Changes to microbial nitrogen-fixation, respiration, and community structure following land-application of municipal biosolids


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Abstract The effects of municipal biosolids application on microbial communities in agricultural soils were assessed in a three-month laboratory incubation study. Reference agricultural soils in containers were left unaltered, amended with commercially-available organic manure, or amended with municipal biosolids from a southern Ontario Wastewater Treatment Plant. An additional treatment of biosolids-only was also used. Soil N\textsubscript{2}-fixation rates in reference and manure-amended soils were largely similar, and lower than in biosolids-amended soil and biosolids-only treatments over the three-month period. Soil respiration rates showed similar trends. Differences among the three soil treatments decreased over time, with no significant difference at test termination for N\textsubscript{2}-fixation (p = 0.82), but some enhanced respiration remaining in the biosolids-amended soils relative to reference (p < 0.0001). Community structure was assessed using Biolog EcoPlates™. Principle Components Analysis of EcoPlate carbon source utilization patterns corresponded with findings from the activity analyses. Reference and manure-amended soils could be clearly discerned from biosolids-amended soils and biosolids up to two weeks post-amendment (p < 0.0001, factor 2), with no significant difference among the three soil treatments at test termination. In general, the effects on the activity and structure of N-fixing and general bacterial communities were temporary; however, this study evaluated a one-time application. The potential for ecological effects of biosolids contaminants requires further investigation.

Keywords agriculture; land-application; municipal biosolids; nitrogen-fixing bacteria; soil ecology

INTRODUCTION

In the Canadian province of Ontario, land-application of municipal sewage biosolids and other non-agricultural source materials on agricultural lands is regulated by the Ministry of Environment (MOE) under the Environmental Protection Act, and jointly administered by the MOE and Ministry of Agriculture, Foods and Rural Affairs under the Nutrient Management Act. In 2004, the ministries produced a draft Guide for the Beneficial Use of Non-Agricultural Source Materials on Agricultural Land, and this document plainly states: “…(the non-agricultural source materials)… must benefit crop production or soil health and not degrade the natural environment. NASM not meeting these requirements may not be land applied.” (MOE/OMAF, 2004).

Within the soil environment, soil organisms, including bacteria, carry out important processes which impact soil physico-chemical properties and enhance soil fertility and quality (Emmerling et al., 2002). Although it is known that soil organisms impact soil processes, the linkage between the biodiversity of organisms within the soil ecosystem and their ecological functions and processes has not yet been completely explained (Emmerling et al., 2002). It is likely that land-applying municipal biosolids does create a change in the soil environment, with resulting changes to soil microbial communities (potentially positive or negative). However, the nature and severity of biosolids land-application impacts on soil microbial community structure and function have not been adequately characterized, and at present, it has not been unequivocally confirmed that land-application of municipal biosolids from any wastewater treatment plant in Ontario meets the standard of benefiting soil health while not degrading the natural environment.
This study focuses on the impacts of municipal biosolids on nitrogen (N\textsubscript{2}) fixing bacteria because of their agronomic importance. Crop rotation with legumes (which contain symbiotic N\textsubscript{2}-fixing bacteria in root nodules) is a common practice, and the loss of N\textsubscript{2}-fixing ability would potentially result in higher operating costs for farmers, by making them reliant on additional fertilizer applications (Giller et al., 1998). There have been reports of negative impacts associated with municipal biosolids land-application (Giller et al., 1998), and these have included a reduction in (Brookes et al., 1986; Broos et al., 2004), or the complete loss of N\textsubscript{2}-fixing ability (Giller et al., 1989), and reduction in diversity among legume-symbiont Rhizobium species (Giller et al., 1989; Hirsch et al., 1993). Most of these studies have been conducted in Europe, with none found from Canadian research. In addition, most ecological studies to date focus on the effects of the heavy metals in the biosolids, with less attention paid to the effects of organic pollutants, especially emerging contaminants such as pharmaceuticals and personal care products (PPCPs), or the potential community-level effects of competition from viable bacteria remaining in the treated biosolids.

The objective of this research was to evaluate how land-applying biosolids from one southern Ontario municipal wastewater treatment plant affects an agronomically-important soil process (biological N\textsubscript{2}-fixation). Because most studies on the effects of municipal biosolids application on N\textsubscript{2}-fixing bacteria have narrowly focused on the effects of metal contamination (often using ‘uncontaminated’ biosolids-amended soils as reference to metal-spiked biosolids-amended soil treatments), there has not been adequate determination that other chemical constituents of the ‘uncontaminated’ biosolids, or viable bacteria, are not creating inhibitory effects themselves. The results of this study will provide critical information for managers in southern Ontario to determine if land application of municipal biosolids is truly a “beneficial use”.

**METHODS**

**Soil Collection and Test Soil Conditioning**

Reference agricultural test soil was obtained from a pasture site in Oro, ON in February, 2006. Approximately 60 cm of snow was removed prior to soil collection. Soil was removed to a depth of approximately 20 cm using clean shovels. One week after collection the soil was sieved (Ø < 3.35 mm) and large root masses (> 1-3 mm diameter) were removed. Fifteen 1 L plastic containers were filled with approximately 800 mL of soil, and planted with a mixture of alfalfa (*Medicago sativa*) and timothy (*Phleum pratense*) to pre-condition test soils prior to test initiation. Soils were kept at ambient laboratory conditions, under 60 W agrobulbs on a 12:12 light:dark cycle. Containers were watered based on hygrometric measurements (Lincoln Soil Meter, Lincoln Irrigation Inc., Lincoln NE); once soils reached a reading below 40% saturation, they were watered with 100 mL of municipal tap water (City of Toronto). Containers were maintained for a period of 4.5 months prior to test initiation in July, 2006. Vegetative growth in all containers was removed prior to test-initiation.

**Test Set-up and Schedule**

Time-specific measurements of N\textsubscript{2}-fixation, soil respiration and community-level substrate utilization (physiological profile) were taken at six test intervals: at day 0 (pre-test), day 1 (immediately following soil amendment), day 7, week 2, week 6 and month 3 (at test-termination). On day 1, pre-conditioned reference soils remained un-altered (reference treatment), or were amended with organic manure (Green Earth Premium Compost, Nu-Gro IP Inc., Brantford ON; soil+manure treatment) or municipal biosolids (soil+biosolids treatment). Biosolids were added at a rate of 5.5% (dry weight biosolids to soil). The biosolids were from a WWTP in the Region of Waterloo with < 125 000 m\textsuperscript{3} day\textsuperscript{-1} capacity and secondary treatment. The manure amendment was used as a control to differentiate the potential inhibitory effects of biosolids contaminants from the stimulatory effects of organic material enrichment. The application rate for organic manure (7.9% dry weight manure to soil) was based on adding an equivalent quantity of total nitrogen to both manure-amended and biosolids-amended soils. An additional biosolids-only treatment (i.e., containers were filled with the liquid biosolids) was used to determine the microbial activity and physiological profile of biosolids as an inoculum added to soils. Each test treatment had 5 replicates, and all test treatments were planted with the same mixture of alfalfa (*Medicago sativa*) and timothy (*Phleum pratense*) used in the pre-conditioning.
Measurement of N₂-fixation and Soil Respiration

At each test interval, a sawed-off 3 mL plastic syringe was used to collect approximately 5 g of soil (or biosolids) from randomly selected sections of each container (3-5 sub-samples per container). Samples were homogenized and a 2 g sub-sample was transferred into a sterile 20 mL glass serum vial. Vials were sealed with a rubber septum stopper and 100 µL of 15N₂ (Scott Specialty Gases, Plumsteadville, PA) was injected into each vial using a gas-tight syringe (Hamilton Company, Reno, NV). An additional air blank vial (without soil) was also spiked with 15N₂ to serve as an analytical reference. Immediately following injection, the serum vials were analyzed on a GC/MS (AutoSystem XL, Perkin Elmer, Waltham, MA). Gases were separated using a SupelQ™ PLOT capillary column (Supelco, Bellefonte, PA) with a carrier gas (He) flow rate of 0.5 mL min⁻¹, 80°C injection temperature, and 35°C (isothermal) oven temperature. Test replicates were analyzed in a randomized run order, and manually injected on-column using the 100 µL gas-tight syringe. At the initial 0 hr reading, peak height was recorded for molecular mass 40 (Ar) and 44 (CO₂). At the next reading (6 hr), maximum absorbance readings were recorded for molecular mass 30 (15N₂), 40 (Ar) and 44 (CO₂). At the final reading (48 hrs), maximum absorbance values were recorded molecular mass 30 (15N₂) and 40 (Ar). These sample intervals are based on previous incubations with this soil in which CO₂-production was linear over the 0 to 6 hour interval, and N₂-consumption (nitrogen fixation) was linear in the 6 to 48 hour interval. Between GC/MS analyses, septum vials were incubated in the dark at 25°C.

Nitrogen-fixation and soil respiration were based on changes in 15N₂:Ar and CO₂:Ar through time, assuming Ar to be a conservative internal standard. Similar approaches have been used to measure denitrification in marine sediments (increase in 14N₂:Ar) (Kana et al., 1994; Cornwell et al., 1998; Laursen et al., 2002), and this study represents an extension of these techniques. The air blank sample as used to make any necessary corrections to measured gas ratios. 15N₂ and CO₂ concentrations in incubation vessels were then back-calculated from ratio measurements, based on known atmospheric concentrations of Ar. The rate of analyte production or consumption per hour per gram of soil was then determined and the rate of 15N₂ consumption was converted to a total of 15N₂+14N₂. The average rate of N₂-fixation and soil respiration for each treatment was determined at each test interval, and treatments were compared using a single-factor ANOVA and a post-hoc test (Fisher’s Least Significant Difference) of means in SAS 9.1.3 (SAS Institute Inc., Cary, NC).

Biolog EcoPlates™ (Community-level Physiological Profiling)

At each test interval, a 2.5 g sub-sample was taken from the homogenized 5 g bulk samples of each treatment, and transferred to a sterile 50 mL plastic centrifuge tube. An additional treatment blank was also prepared without test soil. Bacteria in the test soils (and biosolids) were extracted using the deflocculating agents 0.01% sodium pyrophosphate and Tween 80 (v/v) as described by Victorio et al. (1996). After the final washing with phosphate buffer, the bacterial pellet was resuspended in 12 mL sterile saline solution (0.85% NaCl w/v), and a multi-pipettor was used to inoculate 100 µL of this suspension in individual wells of the Biolog EcoPlates™ (Biolog Inc., Hayward, CA). Plates were incubated in the dark at 25°C and scanned at regular time intervals (4, 22, 42 and 66 hrs post-inoculation) at 570 nm wavelength on a photometric plate reader (MultiSkan Ascent®, Thermo Fisher Scientific Inc., Waltham, MA). Multiple readings were required to determine the optimal incubation period for all treatments. The run order was the same randomly-generated order as used for GC/MS analyses. For each individual plate, the average well colour development score (AWCD) was calculated for each of the 31 carbon sources and the optimal inoculation duration was determined following Glimm et al. (1997). The AWCD scores at 42 hours post-inoculation were then subjected to Principle Components Analysis. Principal component scores for Factors 1 and 2 were then analyzed using a single-factor ANOVA with a post-hoc test (Fisher’s Least Significant Difference) to determine differences among treatments in substrate utilization.
Soil Chemistry
At test termination, the five replicates of the three-month experimental test soils from all treatments (including biosolids-only) were homogenized, and a bulk sample was analyzed for extractable metals (16 metals, including copper, lead, nickel and zinc), anions (bromide, chloride, fluoride, phosphate and sulphate), nitrogen (as total Kjeldahl nitrogen, ammonia-N, nitrite and nitrate), pH, and particle size. Soil chemistry was conducted by a commercial laboratory following accepted standard operating procedures. In January 2007, bulk samples of the three-month test soils, plus the original liquid biosolids (stored at 4°C since collection) were analyzed for 28 PPCPs (including analgesics, antibiotics, anti-epileptic / anti-depressants, lipid-regulating agents and stimulants).

RESULTS AND DISCUSSION
Following addition of organic manure or biosolids on day 1, both amended soils exhibited some degree of elevated N-fixation activity relative to the reference soils; however, over most of the three-month test period the rate of N-fixing activity in reference and manure-amended soils was similar (i.e., not significantly different, with rates ranging from 0.4 to 3.6 µmoles N·hr⁻¹·g soil⁻¹ between day 1 and week 6). Both treatments exhibited lower fixation rates than the biosolids-amended soil (which peaked at 4.5 µmoles N·hr⁻¹·g soil⁻¹ at week 2) and biosolids-only treatments (which peaked at 6.4 µmoles N·hr⁻¹·g soil⁻¹ at day 7). At most test intervals, the rate of N-fixation in the biosolids-only treatment was significantly higher than in the three soil treatments, and peak N-fixing activity for all treatments occurred between day 7 and week 2. The overall level of N-fixing activity, and differences in the rates among all treatments decreased over time, and were not significantly different than zero at test termination. After three months, there was no significant difference among all treatments (p = 0.82) (Figure 1).

![Figure 1](image)

**Figure 1:** N-fixation rates measured at three of the six test intervals (n = 5; standard error bars shown). Treatments with the same letter are not significantly different based on single-factor ANOVA and t-test of means with Fisher’s LSD (α = 0.05).

In general, soil respiration activity showed similar trends to the N-fixation activity. Over most of the three-month test period, respiration activity in reference and manure-amended soils was similar (not significantly different; ranging from 30.9 to 1598 nmoles CO₂·hr⁻¹·g soil⁻¹ between day 1 and week 6), with both treatments showing lower fixation rates than in biosolids-amended soil (which peaked at 2368 nmoles CO₂·hr⁻¹·g soil⁻¹ at day 7) and biosolids-only treatments (which also peaked on day 7 at 2548 nmoles CO₂·hr⁻¹·g soil⁻¹). At most test intervals, general microbial activity in the biosolids-amended soils and in the biosolids-only treatment was significantly higher than in the reference and manure-amended soils; however, activity in the three soil
treatments became more similar over time, with all treatments exhibiting a large decrease in relative activity towards the end of the test period. When considering all four treatments, there was no significant difference among the three soil treatments at test termination, however, there was some enhanced respiration remaining in the biosolids-amended soils ($p < 0.0001$) relative to the three soil treatments (Figure 2).

Figure 2: Soil respiration rates measured at three of the six test intervals ($n = 5$; standard error bars shown). Treatments with the same letter are not significantly different based on single-factor ANOVA and t-test of means with Fisher’s LSD ($\alpha = 0.05$).

EcoPlate™ carbon source utilization patterns also corresponded with findings from the two measures of bacterial activity. In Principal Components Analysis, control and manure-amended soils could be clearly discerned from biosolids-amended soils and biosolids up to two weeks post-amendment. At test termination there was no significant difference between the three soil treatments of manure and biosolids-amended soils, and reference soils ($p < 0.0001$, factor 2) (Figure 3).

Figure 3: Principle Component Analysis of AWCD scores at three of the six test intervals following inoculation on Biolog EcoPlates™. Ellipses represent groupings of treatments which are not significantly different based on single-factor ANOVA of Factor 2 scores, and t-test of means with Fisher’s LSD ($\alpha = 0.05$).

Metals analysis determined an exceedance of the MOE/OMAFRA ‘more restrictive’ biosolids land application standard for copper (1200 µg g$^{-1}$ compared to 760 µg g$^{-1}$). This would potentially restrict land application of biosolids from this wastewater treatment plant to a maximum application rate of 8 tonnes ha$^{-1}$ 5 yrs$^{-1}$ compared to a higher maximum application rate of 22 tonnes ha$^{-1}$ 5 yrs$^{-1}$. In addition, it was noted that biosolids-amended soil had much higher concentrations of sulphate (659 µg g$^{-1}$) and chloride (388 µg g$^{-1}$) than reference and
manure-amended soils (74 and 252 µg g⁻¹, and 81.4 and 124 µg g⁻¹, reference and manure-amended soil sulphate and chloride concentrations, respectively). Although anions are not regulated (i.e., there are no biosolids land application standards), they can be toxic in high doses (Eaton, 1942; Bright and Addison, 2002). An EC₅₀ value for chloride as low as 301 µg g⁻¹ was found in a study assessing springtail (Folsomia candida) reproductive effects in field soils treated with NaCl (Bright and Addison, 2002). PPCP analysis found relatively high levels of two common analgesics in the biosolids and biosolids-amended soil. Salicylic acid (e.g., Aspirin®) was detected in the liquid biosolids (2.60x10⁻¹ to 3.58x10⁻¹ ng g⁻¹), 3-month weathered biosolids (2.09x10⁻¹ to 2.61x10⁻¹ ng g⁻¹), biosolids-amended soil (2.26x10⁻¹ to 2.60x10⁻¹ ng g⁻¹), and was also detected in the reference soil (1.09x10⁻¹ to 1.86x10⁻¹ ng g⁻¹). Ibuprofen (e.g., Advil®) was detected in the liquid biosolids (below detection to 4.86x10⁻¹ ng g⁻¹) and 3-month weathered biosolids (3.24x10⁻¹ to 4.49x10⁻¹ ng g⁻¹). In addition, carbamazepine (e.g., Tegretol®), an anti-epileptic / anti-depressant, was detected in the liquid biosolids (2.97x10⁻¹ to 3.95x10⁻¹ ng g⁻¹) and the three-month aged biosolids samples (3.77x10⁻¹ to 4.75x10⁻¹ ng g⁻¹), with low levels also detected in the biosolids-amended soil (4.66x10⁻³ to 5.97x10⁻³ ng g⁻¹). Differences in concentrations in liquid versus dried biosolids samples can be related to the compound-specific sorption strengths.

Although the effects of biosolids application on crop yield was not originally included as a major component of this study, at two weeks post-amendment it was observed that alfalfa and timothy in biosolids-amended soils began to exhibit wilting and desiccation. These effects were not observed in the other soil treatments. Soon after, the vegetation on the biosolids-amended soils began to die; giving evidence of phytotoxicity associated with the biosolids (the biosolids-only treatment only had weak timothy germination, with no alfalfa present at any point of the experiment). This effect on crop yield may be due solely to the extremely high levels of chloride and sulphate in the biosolids-amended soil, or to toxicity associated with metals (especially copper), or PPCPs (particularly the analgesics). Another potential issue is the nature of biosolids and their hydrophilicity. Although soil moisture in all treatments was maintained at above 40% saturation, repeated measurements in the biosolids-amended soils gave readings equivalent to 80% saturation, despite the appearance of soil surface as being dry. If the biosolids in the soil were tightly absorbing available moisture, this may have partitioned the water molecules away from the plant roots, creating the potential that the apparent phytotoxicity was simply the result of drought stress.

**CONCLUSIONS**

The test methods used in this examination found a temporary change in bacterial communities following land-application of municipal biosolids. However, at most time intervals there was a significant difference between the manure-amended soils and biosolids-amended soils, indicating there were changes to the bacterial community as a result of some component of the biosolids not present in organic manure (eliminating the potential for organic enrichment to be the sole cause of shifts). Without toxicity identification evaluation testing, it is impossible to conclusively link these changes to a particular contaminant, or group of contaminants (if the contaminants in the biosolids are in fact the causative agents of change), or to competition from bacterial species remaining in the biosolids following wastewater treatment. As noted, the high levels of sulphate and chloride in the biosolids may also be related to the changes in activity and physiological profile.

It is important to emphasize that extrapolation of the test results here should not be made to describe the potential effects of other municipal biosolids sources, which will have unique combinations of industrial, agricultural, commercial and residential inputs to their wastewater treatment systems. In comparison to other biosolids analyzed for PPCPs, these biosolids are relatively clean, emphasizing the potential for widely differing concentrations and numbers of contaminants based on the demographics of the catchment area. It also cannot be summarized that the lack of permanent changes to the bacterial community studied here indicates a lack of ecological effects — this study narrowly focused on one small component of the entire agricultural soil foodweb. Researchers have also shown effects to soil organisms at higher trophic levels; biosolids or sewage sludge application on agricultural soils have been shown to impact earthworms (Tomlin et al., 1993; Peles et
al., 2003), insects (Larsen et al., 1994, 1996; Cole et al., 2001), and protists (Campbell et al., 1997), and based on observations of stimulatory and inhibitory effects seen at these trophic levels, further evaluation of effects at an ecosystem level is warranted. Determination of shifts in the structure of N fixation bacterial communities among treatments (based on nifH-derived phylogeny) is currently on-going, and may in fact illustrate a shift in bacterial community structure that was not shown by these other test methods. Also, our study examined the effects of a single application of municipal biosolids. Sites which are chosen for land application are subject to repeated applications necessitating an evaluation of cumulative loading effects.

Based on the results of this study, the numerous studies indicating the potential for permanent ecological effects, and the mandate of the Ontario provincial government that biosolids must not degrade the natural environment, we believe it is necessary to employ a precautionary approach to the practice of land-applying municipal biosolids before the extent of potential ecological effects, on a more holistic scale, has been clearly defined. This is necessary to confirm that biosolids-land application can unequivocally be deemed a "beneficial use".

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