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Evaluation of the enterococci indicator in biosolids using culture-based and quantitative PCR assays

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ABSTRACT

The utility of the enterococci indicator for measuring biosolids quality was evaluated in biosolids from 22 U.S. wastewater treatment facilities. Enterococci were enumerated using 23S rRNA quantitative PCR (qPCR) and membrane filtration with mEI-agar culture analyses in biosolids collected after mesophilic anaerobic digestion (MAD, class B, 13 treatment plants), composting (class A, 10 treatment plants), and temperature-phased anaerobic digestion (TPAD, class A, six treatment plants). Enterococci qPCR and culture values were not significantly different for a given treatment ($P > 0.05$, paired *t*-test) and both assays showed differences in biosolid treatment effectiveness—anaerobic digestion treatments averaged 5–5.5 log genomic units (GU) and colony forming units (CFU)/dry g while composting decreased enterococci on average to 3.7 log GU and 3.8 log CFU/dry g. Only in class A TPAD biosolids dewatered with a belt-filter press were culture values significantly lower than qPCR values (1.7 log CFU/dry g vs. 5 GU/dry g). Further investigation of compost inactivation was compared for enterococci and other fecal indicators ($n = 5$ treatment plants)—the enterococci indicator was more resistant to compost treatment than fecal coliforms, with reductions averaging only 1–2.5 logs for enterococci, male-specific coliphages, and sulfite-reducing Clostridia while 5-log reductions were observed for fecal coliforms. Lastly, biosolid isolates from culture-based methods were identified using DNA sequencing—these results revealed that non-enterococci, including *Bacillus* spp. and *Vagococcus* spp., were commonly isolated from compost and TPAD biosolids using mEI agar. Given the equivalency of culture- and qPCR-based enterococci concentrations in biosolids and the more conservative inactivation noted for both assays during class A composting, the use of enterococci qPCR monitoring could bypass non-specificity issues with culture-based methods while providing an improved description of pathogen fate in biosolids.

1. Introduction

Before sewage sludge (biosolids) is applied to agricultural land, treatment-based standards and fecal indicator monitoring are used by the U.S. EPA (Part 503 rule) and European Union (EC 1774/2002) to ensure that downstream pathogen exposures are interrupted, USEPA (1999), EU (2002). In the U. S., sewage sludge is classified as class B biosolids after stabilization (e.g. mesophilic anaerobic digestion) to reduce fecal coliforms levels $<2 \times 10^6$/dry g and class A biosolids after treatment by processes that significantly reduce pathogens (PRSP), such as composting or temperature-phased anaerobic digestion, that result in fecal coliforms levels $<10^3$/dry g, USEPA (1999). Historically, the regulatory intent was to modulate the risk of gastrointestinal disease from a biosolids exposure, hence enteric pathogens were represented by the fecal coliform

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indicator and *Salmonella* spp., Yanko (1988). Another possible biosolids exposure pathway, the aerosol route, is not well represented by these Gram-negative bacterial indicators. Previous aerosol studies at biosolids-applied agricultural fields find that although highly enriched in bulk biosolids, fecal coliforms and *Salmonella* are absent in aerosols while more robust fecal indicators like Clostridia and coliphages are still present, Dowd et al. (1997), Paez-Rubio and Peccia (2007). Similarly, international surveys of biosolids quality report that enterococci, coliphages, and Clostridia show greater resistance to inactivation compared to fecal coliforms during thermophilic anaerobic digestion and composting, Gantzer et al. (2001), Wery et al. (2006), Pourcher et al. (2005), Bagge et al. (2005), Christensen et al. (2002), Jepsen et al. (1997).

The enterococci indicator is present in high quantities in sewage sludge and should be further investigated as an indicator of biosolids quality. Enterococci may better represent health risks associated with more resistant pathogens, such as *Listeria monocytogenes* and *Staphylococcus aureus*, Sahlstrom (2003), Berg and Berman (1980), Sidhu and Toze (2009). While no epidemiological evidence related to biosolids is available, a strong correlation is found between enterococci densities and gastrointestinal disease incidence in recreational and beach waters, Cabelli et al. (1982), Wade et al. (2008). Most recently, the U.S. EPA evaluated the use of *Enterococcus* spp. quantitative polymerase chain reaction (qPCR) as an alternative or supplement to culture-based enterococci monitoring of beach and freshwater fecal contamination, Haugland et al. (2005), Siefring et al. (2008), Wade et al. (2008). This major recreational water study found that qPCR monitoring of enterococci exhibited the highest correlation to child gastrointestinal disease at Michigan beaches over both *Escherichia coli* and enterococci culture-based measurements, Wade et al. (2008).

In biosolids, enterococci detection with qPCR could be a useful tool to indicate the concentration and fate of bacterial pathogens. Coupling qPCR with culture-based values could also provide perspective on the culturable fraction of pathogens when Gram-positive pathogen genomes are detected, Viau and Peccia (2009). Further, quantitative PCR is able to detect viable but non-culturable bacteria that would escape detection with purely culture-based monitoring—VBNC bacteria in high quality biosolids are a major concern as they are shown to regain culturability when biosolids are dewatered by high-speed centrifugation, Higgins et al. (2007). Finally, because the qPCR assay is more rapid than culture-based monitoring (5 h vs. 48 h), enterococci qPCR monitoring of biosolids could allow for a more rapid assessment of inactivation, reactivation, and regrowth phenomena occurring during treatment, storage, land application, or in aerosols downwind of a biosolids site.

In 2008, the U.S. EPA put forth its intention to reevaluate the pathogen standards for biosolids based on health risks and new analytical methods—one potential action could be to update the indicator used for monitoring, Grumbles (2008). The purpose of this study is therefore to evaluate whether the enterococci indicator is appropriate for monitoring pathogens in biosolids. Biosolids were collected from 22 geographically diverse U. S. wastewater treatment facilities after treatments that include mesophilic anaerobic digestion (MAD), composting (COM), and temperature-phased anaerobic digestion (TPAD)—these three treatments were targeted as they represent the most common class B and class A treatments used in the U.S. by large municipalities, Godfree and Farrell (2005). Concentrations of enterococci in biosolids were determined by the U.S. EPA recommended membrane filtration and mEI-culture method and compared with concentrations derived from the 23S rRNA gene qPCR method, Haugland et al. (2005). Log reductions of enterococci through composting were then determined for qPCR and culture values and also compared to reductions for fecal coliforms, male-specific coliphages, and sulfite-reducing clostridia. Finally, as a supplement to phenotypic typing of enterococci cultures, biosolids colonies isolated on mEI agar were identified genotypically with 16S rRNA gene sequencing to enable the definitive identification of enterococci isolated from different biosolid treatments.

2. Materials and methods

2.1. Sample collection

Biosolid grab samples were collected from 22 treatment facilities across the U.S. and represented all geographic regions. Biosolids were taken after three treatment processes, including class B mesophilic anaerobic digestion (MAD, 13 plants), class A composting (10 plants), and class A temperature-phased anaerobic digestion (TPAD, six plants). Both a MAD sample and compost sample were collected at five of the facilities. Detailed operational parameters for the biosolids samples used here are described in Viau and Peccia (2009). Briefly, MAD samples undergo 1-stage anaerobic digestion at 35–37 °C for 5–30 days followed by dewatering by high-speed centrifugation ($n = 6$ plants), belt-filter pressing ($n = 5$ plants), or solid-bowl centrifugation ($n = 2$ plants). TPAD configurations include mesophilic–thermophilic, thermophilic–mesophilic, or thermophilic–thermophilic operations, with mesophilic stages running at 35–37 °C for 5–30 days and thermophilic stages operating at 55 °C for 5–30 days, followed by dewatering with high-speed centrifugation ($n = 4$ plants) or the belt-filter press ($n = 2$ plants). Compost samples were collected from piles that contained a mixture of dewatered MAD biosolids, wood chips, yard waste, and/or paper and were treated by agitated windrow or in-vessel composting processes.

Each plant provided five 100 g samples collected after dewatering or from finished compost heaps according to EPA Method 1680 USEPA (2006a). Samples were shipped overnight at 4 °C and mixed together on arrival to form one composite sample. Biosolids were then frozen at −80 °C for future DNA analyses or analyzed immediately for culturable indicators spp. and solids content, Eaton et al. (2005).

2.2. Culture-based enumeration

Two 15–25 g (wet) biosolids samples were eluted in 0.05% Tween 80 sterile peptone water, Lee et al. (2006), by mixing for 1 hr at 150 rpm. Serial dilutions were made in 1X PBS (0.14 M NaCl, 0.01 M phosphate, 0.03 M KCl, pH = 7.4) according to sample type and membrane filtration was performed in