fragment of Ab, which has no active site, it is able to couple IgG onto a surface, leaving the antigen-specific sites free. Currently, two major solid media, magnetic microbeads (diameter 0.8–5 μm) and magnetic nanoparticles (MNP, diameter < 200 nm), are employed for IMS [47, 57, 60]. Nanometer-sized particles were found to be superior compared to microbeads, since re-aggregation of the particle is limited, which improves detection and enumeration accuracy [39]. Furthermore, the primary-Ab-conjugated MNP is believed to have the capability to amplify the signal (Fig. 1). One disadvantage for small-sized particles is that they need stronger magnetic fields to separate the cells out of the complex matrix [40, 56, 57]. IMS has been applied to a wide range of bacteria [2, 13, 34, 56, 58] including *Mycobacterium tuberculosis* [37], *M. paratuberculosis* [18], and *M. ulcerans* [53] in clinical matrices.

Recently, IMS and FCM have been coupled to separate and enumerate *Escherichia coli*, *Salmonella enterica*, and undefined sediment consortium samples [14, 32]. To our knowledge, no systematic comparison of traditional centrifugation techniques and IMS for enumeration of mycobacteria in environmental matrices has been

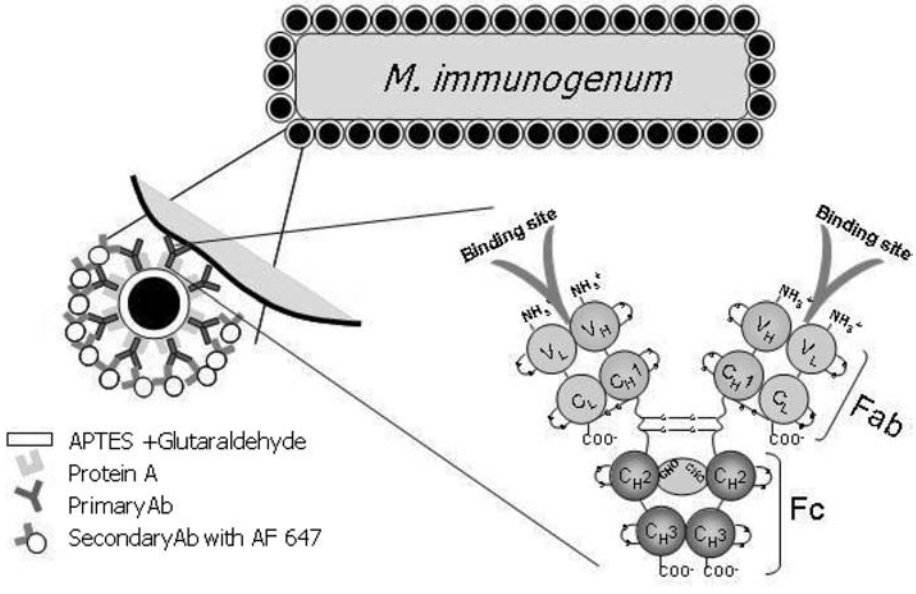

Fig. 1 Schematic representation for immunomagnetic nanoparticle-labeled *Mycobacterium immunogenum* (not to scale). Immunoglobulin G was redrawn from Giacomelli [17]

reported. The objective of this work was to demonstrate the applicability of IMS for separation of mycobacteria from MWFs, and to compare this method with traditional centrifugation techniques.

Materials and methods

Culture conditions and MWF preparation

M. immunogenum (ATCC#700506) was obtained from the American Type Culture Collection (ATCC). *M. immunogenum* was inoculated into Middlebrook 7H9 medium (Fisher Scientific, Pittsburgh, PA) and incubated for more than 59 h before each test until stationary growth phase was reached. A semisynthetic MWF, including formulation and its components (Table 1), was kindly provided by a MWF manufacturer. The MWF solution (pH 8.8–9.1) visually exhibited light yellow color and was slightly translucent, indicating a fine particle emulsion. Before each test, this MWF was sterilized by autoclaving (20 min). Each MWF sample was prepared by adding stationary-phase culture into the model MWF to achieve the initial target cell density ($\sim 10^7$ cells/mL).

Sample preparation

All samples were fixed before any further pretreatment by mixing the cells with 10% (v/v) ice-cold formalin (final concentration 2% formalin) and incubating in a −20°C freezer for 1 h. The impact of fixation on recovery and labeling efficiency will be discussed in the Results and discussion. Since aggregation of mycobacterial cells interferes with accurate optical quantification using both epifluorescent microscopy (EFM) and FCM, the cells were dispersed using a 45-s and power-level-2 exposure of probe sonication (Virsonic 50, Virtis, Gardiner, NY [9]). Sonication after formalin fixation has been suggested to reduce the damage due to strengthened cellular layer [63]. The microcentrifuge tubes were put into an ice-bath while applying probe sonication to prevent sample heating and cavitation [20].

Table 1 Recipe of a semisynthetic metalworking fluid (MWF)

Component	Percentage (w/w)
Mineral oil	0.750
Tall oil fatty acid	0.185
Surfactant, sodium petroleum sulfonate	0.300
Fatty amide	0.400
Borate corrosion inhibitor	0.300
Coupler	0.075
Monoethanolamine	0.095
EDTA	0.008
Deionized water	97.887

Nucleic acid dyes

PicoGreen (P-7581, Molecular Probes, Eugene, OR) was selected after comparing with acridine orange (Fisher Scientific) and SYTO (B-7277, Molecular Probes) using both EFM and FCM (data not shown). PicoGreen was added at 2 μL/mL sample unless otherwise indicated. Those samples were incubated for 30 min in a 37°C water batch before slide preparation and FCM quantification. At high concentrations of MWF, the stained samples were amended with dimethylformamide (DMF, Fisher Scientific) at an optimum 1:1 volume ratio to limit the complexation of dye and surfactants present in the MWF [9]. This step is necessary when the optical interference from MWF components is significant.

Centrifugation speed and buffer system test

An aliquot of the sample was set aside to define the initial cell density. Other samples were prepared either by mixing with phosphate-buffered saline (PBS) or MWF. Different centrifugation speeds (2,000, 3,000, 4,000, 6,000, 8,000, 10,000, and 12,000 *g*) and duration were tested for samples in PBS and MWF. After choosing 3,000 and 12,000 *g* as optimum centrifugation speeds, samples were quantified by EFM and FCM after each wash. Up to six washing cycles were tested to simulate the indirect immunological labeling procedure.

Immunological enumeration by centrifugation

Each sample was subjected to centrifugation in a microcentrifuge (Micro12, Fisher Scientific). The supernatant was decanted and the test buffer was added to resuspend the pellet. After two washing cycles, the cells were incubated with primary Ab (a polyclonal rabbit anti-mycobacterium IgG; #6398-0006, Biogenesis, Kingston, NH) at 4°C for 30 min. Two more washing cycles were then performed to remove the unbound primary Ab, and the cells were incubated with a fluorophore conjugated secondary Ab [Alexa Fluor 647, $F(ab')_2$ fragment of goat anti-rabbit IgG (H + L), A-21246, Molecular Probes]. Finally, two more washing cycles were undertaken to remove secondary Ab. After these steps, the samples were subjected to EFM and FCM quantification.

MNP preparation

MNP were synthesized by the co-precipitation method [11]. The specific area was measured at 18.01 ± 0.04 m^2/g measured by using BET analysis and nitrogen adsorption [12]. By assuming spherical shape, the diameter is computed to be 62.0 nm. The protocol for random conjugation using CDI followed the published method [24]. The protocol for oriented conjugation of primary

Ab onto MNP was modified from the literature [61, 70, 71, 78]. Briefly, 30 mg MNP was transferred into a 50 mL solution of 10% (v/v) APTES in toluene and mixed by vortex and sonication bath (10 min). The solution was incubated at 30°C for 10–14 h. After this incubation, the MNP was rinsed with dry toluene (once), a mixture of dry toluene and methanol (1:1 v/v, once), methanol (once), and milli-Q water (twice) in a GS-6 centrifuge (Beckmann, Palo Alto, CA) at 1,350 *g* for 5 min. Glutaraldehyde [50 mL; 2.5% (v/v) in 0.1 M PBS, pH 7.0] was added and the solution incubated at 4°C for 1 h in the dark. After incubation, the solution was washed with milli-Q water (three times) at 1,350 *g* for 5 min. The pellet was re-suspended in 20 mL ice-cold 0.1 M PBS (pH 7.0) by pipetting and sonicating, if necessary. Freshly prepared protein A reagent (100 µl; 10 mg/ml in PBS) was then added to the solution [7, 35, 64], which was then incubated at 4°C for 12 h in the dark. After this, the MNPs were washed at 1,350 *g* for 5 min with ice-cold PBS three times, to remove unbound protein A. The pellet was re-suspended and 100 µL primary Ab was added. After incubation at 4°C for at least 1 h, the MNPs were washed three times to remove unbound primary Ab with ice-cold PBS at 3,000 *g* (5 min), 6,000 *g* (5 min), and 12,000 *g* (5 min). The pellet was finally re-suspended in 1.0 mL ice-cold PBS and kept under 4°C before use. After this preparation, the primary Ab should be chemically bound to the MNP, with active binding sites facing the solution phase.

Immunomagnetic separation

MNP reagent (10 µL) was added to each vial of sample, mixed well, and incubated at room temperature for 30 min. After incubation, the samples were left on a rare earth neodymium-iron-boron magnet (#5679K15, McMaster Carr, Aurora, OH) for 10 min. The supernatant was carefully removed by pipetting and the pellet re-suspended with PBS to the original volume, and incubated with PicoGreen and secondary Ab at room temperature for 30 min. For samples in MWF, the DMF amendment was applied (1:1 ratio), followed by incubation at 37°C for 15 min before microbial detection and enumeration by EFM and FCM.

Analytical procedures

Epifluorescent microscopy

Samples were vacuum-filtered through a black polycarbonate filter paper (25-mm diameter; 0.22 µm; Osmonics, Pittsburgh, PA). The filter paper was then transferred to a microscope slide and covered with a cover glass for EFM enumeration following a published protocol [28]. A Zeiss Axioplan microscope (Zeiss, Jena, Germany) mounted with a 50-W Osram UV mercury lamp was employed to count the bacteria. Pictures were taken using a Zeiss AxioCam MRc digital camera and processed using AxioVision software Version 4.0 (Carl Zeiss Vision, Hallbergmoos, Germany).

Flow cytometry

A dual laser FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), equipped with a 15-mW air-cooled 488-nm argon-ion laser and a second red-iodide 635-nm laser, was used. Before each test, the instrument was calibrated by using Calibrite bead (Cat.# 340486) solutions. Voltages for each of the photomultiplier tubes were adjusted to achieve good separation between the population of interest and the background. The side-scattered threshold was set at 52, and the acquisition algorithms were set to either "at least 10,000 events or 10 s" or "at least 100,000 events or 10 s" depending on sample conditions. For quantitative enumeration, Trucount (Cat.# 340567) tubes containing a pre-counted number of dye-coated beads were used according to the manufacturer's suggestions. The coated beads have high side-scattered light intensity, and are focused and easily separated from other populations. For PicoGreen-stained and Alex Fluor 647-labeled cells, data recorded from fluorescent light detector 1 (FL1) and detector 4 (FL4) were selected for further data analysis [41]. CellQuest Software (BD Biosciences) was utilized to examine each data file and to ensure that the population of interest was separated from the background. The population of interest was then selected and delineated. Enumeration of the selected events was processed in a batch manner.

Results and discussion

Disaggregation of *M. immunogenum* cells in PBS and MWF

To achieve reliable cell enumeration, a single cell suspension is essential [44]. Probe sonication (45 s) resulted in effective disaggregation of *M. parafortuitum* and *M. immunogenum* based on EFM and FCM quantification, resulting in a good linear correlation between both enumeration methods [9]. By comparing the EFM images of both species, *M. immunogenum* produced more extracellular substances, and exhibited stronger adhesion between cells. Moreover, cells in PBS are slightly more aggregated than those in MWF, especially after centrifugation. Other researchers have attempted to extract surface exposed lipids by incubating *M. tuberculosis* with Tween 80 solution (1% w/v) for 16 h, and the results showed distinct differences between treated and untreated cells. The treated cells exhibited a clean cell surface, and the untreated cells showed clumps with thick mucoid extracellular substances [45]. Since the MWF tested in this study included a comparable concentration of sodium petroleum sulfonate (SPS, 0.3% w/w), and probe sonication provided higher local temperature and shearing force [44], it is plausible that SPS may have washed off

the extracellular substances and also prevented the mycobacterial cells from re-aggregating [6, 45].

Impact of fixation on fluorescent light intensity and recovery

In order to obtain reliable enumeration results, the samples are usually fixed by ice-cold formalin [28]. This standard fixative has been reported to cause cross-linkage, may influence the antigen structure, and may also affect the binding of a polyclonal Ab [52]. To assess the impact of fixation on FCM-based detection, tests following standard indirect immunological labeling as well as nucleic acid dye staining protocols were performed. The results showed that cells with fixation showed slightly weaker light intensity on FL4 by using the same primary and secondary antibodies (Fig. 2a). The mean light intensity of the gated population decreased about 8% in comparison to unfixed cells. This result suggested that the recognition of primary Ab was slightly affected. However, fixed cells showed a much higher FL1 light intensity (Fig. 2b) than cells without fixation, resulting in an amplification of the light intensity by 2.5-fold for PicoGreen-stained mycobacteria, and implying that fixed cells are probably easier for cyanine dyes to penetrate.

Impact of centrifugal speed and duration on recovery

Further tests on different centrifugation speeds and durations were performed for both fixed and unfixed cells in PBS and MWF. Generally, higher recovery yields were achieved for both fixed and unfixed cells at lower speeds (Fig. 3). According to Stokes' law, the major parameters influencing particle sedimentation

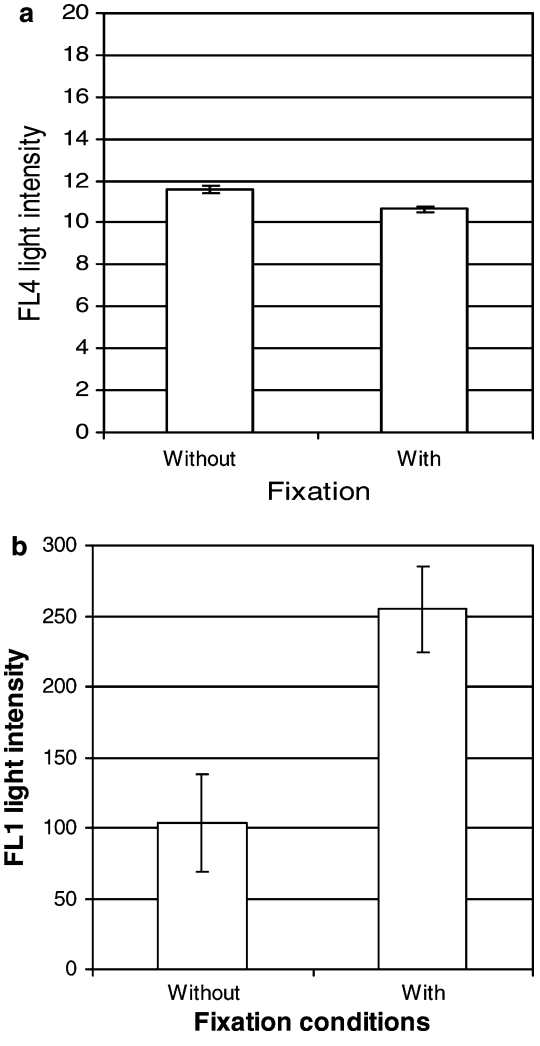

Fig. 2 The impact of fixation light intensity of **a** Alexa Fluor 647-labeled and **b** PicoGreen-stained *M. immunogenum* following an indirect immunological protocol

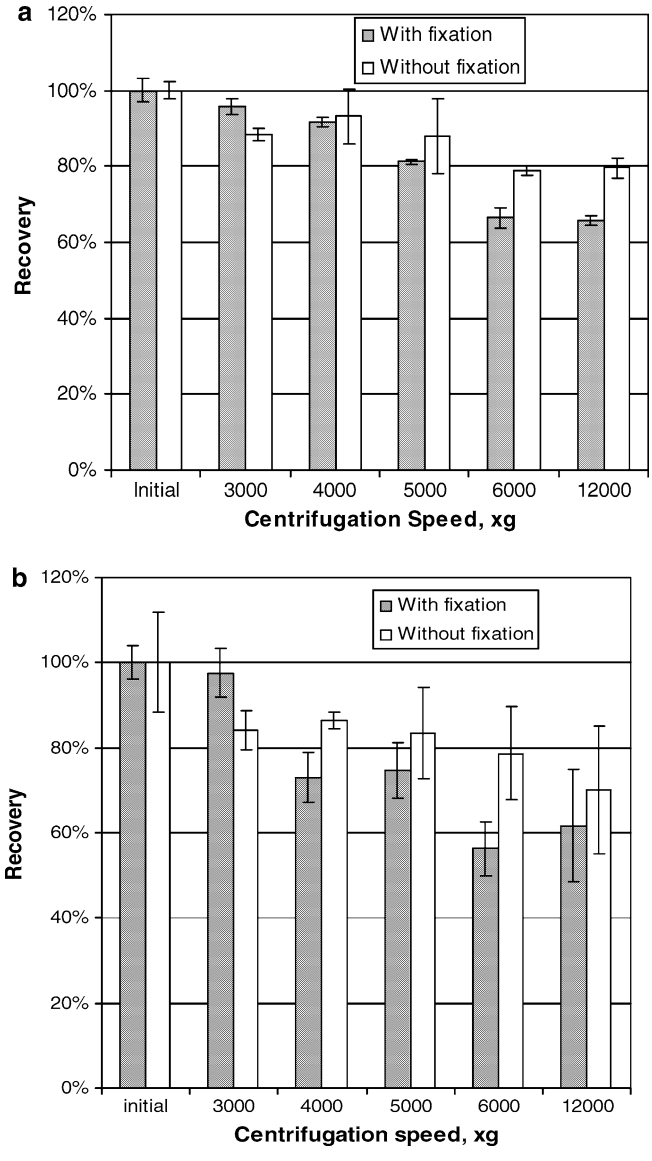

Fig. 3 Impact of fixation on recovery of *M. immunogenum* at different centrifugation speeds in **a** phosphate-buffered saline (PBS) and **b** metalworking fluid (MWF)

velocity are the particle and the liquid density, viscosity of the fluid, particle diameter, and the gravitational force experienced by the particles. The liquid density of MWF (0.9964 g cm^{-3}), is slightly lower than that of PBS (1.0147 g cm^{-3}), and the viscosity of MWF is higher than that of PBS. Assuming cell size is the same in PBS and MWF, the sedimentation velocity will not differ significantly. However, at high centrifugation speeds, the recovery of cells in MWF is significantly lower than that in PBS, which is likely due to matrix properties based on the following rationale. Since the surfactant concentration in MWF, long-side-chain petroleum sulfonate, exceeds the critical micelle concentration [25, 66], micelle-solubilized droplets of hydrophobic components (mineral oils, fatty acids, and fatty amides), and micelles of surfactants, couplers, and mixed components, are likely to be present due to the emulsification characteristics of the MWF. Further, sonication pretreatment in this system may have increased the formation of vesicles [16]. Assuming a vesicle membrane thickness of 5 nm as the bilayer thickness of the cell membrane [1], the calculated specific gravity of selected averaged single-component vesicles is shown in Table 2. However, the situation has been simplified since there are numerous compounds in tall oil fatty acids and SPS [49, 51, 66]. Small oil-in-water emulsion droplets have been shown to undergo coalescence at centrifugal speeds exceeding 1,529 *g* for durations as short as 10 min [54]. Further, re-suspension of MWF samples in the higher ionic strength PBS (relative to MWF) may also increase the droplet size in MWF [79]. Taking all these factors into account, it is plausible for the MWF to have formed a density gradient during centrifugation, resulting in lower recovery at higher centrifugation speeds. A similar trend has been reported on extracting bacteria from freshwater sediment samples using centrifugation techniques [14].

To optimize the centrifugation technique for mycobacterial cells, a centrifugation duration test was performed for fixed cells in PBS and MWF at 3,000 and 12,000 *g*. The 12,000 *g* speed was chosen to benchmark that of 3,000 *g* because the former is commonly widely applied in indirect immunological labeling protocols [46, 77]. The results indicated that, in both PBS and MWF, 3,000 *g* always yielded higher recovery than 12,000 *g* for all tested centrifugation times. A higher recovery of cells is obtained at 5 and 10 min at 3,000 *g* in MFW and PBS, respectively (Fig. 4). Therefore, to recover mycobacterial cells from MWF, a 5-min centrifugation at 3,000 *g* was selected for the first two washes when MWF is still at high concentration, and 10 min at 3,000 *g* for the following rinsing steps.

Low recovery and large variations in cell recovery have been reported in the literature by using centrifugation techniques. Boulanger and Edelstein [5] used 15-min centrifugation at 8,150 and 3,800 *g* for *Legionella pneumophila* recovery from seeded tap water; the mean recovery rate is 32 and 4.2% with a range of 19.7–52.9 and 1.1–44.5%, respectively. Lindqvist [33] used density gradient centrifugation to recover *E. coli* O157:H7 and obtained recovery rates ranging from 20% to 45%. The higher and more steady recovery from our study based on one washing cycle may result from (1) constant-temperature operation (PBS was always kept

Table 2 Pure compound density and computed densities of vesicles and droplets

Species	Pure compound density (g cm^{-3})	Computed vesicle density (g cm^{-3})
Water	0.998	–
Oil	0.920	0.930
Sodium petroleum sulfonate	1.020	1.017
Fatty amide	0.960	0.965
Boron amide	1.010	1.009
Fatty acids	0.905	0.917
Coupler	0.954	0.960

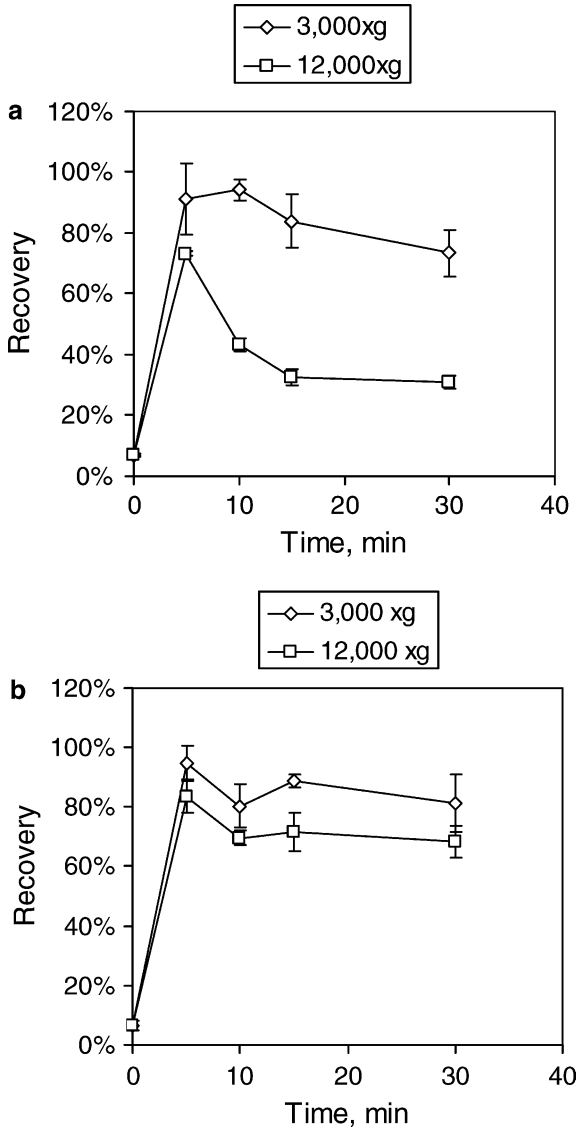

Fig. 4 Recovery for different centrifugation duration in **a** PBS and **b** MWF

at 4°C and vials were always centrifuged at 4°C), (2) smaller volume and use of a conical shaped vial, which significantly reduced the distance for the cell to settle to the bottom of the vial, (3) the characteristics the outer membrane of *Mycobacterium* spp., which tends to aggregate the dispersed cells and accelerate the sedimentation process with an increased particle size, and (4) more optimized centrifugation parameters focused on a specific microbial species.

Comparison of IMS and centrifugation techniques

To compare the recovery rates of IMS with that of centrifugation, the same primary Ab used in the centrifugation technique was used to fabricate Ab-immobilized MNPs. In addition, the recovery rates of CDI and APTES conjugation schemes were compared.

The qualitative differences between IMS and centrifugation methods are apparent from the FCM dot plots (Fig. 5), of the side-scatter-light intensity versus fluorescent light intensity from cells stained either by PicoGreen (FL1) or Alexa Fluor 647 (FL4). By comparing panel a with panel c in Fig. 5, the background (particles with lower fluorescent light intensity) in the centrifuged sample was much lower than in the IMS sample, and the population of interest in the IMS sample was more separated. A higher background in IMS samples was expected, since these samples do not undergo subsequent washing steps. When the events with high FL1 light intensity, representing the mycobacterial cells seeded into MWF, are selected and plotted (Fig. 5b,d), it is apparent that the cells recovered by IMS formed a tighter cluster and emitted higher intensity fluorescent light. This suggests that the cells can be more effectively and evenly stained by conjugated MNP in the 30-min incubation of secondary Ab. Since, in both tests, the same primary Ab and the same concentration of secondary Ab are employed, these results lend support to the hypothesis that IMS amplifies the signal.

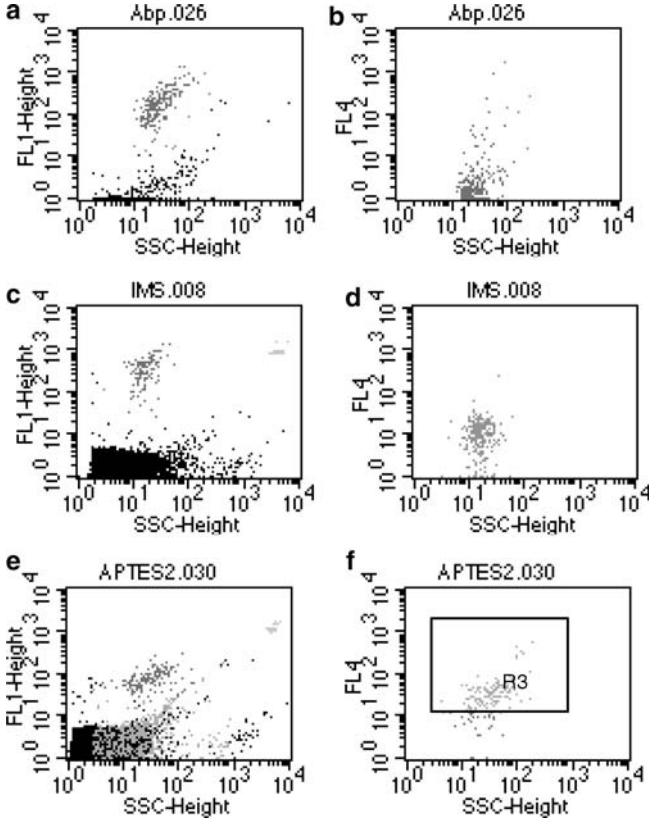

Fig. 5 Flow cytometry (FCM) dot plots of *M. immunogenum* processed by centrifugation (**a**, **b**) and immunomagnetic separation (IMS) after a 30-min (**c**, **d**) or 10-h (**e**, **f**) incubation. Note that the FL4 light intensity of samples using the IMS method was much higher

The quantitative recovery in PBS and MWF by IMS was much higher than that achieved by centrifugation (Table 3). The recovery rate using CDI conjugated MNPs showed one order of magnitude improvement over the centrifugation-based method. However, it is still much lower than that based on APTES-conjugated MNPs. Turková [65] tested free, randomly bound and oriented covalently bound trypsin on 45–165 μm Sepharose 4B beads, and found that oriented bound trypsin retained 96.3 and 98.6% of relative specific proteolytic and amidolytic activity, whereas the randomly bound trypsin showed 15 and 70%, respectively. Giacomelli [17] reviewed the immunoactivity of antibodies immobilized through oriented immobilization and concluded that the activity achieved is always higher than that obtained from random covalent coupling. The dependence of recovery rate on CDI (random) and APTES (oriented) conjugation is consistent with these previous studies.

By comparing the recovery rates of different methods in PBS, it is apparent that the correlation between EFM and FCM enumeration for centrifuged samples is better than those separated using IMS, despite initial disaggregation of the cells using sonication. It is hypothesized that after six washing cycles in PBS, all cells are more dispersed than when using the IMS technique, because PBS itself is regarded as a dispersant [28]. Moreover, the inherent limitations in resolution of PicoGreen-stained particles using FCM may have resulted in under-counting of the true number, relative to microscopic techniques. The reason why this was not observed for samples in MWF by IMS is that the SPS may have helped to disperse the cells and may also have prevented them from re-aggregating [6, 45]. Therefore, the correlation between the recovery rates by EFM and FCM in MWF is improved over those in PBS. Previously, a good linear correlation ($R^2 = 0.99$) between EFM and FCM counts in MWF samples was reported for mycobacterial cells stained by nucleic acid dye after treatment with resolving agents over four orders of magnitude of cell densities [9]. Further testing should include evaluating this enhancement, provided that the polar solvent resolving agents do not interfere with the coated MNP.

Table 3 Total cell recovery in buffer and MWF solution using centrifugation and immunomagnetic separation (*IMS*) techniques. *PBS* Phosphate-buffered saline, *EFM* epifluorescent microscopy, *FCM* flow cytometry, *CDI* 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide, *APTES* (3-aminopropyl) triethoxysilane

	In PBS		In MWF	
	EFM	FCM	EFM	FCM
Centrifugation	13.5 ± 2.6%	10.7 ± 4.0%	1.6 ± 1.5%	0.54 ± 0.18%
IMS (CDI)	–	–	14.9 ± 4.8%	–
IMS (APTES)	29.7 ± 3.0%	12.7 ± 2.4%	40.1% ± 3.4%	40.2 ± 14.3%

It is intriguing that the cell recovery by IMS is always higher from MWF than from PBS. The recovery is 40.1 ± 3.4% (average ± SD, $n = 18$) in MWFs and 29.7 ± 3.0% ($n = 8$) in PBS. It is probable that, in MWF, due to the presence of surfactants and EDTA, without any input of other divalent cations, the cell membrane allowed the targeted antigen to be more exposed to the primary Ab. Evidence that high concentrations of sodium dodecyl sulfate (SDS, which is an anionic surfactant like SPS) solubilize membrane proteins, and the fact that EDTA acts as a cell membrane destabilizing agent, may lend support to this hypothesis [1]. Consequently, in MWF, larger amounts of primary Ab-conjugated MNPs can be bound onto the cell surface, and higher recoveries are achievable.

The resultant FL4 light intensities from cells recovered by the two different methods from PBS and MWF are presented in Fig. 6. Evidently, cells recovered by IMS showed a 2- to 3-fold higher FL4 light intensity than those recovered by centrifugation. This effect may be explained based on the theoretical binding opportunity between the MNP and target proteins. Since the surface density of membrane proteins is high (on the order of ~100,000/μm^2; [8]), the ratio of available binding chains (both heavy and light) on MNP to the number of cell membrane proteins covered by a single bound MNP is low (less than unity). Hence, a 62-nm diameter MNP exhibits a theoretical ratio of 1:3.13 (MNP binding sites/proteins), assuming an APTES film thickness of < 0.7 nm [23, 31, 74, 76], 3 nm for globular protein A, and a 10.4 nm height for IgG [15]. Changing the MNP to a smaller diameter (e.g., 20 nm) may not improve signal amplification, as the theoretical ratio would be 1:5.90. The binding efficiency expected in the centrifugation method may be even lower considering the multiple rinsing steps and the observed lower FL4 light intensity.

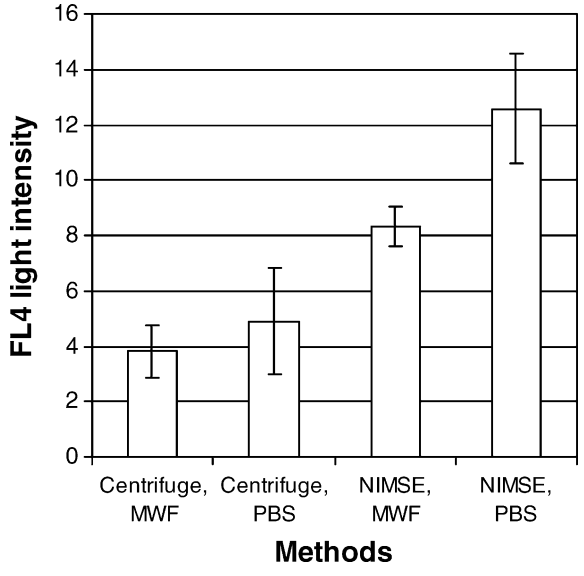

Fig. 6 Comparison of FL4 light intensity of samples processed by centrifugation and IMS methods

Using commercial 2.8-μm-diameter magnetic Dynal beads conjugated with secondary Ab and incubated overnight with primary Ab-labeled *Pseudomonas stutzeri* (ATCC14405), Bard and Ward [2] reported an average recovery rate of 75 ± 5.8% in PBS-diluted human serum (1:20 to 1:1,000). By using column-type IMS and commercially available ~50-nm diameter MACS Microbeads, Jacobsen et al. [27] achieved 23 ± 13–91 ± 9% recovery distribution in the third of three eluted fractions, after six washing cycles for *Listeria* spp. in Tris-HCl buffer suspension. However, these authors did not enumerate the cell density beforehand and, therefore, did not report the exact overall recovery. Thus, the 40% recovery efficiency in the current IMS protocol, which requires no washing steps, is quite efficient in the rapid detection and enumeration of mycobacteria in MWF.

Conclusion

M. immunogenum has been linked to several severe HP outbreaks; a rapid identification and enumeration method will help establish cause-effect relationships and management of this health-hazardous bacterial species in MWF operations. In this study, we reported an IMS method based on in-lab synthesized and conjugated nanometer-sized magnetic particles and compared the recovery rate of this method to an optimized conventional indirect immunological technique by using both EFM and FCM. Centrifugation methods were found not to be suitable for rapid quantification of *M. immunogenum* in MWF due to high variation in recovery, low total cell recovery, and low signal of secondary Ab (FL4 light intensity). IMS appears to be a promising tool to rapidly detect and enumerate *M. immunogenum* in MWF with an overall cell recovery of ~40%.

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References

1. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular biology of the cell, 4th edn. Garland, New York, pp 470, 584, 599
2. Bard DG, Ward BB (1997) A species-specific bacterial productivity method using immunomagnetic separation and rediotracer experiment. J Microbiol Methods 28:207–219
3. Bennett EO (1972) The biology of metalworking fluids. Lubr Eng July:237–247
4. Bernstein DI, Lummus ZI, Santilli G, Siskosky J, Berstein IL (1995) A hypersensitivity pneumonitis disorder associated with exposure to metalworking fluid aerosols. Chest 108:636–641
5. Boulanger CA, Edelstein PH (1995) Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation. Appl Environ Microbiol 61:1805–1809
6. Calleja GB (1984) Microbial aggregation. CRC, Boca Raton, Fla., pp 49–52
7. Caruso F (1999) Fabrication of immunoglobulin mono- and multilayers and their application for immunosensing. In: Lvov YM, Möhwald H (eds) Protein architecture: interfacing molecular assemblies and immobilization biotechnology. Dekker, New York, p 201
8. Cascales E, Bernadac A, Gavioli M, Lazzaroni JC, Lloubes R (2002) Pal lipoprotein of *Escherichia coli* plays a major role in outer membrane integrity. J Bacteriol 184:754–759
9. Chang SC, Rihana A, Bahrman S, Gruden CL, Khijniak AI, Skerlos SJ, Adriaens P (2005) Flow cytometric detection and quantification of mycobacteria in metalworking fluids. Int Biodeterior Biodegrad 54:105–112
10. Childers JC (1994) The chemistry of metalworking fluids. In: Byers JM (ed) Metalworking fluids. Dekker, New York, pp 165–189
11. Cornell RM, Schwertmann U (2003) The iron oxides. Wiley, Weinheim, Germany, pp 536–537
12. Danielsen KM (2004) Reductively dechlorination of carbon tetrachloride by magnetite: the importance of geochemical conditions. Dissertation, University of Michigan
13. Favrin SJ, Jassim SA, Griffiths MW (2003) Application of a novel immunomagnetic separation-bacteriophage assay for the detection of *Salmonella enteritidis* and *Escherichia coli* O157:H7 in food. Int J Food Microbiol 85:63–71
14. Furtado ALD, Casper P (2000) Different methods for extracting bacteria from freshwater sediment and a simple method to measure bacterial production in sediment samples. J Microbiol Methods 41:249–257
15. Galli C, Coen MC, Hauert R, Katanaev VL, Gröning P, Schlapbach L (2002) Creation of nanostructures to study the topographical dependency of protein adsorption. Colloids Surf B: Biointerfaces 26:225–267
16. Garcia MED, San-Medel A (1986) Dye-surfactant interaction: a review. Talanta 33:255–264
17. Giacomelli CE (2002) Adsorption of immunoglobulins at solid-liquid interfaces. In: Hubbard AT (ed) Encylopedia of surface and colloid science. Dekker, New York, pp 418–439
18. Grant IR, Ball HJ, Rowe MT (1998) Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. Appl Environ Microbiol 64:3153–3158
19. Gruden CL, Skerlos SJ, Adriaens P (2004) Flow cytometry for microbial sensing in environmental sustainability applications: current status and future prospects. FEMS Microbiol Ecol 49:37–49
20. Harrison STL (1991) Bacterial cell disruption: a key unit operation in the recovery of intracellular products. Biotechnol Adv 9:217–240
21. Hills EC (1977) Microbial infection of cutting fluids. Tribol Int 10: 49–54
22. Hodgson MJ, Bracker A, Yang C, Storey E, Jarvis BJ, Milton D, Lummus Z, Bernstein D, Cole S (2001) Hypersensitivity pneumonitis in a metal-working environment. Am J Ind Med 39:616–628
23. Horne JC, Huang Y, Liu G-Y, Blanchard GJ (1999) Correspondence between layer morphology and intralayer excitation transport dynamics in Zirconium-phosphonated monolayers. J Am Chem Soc 121:4419–4426
24. Huang S-H, Liao M-H, Chen D-H (2003) Direct binding and characterization of lipase onto nanoparticles. Biotechnol Prog 19:1095–1100
25. Huibers PDT, Labonav VS, Karitzky AR, Shas DO, Karelson M (1997) Prediction of critical micelle concentration using a quantitative structure-property relationship approach. J Colloid Interface Sci 187:113–120
26. Independent Lubricant Manufacture Association (2000) ILMA Report, in Lubricants World, November, p 10
27. Jacobsen CN, Fremming C, Jacobsen M (1997) Immunomagnetic separation of *Listeria monocytogenes* for flow cytometric determination of viable cells in liquid. J Microbiol Methods 31:75–81
28. Kepner RL Jr, Pratt JR (1994) Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol Rev 58:603–615
29. Konerachá M, Kopčanský P, Timbo M, Ramchand CN, de Sequeira A, Trevan M (2002) Direct binding procedure of proteins and enzymes to fine magnetic particles. J Mol Catal B: Enzymatics 18:13–18
30. Kreiss K, Cox-Ganser J (1997) Metalworking fluid-associated hypersensitivity pneumonitis: a workshop summary. Am J Ind Med 32:423–432
31. Kurth DG, Bein T (1995) Thin films of (3-aminopropyl)triethosysilane on aluminum oxide and gold substrates. Langmuir 11:3061–3067
32. Kusunoki H, Bari ML, Kita T, Sugii S, Uemura T (2000) Flow cytometry for the detection of enterohaemorrhagic *Escherichia coli* O157:H7 with latex beads sensitized with specific antibody. J Vet Med Ser B Infect Dis Vet Publ Health 47:551–559
33. Lindqvist R (1997) Preparation of PCR samples from food by a rapid by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli* O157:H7. Int J Food Microbiol 37:73–82
34. Liu Y, Che Y, Li Y (2001) Rapid detection of *Salmonella typhimurium* using immunomagnetic saparation and immunooptical sensing method. Sens Actuators B 72:214–218
35. Lu B, Smyth MR, O'Kennedy R (1996) Immunological activities of IgG antibody on pre-coated Fc receptor surfaces. Anal Chim Acta 331:97–102
36. Mattsby-Baltzer I, Sandin M, Ahlstrom B, Allenmark S, Edebo M, Falsen E, Pedersen K, Rodin N, Thompson RA, Edebo L (1989) Microbial growth and accumulation in industrial metalworking fluids. Appl Environ Microbiol 55:2681–2689
37. Mazurek GH, Reddy V, Murphy D, Ansari T (1996) Detection of *Mycobacterium tuberculosis* in cerebrospinal fluids following immunomagnetic enrichment. J Clin Microbiol 34:450–453
38. Mehta RV, Upadhyay RV, Charles SW, Ramchand CN (1997) Direct binding of protein to magnetic particles. Biotechnol Tech 11:493–496
39. Miltenyi S, Muller W, Weichel W, Radbruch A (1990) High-gradient magnetic cell-separation with MACS. Cytometry 11:231–238
40. Molday RS, Molday LL (1984) Separation of cells labeled with immunospecific iron dextran microspheres using high-gradient magnetic chromatography. FEBS Lett 170:232–238
41. Molecular Probes Handbook. http://www.probes.com/handbook

42. Moore JS, Christensen M, Wilson RW (2000) Mycobacterial contamination of metalworking fluids: involvement of a possible new taxon of rapidly growing mycobacteria. Am Inst Hyg Assoc J 61:205–213
43. Muilenburg ML, Burge HA, Sweet T (1993) Hypersensitivity pneumonitis and exposure to acid-fast bacilli in coolant aerosols. J Allergy Clin Immunol 91:311
44. Nebe-von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA (2000) Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J Microbiol Methods 42:97–114
45. Ortalo-Magné A, Lemassu A, Lanéelle M-A, Bardou F, Silve G, Gounon P, Marchal G, Daffé M (1996) Identification of the surface-exposed lipids on the cell envelopes of *Mycobacterium tuberculosis* and other mycobacterial species. J Bacteriol 178:456–461
46. Ozane V, Ortalo-Magné A, Vercellone A, Fournié J-J, Daffé M (1996) Cytometric detection of mycobacterial surface antigens: exposure of mannosyl epitopes and of the arabinan segment of arabinomannans. J Bacteriol 178:7254–7259
47. Pankhurst QA, Connolly J, Jones SK, Dobson J (2003) Applications of magnetic nanoparticles in biomedicine. J Phys D Appl Phys 36:R167–R181
48. Passman FJ, Rossmoore HW (2002) Reassessing the health risk associated with employee exposure to metalworking fluid microbes. Lubr Eng 58:30–38
49. Pine Chemical Association (2001) Test plan for tall oil and related substances. http://www.epa.gov/chemrtk/tofars/c13056tc.htm
50. Porter J, Deere D, Hardman M, Edwards C, Pickup R (1997) Go with the flow—use of flow cytometry in environmental microbiology. FEMS Microbiol Ecol 24:93–101
51. Porter MR (1994) Handbook of surfactants. Chapman & Hall, New York, pp 155–159
52. Rait VK, Xu L, O'Leary TJ, Mason JT (2004) Modeling formalin fixation and antigen retrieval with bovine pancreatic RNase A. II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. Lab Invest 84:300–306
53. Roberts B, Hirst R (1997) Immunomagnetic separation and PCR for detection of *Mycobacterium ulcerns*. J Clin Microbiol 35:2709–2711
54. Roland I, Piel G, Delattre L, Evrard B (2003) Systematic characterization of oil-in-water emulsions for formulation design. Int J Pharm 263:85–94
55. Rossmoore HW, Holtzman GH, Kondek L (1976) Microbial ecology with a cutting edge. In: Miles J, Kaplan AM (eds) Proceedings of the third international biodegradation symposium. Applied Science Publishers, London, UK
56. Šafařík I, Šafaříková M (1999) Use of magnetic techniques for isolation of cells. J Chromatogr B 722:33–53
57. Šafařík I, Šafaříková M (2002) Magnetic nanoparticles and biosciences. Monatshefte für Chemie 133:737–759
58. Seo KH, Brackett RE, Frank JF (1998) Rapid detection of *Escherichia coli* O157:H7 using immunomagnetic flow cytometry in ground beef, apple juice, and milk. Int J Food Microbiol 44:115–123
59. Shelton BG, Flanders W, Morris GK (1999) *Mycobacterium* sp. as a possible cause of hypersensitivity pneumonitis in machine workers. Emerg Infect Dis 5:270–273
60. Shinkai M (2002) Functional magnetic particles for medical application. J Biosci Bioeng 94:606–613
61. Shinkai M, Wang J, Kamihira M, Iwata M, Honda H, Kobayashi T (1992) Rapid enzyme-linked immunosorbent assay with functional magnetite particle. J Ferment Bioeng 73:166–168
62. Tant CO, Bennett EO (1956) The isolation of pathogenic bacteria from used emulsion oils. Appl Microbiol 4:332–338
63. Tso SF, Taghon GL (1997) Enumeration of protozoa and bacteria in muddy sediment. Microb Ecol 33:144–148
64. Turková J (1999) Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function. J Chromatogr B 722:11–31
65. United States Department of Health and Human Services (1998) Criteria for a recommended standard: occupational exposure to metalworking fluids. HIOSH, Cincinnati, Ohio
66. Van Os NM, Haak JR, Rupert LAM (1993) Physico-chemical properties of selected anionic, cationic, and nonionic surfactants. Elsevier, Amsterdam, pp 90–93
67. Veal DA, Deere D, Ferraro B, Piper J, Attfield PV (2000) Fluorescence staining and flow cytometry for monitoring microbial cells. J Immunol Methods 243:191–210
68. Vives-Rego J, Lebaron P, Nebe-von Caron G (2000) Current and future applications of flow cytometry in aquatic microbiology. FEMS Microbiol Rev 24:429–448
69. Wallace RJ Jr, Zhang Y, Wilson R, Mann L, Rossmoore HW (2002) Presence of a single genotype of the newly described species *Mycobacterium immunogenum* in industrial metalworking fluids associated with hypersensitivity pneumonitis. Appl Environ Microbiol 68:5580–5584
70. Weetall HH (1976) Covalent coupling methods for inorganic supports. Methods Enzymol 44:134–148
71. Weetall HH (1992) Preparation of immobilized proteins covalently coupled through silane coupling agents to inorganic supports. In: Proceedings of the Mosbach symposium on biochemical technology, 2–4 December 1992, Lund, Sweden
72. Weiss L, Pue C, Rossmoore HW, Fink J, Harney J, Trout D (2001) Respiratory illness in workers exposed to metalworking fluid contaminated with nontuberculous Mycobacteria—Ohio, 2001. J Am Med Assoc 287:3073–3074
73. Omitted
74. Wetterö J, Askendal A, Tengvall P, Bengtsson T (2003) Interactions between surface-bound actin and complement, platelets, and neutrophils. J Biomed Mater Res Part A 66A:162–175
75. Wilson RW, Steingrube VA, Bottger EC, Springer B, Brown-Elliott BA, Vincent V, Jost KC Jr, Zhang Y, Garcia MJ, Chiu SH, Onyi GO, Rossmoore H, Nash DR, Wallace RJ Jr (2001) *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 51:1751–1764
76. Xiao SJ, Textot M, Spencer N, Wieland M, Keller B, Sigrit H (1997) Immobilization of the cell-adhesive peptide Arg-Gly-Asp-Cyc (RGDC) on titanium surfaces by covalent chemical attachment. J Mater Sci Mater Med 8:867–872
77. Yi WC, Hsiao S, Liu J-H, Soo P-C, Horng Y-T, Tsai W-C, Lai H-C, Teng L-J, Hsueh P-R, Hsieh R-F, Luh K-T, Ho S-W (1998) Use of fluorescein-labeled antibody and fluorescence activated cell sorter for rapid identification of *Mycobacterium* species. Biochem Biophys Res Commun 250:403–408
78. Zhang Y, Kohler N, Zhang M (2002) Surface modification of superparamagnetic magnetite nanoparticles and their intracellular uptake. Biomaterials 23:1553–1561
79. Zimmerman JB, Hayes KF, Skerlos SJ (2004) Influence of ion accumulation on the emulsion stability and performance of semi-synthetic metalworking fluids. Environ Sci Technol 38:2482–2490