THE ECOLOGICAL EFFECTS OF LAND-APPLYING MUNICIPAL BIOSOLIDS ON NITROGEN-FIXING BACTERIA

by

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A thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Applied Science

in the Program of

Environmental Applied Science and Management

Toronto, Ontario, Canada, 2007

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Abstract

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Masters of Applied Science
Environmental Applied Science and Management
July 2007
Ryerson University

The effects of municipal biosolids on nitrogen-fixing bacteria were assessed in a three-month soil incubation study. Treatments included reference agricultural soils, soil amended with municipal biosolids or manure, and biosolids without soil. Nitrogen-fixation rates in reference and manure-amended soils were similar, and lower than in biosolids treatments; respiration rates showed similar trends. At test termination there was no difference between soil treatments for nitrogen-fixation, but some enhanced respiration in the biosolids-amended soils. Community structure was assessed using Biolog EcoPlates™ and denaturing gradient gel electrophoresis with a nitrogen-fixing gene (nifH). EcoPlate™ carbon utilization patterns corresponded with activity measures, with no difference among soil treatments at test termination. Nitrogen-fixing gene patterns showed a potential shift in community structure of biosolids-amended soils three months post-amendment. In general, the effects on the activity and structure of nitrogen-fixing communities were largely temporary; however, this study evaluated a one-time biosolids application. The potential for cumulative effects requires further investigation.

Acknowledgements

My research was funded by an Ontario Graduate Scholarship in Science and Technology (OGSST), Ontario Graduate Scholarship (OGS), Ryerson Graduate Award, Golder Associates Ltd. (Continued Learning Awards for 2005 and 2007, and funding of chemical analyses), NSERC Operating Grants to Dr. Lynda McCarthy and Dr. Andrew Laursen, and the Ryerson Department of Chemistry and Biology. Technical assistance from Joseph Bautista, Dennis Walmsley, and Max Niedzielski was greatly appreciated, and essential to the success of my experiment. In addition, I would like to thank Michael Payne of the Ontario Ministry of Agriculture, Foods and Rural Affairs for facilitating the collection of our experimental biosolids, and Kristen and Wayne Varcoe for providing access to reference agricultural soil with no recent synthetic fertilizer or pesticide application, and no history of biosolids application.

On a personal note, I would like to offer my greatest appreciation to my fiancé, Andrew Hough - who left Vancouver, moved across the country, fought his way into the Ontario public education system, and provided unwavering support of my pursuit of higher education. I would also like to thank my parents, Jim and Gwen Holt, who have always provided unique (not equal) support and encouragement to myself and my sister Melissa, in all our academic and professional endeavours.

Finally - I would like to express gratitude to my three supervisors (the "Trinity"), Drs. Andrew Laursen, Lynda McCarthy and Vadim Bostan. You have provided a challenging, supportive, and most importantly, fun environment in which to learn.

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List of Abbreviations

AM	Arbuscular Mycorrhizae
AOB	Ammonia-oxidizing Bacteria
AOX	Adsorbable Organic Halides
APLR	Annual Pollutant Loading Rate (US EPA)
AWCD	Average Well Colour Development
BMP	Best Management Practice
BNQ	Bureau de Normalisation du Québec
BOD	Biological Oxygen Demand
BSA	Bovine Serum Albumin
BSAF	Biota-soil Accumulation Factor
CA	Certificate of Authorization (Quebec)
CEC	Cation Exchange Capacity
CFIA	Canadian Food Inspection Agency
CLPP	Community-level Physiological Profile
CPLR	Cumulative Pollutant Loading Rate (US EPA)
DEHP	Di-(2-ethylhexyl) phthalate
DGGE	Denaturing Gradient Gel Electrophoresis
DHA	Dehydrogenase
DNA	Deoxyribonucleic Acid
EC_{10} / EC_{50}	Effective Concentration of a compound associated with a measurable effect in 10 or 50% of all test organisms, within the specified toxicity test duration
EEM	Environmental Effects Monitoring (Environment Canada)
EPA	Environmental Protection Act (Ontario)
EQ	Exceptional Quality (US EPA)
EU	European Union
G+C	Guanine + Cytosine
GC/MS	Gas Chromatography / Mass Spectrometry
GPI	Glucose Phosphate Isomerase
ITS	Intergenic Spacer

LAS	Linear Alkylbenzene Sulfonate
LOEC	Lowest Observed Effective Concentration
MENV	Ministère de l'Environnement (Quebec)
MOE	Ministry of Environment (Ontario)
MPN	Most Probable Number
N	Nitrogen
N_2	Nitrogen gas
NASM	Non-agricultural Source Material (Ontario)
NMA	Nutrient Management Act (Ontario)
NP	Nonylphenol
NPDES	National Pollutant Discharge Elimination System (US EPA)
OD	Optical Density
OMAF / OMAFRA	Ontario Ministry of Agriculture and Foods / Ontario Ministry of Agriculture, Foods and Rural Affairs
OMRR	Organic Matter Recycling Regulation (British Columbia)
OTU	Operational Taxonomic Unit
P	Phosphorus
PAH	Polycyclic Aromatic Hydrocarbon
PBDE	Polybrominated Diphenyl Ether
PC	Pollutant Concentration (US EPA) / Principal Component
PCA	Principal Component Analysis
PCB	Polychlorinated Biphenyl
PCDD / PCDF	Polychlorinated Dibenzo-p-Dioxin (dioxin) / Dibenzofuran (furan)
PCR	Polymerase Chain Reaction
PLFA	Phospholipid Fatty Acid
PPCP	Pharmaceuticals and Personal Care Products
RFLP	Restriction Fragment Length Polymorphism
TEQ	Toxic Equivalent
TIE	Toxicity Identification Evaluation
US EPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant

1 Introduction

Human waste (also known as "night soils") has been used as fertilizer treatment on agricultural lands for centuries. The first known irrigation system designed for sewage disposal was built in Prussia in 1559, and in 1859, the English Royal Commission on Sewage Disposal recommended that town sewage be land-applied in order to minimize river pollution (Webber and Hilliard 1974). In the last few years, increasing concerns over disposal costs, and recognition of the need to improve the sustainability of waste disposal practices (e.g., landfill diversion and reduction of incineration), has increased pressure on municipal wastewater treatment plant managers to employ land application of sewage waste as a disposal method in Canada (Vasseur *et al.* 2000, LeBlanc *et al.* 2004), the US (Meyer *et al.* 2001) and Europe (Wang and Jones 1994). Despite this industry shift, most members of the public are not aware that land-application of municipal sewage waste on agricultural lands is still a common practise, even in developed countries like Canada (InfraGuide 2005); this explains why public awareness and education campaigns are considered necessary to continue and expand this practice in North America (Fitzhugh *et al.* 1994, Draman 1995, Hodson 1996).

The term "biosolids" was coined by Dr. Bruce Logan of the University of Arizona in the early 1990s, in an effort by the wastewater treatment industry to create a "more favourable term" to describe stabilized sewage sludge meeting U.S. federal land-application regulations (WEF, 1997). Disposal of biosolids on land is termed a "beneficial use", with biosolids promoted as a soil amendment that supplies nitrogen (N), phosphorus (P) and other micro-nutrients that promote crop growth, provides increased water retention capacity, and provides water-stable aggregates (Tierney *et al.* 1997, Meyer *et al.* 2001, Selvaratnam and Kunberger 2004). However, because biosolids are a waste product of the municipal wastewater treatment process, the use of biosolids has drawbacks compared to conventional fertilizers and soil improvement techniques (McBride 2003). Although primarily designed to treat sewage, conventional wastewater treatment plants (WWTPs) serve developed areas with unique combinations of land uses; these can include residential, commercial and industrial uses, which produce a mixture of anthropogenic contaminants including heavy metals, pathogens and organic chemicals. Heavy metals in biosolids are often described as valuable micro-nutrients (Schut 2005); however, there are maximum plant tissue concentrations at which they are of beneficial use to plants (in the 20

to 200 mg kg^{-1} DW range for copper, boron and zinc) (Hopkins 1999). As with any nutrient, once the species-specific critical concentration of these heavy metals is exceeded, they can become phytotoxic (Hopkins 1999) and toxic to soil organisms (Giller *et al.* 1998). Organic anthropogenic chemicals - including dioxins and furans (PCDD/Fs), polychlorinated biphenyls (PCBs) and the emerging pharmaceuticals and personal care products (PPCPs) - comprise a second class of important biosolids contaminants, especially in light of the fact that most existing WWTPs are not typically designed to treat them, and they are known to accumulate in municipal biosolids (Stevens *et al.* 2001, Overcash *et al.* 2005). Pathogens and other microorganisms are also concentrated in activated sludge (O'Connor *et al.* 2005), and are not fully inactivated following digestion to create biosolids (Gibbs *et al.* 1997). Once biosolids are land-applied, viable bacteria may also interact with indigenous soil organisms, potentially altering the ecological equilibrium of the agricultural soils being amended. The potential for runoff and leaching of biosolids contaminants from agricultural lands into surface and groundwater makes nutrient loading (leading to eutrophication) (Kelling *et al.* 1977, Cornu *et al.* 2001), and the release of anthropogenic contaminants (Cornu *et al.* 2001) or pathogens (Selvaratnam and Kunberger 2004) additional sources of environmental concern.

In the province of Ontario, municipal biosolids have been applied to agricultural lands for over 40 years. A 1974 quote by Ontario Ministry of Environment and Environment Canada staff highlights the need for ecological effects assessment of land-application of municipal biosolids at that time (Black and Schmidtke 1974):

> *"Sewage sludge application on agricultural lands has been practiced in Ontario for many years without the identification of any specific problems. The ultimate disposal of sludge was generally of no concern to the sewage treatment plant operator and no particular effort was placed on evaluating this method of disposal. The potential hazards and environmental limitations associated with its usage were never truly considered. In the environmentally conscious society of today, such a practise is no longer acceptable. Land application of sewage sludge for its soil amendment and fertilizing properties can only be a viable process if the potential hazards and limitations of its usage are clearly defined."*

Over thirty years later, the potential hazards and limitations of biosolids usage have not been clearly defined, the population (and source of biosolids) in southern Ontario has grown exponentially, and the appropriate disposal method, or "management option", for Ontario

municipal biosolids remains a contentious issue. In 2004, the City of Toronto produced the "City of Toronto Biosolids and Residuals Master Plan"; a future plan for biosolids and water residuals management up to the year 2025 (KMK Consultants *et al.* 2004). This document outlined the management option for Ashbridges Bay WWTP, the largest in Canada, as being 100% beneficial use, with 50% of biosolids applied to agricultural lands and 50% pelletized (to create a commercially-sold fertilizer product). Unfortunately, this plan did not consider the ecological impacts of land-applying biosolids, nor did it recommend future studies to evaluate potential impacts. Disregard for the ecological effects of this disposal method is not in keeping with the criteria for "beneficial use" described in Ontario's 2004 draft guide for biosolids land application (where biosolids are referred to as "non-agricultural source materials"). This document plainly states (Ontario Ministry of Environment and Ontario Ministry of Agriculture and Food 2004):

> *"...(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."*

Although it is only considered the contingency management option, most of the biosolids from Ashbridges Bay are not land-applied, and until late 2006 have been transported to a landfill in Michigan along with most of the City of Toronto's municipal waste (Kurth 2005, Truini 2006). This situation will change as a result of recent laws passed by the Governor of Michigan designed to limit the volume of municipal waste exported to Michigan (CTV.ca News Staff 2003, Hansen 2004). Closure of the border and the current lack of local landfill options will leave land application and pelletization as the two main disposal options. This situation is further complicated by public resistance to land application, and concerns over human health effects, with symptoms of nosebleeds, headaches, stomach pains and eye irritation reported by residents living adjacent to farms receiving Toronto biosolids (Landsberg 2000, McLeod 2003, CTV.ca News Staff 2004, McLeod 2004). Public education is described as the best way to win public approval (Hodson 1996); however, it is extremely important to differentiate education from marketing or propaganda.

Without the option of exporting large quantities of biosolids to Michigan, and potentially fewer local areas accepting biosolids for land-application, waste managers with the City of Toronto are at risk of being in a situation where they are without sufficient disposal options. Although it may

hinder WWTP managers in Southern Ontario, determining the potential for ecological damage as a result of land application of municipal biosolids is now very important, if increased land-application remains as the intended direction for policy makers. The information gained will benefit all parties; whether it bolsters the argument for beneficial use, demonstrates ecological effects are possible, finds that existing land-application regulations require strengthening, or suggests alternate routes of disposal are necessary.

The overall objective of this thesis is to determine the effects of land-applying municipal biosolids on the structure and function of nitrogen-fixing bacterial communities in agricultural soil. The objective of the first section of this thesis, the literature review, is to put research on the effects of municipal biosolids land-application on indigenous soil microorganisms into the context of the biosolids production process (wastewater treatment), regulations for biosolids land application, and the state-of-knowledge on the ecological impacts of land application. The objective of the research experiment is to evaluate how land-applying biosolids from one southern Ontario municipal wastewater treatment plant affects one important component of the soil ecosystem, the nitrogen-fixing bacteria. Specifically, the hypothesis tested is that land-application of municipal biosolids is associated with a decrease in nitrogen-fixing activity. These bacteria have been shown to be negatively affected by biosolids land-application; however, the depth of investigation has not been sufficient to-date. A second hypothesis to be tested is that land-application of municipal biosolids results in a change in nitrogen-fixing community structure, based on the potential toxicity of biosolids contaminants or the influx of viable bacteria and other microbes from biosolids. Additional measures of how land-application affects overall microbial community health are included to more fully describe the changes in indigenous microbial ecology. The third hypothesis tested is that general bacterial activity is stimulated by biosolids application, based on the stimulatory effect of nutrient addition. The results of this study will provide critical information for WWTP managers in southern Ontario, and the general public, in determining if land application of municipal biosolids is truly a "beneficial use", and if the policy of 100% beneficial use is ecologically sound.

2 Contextual Background

2.1 The Wastewater Treatment Process

Biosolids are significantly more complex (in terms of composition, and spatial and temporal variability) than wastes from process-specific industries (e.g., factories with known inputs of chemical and biological reagents which carry out routine production methods, and produce similar outputs of waste through time). In North America, WWTPs treat wastewater originating from residential, commercial and industrial sites before releasing treated effluent into receiving environments. In order to fully understand the characteristics of biosolids and how they might affect terrestrial and aquatic ecosystems, it is important to understand the processes involved in creating biosolids. The wastewater treatment process utilizes up to three basic levels of treatment; primary, secondary, and less frequently, tertiary treatment.

Primary treatment consists of the mechanical separation of insoluble particulates from wastewater through the processes of screening, precipitation, and settling (Prescott *et al.* 1996). This process reduces the biological oxygen demand (BOD) of wastewater by 20-30%. Treated water then flows into a secondary treatment reactor tank, and remaining solids settle to the bottom. These solids are considered "raw" sludge, and can be conveyed to a digester for further stabilization.

Secondary treatment is an aerobic, biologically-mediated process where a consortium of microorganisms, primarily bacteria (up to 10^{11} cells mL^{-1}) (Wagner 2005), are used to further reduce dissolved organic matter and nutrients (i.e., nitrogen and phosphorus) (Prescott *et al.* 1996). Until the advent of molecular techniques, the composition of activated sludge communities was poorly understood (Wagner 2005, Gilbride *et al.* 2006). The bacteria involved in this process can include *Nitrospira*-like sp. and *Candidatus* sp. (Wagner 2005), as well as *Nitrobacter*, *Nitrosomonas*, *Proteobacteria*, and *Acinetobacter* sp., and *Zooglea ramigera* (as summarized by Gilbride *et al.* 2006), with protozoans including ciliates, and a smaller number of amoebae and zooflagellates (Curds and Cockburn 1970). The specific community of microbial species depends on the characteristics of the sludge material and treatment conditions. The types of secondary treatment reactors vary, and include activated sludge with microbial biomass recycling, tricking filter, and extended aeration without biomass recycling (Prescott *et al.* 1996).

Secondary treatment reduces 90-95% of the remaining BOD, and removes many of the bacterial pathogens from wastewater (Prescott *et al.* 1996). After secondary treatment, the remaining bacterial biomass flocculates and sinks to the bottom of the tank. The biomass (or sludge) from this process is considered "waste activated" (containing bacterial biomass and other recalcitrant organic pollutants). Along with the primary treated sludge, the waste activated secondary sludge is conveyed to an anaerobic digester for further stabilization.

Wastewater from the secondary treatment may then be subject to tertiary treatment, which further reduces the most problematic pollutants such as N and P (which cause eutrophication), non-biodegradable contaminants (e.g., PCBs and other persistent organics), heavy metals, and minerals (Prescott *et al.* 1996). Tertiary treatment is more expensive than primary and secondary treatment, and is not commonly used except in areas with sensitive receiving environments (Prescott *et al.* 1996).

Following tertiary or secondary treatment, anaerobic digestion of sludge is carried out in large fermentation tanks under de-oxygenated conditions (aerobic digestion is also possible). Primary, secondary and tertiary sludge is fed into the digester at a constant rate and undergoes three digestion steps: fermentation to form organic acids, production of methanogenic substrates (acetate, CO_2 and hydrogen), and methanogenesis by methane-producing bacteria (Prescott *et al.* 1996). Methane can then be used as a fuel for heating and electricity. As the degree of treatment increases beyond the primary level, the volume of biosolids also increases. Following digestion, the sludge is considered stabilized due to the reduction of organic matter (to a state at which it is no longer readily decomposed), and reduction in pathogens and putrecible solids (which cause foul odours and attract pathogen vectors such as rodents, insects and birds) (Jacques Whitford 2004, Ontario Ministry of Environment and Ontario Ministry of Agriculture and Food 2004). At this point, the sewage sludge can technically be called "biosolids". It is important to note that although anaerobic digestion removes volume from the biosolids, the heavy metals and other organic contaminants are concentrated (Prescott *et al.* 1996).

After digestion, biosolids are then disposed of through incineration (often generating power) or landfilling, or can be applied to agricultural or other types of soils (e.g., mine rehabilitation sites or forests). It is important to reiterate that the biosolids produced on a particular day from one WWTP will not be identical to biosolids produced at the same plant on another day, or to

biosolids produced from another WWTP; there are intrinsic temporal and spatial variations in waste input from the many types of wastewater sources (i.e., residential, commercial and industrial).

2.1.1 Ashbridges Bay WWTP

The City of Toronto's Ashbridges Bay WWTP was built in 1912 (Salib 1974), and has undergone numerous upgrades and expansions since that time. Ashbridges Bay is currently the largest WWTP in the Greater Toronto area, serving Toronto, North York, East York and Scarborough, and is also the largest WWTP in Canada (KMK Consultants *et al.* 2004). Ashbridges Bay treats raw wastewater from residential, industrial and commercial sources, and raw primary sludge from Humber and North Toronto WWTPs (KMK Consultants *et al.* 2004). The plant provides secondary treatment (using activated sludge aeration), with anaerobic digestion of primary and secondary sludges carried out in 16 primary digesters and four secondary digesters (Jacques Whitford 2004, KMK Consultants *et al.* 2004). Following anaerobic digestion, a stabilizing polymer solution is added to the biosolids before dewatering through centrifugation (KMK Consultants *et al.* 2004). As of 2002, Ashbridges Bay processed 695 000 $m^3 day^{-1}$ of wastewater (83% of their 818 000 $m^3 day^{-1}$ capacity), and generated approximately 126 000 kg dry solids day^{-1}, and 50 000 dry tonnes $year^{-1}$ of dewatered biosolids (25-30% solids) (Nazareth *et al.* 2001, KMK Consultants *et al.* 2004).

2.1.2 Kitchener WWTP

Although it was intended that Ashbridges Bay WWTP biosolids would be evaluated in this experiment, biosolids samples were ultimately not obtained due to the change in political climate. The City of Toronto had originally agreed to provide biosolids for this research in September 2005; however, by summer 2006 the closure of the Michigan border to Ontario's biosolids was underway, and use of Toronto biosolids for ecological research was prohibited. An alternate biosolids source was sought, and biosolids from the Kitchener WWTP in Kitchener, ON (in the Regional Municipality of Waterloo) were provided and used as a contingency. As of 2004, the Kitchener WWTP provided secondary treatment though conventional activated sludge treatment (Region of Waterloo and Earth Tech 2004). Treatment upgrades, including enhanced removal of ammonia (through nitrification) and phosphorus, and UV disinfection are planned over the next 10 years under the Regional Municipality of Waterloo Wastewater Treatment

Master Plan (Region of Waterloo and Earth Tech 2007). As of 2006, the Kitchener WWTP served a catchment population of 202 213 people with a rated capacity of 122 700 m^3 day^{-1} (Region of Waterloo and Earth Tech 2007). This capacity is considered sufficient to serve a maximum population of 358 000 residents (Region of Waterloo 2006). As of 2005, the average throughput was well below capacity (68 224 m^3 day^{-1}) (Region of Waterloo 2006).

It is important to reiterate that the composition of biosolids from the Kitchener plant will differ from Ashbridges Bay biosolids; although they both provide secondary treatment through activated sludge aeration, there are large differences in total throughput of wastewater (the Kitchener plant treats 10% of the total volume treated at Ashbridges Bay), and in catchment area land use.

2.2 *Biosolids Disposal Methods*

In the past, options for municipal biosolids disposal included ocean dumping, incineration, landfilling, mine site rehabilitation, and use as a soil amendment (i.e., land-application) (Gibbs *et al.* 1997). Of these disposal options, ocean dumping, landfilling, incineration and land application have historically constituted the major routes, although ocean dumping has been banned in the US (Vaccaro *et al.* 1981) and Europe (Eljarrat *et al.* 1997). Table 1 provides an overview comparison of the municipal biosolids disposal methods used in Ontario, Quebec and British Columbia, the US and Europe.

Table 1: Comparison of municipal biosolids disposal methods in Canada, US and Europe.

Jurisdiction	Land Application amount (tonnes)	Application area (hectares)	Biosolids Disposal Method (as %)			
			Land Applied	Incinerated	Landfill Disposal	Ocean Disposal
Ontario (2000)*	1 500 000 (wet)	13 000				
Quebec (1999)†	500 000		8	80	12	
Quebec (2001-2002)‡	70 000					
British Columbia§			90			
USA (1980s)**			20	25	40	15
USA (1997)	6 000 000		36-40			
France (1995)††			60	15-20	20-25	
UK (1996)‡‡	500 000					

*(Sidhwa 2000)
†(Davey 2000)
‡(Hébert 2004)
§(Davey 2000)
**(Vaccaro *et al.* 1981)
††(Jacquot *et al.* 2000)
‡‡(Cole *et al.* 2001)

It is apparent from these data that over time, land application of biosolids has decreased in volume in Quebec, but increased as a disposal method in the US (from 20% up to 40%). The province of BC currently land-applies 90% of their biosolids (of which, 90% is used in land reclamation, with the rest applied on agricultural soils) (Davey 2000), with France land-applying only 60% (as of 1995). The break-down in disposal methods used in Ontario was not available, although the total amount land applied as of 2000 was 1.5 million tonnes, over an area of 13 000 hectares (Sidhwa 2000).

2.2.1 Ashbridges Bay WWTP Biosolids Disposal

Until the late 1990s, the majority of the biosolids from Ashbridges Bay WWTP were incinerated, with only a small fraction land-applied (Nazareth *et al.* 2001). In the fall of 1998, the City of Toronto initiated a search for alternate disposal methods to incineration, with a Biosolids Multi-Stakeholder Committee determining land application to agricultural land and pelletization as the two best options. Other options under consideration included alkaline stabilization (i.e., liming) and composting (Nazareth *et al.* 2001).

Pelletization of biosolids began in 2002, and the plant produced 4416 tonnes of pellets (approximately 20% of capacity) that year, which were to be sold commercially as a low-nutrient fertilizer product "Nutripel", or potentially used as a component of landfill topdressing (Jacques Whitford 2004). The pelletizer plant was damaged by fire in August 2003, and was not re-commissioned. Very recently, the City of Toronto decided to re-commission the pelletizer plant, and according to a January 2007 budget brief, once operational, will function at pre-fire levels (i.e., will process up to 89 000 tonnes of biosolids annually, or at 28% solids, 22 250 dry tonnes per year) (Fleming 2007).

In the years since the pelletizer fire, the only two disposal options for Ashbridges Bay biosolids have been land application and landfill disposal. The City of Toronto has held an operating contract with Terratec Environmental for agricultural land application since 1996, and as of 2004, biosolids application sites were located in Dufferin, Wellington, Grey and Northumberland counties (KMK Consultants *et al.* 2004). The volume of Ashbridges Bay biosolids applied to agricultural lands, and the area of lands receiving biosolids application from the years 1998 to 2003 are provided in Table 2.

Table 2: Land application of biosolids from Ashbridges Bay WWTP from 1998-2003 (KMK Consultants *et al.* 2004).

Year	Land Application amount (dry tonnes)	Biosolids application area (hectares)
1998	18 010	2100
1999	20 740	2600
2000	23 540	1600
2001	17 310	2250
2002	5120	800
2003	1480	250

Of particular note is the drastic reduction in land application from 2001 to 2002. The City has attributed this decline to a lack of seasonal storage (the Region of Halton terminated an arrangement with Terratec Environmental based on public odour concerns in 2001), stricter setback distances from biosolids regulations which came into effect in 2003 (discussed further in Section 2.3.1.1; which were also prompted by public odour concerns), weather (application is restricted to dry weather conditions) and public perception (partially attributed to the Walkerton tainted drinking water incident) (KMK Consultants *et al.* 2004). One additional explanation provided by the Toronto Commissioner of Works and Emergency Services is that some rural municipalities (no names provided) where Toronto biosolids were applied had enacted bylaws prohibiting the spreading of biosolids in their jurisdictions (Commissioner of Works and Emergency Services 2003). Biosolids land-application data for 2004 and beyond were not available for Ashbridges Bay WWTP.

Although it is only considered the contingency plan for disposal, landfilling has been the main route of Ashbridges Bay biosolids disposal since the decommissioning of the incinerators in 2002. Since the closure of the Keele Valley landfill in 2003, a large proportion of the biosolids from Ashbridges Bay, and Toronto's municipal solid wastes have been transported 430 km to a landfill in Michigan (Kurth 2002, KMK Consultants *et al.* 2004, McLeod 2004). As mentioned, in 2006, the Governor of Michigan signed laws designed to limit the volume of municipal waste exported to Michigan, effectively removing this disposal option (CTV.ca News Staff 2003, Hansen 2004). As of August 2006, the City had secured contracts with Environmental Management Solutions Inc. and Ferti-val Inc. to dispose of about seven truckloads of Toronto biosolids per day, plus a third company that will handle another truck-and-a-half (to account for

the daily total amount of biosolids produced by Ashbridges Bay WWTP), however the destination of the biosolids was not reported (Truini 2006).

2.2.2 Kitchener WWTP Biosolids Disposal

As of 2003, the Region of Waterloo also held a biosolids land-application contract with Terratec Environmental, which was potentially to be extended to 2005, based on a lack of additional bidders (Andrews 2003). The Region land-applies the majority of biosolids from all 13 of their WWTPs. In 2002, there were 186 471 m^3 (note: provided as a volume, not weight) of anaerobically stabilized biosolids applied to 2982 acres of land (1206.8 hectares), and 14 447 m^3 of aerobically stabilized biosolids applied to 142 acres of land (57.5 hectares), for a total of 200 918 m^3 over 1264 hectares (Andrews 2003). No specific information was available for the Kitchener WWTP. For the year 2002, the Region land-application area was greater than Ashbridges Bay (800 hectares). As of 2007, the region land-applies a total of 250 000 m^3 of municipal biosolids from its 13 WWTPs (Region of Waterloo 2007). The majority of biosolids are applied and immediately incorporated by subsurface injection, with a small amount spray-irrigated to provide nutrients to emerged crops (Andrews 2003).

As with the City of Toronto, the Region's municipal biosolids land-application programs faced increased public scrutiny in 2002, which resulted in delays in approvals and municipal restrictions on the application of biosolids (Andrews 2003). In order for the Region to maintain its application schedule, additional land-application sites were obtained at greater distances (of all 64 sites which received biosolids, 59% were located outside the Region). This difficulty in obtaining land application sites resulted in Terratec's inability to drain the storage lagoon, leaving approximately 30 000 m^3 (close to 15% of the total biosolids produced) at the end of the spreading season (Andrews 2003). The Region currently maintains a number of centrally-located storage lagoons with a total capacity of 200 000 m^3 (Region of Waterloo 2007).

It was postulated in a 2003 Regional report that the regulations associated with the Ontario Nutrient Management Act (discussed further in Section 2.3.1.1) may put additional pressure on existing biosolids land-application programs, once enforced (Andrews 2003). In 2003, the Region of Waterloo began work on its Biosolids Master Plan which will consider the biosolids

land-application issues, and provide direction on biosolids management for the next 20 years (Andrews 2003).

2.3 Regulations Governing Biosolids Land Application

The most commonly studied, and as a result, most highly regulated biosolids contaminants are heavy metals (notably cadmium, copper, lead, nickel and zinc) and pathogens (fecal coliforms like *E. coli*, and *Salmonella*) (Sterritt and Lester 1981, United States Environmental Protection Agency 1994, European Union 2000, Ontario Ministry of Environment and Ontario Ministry of Agriculture and Food 2004). Organic pollutants (such as PPCPs, dioxins and furans, pesticides, and other persistent organic contaminants) are a relatively more recent concern (Wang and Jones 1994, Chaudri *et al.* 1996, Debosz *et al.* 2002, Harrison *et al.* 2006, Kinney *et al.* 2006) with fewer jurisdictions providing land application regulations (European Union 2000, Hébert 2004).

Because of concerns regarding the risks of various contaminants (chemical and biological) in biosolids, most jurisdictions practicing land application have created land application guidelines and regulations. These include standards outlining the maximum allowable concentrations of major biosolids contaminants, the allowable rate of land application, setbacks from sensitive landscape features (such as vertical distance to groundwater and horizontal distance to surface waters), and bans on application during inclement weather, or while there is snow cover. A comparison of the standards for maximum levels of major biosolids pollutants in a selection of Canadian provinces (Ontario, Quebec and British Columbia), the US and Europe is provided in Table 3.

Table 3: Comparison of municipal biosolids land-application standards for metals, organic pollutants and pathogens in Canada, the US and Europe.

Constituents	Ontario More Restrictive*	Ontario Less Restrictive†	Quebec C1P1‡	Quebec C2P3§	BC Growing Medium**	BC Class A	BC Class B	US EPA EQ and PC††	US EPA Ceiling Conc^{n}.	EU‡‡	EU (Proposed)
Metals (mg dry kg^{-1})											
Arsenic	75	170	13	40	13	15	75	41	75		
Cadmium	20	34	3	10	1.5	4	20	39	85	20 - 40	10
Chromium	1060	2800	210	1060	100		1060	1200	3000		1000
Cobalt	150	340	34	150	34	30	150				
Copper	760	1700	400	1 000	150		2200	1500	4300	1000-1750	1000
Lead	500	1 100	150	300	150	100	500	300	840	750-1200	750
Mercury	5	11	0.8	5	0.8	1	15	17	57	16-25	10
Molybdenum	20	94	5	20	5	4	20		75		
Nickel	180	420	62	180	62	36	180	420	420	300-400	300
Selenium	14	34	2	14	2	2.8	14	36	100		
Zinc	1850	4200	700	1850	150	370	1850	2800	7500	2500-4000	2500
Organics (mg dry kg^{-1} except where noted)											
Adsorbable organic halides (AOX)										500	
Linear alkylbenzene sulfonates (LAS)										2600	
Di-(2-ethylhexyl) phthalate (DEHP)										100	
Nonylphenol ethoxylates (NPE)										50	
Total polycyclic aromatic hydrocarbons (PAHs)										6	
Total polychlorinated biphenyls (PCBs)										0.8	
Dioxins and furans (PCDD/F ng TEQ dry kg^{-1})			17	50						100	
Pathogens (MPN g^{-1})											
Fecal coliforms	2 000 000 (*E. coli*)	2 000 000 (*E. coli*)	1000 (*E. coli*)	2 000 000 (*E. coli*)	1000	1000	2 000 000	1000 (Class A)	2 000 000 (Class B)	500 (*E. coli*)	
Salmonella			3 MPN 4 g^{-1}					3 MPN 4 g^{-1} (Class A)		0 50 g^{-1}	

*For biosolids applied at a rate up to 22 tonnes ha^{-1} 5 yrs^{-1} under O.Reg. 267/03 (Ontario Ministry of Environment and Ontario Ministry of Agriculture and Food 2004)
†Biosolids applied at a rate up to 8 tonnes ha^{-1} 5 yrs^{-1}.
‡(Hébert 2004)
§Maximum application rate of 22 tonnes ha^{-1} 5 yrs^{-1}.
**Class A standards based on CFIA Trade Memorandum T-4-F3 (McDougall *et al.* 2002)
††(United States Environmental Protection Agency 1994)
‡‡Annex III – metals, Annex IV – organics (European Union 2000)

From Table 3, it is apparent that most jurisdictions regulate metals, and many regulate pathogens (most commonly fecal coliforms and *Salmonella*), but few, with the exception of Quebec and the European Union, regulate any organic contaminants. This situation has been attributed to a lack of information to support scientifically based restrictions for organics (Wang and Jones 1994), while traditionally, more has been known about the fate and effects of metals and pathogens in land-applied biosolids (Lester 1982). Ontario's more restrictive guidelines (for biosolids to be land-applied at a maximum rate of 8 tonnes ha^{-1} every 5 years), are among the least stringent for many metals (i.e., arsenic, cadmium, chromium, cobalt, and molybdenum) and fecal coliforms, with no land-application guidelines for organic pollutants. Information on the derivation of land-application standards for municipal biosolids in Ontario was not found.

The following sections describe the municipal biosolids land application guidelines and regulations for these jurisdictions in greater detail.

2.3.1 Canada

In Canada, land application of municipal biosolids is not specifically regulated by federal legislation. Individual provinces (including British Columbia, Alberta, Ontario, Quebec, Nova Scotia and New Brunswick) enforce their own regulations, and each province determines the appropriate setback distances, allowable rates of application and maximum concentrations of biosolids contaminants (Jacques Whitford 2004). There are some federal laws (e.g., the *Fisheries Act*) that could be triggered, if for example, land-application of biosolids were to impact a fish-bearing watercourse through surficial run-off or some other pathway (Lewis 2006).

Although there are no federal guidelines regarding biosolids land application, if biosolids are commercially sold as a fertilizer product (e.g. as by the Greater Vancouver Regional District in BC, or the City of Calgary in Alberta), they may be regulated under the Canadian *Fertilizers Act*, which is administered by the Canadian Food Inspection Agency (CFIA). In December, 2004 a Notice was placed in the Canada Gazette which proposed to remove sewage products from the *Fertilizers Regulations* Schedule II exemption list (Lewis 2006). These regulations are supplemented by a series of Trade Memoranda, including the T-4-93 "Standards for Metals in Fertilizers and Supplements", which was developed to minimize the risk of adverse effects from metal contamination. Some provinces, like British Columbia, use the T-4-93 standards for their

higher quality (Class A) biosolids. Of the provinces which regulate land application of biosolids, further information is provided for Ontario, Quebec and British Columbia, as these provinces contain the highest population densities in Canada, producing the largest quantities of biosolids.

2.3.1.1 Ontario

There are two provincial agencies involved in the regulation of biosolids land application in Ontario and two main pieces of legislation. The Ministry of Environment (MOE) has been the approval-granting agency for land application of biosolids and other non-agricultural source materials for over 25 years, and the *Environmental Protection Act* (EPA) (Part V and Regulation 347) has been the main piece of governing legislation. The EPA requires Certificates of Approval outlining requirements for the protection of the environment and human and animal health for hauling and land application. This legislation is considered a "material-specific approach", and the 1996 document "Guidelines for the Utilization of Biosolids and Other Wastes on Agricultural Land" provides program criteria and standards.

More recently, MOE and the Ministry of Agriculture and Foods (OMAF, now Ontario Ministry of Agriculture, Foods and Rural Affairs, or OMAFRA) conducted a review of the land application program, and developed enhanced land application requirements in the jointly administered *Nutrient Management Act* (2002) and *Nutrient Management Regulations* (2003; O.Reg. 267/03). Both the EPA and NMA may apply to the application of biosolids on agricultural lands. As of 2004, there were still land application sites not regulated by the NMA, meaning Certificates of Approval under the EPA would still be required.

The NMA and NMR regulate the production and land application of agricultural source (i.e., manure) and non-agricultural source materials (NASM) using what is considered to provide a broader approach than the EPA. Biosolids fall under the category of NASM. In 2004, MOE and OMAF produced the "Draft: Guide for the Beneficial Use of Non-Agricultural Source Materials on Agricultural Land", which includes enhancements to the regulation of NASM application such as changes to storage requirements, buffers, set back distances, incorporation requirements, protection for municipal wells, winter spreading restrictions, agronomic application rate restrictions, and quality and pathogen standards. As of September 2003, the ban on spreading biosolids between December 1 and March 31 (or during periods of snow cover), a ban on high-trajectory irrigation guns spraying a distance greater than 10 m (except if applying a liquid more

than 99% water), and a minimum 20 m setback from top-of-bank for surface waters were enforced. The other enhancements were to be phased in incrementally. The NMA also contains provisions for the development of new standards, review and approval of nutrient management strategies and plans, certification of haulers, brokers and land applicators and a new registry system for all sites receiving non-agricultural source materials.

Briefly, the guidelines for land application of biosolids and other NASM in Ontario include (Ontario Ministry of Environment and Ontario Ministry of Agriculture and Food 2004):

- Confirmation that application rates will be beneficial to agricultural crops and will not pose an undue risk to the environment or plant, animal and human health (based on the opinion of the owner or agent of the material);
- Sampling and analysis requirements for soils (nutrients and metals), biosolids (nutrients such as total Kjeldahl N (TKN), NH_4, NH_4-N, NO_3-N, NO_2-N, Total-P, total solids, volatile solids, metals, and *E. coli.*;
- Use of nutrient management planning software for calculation of N, P and K loading, or if metals are high, maximum loading rates will be enforced (22 tonnes ha^{-1} 5 $years^{-1}$ if there are no metal exceedances, or 8 tonnes ha^{-1} 5 $years^{-1}$ if one metal exceeds the guidelines);
- Further restrictions for application rates based on the runoff potential of various soil types and the slope of the land;
- Setback distances including:
    - 450 m to residential areas (if surface applied, 50 m if injected or incorporated);
    - 100 m to municipal wells (90 m to all other wells including dug wells, 15 m to drilled wells);
    - 90 m to individual residences (if surface applied, 25 m if injected or incorporated);
    - 20 m to surface water;
    - 1.5 m depth to bedrock and 0.3 m depth of unsaturated soil (depth to water table);
- Pre-harvest waiting periods between 3 weeks (for hay and haylage) and 15 months (for small fruits);
- Pre-grazing waiting periods of 2 months for horses, beef or dairy cattle and 6 months for swine, sheep or goats;
- Winter application restrictions (as mentioned above);
- Storage requirements (for temporary field storage);

- Record-keeping requirements (including locations of fields receiving biosolids, amount applied, material analysis, complaints about land application operations, spills), material analysis, monitoring application rates and contingency planning by biosolids generators (i.e., WWTPs).

2.3.1.2 Quebec

In the province of Quebec, biosolids are referred to as "fertilizing residuals" or FRs which constitute biosolids and other soil amendments. Land application of biosolids is administered by the Ministère de l'Environnement (MENV) under the provincial *Environmental Quality Act* (Hébert 2004). Guidelines for land application are provided in "The Guidelines for the Beneficial Use of Fertilizing Residuals". These include applicable standards and criteria, which are used to determine if a certificate of authorization (CA) is required for the reclamation of specific fertilizing residuals. A CA is typically required if there are no regulatory or administrative exemptions, or if there is considerable ecological risk in application of biosolids.

For municipal biosolids to qualify as a reclaimable FR, they must have proven fertilizing properties and satisfy provincially defined environmental protection criteria. Quebec uses a C-P-O classification scheme for every FR, which measures the chemical contaminant content (C), pathogen content (P) and odour (O). Restrictions for land application of biosolids onto agricultural lands are outlined in the agro-environmental reclamation plans (AERP) that are designed by agrologists for each site. The AERP determines the application limits N and P inputs and provides measures to protect the environment. Environmental protection measures in the AERP include (Hébert 2004):

- Design of a location plan by an agrologist;
- Incorporation of storage criteria;
- Incorporation of separation distances (i.e., setbacks) including:
    - 30 m to potable groundwater sources (or 100 m if FR contains fecal matter, except if already certified by the Bureau de Normalisation du Québec (BNQ), with 100 m to bacteriological protection area and 300 m to virological protection area);
    - 1 m to agricultural ditches or ditches in non-agricultural environments;
    - 3 m to watercourses such as lakes swamps or ponds;

- For protection of air from bioaerosols, 10 m to property lines or roads (for air pollution), 100 m to dwellings, 200 m to protected immovables (e.g., recreational areas, churches, campgrounds), and 500 m to municipal urbanization perimeters; and
- For protection of air from odours, 75 m for O2 and 500 m for O3 from dwellings.

- Incorporation of spreading constraints, including some of the following:
  - Maximum hydraulic load of $< 100\ m^3\ ha^{-1}\ day^{-1}$;
  - Spreading of liquids from June 15 to August 15 (if irrigation of plants is the main value);
  - Prohibition of land application on frozen or snow-covered soil;
  - Prohibition of application on some types of crops (e.g., human consumption);
  - Delay before crop harvest; and
  - Delay before allowing public access to the site.
- Incorporation of minimal measures to provide public information and awareness (including signage and phone or fax/email information packages to municipalities and neighbours); and
- Commitment by the agrologist to complete at least 2 monitoring visits, with one made during spreading equipment calibration.

2.3.1.3 British Columbia

Land application of municipal biosolids in British Columbia is administered by the Ministry of Environment (formerly Ministry of Water, Land and Air Protection), under the *Organic Matter Recycling Regulation* (OMRR). Guidelines for land application are provided in the "Best Management Practices Guidelines for the Land Application of Managed Organic Matter in British Columbia" (McDougall *et al.* 2002).

In BC, biosolids are classified as Class A or B depending on the degree of pathogen, vector attraction and trace element reduction achieved, in a manner similar to the US EPA approach. There is an additional category of "biosolids growing medium" which consists of Class A or pathogen-free Class B biosolids plus an organic mixture, which can be marketed and sold as a consumer product, or distributed free-of-cost.

Briefly, the best management practices (BMPs) for land application provided in the BC guidelines include (McDougall *et al.* 2002):

- A Land Application Plan (for Class A biosolids sold or distributed in a volume > $5m^3$ and for all Class B applications) prepared by a "qualified professional" which outlines the rate, timing and requires a site visit during or after application to confirm application was carried as per the plan;
- Notification of neighbours regarding application (including signage);
- Calculation of application rate matching the crop N requirements in the year of application;
- Insurance that P application rates (in combination with background levels) do not result in elevated levels, sometimes requiring P to be the rate limiting nutrient;
- Insurance that post-application trace elements in soil do not exceed allowable limits;
- Immediate injection or incorporation of biosolids;
- Application during the regional-specific timing windows and not during inclement weather conditions;
- Observation of required setbacks for Class B biosolids (recommended for some Class A applications) of:
    - 30 m for potable water and irrigation wells, surface water, on-site dwellings, off-property dwellings, boundaries of residential or recreational properties;
    - 20 m to major arterial roads and highways; and
    - 10 m to minor public roads;
- Observation of depth to groundwater requirements (> 1 m depth to water table);
- Avoidance of sites with slope or soil stability issues;
- Avoidance of sites in heavily populated areas or near recreational or environmentally sensitive areas;
- Observation of post-application waiting periods and BMPs regarding livestock grazing and public access restrictions (Class B); and
- Design of a post-application monitoring plan addressing site-specific environmental concerns.

2.3.2 United States

Unlike Canada, a federal agency oversees biosolids management in the US. The Unites States Environmental Protection Agency (US EPA) manages biosolids land application under the *Clean Water Act* "Standards for the Use or Disposal of Sewage Sludge", Title 40 of the Code of Federal Regulations, Part 503 published in 1993 (United States Environmental Protection Agency

1994)*. In 1994, the US EPA produced a document titled: "A Plain English Guide to the EPA Part 503 Biosolids Rule" which outlines the requirements for land application and other disposal methods (including surface disposal and incineration), pathogen and vector attraction reduction, and biosolids and soil sampling and analysis methods. The regulations for biosolids land application include standards for pollution limits, management practices, monitoring requirements, operation standards and record keeping and reporting (Jacques Whitford 2004). Biosolids are classified according to three criteria: chemical concentrations (ceiling pollutant concentrations), pathogen content (as Class A or B) and the specific process used to control vector attraction. There are four options for meeting pollutant limits and pathogen and vector attraction reduction requirements for land application. If all criteria are met, the biosolids are considered "Exceptional Quality" (EQ) and have unrestricted use and distribution. "Pollutant Concentration" (PC) biosolids generally have higher pathogen loads and/or fewer vector attraction reduction requirements than EQ biosolids. "Cumulative Pollutant Loading Rate" (CPLR) and "Annual Pollutant Loading Rate" (APLR) biosolids are subject to cumulative and annual pollutant loading rates, respectively, and various vector attraction requirements. The PC and CPLR biosolids have application restrictions for agricultural land, forests, public contact sites or reclamation sites including (United States Environmental Protection Agency 1994):

- Application is not permitted to flooded, frozen or snow-covered land, and cannot be applied in such a way that the biosolids enter a wetland or other waters (including tidal waters, interstate and intra-state waters, tributaries and wetlands) except if permitted by a Section 402 (National Pollutant Discharge Elimination System, or NPDES permit) or Section 404 (Dredge and Fill permit) of the Clean Water Act;
- Biosolids cannot be applied within 10 m of US waters, unless otherwise specified by the permitting authority;
- Application must be at a rate equal to or less than the agronomic N rate for the crop to be grown; and
- Biosolids must not harm or contribute to the harm of a threatened or endangered species or result in destruction of their habitat.

* The US EPA biosolids regulations are most commonly referred to as "the Part 503 rule" or "Part 503".

2.3.3 Europe

Although individual member states of the European Union (EU) enforce national environmental regulations, the formation of the European Union has engendered the creation of continent-wide laws, with the European Commission creating harmonized environmental rules and regulations. European laws have already banned ocean dumping of sewage sludge as of 1998, and in the future, will require wastewater treatment in all populated areas over 2000 people (91/271/EEC), and aim to reduce dumping of biodegradable wastes in landfills (99/31/EC). It has been predicted that new European regulations will increase the supply of biosolids (by increasing the number of WWTPs), but also restrict the areas for disposal by the creation of increasingly restrictive use and disposal laws (Eljarrat *et al.* 1997, Debosz *et al.* 2002).

Council Directive 86/278/EEC* specifically regulates land application of biosolids onto agricultural lands. As mentioned, numerous member states (e.g., UK and Germany) previously developed national land application guidelines; under European law, member states' guidelines must meet the minimum standards set out in CD 86/278/EEC, or may be further restrictive. The goals of the 1986 Directive are to prevent harmful effects on soil, vegetation, animals and man, with specific consideration of metals by limiting concentrations of heavy metals in soils and sewage sludge and limiting annual metal loading rates (European Union 2000). The 2000 document: "Working Document on Sludge, 3rd Draft" provides a comprehensive guide to land application including: definitions of sewage sludge and the intended uses (including expansion from agricultural lands to include silviculture, green areas and reclaimed lands); limit values for heavy metals and organic compounds; requirements for treatment (including thermal drying, thermophilic aerobic stabilization, liming and others); descriptions of sludge producer responsibility and certification; information requirements; the land-application code of practice; and pollution prevention. The land-application requirements are as follows (European Union 2000):

- Sludge should not be used on soils with pH < 5, on water saturated, flooded, frozen or snow-covered ground, and should be spread in such a way as to not cause run-off and minimize soil compaction and the production of aerosols; and
- Sludge application must meet the following conditions:

* The full legal title of Council Directive 86/278/EEC is "Council Directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture".

- Load limits (from Annex V) are not exceeded;
- There is an agronomic interest for nutrients or for improvement or organic matter in soil;
- The quantity of nutrients introduced is adapted to the needs of the crops or soil according to best practice; and
- The use does not cause unreasonable odour nuisance to the nearest dwellings.

It does not appear that this EU directive provides specific setback distances to surface waters or areas of human activity, or allowable application windows, and it is assumed that individual member states may determine these measures based on local constraints.

2.3.4 Comparison of Other Land Application Guidelines and Regulations to Ontario

From evaluation of the five jurisdictions here, it appears that although the metal and pathogen limits for biosolids in Ontario are among the least stringent, the land application requirements in Ontario (specifically, as part of the more recent *Nutrient Management Act* and *Regulations*) are among the most stringent. The Ontario regulations are relatively more comprehensive in some areas, especially with regard to minimizing the potential for off-site migration of biosolids contaminants and odour. Land-application setback distances are larger than most jurisdictions, and include consideration of surface waters, groundwater, residential areas, and also vertical distance to bedrock and the water table.

2.4 *Global Comparison of Biosolids Contaminants*

Restrictions and regulations for land application of biosolids originate from the known presence of metals and pathogens in biosolids, and the known hazards associated with these contaminants. For the most part, routine monitoring programs focus on nutrients (as the nutrient content determines the value as a fertilizer product), metals, and pathogens. It is important to note that contaminants cannot be detected if they are not tested for, and there may be more contaminants of concern in municipal biosolids than those analyzed in routine testing programs. For example, although they are not a requirement of many routine monitoring programs, organic contaminants are known to exist in biosolids, and the most common groups of organic contaminants include: phthalate esters, monocyclic aromatics (e.g., chlorobenzenes), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and

dibenzofurans (PCDFs), chlorinated aliphatics (short chain), triaryl phosphate esters, aromatic and alkyl amines, phenols, petroleum hydrocarbons, flame retardants, and chlorinated pesticides (Wang and Jones 1994, Harrison *et al.* 2006, Kinney *et al.* 2007). Examples of municipal biosolids constituents from a selection of Canadian, US and European jurisdictions are provided in Table 4. Exceedances of the more restrictive Ontario land-application guidelines were found for two metals (copper and mercury) in the Toronto Ashbridges Bay, Greater Vancouver Regional District (GVRD) and 1990 New York State examples. Although there are no Ontario standards for organic contaminants, in these examples, PAHs, PCBs, organo-chlorine pesticides, dioxins (PCDD) and furans (PCDF) were detected in biosolids analyzed for organics from Toronto Ashbridges Bay, GVRD, UK and Germany.

Table 4: Comparison of average constituent concentrations of biosolids from the City of Toronto Ashbridges Bay WWTP and jurisdictions in Canada, the US and Europe (exceedances of Ontario land application standards are indicated).

Constituents	Ontario Standards (more restrictive)	Toronto - Ashbridges Bay (1996 – 2003)[*****]	GVRD – Lions Gate (2002 "Typical")[††††]	GVRD – Lulu Island (2002 "Typical")	US (1988) [‡‡‡‡]	New York State (1990)[§§§§]	EU[******] – (1992)	EU[††††††] – (1997-1998)	UK (1995-1996)[‡‡‡‡‡‡]	Germany (1995-1997)[§§§§§§]
Biosolids type	-	2° anaerobic dewatered	1° anaerobic dewatered	2° anaerobic dewatered	Various	Various	Various	Various	1° and thermophillic	1° and higher
Metals (mg dry kg^{-1})										
Arsenic	75	7.3	2.7	5	10	6			2.5[********]	
Cadmium	20	4.6	2	13.4	7	7	4.0	2.2	3.5	1.5
Chromium	1060	150	53	59	119	86	145	74	159.5	50
Cobalt	150	2.9	3.3	3.9						
Copper	760	**1100**	**1400**	**1670**	741	**763**	380	365	562	275
Lead	500	88	76	62	134	152		97	221.5	67.7
Mercury	5	1.6	**6.2**	3.2	**5**	2.7	2.7	2.0	2.5	1.2
Molybdenum	20	12	10	12	9	18				
Nickel	180	36	39	47	43	44	44	33	58.5	23.3
Selenium	14	2.9	4.5	5.4	5	5			1.6	
Zinc	1850	880	564	1024	1202	887	1000	817	778	834
Organics (mg kg^{-1})										
PAHs			1.2x10^{-5}-0.22[†††††††]						0.09-16	0.09-4.39
PCBs		1.65x10^{-4} [‡‡‡‡‡‡‡]	0.17-0.82						0.02-0.46	
Organo-chlorine pesticides			2.5x10^{-3}-0.22						0.01-1.26[§§§§§§§]	
Dioxins (PCDD)			3.0x10^{-7}-0.015						<8.0x10^{-5}-0.03	1.30x10^{-4}-0.136
Furans (PCDF)			1.6x10^{-5}-6.7x10^{-4}						1.0x10^{-3}-5.0x10^{-3}	3.8x10^{-4}-7.5x10^{-4}
Pathogens										
Fecal coliforms (MPN g^{-1})[*********]	2 000 000		12 600	356 000						

XXX: indicates an exceedance of an Ontario more restrictive biosolids land application standard (O.Reg. 267/03)

[*****](Jacques Whitford 2004)

[††††](Greater Vancouver Regional District 2003)

[‡‡‡‡](Harrison *et al.* 1999)

[§§§§](Harrison *et al.* 1999)

[******]EU 1992 values are for B, DK, F, D, EL, IRL, I, L, NL, P, ESP, UK (I C Consultants 2001)

[††††††]EU 1994-1998 values are for AT, D, DK, F, FI, IRL, L, SE, UK and NO

[‡‡‡‡‡‡]Metals are averages from 1995-1996 (I C Consultants 2001); organics are historical (Lester 1982); PCDD/PCDF from a 1989 report (Rogers 1996)

§§§§§§Metals are averages from 1995-1997 (I C Consultants 2001); organics are historical (Lester 1982); PCDD/PCDF from a 1986 report (Rogers 1996)

[********]Median value, from 1996-1997

[†††††††]All organics are ranges of average concentrations of individual chemicals for "historical GVRD samples" from 1986-1998 (Healey and Bright 2000)

[‡‡‡‡‡‡‡]Value is from a single measurement in 2000 (Jacques Whitford 2004)

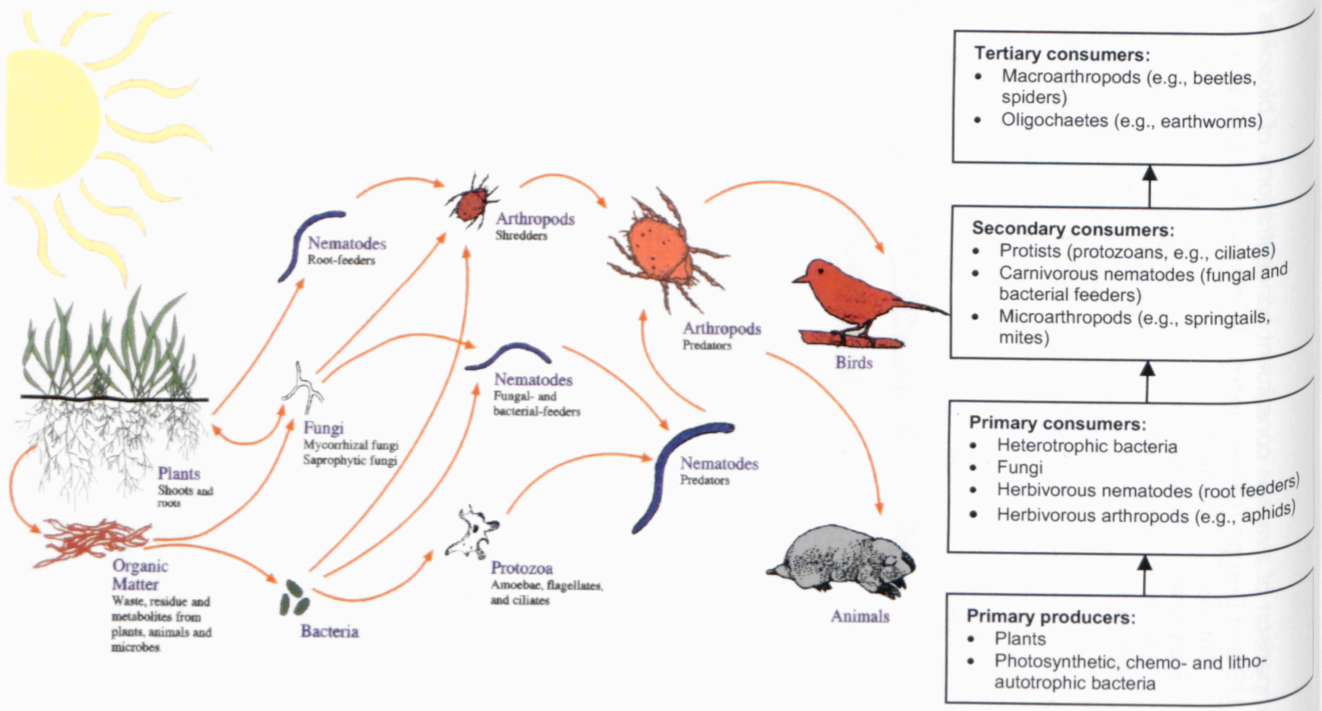
[§§§§§§§]Includes lindane, DDE and dieldrin

[*********] MPN is Most Probable Number

3 State-of-Knowledge on the Ecological Effects of Land-Applying Municipal Biosolids

Because soil ecosystems include complex biological, chemical and physical interactions, it is essential to consider the trophic structure of the entire soil food web and the soil characteristics that affect the fate of land-applied biosolids. Through their interactions, soil organisms carry out important soil processes including: humification, recycling and mineralization of organic residues (by microorganisms); mechanical separation of organic residues; stabilization of soil aggregates; and bioturbation (i.e., mixing) of organic and mineral substances (by larger organisms such as springtails and earthworms) (Emmerling *et al.* 2002) (Figure 1). These processes influence the physico-chemical properties of soil and in turn, increase soil fertility and quality (Emmerling *et al.* 2002). The level of soil organism activity largely determines the chemical and physical properties of the soil (Lee and Pankhurst 1992).

Figure 1: Soil food web showing five food web trophic levels, from primary production to quaternary consumption (Source: US Department of Agriculture 2007). The literature review is limited to invertebrates (i.e., does not include higher organisms such as birds or mammals), as described in the flow diagram on the right.



Once biosolids are land-applied, the rate of nutrient, metal and/or organic contaminant uptake by plants and microbes is affected by a number of physical soil properties including: pH, organic matter content, cation exchange capacity (CEC), iron and aluminum oxides content, texture, aeration, specific sorption sites, and moisture. These physical properties of soil are interrelated and are in turn based on numerous chemical and microbial processes (Somers *et al.* 1987). The natural characteristics of soils that are relatively stable include: texture, CEC (which is dependent on organic matter, pH, soil type and % clay), organic matter content, and iron and aluminum oxides content (Somers *et al.* 1987). The more variable physical characteristics of soils are pH, moisture and aeration. Following biosolids application, soil pH may decrease over time due to the generation of cations during the oxidation of reduced forms of N and S, which are mineralized from organic matter in the biosolids.

Metal salts are taken up more readily than metals in biosolids (i.e., they are more bioavailable). Metals in biosolids are often associated with insoluble inorganic components like phosphates, sulfides and carbonates (Somers *et al.* 1987). This is important to note, as many of the studies used to determine the ecological effects of land-applying municipal biosolids have used spiking with metal salts to reach maximum land-application guidelines (in order to determine a worst-case, or cumulative loading effect). In addition to the physical properties of the soil, the fate and effects of sludge constituents are also influenced by environmental factors including: climate (precipitation and temperature), land management characteristics including the irrigation type and rate, site drainage pattern, use of lime, fertilization type and rate, and addition of other soil amendments (Somers *et al.* 1987). These factors will be of importance in evaluating the studies conducted on the impacts to soil organisms following biosolids land application.

The objectives for this literature review were to determine the state of knowledge on the ecological effects of land-applying municipal biosolids on agricultural soil ecosystems. An effort has been made to include relevant studies with unique findings, from a variety of countries. The focus is further narrowed down to studies on N_2-fixing bacteria, as a large body of research has amassed on this agronomically-important bacterial group. This review briefly describes the types of test methodologies used, the contaminants or groups of contaminants implicated in causing deleterious impacts on soil organisms, and discusses gaps in our total understanding of ecological effects. In keeping with the original intent for the term biosolids, unless the method of

sewage sludge stabilization was described in the study, the term sewage sludge is used. However, if some method of stabilization prior to land application was mentioned, the term biosolids is used.

3.1 Effects of Biosolids Land Application on General Microbial Processes

Studies on the effects of applying municipal biosolids (or sewage sludge) on non-specific bacterial and fungal communities in agricultural soils can be divided into six major types: 1) those focusing solely on direct toxicity, 2) changes in microbial activity, 3) changes in diversity, 4) changes in metal tolerance, and 5) changes in microbial biomass. A final category 6) focuses on studies evaluating changes to what has been termed the 'black box' relationship of microbial community structure and biological function (Tiedje *et al.* 1999), by simultaneously evaluating the combined changes to microbial diversity and activity. It must be noted that our primary interest in this literature review was to examine the potential impacts of unadulterated biosolids land-application on microbial ecology. A large proportion of the studies reviewed employed spiking with additional contaminants (often to reach legislated criteria values); however, it was decided to include these studies to illustrate the breadth of research relating to microbial ecology. A summary table of all studies and key information on the biosolids sources, soil types and key findings is provided in Appendix A.

3.1.1 Direct Toxicity

Jacquot *et al.* (2000) conducted a pilot study to determine the best test species of arbuscular mycorrhizal (AM) fungi for evaluation of the ecotoxicity of municipal sewage sludge, using colonization of alfalfa (*Medicago truncatula*) as a measurement endpoint. Application of unadulterated sewage sludge and polycyclic aromatic hydrocarbon (PAH)-spiked and heavy metal-spiked sewage sludge was associated with a reduction in colonization by AM fungal species, leading the authors to suggest this was a good test method for evaluating the ecotoxicological effects of sewage sludge application. Elsgarrd *et al.* (2001) amended soils with linear alkylbenzene sulfonate (LAS)-spiked sewage sludge to determine short-term EC_{10} and EC_{50} (the effective concentrations of a compound that produce a measurable effect in 10 and 50% of all test organisms, within the specified test duration) on soil bacteria using microbial biomass C, dehydrogenase and arylsulfatase activity, and iron reduction as measurement

endpoints. While unamended biosolids containing LAS were not examined, the importance of this study lies in the fact that these anionic surfactants are widely found in sewage treatment plants as by-products of the production of detergents (Elsgaard *et al.* 2001). While the EC_{10} values were higher for most endpoints (except arylsulfatase activity) in LAS-spiked sludge compared to aqueous LAS samples, the potential for microbial recovery for most parameters was considered likely based on the buffering effects of the sewage sludge, and the reduction in effects after prolonged incubation. Gejlsbjerg *et al.* (2001) amended two soil types individually with LAS and nonylphenol (NP) to determine short-term EC_{10} and EC_{50} on soil bacteria (along with the invertebrates *Folsomia candida* and *Enchytraeus albidus*). Denitrification, aerobic respiration, nitrification, and anaerobic C mineralization were used as measurement endpoints for microbial processes. For LAS and NP, stimulation was seen in denitrification and aerobic respiration, and reductions were seen in nitrification and anaerobic C mineralization rates. It should be reiterated here that the effects of un-spiked biosolids containing LAS and/or NP were not evaluated.

These three studies were the only ones found to be directly concerned with establishing toxicity threshold values for land-application of municipal biosolids. The utility of these studies are limited to biosolids-specific and site-specific evaluations, as the exact concentration of causative agents of toxicity will vary based on biosolids and soil conditions at the site, and the potential for synergistic effects of multiple contaminants, which were not evaluated in these studies.

3.1.2 Changes in Microbial Activity

Numerous studies were found which focused on the effects of land-applying municipal sewage sludge or biosolids on microbial activity in agricultural soils. From these studies, there is a general trend of elevated microbial activity following biosolids or sewage sludge amendment. For example, in Sonoran Desert clay loams, high rates of biosolids application increased dehydrogenase activity and initial CO_2 production (Brendecke *et al.* 1993). Similarly, Varanka *et al.* (1976) found the activity of some microbial enzymes (protease, amylase, and dehydrogenase) were elevated in soil with long-term (6 years) application of anaerobically-digested biosolids, and Dar (1997) found that un-spiked sewage sludge amendment increased C and N mineralization relative to rates in unamended soil. Debosz *et al.* (2002) conducted an 11-month incubation experiment (with laboratory and field incubations) and a three-year field trial in sandy

loam. In the incubation experiment, enzyme activities (β-glucosidase and FDA hydrolysis) were stimulated by biosolids application in the short-term, eventually reaching a similar level to un-amended control soils. Similarly, CO_2 production was briefly stimulated and gradually decreased (due to substrate depletion). In the three-year field study, respiration and FDA hydrolysis activity were not significantly altered in the biosolids-amended soils relative to un-amended control, and the authors suggested the effects of the biosolids amendment on soil microbial activity were moderate and temporary. Sánchez-Monedero *et al.* (2004) found elevated enzyme activity (FDA hydrolysis) and respiration (qCO_2) in biosolids-amended soils (using biosolids with varying levels of stabilization through compost treatments). Basal respiration in the most stabilized biosolids-amended soils returned to pre-amendment equilibrium status (as measured by qCO_2) after 18 days, while the fresh (non-composted) biosolids-amended soils exhibited respiration and mineralization rates double that seen in the most stabilized biosolids-amended soils, even at the termination of the experiment (after 60 days). Sheppard *et al.* (2005) found biosolids amendment stimulated both CO_2 and CH_4 production in the short-term, but soil limed prior to biosolids amendment showed prolonged stimulation of CO_2 production. Stamatiadis *et al.* (1999) found single (short-term, high-N), and long-term liquid biosolids applications caused a significant increase in soil respiration and N mineralization, and improved soil fertility, with the single application having the greater short-term effect. Brendecke *et al.* (1993) found no relationship between soil microbial activity and cotton yield in desert clay loam soils amended with biosolids, and suggested soil microbial activity was not a good predictor of changes to soil fertility following biosolids application.

In contrast to the findings above, other studies have found evidence for a critical threshold rate of biosolids or sewage sludge-amendment providing optimal soil improvement (in terms of promoting soil fertility and bacterial activity). Indigenous soil bacteria can be negatively affected by numerous factors including: changes to soil quality, competition with biosolids bacteria, or the toxic effects of heavy metals and other sewage contaminants. For example, when sewage sludge was spiked with lead (particularly at quantities above 350 mg kg^{-1}), Dar found C and N mineralization rates were inhibited (Dar 1997). Stamatiadis *et al.* (1999) found application of excessive amounts of ammonium salts in biosolids, and the consequent stimulation of nitrification, resulted in excess nitrate formation and soil acidification, and a reduction in soil

productivity. Wong *et al.* (1998) found that high application rates of anaerobically-digested biosolids (above 350 g kg^{-1}) were associated with decreased carbon mineralization and decreased efficiency of N and P mineralization. These decreases were thought to be associated with the degraded soil structure and higher moisture content (limiting oxygen availability) following amendment. However, the authors also suggested microbial activity was not directly affected by heavy metals, as the bioavailability of heavy metals in each biosolids treatment (regardless of composting treatment) was similar, but microbial activity varied with composting treatment.

3.1.3 Changes in Microbial Diversity

Numerous studies have focused on the effects of municipal sewage sludge or biosolids application on microbial diversity. From these, it is apparent that the techniques used to evaluate shifts in microbial diversity have a strong effect on a study's findings; older studies conducted prior to the advent of molecular techniques were limited in their level of detail. For example, Varanka *et al.* (1976) found mixed results in their evaluation of diversity, with sludge-amended soils showing increased percent composition of denitrifying bacteria, but decreased percent composition of *Azotobacter* (an N_2-fixing genus). In addition, Barkay *et al.* (1985) found the Shannon species diversity index was unchanged for the entire soil community; however, there was an increase in the diversity among cadmium-resistant species attributed to the dominance of cadmium-resistant strains of *Pseudomonas* and Gram-negative fermenters. Both of these studies employed plate culture techniques.

In comparison, the advancement of molecular techniques has provided useful tools for more accurately assessing the effects of biosolids or sewage sludge amendment on soil microbial community structure. Bååth *et al.* (1998) evaluated phospholipid fatty acid (PLFA) patterns using principal components analysis (PCA) at two agricultural sites, and found PLFA patterns exhibited significant shifts in the population structure in metal-spiked sludge-amended soils relative to un-spiked sludge-amended controls. In 2000, Witter *et al.* (2000) also evaluated PLFA profiles and found a small difference in patterns from the un-spiked sludge treatment relative to the un-amended control, and larger difference from the metal spiked sludge soils at the highest rate of application. Results were compared to those from Bååth *et al.* (1998), and the authors found the PLFAs most affected by metals in their study did not correspond to the earlier work, leading them to conclude there is a lack of consistent metal effects on PLFA profiles across

agricultural soil bacterial communities. In 1999, Sandaa *et al.* (Sandaa *et al.* 1999) used multiple methods including: analysis of DNA reassociation times and guanine and cytosine (% G+C) content, hybridization with numerous probes, and 16S rDNA sequencing. They found a significant decrease in bacterial diversity (using reassociation times), with 16,000 bacterial genomes g^{-1} in control soil, compared to 6,400 genomes g^{-1} in low-metal sewage sludge-amended soils, and 2,000 genomes g^{-1} in high-metal (spiked) sewage sludge-amended soil. Based on their relatively higher abundance in sewage sludge-amended soils, the authors recommended use of two specific groups as useful indicators of metal contamination effects: the α-subdivision of Proteobacteria, and Gram-positive bacteria with high G+C content. Sandaa *et al.* (2001) later used dot-blot hybridization and RFLP analysis, and found the analysis of isolates (the culturable bacteria) and clones (total bacteria, as determined by clones in a 16S rDNA library) resulted in contrasting findings; isolates had lower diversity in soil amended with low-metals biosolids, whereas clones exhibited lower diversity in soil amended with high-metal sewage sludge. Moffett *et al.* (2003) conducted similar work to Sandaa *et al.* using 16S rDNA sequencing and RFLP analysis at an English site (ADAS Gleadthorpe), and found the number of operational taxonomic units (OTUs) was higher in the control (uncontaminated sewage sludge-amended) soil relative to zinc-spiked sewage sludge-amended soils (120 compared to 90), which indicates a decrease in bacterial diversity in zinc-contaminated soils. They also found the number of single-occurrence OTUs was also higher in control soil relative to zinc-contaminated soils (82 compared to 52), indicating a decrease in evenness, as there were more rare species in the control soil, and a higher proportion of a few species in the zinc-contaminated soils. For example, Gram positive bacteria with high G+C content (specifically, *Rubrobacter radiotolerans*) were found in highest abundance in both the control and biosolids-amended soils; however, they constituted a higher proportion of the total bacterial community in the zinc-spiked sewage sludge-amended soils compared to control (uncontaminated) sewage sludge-amended soils. Chander *et al.* (2001) also evaluated the effects of metals (in sewage sludge-amended soils, and other metal contaminated sites including river sediment and dump soils) using the ratio of ergosterol to total biomass carbon to determine the relative proportion of fungi. They determined heavy metal contamination caused a shift in microbial community structure towards fungi.

With regards to the utility of culturing techniques in determining shifts to bacterial community structure in biosolids-amended soils, the study by Sandaa *et al.* (2001) shows the use of the

culturable fraction is inadequate for determining the true effects of a soil perturbation on the entire community of soil bacteria. A common finding of studies using molecular techniques is that a combination of biosolids and/or the heavy metal biosolids contaminants (or other as-yet un-measured contaminants) is associated with a decrease in bacterial diversity. This may be due to numerous factors, including the direct toxicity of sewage contaminants, competition between indigenous soil bacteria and biosolids bacteria, changes to the nutrient status of soil causing promotion of specific species adapted for specific nutrient requirements, or a community shift caused by differences in the metal tolerance (or tolerance to other contaminants not yet considered).

3.1.4 Changes in Metal Tolerance

Many studies on the effects of biosolids or sludge amendment on soil microbial communities focus solely on metal contamination (often employing metal spiking to reach federal or international limits for land application). Metal tolerance was found to be significantly elevated in soils amended with copper, nickel, or zinc spiked sludge, compared to soils with un-spiked control sludge amendment (Bååth *et al.* 1998). Metal tolerance was also shown to increase with increasing metal concentrations in soil, even at concentrations below current EU limits (Witter *et al.* 2000). Metal tolerance has also been shown to be species-specific; for example, Barkay *et al.* (1985) found increased cadmium toxicity in Gram-positive bacteria and increased tolerance in Pseudomonads, *Flavobacterium* spp., and Gram-negative fermenters. Later studies have found metal tolerance in α-Proteobacteria (Sandaa *et al.* 1999, Sandaa *et al.* 2001), in Gram-positive bacteria with high G+C content in metal-spiked sludge-amended soils (Moffett *et al.* 2003), or low-metal sludge-amended soils (Sandaa 1999) and in fungi (Chander *et al.* 2001), all leading to shifts in microbial community structures. Bååth *et al.* (1998) recommended the use of bacterial community tolerance as a direct measure of toxic effects of metal-rich sewage sludge, as they suggest it reflects a selection pressure due directly to metals toxicity, and not other unrelated environmental factors. They did not consider the potential for non-metal toxicity, for example from other organic pollutants or competition with biosolids bacteria.

It is striking that only metal tolerance has been evaluated to date, when many other known contaminants (particularly organic pollutants) have not yet been correlated to shifts in microbial community structure. It is also notable that although species-specific metal toxicity has been

observed, it has not been related back to effects on the function of these species. For example, an examination of how the loss of these species may alter the larger microbial community structure and processes relating to soil quality was not found.

3.1.5 Changes in Microbial Biomass

Changes in microbial biomass as a result of biosolids or sewage sludge application was not the sole focus of any studies found; however, it was included as one measure of effects on microbial communities in numerous studies. Varanka *et al.* (1976) found a slight increase in biomass in biosolids-amended soils relative to un-amended control soils and found abundance had a slight positive correlation to the level of biosolids amendment. Dar (1997) also found sewage sludge amendment was associated with a slight increase in biomass C relative to un-amended control soil. The increased microbial biomass C following sewage sludge amendment was thought to be caused by the loading of the bacteria originating from the sewage sludge, although this hypothesis was not tested. Chander *et al.* (2001) evaluated biomass C and the ratio of biomass C to soil C in Braunschweig (Germany) test site soils, and other contaminated sites, and they found the average biomass C and average biomass C to soil C ratio was significantly higher in sludge-amended soils compared to other contaminated sites. They did not find a definitive negative correlation between increasing heavy metals (copper, lead, and zinc) and decreasing biomass, which led them to conclude other environmental factors were also important. Debosz *et al.* (2002) performed an 11-month incubation and three-year field trial study and found biomass C in the field incubation was initially higher in biosolids-amended soils, with a secondary increase in the spring (biomass C in the laboratory was less dynamic and unrelated to trends seen in the field). The field trial found no significant differences between biomass C in the un-amended control, compost, or biosolids-amended soils, but biomass N in biosolids-amended soil decreased steadily during summer and fall. In the laboratory incubations, biomass N increased for several months, but showed a significant decrease in the last sampling date.

The Sánchez-Monedero *et al.* (2004) study also indicated microbial biomass C increased in all treatments after addition of biosolids, with the greatest increase associated with fresh biosolids amendment, and decreasing effects associated with increasing levels of composting stabilization. Microbial biomass was relatively higher in biomass in the fresh, un-composted biosolids and was

thought to be caused by greater survival of biosolids bacteria in biosolids not undergoing stabilization treatments.

In contrast to the aforementioned studies, other studies found biomass or abundance was unchanged, or decreased following biosolids or sewage sludge amendment. Brookes and McGrath (1984) found the average soil biomass in biosolids-amended and composted biosolids-amended soils measured almost 20 years post-amendment was much lower relative to inorganic fertilizer or farmyard manure-amended soils. These results were confirmed from studies at a site with similar biosolids land-application history. Brendecke *et al.* (1993) in Tucson, Arizona found bacterial, actinomycete, and fungal abundance showed no significant difference in the low or high-rate biosolids amended soils, relative to control. Bååth *et al.* (1998) found high-copper sludge-amended soils had significantly lower biomass relative to the uncontaminated sludge-amended soils. Sandaa *et al.* (1999) also found a slight decrease in abundance following amendment with heavy metal contaminated sewage sludge; there were $2.6x10^9$ bacteria g^{-1} dry soil in control soils, compared to $2.4x10^9$ bacteria g^{-1} in low-metal soils, and $2.0x10^9$ bacteria g^{-1} in high-metal soils. Moffett (2003) also found a decrease in biomass following amendment with spiked and naturally zinc-contaminated biosolids.

The contradictory results from these studies on microbial biomass convey the importance of understanding the differences between the site histories, and methodologies employed in each study. A wide range of sewage sludge or biosolids amendment rates have been evaluated, with diverse concentrations of heavy metals (sometimes spiked in biosolids) and other unanalyzed organic contaminants, differing lengths of application periods and duration since last application, and differing site conditions. All of these factors can have significant effects on the growth of indigenous soil microbes. There is also most likely an effect of season on microbial growth; the frequency and timing of sludge application is an important consideration, as microbial loading may also occur through addition of viable organisms contained in the biosolids, and may constitute a major component of the shift in microbial biomass following land application. There have been numerous studies which confirm pathogens (including viruses, bacteria, and protists) survive the wastewater treatment process, survive in soil following land application (Sawyer 1989, Sastre *et al.* 1996, Vasseur *et al.* 1996, Gibbs *et al.* 1997, Tierney *et al.* 1997, Pourcher *et*

al. 2007), and even survive in composted biosolids (Hussong *et al.* 1985, Millner *et al.* 1987, Sidhu *et al.* 2001).

3.1.6 Changes in the Structure and Function of Microbial Communities

Evaluation of both the structure and function of the bacterial community after amendment with sewage sludge or biosolids constitutes a fundamental leap in the ecological relevance of a study's findings. Despite the fact that the ecological functions carried out by soil microbes are extremely important to the profitability of agricultural operations, the structure-function relationship of microbial communities is widely acknowledged to be poorly-understood. Gray *et al.* (2003) conducted a study on the effects of municipal anaerobically-digested biosolids and/or lime application on the community structure and activity of ammonia-oxidizing bacteria (AOB) in soils. Their results indicated significant changes on both general and specific AOB community structure in all soil treatments compared to control. Time-series showed that untreated control soils had the most stable AOB communities, while all other treated soils showed unstable AOB communities, even after two months of sampling (possibly reflecting the short-term response of bacteria to perturbation). With regards to function, biosolids amendment had little effect on overall respiration, but was associated with a significant increase in ammonia oxidation rate potential in biosolids and limed biosolids-amended soils relative to the un-amended control. The authors suggested AOB populations may exhibit functional redundancy; AOB community structures became more dynamic over time, and AOB activity was enhanced following biosolids-amendment, suggesting sub-groups of AOB were responding at different times.

Marschner *et al.* (2003) evaluated the long-term effects of sewage sludge-amendment on the general structure (using PLFA pattern analysis and PCR-DGGE methodology), and function (enzyme activity) of the total microbial community. There were no significant differences among the total concentration of PLFAs in all treatments; however, total bacteria PLFAs, Gram-positive bacteria, and the ratio of Gram-positive to Gram-negative, and bacteria to fungi were significantly higher in the sewage sludge-amended soils relative to control soils treated with mineral fertilization. PCR-DGGE revealed two distinct bacterial communities in the manure and straw treatments, and in the sewage sludge treatment, with the other two mineral treatments grouped in-between. Conversely, the eukaryotic (fungal, protozoan, red and green algal) community structure and enzyme activities did not exhibit treatment-related trends; among

several enzymes tested in the sewage sludge treatment, only protease activity was significantly greater than in the control. No relationship between eukaryotic community structure and function could be established, and the authors suggested functional redundancy as the cause.

It is not unexpected that Marschner's study focusing on more diverse groups of microorganisms would find redundancy in the number of species capable of carrying out less specific ecological functions, as there are a myriad of species capable of those activities. On the other hand, Gray *et al.* (2003) found AOB, a select group carrying out a specific ecological function (nitrification), may also show functional redundancy. From an ecological standpoint, in order to truly determine the effects of biosolids land-application on soil bacteria and their role in maintaining soil fertility, it is important for future studies to continue to narrowly focus on the relationship between structure and function of specific bacterial guilds such as ammonia-oxidizers and N_2-fixers. This is discussed in more detail in the following sections.

3.2 *Effects of Land Application of Biosolids on N_2-fixing Bacteria*

Many studies have narrowed their evaluation of the effects of biosolids or sewage sludge application to the nitrogen (N_2)-fixing bacterial community, warranting a separate category for studies on this guild of agronomically-important bacteria. The N_2-fixing bacteria studies can be sorted into the same general sub-categories as used for the general microbial studies: 1) changes in microbial activity (specifically the rate of N_2-fixation or effectiveness in nodulation of host legume species), 2) changes in microbial diversity, 3) changes in biomass and/or abundance, 4) changes in metal tolerance, and 5) the effects on the structure-function relationship in N_2-fixing bacteria (primarily focusing on the *Rhizobium* genus).

3.2.1 Changes in Microbial Activity – N_2-fixing Ability

Evaluation of the effects of biosolids and sewage sludge on N_2-fixation has mostly focused on metal contamination. For example, Brookes *et al.* (1986) evaluated the effects of metal-rich biosolids on cyanobacteria development and N_2-fixing ability in soil (using acetylene reduction and $^{15}N_2$ incorporation). N_2-fixation was initiated much later in biosolids-amended soils than manure-amended soils, and over the duration of the experiment, N_2-fixation in the biosolids-amended soils was one-third that in the manure-amended soils. Soil samples collected along an increasing metal gradient (from the manure- to biosolids-amended soils) found a negative

correlation between N_2-fixation and heavy metal concentrations. Mårtensson and Witter (1990) evaluated the effects of long-term metal-rich sewage sludge application on the activity and abundance of N_2-fixing bacteria in soils. They found the nodulation of white clover (*Trifolium repens*) was delayed in soil amended with sewage sludge, but found no effect on the number of nodules or N content of nodulated plants. The rate of N_2-fixation in free-living diazotrophic bacteria (measured as acetylene reduction) was similar in sludge-amended soils and reference soils; however, the rate of N_2-fixation after glucose addition increased more in reference soils than in sludge-amended soils. Cyanobacterial growth was also delayed in soils amended with sludge, and the N_2-fixation rate was 100 times lower than in reference soil. Similarly, Munn *et al.* (2001) found the effects of municipal biosolids application on N_2-fixation in subterranean clover (*Vicia sativa*) were highly site-specific, with clover grown in some soils showing reductions in average shoot weight (used as a measure of N_2-fixing effectiveness) and N_2-fixation (^{15}N dilution). Broos *et al.* (2004) used soils from numerous sites with metal-rich sewage sludge-amendment, or from sites near metal smelters. Effects of metal toxicity on the N_2-fixing ability in white clover (^{15}N dilution) were apparent only at low abundance of *Rhizobium leguminosarum* biovar *trifolii* (log MPN < 3), and metal toxicity to N_2-fixation was confirmed in only one of the sludge-amended sites. Horswell *et al.* (2003) conducted a study using soils having naturally metal-rich raw sewage sludge applied. N_2-fixation by *Rhizobium* (^{15}N natural abundance) in one sewage sludge-amended plot was significantly lower than in the un-amended reference. However, confounding effects of inter-site differences in soil characteristics were considered to be too great to attribute this reduction in N_2-fixation to heavy metals in sewage sludge. Obbard and Jones (2001) evaluated the effects of metal-rich biosolids on the rate of N_2-fixation ($^{15}N_2$ dilution and ^{15}N incorporation) in three different legumes (*T. repens*, *V. faba*, and *Pisum sativum*). In general, the rate of N_2-fixation was lower in biosolids-amended soils than in reference soils. Spiking of biosolids with metals further reduced N_2-fixation. While there were clear effects of biosolids on N_2-fixation in *V. faba*, and *P. sativum*, there was no corresponding effect on yield, and the authors suggested the importance of the loss of N_2-fixing ability depends on the quantity of nitrogen fertilizers applied in the years after biosolids amendment. While application of biosolids and sludge was thought to introduce metal toxicity, the inhibition of N_2-fixation by metals and the agricultural significance of this inhibition were unclear. Obbard and Jones also suggested the effects of biosolids may be related to contaminants other than metals

(e.g. organic contaminants), with the impact of reduced N_2-fixation depending on the soil nutrient status of a site.

In contrast to the negative effects of biosolids on N_2-fixation seen above, Heckman *et al.* (1986), found nodulation and N_2-fixation in soybeans (*Glycine max*) following long-term biosolids-amendment were significantly higher in biosolids-amended soils, than in reference soil. Nodulation and N_2-fixation were also greater in soils with neutral pH than in mildly acidic soil, which was thought to be related to the enhanced bioavailability of metals at lower pH. In further studies, Heckman *et al.* (1987) found little evidence for sewage sludge toxicity on N_2-fixation; with N_2-fixation shown to decrease with increasing sewage sludge application rates at one site, presumably due to metals toxicity. Based on these findings, the authors suggested the beneficial effects of sludge amendment on soybean growth may be restricted to sludges with low metals concentrations.

There appears to be a common trend of decreased N_2-fixation in clover with *R. leguminosarum* symbionts in these studies evaluating changes to the activity of N_2-fixing bacteria following biosolids or sewage sludge amendment; however, the findings by Heckman *et al.* (1986) using soybeans found conflicting evidence (i.e., enhanced N_2-fixation). In order to fully examine this aspect of N_2-fixing bacterial function it is critical to use more direct measures (e.g., ^{15}N), with a more holistic determination of all potential causative agents, not just metals.

3.2.2 Changes in N_2-fixing Bacterial Diversity

There is a general paucity of research on the effects of biosolids or sewage sludge on the N_2-fixing bacterial community structure. More research is required before meaningful conclusions can be drawn about the relationship between biosolids application and N_2-fixing community structure. In one study by Kinkle *et al.* (1987), the distribution of eleven *Bradyrhizobium japonicum* serogroups (determined by agglutination tests) was similar in soybean nodules collected from plots amended with 0, 56 and 112 tonnes ha^{-1} anaerobically-digested biosolids. Lakzian *et al.* (2002) used PCR-RFLP analysis of an intergenic spacer (ITS) region and found significant shifts in *R. leguminosarum* biovar *viciae* isolates colonizing hairy vetch (*V. hirsuta*) in long-term, metal-spiked biosolids-amended soils. There were a total of 20 ITS groups (called 'strains') identified, ranging from a high of 10 groups isolated from un-amended control plots, to

a low of two groups isolated from a plot amended with metal-spiked biosolids. Evenness was also lower in high-metal plots. Conversely, the number and complexity of plasmid profiles were greater in metal-spiked biosolids-amended soils, compared to un-amended controls, and the authors concluded that the lack of a significant relationship between metal levels and microbial diversity suggested the assumption that increasing stress leads to reduced diversity should be revisited. They did not consider the possibility of other contaminants having effects on diversity.

The differences in findings between these studies can be attributed to differences in test methods and tools available (pre- and post-molecular techniques availability), differences in the contaminants contained in the biosolids, the physical or chemical characteristics of the microbial community studied, or the physical and chemical characteristics of the soils in each study site. In general, more focus is needed in determining how biosolids land-application can affect diversity in N_2-fixing bacteria.

3.2.3 Changes in N_2-fixing Bacterial Biomass and/or Abundance

In the following studies, application of biosolids or sewage sludge was generally associated with a decrease in N_2-fixing rhizobia and cyanobacteria, and this impact was frequently attributed to heavy metals contamination. The effects on the abundance or survival of one species, *R. leguminosarum* biovar *trifolii,* have been evaluated in numerous studies. For example, Mårtensson and Witter (1990) found abundance of *R. leguminosarum* biovar *trifolii* was significantly lower in soil amended with sewage sludge. Similarly, Chaudri *et al.* found abundance was significantly lower relative to reference plots in ex-woodland soils amended with biosolids (either spiked or un-spiked with metals), and in soils amended with metal-spiked biosolids at an old arable site (Chaudri *et al.* 1993). In another study, *R. leguminosarum* biovar *trifolii* abundance in metal-rich sewage sludge-amended soils decreased with increasing metal concentrations (Broos *et al.* 2004). Horswell *et al.* (2003) found that abundance was significantly lower in one study block having sewage sludge-amended soil compared to reference soil, and it was suggested that elevated zinc levels were associated with the decrease. However, in their remaining four sludge-amended blocks, no decrease in abundance was observed, and the authors related this to the confounding effects of varying cultivation methods and application rates.

Other rhizobia have also shown changes to abundance following biosolids or sludge amendment. Reddy *et al.* (1983) found application of metal-rich sewage sludge at a 1:5 ratio to soil was associated with significantly reduced survival of *B. japonicum*, and the authors suggested the decrease was due to the presence of heavy metals. Lakzian *et al.* (2002) found abundance of *R. leguminosarum* biovar *viciae* (as log MPN) decreased linearly with increasing soil zinc levels following amendment with metal-rich biosolids. Additionally, Brookes *et al.* (1986) found rapid cyanobacteria development in manure-amended soils, but few cyanobacterial colonies in metal-rich biosolids-amended soils over a 118-day incubation period.

Not all studies have consistently shown a reduction in abundance of N_2-fixing bacteria with biosolids application. Although Obbard and Jones (2001) found decreased rhizobia abundance in metal-spiked sewage sludge-amended soils relative to reference soil, they also found elevated abundance following the application of non-spiked sewage sludge. Kinkle *et al.* (1987) found the abundance of *B. japonicum* increased with increasing biosolids application, and the authors concluded that application of heavy metal-rich biosolids did not cause a long-term effect on abundance nine years post-application.

From the studies above, it is apparent that the severity of effects on N_2-fixing bacteria species is influenced by the concentration of metals (and presumably, other contaminants) in the biosolids or sewage sludges applied to soils. In order to determine the true effects of potential contaminants, without the stimulatory effects of the nutrient contents, either manure controls should be employed (to control for the addition of N and other nutrients), or long-term field sites with no biosolids application for at least one previous season should be used (to allow most nutrients to be taken up by crop plants). This will remove potential stimulatory effects, and determine any true inhibitory effects associated with residual biosolids contaminants.

3.2.4 Changes in N_2-fixing Bacterial Metal Tolerance

Studies on metal tolerance of N_2-fixing bacteria have found either no evidence of enhanced metal tolerance following long-term biosolids application (Kinkle *et al.* 1987, Mårtensson and Witter 1990), or mixed results (Lakzian *et al.* 2002). However, based on the limited study of this phenomenon, and the limitations associated with the culturing techniques used in these studies, it is difficult to draw definitive conclusions. In addition, tolerance to metals may not be the key

driver in causing shifts to community structure; organic contaminants may also be a factor, and this has not been a focus of any studies found.

3.2.5 Changes in N_2-fixing Community Structure and Function

Only two studies were found which evaluated the effects of biosolids application on the structure-function relationship of a N_2-fixing bacterial species. Giller *et al.* (1989) evaluated the effects of metal-rich anaerobically-digested biosolids on the diversity, abundance, and activity of *R. leguminosarum* biovar *trifolii*, and found decreased plasmid diversity and abundance in biosolids-amended soil relative to manure-amended soil. All nodule isolates from biosolids-amended soils showed no N_2-fixation activity (as measured by acetylene reduction), whereas all isolates from manure-amended soils were able to fix N_2. Hirsch *et al.* (1993) used RFLP analysis in the same soils to confirm that the diversity of *R. leguminosarum* isolated from white clover nodules was reduced in biosolids-amended soil, and strains capable of forming effective nodules were absent. In comparison, the *R. leguminosarum* isolates in manure-amended plots were largely unrelated to those in biosolids-amended soils, and were able to nodulate and fix N_2 in white, red, and subterranean clover roots.

These two studies confirm that a loss of diversity in *R. leguminosarum* can be associated with a loss of N_2-fixing capacity in biosolids-amended soils (a distinct structure-function relationship). However, these studies both narrowly focused on single-species effects in relation to metal contaminants of municipal biosolids and sewage sludge. In future studies, expansion of the evaluation to a larger number of contaminants (including organic pollutants and pathogens) and effects on the entire community of N_2-fixing bacteria (which includes symbiotic rhizobia and also free-living cyanobacteria and *Azotobacter* sp.) can further advance the understanding of potential impacts of biosolids-amendment on the entire community of these agronomically-important species.

3.3 *Potential for Competition Among Biosolids Pathogens and Indigenous Bacterial Populations*

There have been numerous studies conducted that confirm pathogens (including viruses, bacteria and protists) survive the wastewater treatment process, survive in composted biosolids (Hussong *et al.* 1985, Millner *et al.* 1987, Sidhu *et al.* 2001), and also survive in soil following land application (Sawyer 1989, Sastre *et al.* 1996, Vasseur *et al.* 1996, Gibbs *et al.* 1997, Tierney *et al.* 1997). Survival of pathogens through the composting process is especially problematic, as composting is specifically used as a method of pathogen attenuation (Hussong *et al.* 1985, Millner *et al.* 1987).

Edmonds (1976) found fecal coliform counts from biosolids applied to clear-cut forest soils in July fell from $1.08x10^5$ g^{-1} to 0 g^{-1} in approximately nine months (in April), but winter-applied sludge had a more drastic die-off ($1.2x10^5$ g^{-1} to 20 g^{-1}) in approximately five months. Initial death rates were attributed to higher biosolids temperature and moisture (relative to soil), lower pH, physical composition and microbial competition. Re-population of coliforms occurred in warm summer and fall months, to a level lower than initial, but similar to background forest soil levels (54 g^{-1}). Sastre *et al.* (1996) also found biosolids application introduced a large number of microorganisms, and stated that indigenous populations attempt to maintain homeostatic conditions causing pathogens to die rapidly (although the mechanism was not provided).

Vasseur *et al.* (1996) found fecal coliform abundance in biologically activated and de-phosphated biosolids applied to coniferous or mixed regenerative forests did not differ between biosolids type or plots. This led them to believe coliforms rapidly died out within the first two weeks after application, and despite having abundant food sources (biosolids organic matter), the bacteria were stressed by the unfavourable moisture, pH, temperature, sunlight and nutrient level conditions of the soil (Vasseur *et al.* 1996). Although there was a rapid die-down after the first season, Vasseur *et al.* also found a re-emergence of fecal coliforms the following season (seen in the biologically treated biosolids on hardwood forest soils), indicating a period of dormancy during the unfavourable winter months. The study by Vasseur *et al.* (1996) was conducted at the end of the season (August 2nd to September 15th), when temperatures can drop rapidly, and on forest soils with a pH of pH 4.2, which is generally more acidic than agricultural soils; two factors which may limit the relevance of this study to agricultural application of biosolids.

Gibbs *et al.* (1997) studied fecal coliform survival in biosolids applied to agricultural soils during the summer. They also found fecal coliforms and *Salmonella* in land-applied biosolids and compost piles were undetectable in the extended hot, dry summer period, but following a rainfall event, re-populated and were more abundant than at the beginning of the experiment. The authors suggested environmental changes caused viable but non-culturable bacteria to be converted to a culturable state, which created the apparent re-emergence. Bacteria were only measured by culture methodology, which has been estimated to approximate <1-5% of a true soil bacterial community (Torsvik *et al.* 1990, Amann *et al.* 1995). Selvaratnam and Kunberger (2004) also report that bacteria can enter a dormant viable, but non-culturable state under certain environmental conditions.

From what was found in the literature, there may be a potential for competition effects on indigenous bacteria from pathogens in biosolids, but this was not a key component of any studies found (i.e., most focused strictly on pathogen survival following land application). Fecal coliform survival is influenced by the presence of competitive organisms and other factors including sunlight, temperature, moisture, and organic matter (Edmonds 1976), and numerous studies have found pathogens are rapidly out-competed by indigenous bacteria, although they may enter a second growth phase during subsequent years. In summary, the consensus among all authors is that soils amended with biosolids are not fully pathogen-free for at least one year post-amendment.

Conveyance of antibiotic resistance is another phenomenon related to competition among biosolids pathogens and indigenous agricultural soil bacteria, in that it may have an impact on the composition of the soil bacterial community. A study conducted by Radtke and Gist (1989) evaluated antibiotic resistance in bacteria isolated from biosolids samples from a WWTP in Tennessee. They found over 60% of the species isolated were resistant to at least one of the 11 antibiotics tested, and that antibiotic resistance was transferable (50% in *Klebsiella* spp. up to 72.2% in *Enterobacter* spp.). These authors caution that further quantification of antibiotic resistant bacteria in biosolids is necessary to determine the effects of releasing large numbers of antibiotic resistant bacteria into the environment, potentially exacerbating the problem of antibiotic resistance in general.

3.4 *Effects of Biosolids Land-Application on Macroorganisms*

Summaries of studies evaluating the ecological impacts of land application of biosolids are generally arranged according to trophic levels (as seen in Figure 1), from secondary consumers (i.e., protists) to secondary or tertiary consumers (i.e., earthworms and insects). A summary table of all studies and key information on the biosolids sources, soil types and key findings is provided in Appendix A.

3.4.1 Effects on Protists (Ciliates)

Two studies were found that focused on the effects of municipal biosolids application on one species of soil ciliate (Kingdom: Protista). Forge *et al.* (1993) developed a toxicity test method suitable for evaluating the effects of municipal biosolids application on agricultural soils using *Colpoda steinii* abundance (measured as cells mL^{-1}) as an endpoint. They extracted soil solutions from un-amended control soils, as well as from soils subject to unaltered biosolids- and metals spiked biosolids-amendment three years prior to the experiment (metals were spiked to the maximum soil-application limit or double the European limits). A suspension of approximately 100 cells of *C. steinii* were added to cell culture plates containing soil solution, and incubated for 24 hours before being enumerated. No treatments showed a change in abundance of *C. steinii* in soil solutions extracted from test soils sampled during the winter (February); however, the nickel (Ni) and zinc (Zn)-spiked biosolids-amended soils at twice the European limit showed significantly lower abundance than un-amended control soils in the summer (July) samples. Campbell *et al.* (1997) also evaluated the effects of metal-rich biosolids using the same general *C. steinii* bioassay developed by Forge *et al.* (1993). They found abundance was significantly lower in metal-spiked biosolids samples from two test sites relative to un-amended control sites, but found no difference in growth between uncontaminated biosolids-amended soils and control. Metal EC_{50} values were derived for each test site, with the relative toxicity being: Cu > Ni > Zn (Luddington soils had equally high Cu and Ni toxicity). The authors further noted *C. steinii* was more sensitive to metal-related effects than microbial bioassays performed at the same test sites.

Although ciliates have not been widely used as a test organism, they do show effects of heavy metal contamination in biosolids (notably Ni, Zn and Cu). As suggested by Forge *et al.* (1993), ciliates may prove useful as an indicator organism for this trophic level in future biosolids ecological effects studies. However, because these studies were based on laboratory-based

toxicity tests, they do not take into account community-level interactions that would occur *in situ*. Field studies would provide greater ecological relevance. Additionally, the potential toxicity of organic contaminants, not just heavy metals, and the effects of viable bacteria from biosolids (as it relates to shifts in food sources for ciliates) on soil ciliates should be evaluated.

3.4.2 Effects on Arthropods

Compared to protists, much more attention has focused on the potential effects of municipal biosolids land application on insects (Phylum: Arthropoda); these have included multiple species and trophic levels. The following studies have been arranged in order of size and trophic level (as arthropods represent both secondary and tertiary consumers).

Numerous studies have focused specifically on the smallest group, the microarthropods, which includes springtails (Order: Collembola). Cole *et al.* (2001) found application of uncontaminated biosolids was associated with an increase in springtail total abundance (specifically, *Heteromurus nitidis* and *Isotomurus maculatus*), relative to un-amended control plots. There were no increases in the abundance of springtails in biosolids with naturally high levels of cadmium (Cd) and Zn, indicating an overall inhibitory effect of metals relative to the stimulatory effects of organic enrichment. In addition, Cd-rich biosolids caused a decrease in the abundance of two particular species (*Lepidocrytus cyaneus* and *Isotoma viridis*), relative to the uncontaminated biosolids-amended plots, indicating species-specific sensitivity to Cd. Other species were found in significantly higher abundance in the metal-rich biosolids (*Isotomurus palustris*) relative to un-amended control plots, indicating the potential for overall shifts in springtail community structure following metal-contaminated biosolids land application on agricultural fields. Crouau *et al.* (2002) used *Folsomia candida* laboratory bioassays to determine the toxicity of numerous wastes commonly land-applied (including biosolids). Biosolids were added to artificial soils at the following rates: 0, 12.5, 25 and 50%; the reproductive (chronic) endpoint had a lowest observed effective test concentration (LOEC) of 12.5%, with an EC_{50} of > 50%. There was no significant mortality observed (i.e., no acute effects at any concentration). Scott-Fordsmand and Krogh (2004) assessed acute and chronic endpoints in *F. fimetaria* L. following exposure to nonylphenol (NP) spiked biosolids in soil cores. They employed two methods of biosolids incorporation, the first used homogeneous mixing of NP in biosolids and soil (0 to 200 mg NP kg dry $soil^{-1}$), and the other attempted to mimic the patchy distribution of

biosolids in agricultural soils using NP-spiked biosolids pellets (0 to 285 mg NP kg dry soil^{-1}). Endpoints included mortality (acute toxicity) and chronic endpoints including adult and juvenile growth (male and female), and reproduction. For the homogeneously mixed NP-spiked biosolids they found acute toxicity at an EC_{10} equivalent to 45 mg NP kg soil^{-1} (with 100% mortality at 200 mg NP kg soil^{-1}). The lowest chronic toxicity concentration was for reproduction at 6 mg NP kg soil^{-1}, with 100% reduction at 100 mg NP kg soil^{-1}. For the NP-spiked biosolids pellets there was no mortality at any concentration (no acute toxicity), but chronic effects at an EC_{10} equivalent to 19 mg NP kg soil^{-1} (reproduction). These results were not reproducible in field studies (application of NP-spiked biosolids at 150 mg NP kg soil^{-1} was associated with no change, or an increase in Collembola density). Petersen *et al.* (2003) evaluated the potential for effects of biosolids land-application (as well as compost and manure amendment) on two different microarthropod groups (springtails and mites), using biosolids with naturally high- and low-levels of metals and various organic contaminants in a three-year field trial conducted at two sites. They found microarthropod total abundance (springtails and mites) in high- and low-contaminant biosolids amended soils was not significantly different from un-amended control plots at one site (Askov), but significantly higher at the other (Lundgaard) in high-contaminant sludge, compost and manure-amended soils. Within species of springtail, there were significantly higher numbers of *F. fimetaria* in the high-contaminated biosolids-amended and manure-amended soils relative to un-amended control soils. In the Lundgaard site there was significantly higher total abundance of springtails in the high- and low-contaminated sludges and compost and manure-amended soils, with greater numbers of *F. fimetaria* in the high-sludge (and compost and manure-amended soils), and *I. notabilis* in biosolids, compost and manure-amended soils. Mites showed no significant differences in abundance in the Askov site (relative to control), with the Lundgaard site exhibiting significantly higher mites (order: Gamasida) in one treatment only (manure-amended soils). In general, they found either no change or stimulatory effects in springtail and mite abundance following biosolids application, with differences between sites relating to site-specific soil conditions.

There were only three studies found that focused on macroarthropods. Culliney and Pimental (1986) determined the effects of sludge and other soil amendments (manure and fertilizer) on the population densities (i.e., abundance) of three insect guilds in an agricultural field plot. Data on organism density on collard leaves (*Brassica oceracea* var. *acephala*) was collected over a 14-

week period for pre-determined insect guilds and species including: aphids (*Lipaphis erysimi*, *Myzus persicae* and *Brevicoryne brassicae*), flea beetles (*Phyllotreta cruciferae* and *P. striolata*), and caterpillars (various Lepidopteran species at late instar stage). Density was reported as number individuals per 1.3 x 10^5 cm^{-2} leaf surface area at weekly increments. Density of each guild fluctuated throughout the test period, with flea beetles showing highest density in the biosolids-amended soils through half of the test duration (with a decrease relative to the other treatments mid-test), and at test termination (14 weeks). Alate aphids and caterpillars (Lepidopterans) showed a different trend, with density in collards grown on biosolids-amended soils often lowest compared to other soil treatments through the test duration, and at test termination (caterpillar density on collards in biosolids- and manure-amended soils was not significantly different throughout the test duration). Interestingly (and possibly relating to the date of research), instead of considering that the lower density of aphids and caterpillars on biosolids-amended soils was related to the potential inhibitory effects of biosolids contaminants, the authors concluded that organic material amendment (from biosolids and manure) created a favourable balance of nutrients required for collard growth, and caused a suppression of these insect species (potentially through enhanced plant production of allelochemics). Larsen *et al.* (1994) evaluated the rate of heavy metal bioaccumulation in spiders (Class: Arachnida) inhabiting an old-field biosolids amendment test plot (biosolids were applied for 10 years, three years prior to test initiation), and evaluated the potential for feeding habits (i.e., airborne versus terrestrial prey capture) to affect the level of metals bioaccumulation over a two year test period. In the first year, they found significantly higher concentrations of Cd, Cu and Zn (in ground and web spiders), and lead (Pb) (in web spiders only) relative to spiders in un-amended control soils. In the second year, they found significantly higher bioaccumulation of Cd (in ground and web spiders) and Cu and Pb (in ground spiders only), relative to un-amended control soils. The authors recommended further research on the ecological ramifications of metal bioaccumulation on the spider community, as well as other components of the soil foodchain. Larsen *et al.* (1996) later determined the effects of municipal biosolids application on abundance, diversity, and bioaccumulation of metals in ground beetles (family: Carabidae), using the same site and during the same 2-year period as the study evaluating metal bioaccumulation in spiders.

Bioaccumulation of Zn in biosolids-amended soils relative to un-amended control soils was observed in the first year, and bioaccumulation of Cd and Pb was observed in the second year of

the experiment. Despite the occurrence of bioaccumulation, beetle abundance, species richness and diversity were significantly higher in biosolids and fertilizer-amended plots relative to control. Increased diversity was attributed to the level of nutrient enrichment, which increased plant growth and created larger herbivore populations to provide food for ground beetles. Although there were no negative impacts observed in the biosolids-amended plots through the experimental methods used, the authors suggested the limited duration of their study (2 seasons) was not sufficient to show differences caused by heavy metals. They also proposed that a less abundant species (such as *Scarites* spp., a predator) might be a more significant accumulator of heavy metals, and a more useful indicator species to determine the potential for heavy metal contamination than *P. lucublandus*, the most abundant species found in biosolids plots. The authors hypothesized that *P. lucublandus* may preferentially feed on herbivore species that are specialists towards plant species growing only in the biosolids-amended plots.

In order to accurately summarize the numerous studies on arthropods, it is important to note the wide differences in application methods, application rates, background concentrations of biosolids contaminants and inclusion of metal or organic pollutant spiking of biosolids (to reach maximum limits for land application), and differences in the complexity of ecological measures (i.e., simple bioaccumulation, versus more complex community-level shifts in relative species abundance). Two studies (Larsen *et al.* 1994, Larsen *et al.* 1996), illustrated the potential for heavy metal bioaccumulation from a commercially-available biosolids product in macroarthropods. Studies on springtails and mites (the microarthropods) found mixed results, ranging from increased overall abundance in relatively un-contaminated biosolids-amended soils (Cole *et al.* 2001) and naturally contaminated biosolids-amended soils (Petersen *et al.* 2003), to occurrences of reproductive impairment (Crouau *et al.* 2002), to acute toxicity in NP-spiked biosolids (Scott-Fordsmand and Krogh 2004). Arthropod studies also described a potential for community-level shifts in species composition (Culliney and Pimentel 1986, Cole *et al.* 2001, Petersen *et al.* 2003). The studies here may not have fully explored the intricate relationships among the arthropod trophic levels and other ecologically-relevant endpoints, but they do show a potential for effects of municipal biosolids. More research is necessary to determine ecologically-relevant measures of potential effects of biosolids land-application (i.e., community-level shifts and the resulting effects on soil fertility), and also to consider other classes of biosolids contaminants than heavy metals.

3.4.3 Effects on Earthworms and Nematodes

Earthworms at the higher end of the agricultural soil food web and have been the most widely-studied organism group (other than bacteria) in studies of the ecological effects of biosolids land-application. The majority of these studies have focused on the potential for bioaccumulation of heavy metals. Diercsxens *et al.* (1985) found concentrations of heavy metals in anecic earthworm tissue present in sludge-amended fields ranging from $1.12x10^3$ μg g^{-1} (Cd) up to $1.39x10^5$ μg g^{-1} (Zn) (fresh weight) in soils containing from $1.06x10^3$ μg g^{-1} Cd up to $7.87x10^4$ μg g^{-1} Zn (dry weight). They concluded there was significant bioaccumulation of Cd and Zn in earthworm tissues following sludge application, with Pb, Cu and chromium (Cr) not resulting in the same level of bioaccumulation. Kruse and Barrett (1985) determined the rate of bioaccumulation in one species of earthworm (*Lumbricus rubellus*) collected from a long-term biosolids land application site. Concentration factors for Cd were highest (104.3), with lower factor values for Cu (1.2), Pb (0.4), and Zn (7.9). They found the maximum average concentration of Cd (136 μg g^{-1}) was significantly higher in earthworm tissue in biosolids-amended plots relative to un-amended control, with Cu (20.8 μg g^{-1}) and Pb (8.78 μg g^{-1}) also significantly higher, but Zn ($1.09x10^3$ μg g^{-1}) not significantly higher than in un-amended control. Average soil metal concentrations were 1.29, 16.9, 23.1, and 136.9 μg g^{-1}, respectively, for Cd, Cu, Pb and Zn in biosolids-amended soils. The authors recommended *L. rubellus* as an indicator species for the effects of biosolids land application on this trophic level, based on the evidence of its role as a sink for heavy metal contamination in soil. Tomlin *et al.* (1993) evaluated bioaccumulation rates and biomass in earthworms inhabiting a long-term biosolids application test site. They found *L. terrestris* biomass was higher in biosolids-amended plots relative to control, with no significant difference in earthworm abundance or biomass between treatments or increasing loading rates. The Al-precipitated biosolids-amended plots were the only to have elevated tissue concentrations of Cd in earthworms (up to 570 μg g^{-1} ashed weight), with body mass concentrations correlated to soil Cd concentrations (illustrating bioconcentration, with tissue concentration of Cd up to 200 times above soil concentrations).

Beck *et al.* (1996) discussed bioavailability of organic pollutants to various trophic levels, including earthworms, and used two reviewed studies to estimate the throughput of organic contaminants ingested by earthworms (using a theoretical soil ingestion rate of 400 tonnes ha^{-1} $year^{-1}$). Estimated throughput following a single biosolids application at a rate of 50 tonnes ha^{-1},

from highest to lowest they was: benzo[ghi]perylene ($1.67x10^7$ μg ha^{-1} $year^{-1}$), PCB 153 ($1.04x10^6$ μg ha^{-1} $year^{-1}$), OCDD ($6.35x10^5$ μg ha^{-1} $year^{-1}$), and 13-DCB ($2.30x10^5$ μg ha^{-1} $year^{-1}$). Based on a number of studies reviewed, the authors suggest earthworms are involved in releasing organic non-ionic compounds from soil particles. Matscheko *et al.* (2002) evaluated bioaccumulation of organic biosolids contaminants such as dioxins and furans, PCBs and polybrominated diphenyl ethers (PBDEs) in earthworms (*L. terrestris*, *L.* spp., *A. caliginosa*, *A. rosea* and *Allolobophora chlorotica*) inhabiting four long-term biosolids test plots. They found evidence of bioaccumulation in earthworm tissue, and determined the biota-soil accumulation factors (BSAFs) for these organic contaminants in decreasing order were: ortho-PCBs ~ PBDEs > non-ortho-PCBs > 2,3,7,8-PCDD/Fs, with the average BCAF for ortho-PCBs being 5 (organic matter:lipids) and the lowest values for octachlorodibenzo-p-dioxin (0.1 to 0.8).

Relatively fewer studies have evaluated more ecologically-relevant measures than simple bioaccumulation in earthworms; however, these may only evaluate as much as abundance, with even fewer considering more descriptive measures such as community composition and diversity. In 2002, Baker *et al.* (2002) evaluated the effects of biosolids on earthworm abundance and found significant increases in total earthworm abundance in biosolids-amended plots (of all application rates, from 30 to 120 dry tonnes ha^{-1}), relative to un-amended control soils. Species composition showed effects in biosolids-amended soils; low-rate amended biosolids-amended soils (30 and 60 tonnes ha^{-1}) and control earthworm communities were dominated by *S. macleayi*, while in the high-rate biosolids-amended soils (120 tonnes ha^{-1}), the community was dominated by *M. dubius*. Caged bioassays (using *Aporrectodea longa* and *A. caliginosa*) found no effect of biosolids application on the survival or biomass of caged organisms. Peles *et al.* (2003) used the same Ohio research site as Larsen *et al.* (1994, 1996) to determine and compare allele and genotype frequencies and multi- and single-locus heterozygosity in earthworms (*L. rubellus*) inhabiting a long-term biosolids test site. They found shifts in earthworm genotypes in sludge-amended soils attributed to the sub-lethal concentrations of heavy metals; the frequency of common enzyme genotypes for glucose phosphate isomerase (GPI) were reduced by 15% in biosolids-amended earthworms relative to control, and the most common enzyme genotype phosphoglucomutase (PGM) was 25% lower relative to control. The biosolids population was characterized by alleles that were absent in the control population and a higher frequency of other alleles. The authors suggest their findings provide evidence that certain genotypes/alleles

are more sensitive to heavy metals, and that evaluation of the genetic structure of earthworm communities is a useful biological indicator for ecological effects from pollution. Kizilkaya and Hepşen (2004) evaluated the effects of biosolids application on nutrient content and enzyme activity in *L. terrestris* casts and in the surrounding agricultural soils. They found some enzymes (e.g., the hydrolytic urease and alkaline phosphatase) were positively correlated with the level of biosolids application, and activity was significantly higher in biosolids-amended soils; however, dehydrogenase (DHA), an intracellular enzyme, and arylsulfate (in soils with biosolids application rates > 100 g kg^{-1}) were negatively correlated with the level of biosolids application, and were significantly lower than in un-amended control soils. The decrease in DHA was specifically attributed to heavy metal contamination of the biosolids.

Two studies evaluated the long-term effects of metal-enriched biosolids on the community structure of nematodes. Georgieva *et al.* (2002) found richness (number of taxa) was highest in un-amended soils, and lowest in Cu, Zn+Cu and Zn-spiked biosolids amended soils. Conversely, the total abundance was low in the un-amended soils relative to the metal-spiked biosolids amended soils. Analysis of maturity index scores (based on Colonizer-Persister values) found trends indicative of stressed ecosystems, whereby the r-strategists were in higher abundance and K-strategists were in lower abundance in some of the Cu, Zn+Cu and Zn-spiked biosolids amended soils. Banks *et al.* (2006) employed bioassays with earthworms (*Eisenia foetida*) and nematodes (*Caenorhabditis elegans*) on 19 WWTP biosolids applied to two long-term and five short-term field sites. Earthworm bioassays found significant reduction in growth (chronic endpoint) in two sites (from the short-term trials), and a significant increase in cocoon production (reproductive, chronic endpoint), in two of the long-term application sites. Nematode bioassays found mortality (acute endpoint), in two long-term sites and one short-term application site. The authors concluded that the toxicity observed in their experiment was within the normal range of statistical variability, and that the existing land-application regulations in the US are adequately protective of agricultural resources.

From the numerous earthworm bioaccumulation studies described here, it can be concluded that earthworms are significant bioaccumulators, or sinks, of heavy metals (Cd, Cr, Cu, Pb, Zn) and organic contaminants (PBDEs, PAHs, PCBs, PCDD/DFs). It is interesting to note there is a burgeoning form of biosolids treatment termed "vermiculture"; biosolids not suitable for land

application (based on exceedances of land-application standards) are treated by incorporating earthworms (*Eisenia* spp.), and allowing them to bioaccumulate the heavy metals and other contaminants present (Craig and Ankers 2006). The end product is called vermicompost, and it is claimed that this process is highly effective. The fact earthworms are used as a biosolids treatment, and effectively "clean" biosolids to make them suitable for land application illustrates the potential for significant uptake in the environment, with potential impacts on earthworm predators (such as mammals or birds), and further movement up the terrestrial food web. In general, it also appears biosolids land application can result in an increase in total abundance of earthworms and nematodes; however, this measure can mask the more subtle effects such as shifts to community structure, with the potential for opportunistic species to dominate, as shown by Georgieva *et al.* (2002).

3.4.4 Bioaccumulation and Biomagnification in Multiple Trophic Levels

A number of multi-level bioaccumulation and biomagnification studies were found from one group of researchers in the UK; two of these are described here. Winder *et al.* (1999) determined the level of Zn bioaccumulation and potential biomagnification in three trophic levels: a primary producer, winter wheat (*Triticum aestivum* cv. *Brigadier*); a primary consumer, grain aphids (*Sitobion avenae*); and a secondary consumer, the carabid beetle (*Bembidion lampros*). Soil, wheat and aphid samples were collected from biosolids amended soil plots (with 0, 10 and 15 tonnes ha^{-1} rates of application, and 2 application periods), then analyzed for Zn. They also used additional aphids from the site for a 9-day beetle feeding trial (beetles were subsequently analyzed for Zn). Maximum concentrations of Zn in each trophic level were as follows: winter wheat (31.7 $\mu g\ g^{-1}$), aphids (116.0 $\mu g\ g^{-1}$), and beetles (112.2 $\mu g\ g^{-1}$), which suggests biomagnification might occur up the trophic levels in this experimental food web; however, concentrations of Zn in each trophic level were only significantly different among the biosolids amendment treatments in 2 of 4 winter wheat harvests, and not in aphids or beetles. This study evaluated bioaccumulation following relatively low rates of application and only 2 application times, suggesting a cumulative loading effects study may find greater rates of biomagnification. Green *et al.* (2003) tested soils from the same study site as Winder *et al.* (1999) (with 0, 10 and 30 tonnes ha^{-1} rates of biosolids application), and expanded the study of bioaccumulation to Cd as well as Zn in a second tri-trophic level study. They used the same primary producer and consumer species, spring wheat (*T. aestivum* cv. *Alexander*) and grain aphids (*S. avenae*), but a

different secondary consumer, ladybirds (*Coccinella septempunctata*), in a greenhouse culture study. Spring wheat was planted in test soils and 200 individual aphids were seeded per pot. Ladybird larvae were kept in separate Petri dishes and fed known quantities of aphids from each of the three biosolids treatments. Once larvae pupated they were analyzed for Cd and Zn. They found average concentrations of Zn in each trophic level were as follows: winter wheat (115.0 $\mu g\ g^{-1}$), aphids (248.5 $\mu g\ g^{-1}$), and adult ladybirds (217.1 $\mu g\ g^{-1}$), with significantly higher levels in biosolids-amended soils relative to control; however these were not significantly different among treatments for ladybird Zn concentrations. Average concentrations of Cd in each trophic level were much lower than for Zn: winter wheat (0.314 $\mu g\ g^{-1}$), aphids (0.419 $\mu g\ g^{-1}$), and adult ladybirds (0.223 $\mu g\ g^{-1}$), however these were not significantly different between control and biosolids-amended soils for any trophic level. Although biomagnification of Zn was evident in aphids, it was not associated with a corresponding reduction in abundance in biosolids-amended plots relative to un-amended control plots.

3.5 *Summary of the State-of-Knowledge on Ecological Effects*

Many of the inconsistencies among the findings of the studies reviewed here can be related to a few common factors. First, results can sometimes be an artefact of the research date; for example, studies on bacterial diversity conducted before the advent of molecular techniques were only capable of viewing effects on the culturable fraction of the entire soil community, which is estimated to constitute as low as 0.01 to 1% of the total bacterial population in soil (Torsvik *et al.* 1990, Torsvik *et al.* 1996). Also, studies often focus on one or a few aspects of general microbial ecology (i.e., the diversity, abundance, or activity), and these can be misleading. General soil respiration or biomass gives an indication of the general size of the microbial population, but no information on the ecological function or community composition. Measurement of some form of activity (i.e., nutrient mineralization) does not provide a description of the underlying species responsible for that function. Also, some papers have noted "conflicting evidence" over findings of the severity of effects, specifically relating to metal toxicity (Broos *et al.* 2004). Often, the differences in the findings can be attributed to comparing results from sites with different soil conditions, different municipal biosolids sources (each with a unique mixture of potential contaminants and synergistic effects), and comparing studies which may not have employed consistent methodologies or ecological endpoints. Munn *et al.* (2001) go so far as to recommend using site-specific evaluations for biosolids-application sites in the absence of consistent thresholds of metal toxicity across soil and biosolids types.

The majority of studies on the effects of municipal sewage sludge or biosolids on soil microbial communities studies specifically focus on effects associated with the heavy metal constituents of the biosolids applied, and do not describe the potential organic pollutants. There have been a few later studies which include discussion of organic pollutants and the impacts to soil organisms (Jacquot *et al.* 2000, Elsgaard *et al.* 2001, Gejlsbjerg *et al.* 2001, Debosz *et al.* 2002), with an additional study by Chaudri *et al.* (1996) determining threshold concentrations of organic contaminants (singly, and not in biosolids) associated with shifts in *R. leguminosarum* biovar *trifolii* abundance. Many studies that focus on organic pollutants (Wang and Jones 1994, Rogers 1996, Overcash *et al.* 2005) consider indigenous soil microbes in terms of their role as a degradation pathway, instead of being ecosystem components. As such, an in-depth analysis of the effects of organic pollutants on soil organisms is still needed to determine the potential for

causative links for the toxicity of organic pollutants in addition to those previously determined for heavy metals.

Studies concluding that a single contaminant or class of contaminants (i.e., heavy metals) in municipal biosolids or sewage sludge do not show correlation to some ecological effect cannot state there are no effects; in reality, the lack of an obvious relationship between increasing concentrations of a contaminant and increased perturbation to the soil microbial community may be a result of not having measured the true causative agent or groups of causative agents, or that variability in the data are too great to prove or disprove the correlation (McBride 1995). Determination of the trends in contaminant concentrations and ecological effects can be carried out by conducting multivariate analysis (e.g., Principal Component Analysis, or PCA) using all ecological endpoints and contaminant concentrations. However, to truly understand the relationship between the potential effects of a municipal sewage sludge source and soil microbial communities, it is necessary to conduct toxicity identification evaluations (TIEs) on biosolids from each individual wastewater treatment source to determine which contaminant, or groups of contaminants, may be creating toxic effects. It is also important to consider that when biosolids are spiked with metals, attention is limited to the relationship between metal concentrations and ecological endpoints; there may be other metals or contaminants not specifically spiked (and as a result, not specifically analyzed) that may create ecological effects, but would be overlooked. If a chemical is not tested for, it cannot be measured, nor correlated to some ecological endpoint.

There also appears to be a need to separate out the toxic effects of pollutants from the stimulatory effects of the organic matter and nutrients contained in biosolids (Witter *et al.* 2000). Some authors stated that toxicity may be more apparent in the long-term, because organic content and nutrients supplied by biosolids may initially stimulate plant growth and soil microbial activity, and alter the bioavailability of the metals. Site-specific soil properties like pH, organic matter, and clay content also affect bioavailability of metals (Campbell *et al.* 1997) and the pre-existing status of the soil community, and it is important to consider these properties when comparing the effects of biosolids land application across study sites and studies (Appendix A).

One note of caution is to weight any effects found by the nature of biosolids being evaluated; if metal salts were used to spike biosolids to maximum regulated levels, metals would most likely have been more bioavailable than bound metals, and may show artificially higher toxicity effects or bioaccumulation levels than un-altered biosolids from a WWTP (Giller *et al.* 1998, Cole *et al.* 2001).

Regardless of contaminant bioavailability or analytical issues, some generalities can be made. Often, the abundance of a particular group of organisms may not change, or increase following biosolids land application – this measure would seem to indicate a stimulatory, potentially positive effect of biosolids land application. However, when other, potentially more ecologically-relevant measures are taken into consideration (such as diversity, richness or other community-level effects), the apparent stimulatory effect can often mask more subtle changes to the community structure. Biosolids impacts on sensitive species (as a result of metal, organic contaminants, or shifts in food resources) in turn results in a decrease of one group, and the potential creation of an opening in their ecological niche, which is often filled by r-selected, or 'weedy' species. This phenomenon was specifically evaluated in nematodes by Georgieva *et al.* (2002). Based on the paucity of studies evaluating the full, complex ecological effects of biosolids land-application, at multiple trophic levels, it is apparent that more should be done to evaluate ecosystem-level changes in soil amended with municipal biosolids. In terms of the effect of biosolids land application on the entire soil ecosystem, it appears that the loss of specific ecological function (most notably, N_2-fixing capability), acute toxicity (resulting in shifts in community structure or changes to biodiversity), and the bioaccumulation of biosolids contaminants (and potential movement up the food chain) are the most significant ecological impacts found to date.

The most striking economic concern with land application of municipal biosolids is the potential loss of N_2-fixation capability in long-term biosolids-amended agricultural sites. This may create a serious setback for farmers practicing legume crop rotation to increase bioavailable N levels. Biosolids may create a long-term and potentially irreversible loss of N_2-fixing capability and result in higher costs and reliance on fertilizers. For this reason, N_2-fixing bacteria were chosen as the focus of this thesis research.

4 Rationale for Thesis Research Experiment

The following sections provide a rationale for the thesis research on multiple levels, from an overall need (why ecological effects assessment is required), to the organism studied (why N_2-fixing bacteria are suitable), and specific analytical techniques chosen.

4.1 Why Ecological Effects Assessment is Necessary

In the province of Ontario there is significant pressure to greatly increase the scale of municipal biosolids land-application. This makes determination of the ecological effects of a "100% beneficial use" of biosolids policy very timely. Regrettably, the City of Toronto's Master Plan, which would see up to 25 000 dry tonnes of biosolids from Canada's largest WWTP being land-applied annually, did not consider the ecological effects of this activity when determining policy direction. The Master Plan also does not recommend the use of ecological assessment to ensure this policy is ecologically sound, and does not recommend biological monitoring as part of routine monitoring programs, despite the clear wording of the Ontario draft guide for biosolids land-application that states materials not benefiting crop production or soil health, or that degrade the natural environment, may not be land-applied (Ontario Ministry of Environment and Ontario Ministry of Agriculture and Food 2004). Without biological effects assessment in the soil receiving environment, it cannot be confirmed that degradation to the natural environment is not occurring.

Strictly extrapolating the results of ecological effects assessments from other studies is not sufficient. Although it may appear that sufficient work has been done to evaluate the ecological effects of land-applying municipal biosolids, the sum of information does not apply to each situation; the studies reviewed did not apply equivalent volumes of biosolids nor did they follow equivalent application frequencies. There are also unique, site-specific factors in the soil receiving environment, and site-specific factors in the composition of the biosolids (and associated levels of metallic, organic and pathogenic contaminants), which could have a great impact on the changes seen in the soil environment (McBride 2003). In addition, a large number of the studies employed contaminant spiking to reach maximum loading levels. If metal salts are used to spike biosolids, effectively, what is evaluated is the effect of metal salts plus municipal biosolids (which also include metals complexed with inorganic residues), not the specific effect

of the municipal biosolids. Finally, very few ecological effects studies were found from Canada, or southern Ontario, increasing the need for more local research.

In a more general sense, there are also significant potential contaminant issues associated with municipal biosolids; high levels of copper and mercury were reported in numerous examples of biosolids from around the world (Table 4). Existing land-application standards have been criticized in the US for not being suitably protective (McBride 1995, Harrison *et al.* 1999), and the most stringent Ontario standards for some metals (e.g., arsenic, lead, molybdenum) and fecal coliforms are higher than the most stringent US standards. The emergence of contaminants like PPCPs and other organic contaminants dictate that ecological studies not only consider metals. In order to capture the true ecological effects and integrate the changes occurring in time since biosolids are applied, biological studies are necessary. Biological monitoring, which use indigenous organisms (or bioindicators) as monitoring tools, temporally incorporates all chemical and physical aspects of the environment, and embodies a holistic evaluation of effects of contaminants, more than chemical analysis can alone (Golder-EVS 2006).

Ecologically-relevant research on the effects of land-applying municipal biosolids is scarce; often studies evaluate bulk abundance or general activity, without concern for subtlety of ecological processes. By evaluating the structure (i.e., community diversity of N_2-fixing bacteria) in concert with their function (i.e., rate of N_2-fixation), this research simultaneously examines the poorly understood structure-function relationship of microorganisms, determining the effects of biosolids land application on an agronomically important group of organisms, and determining locally whether biosolids land application should be expanded and promoted, or if other disposal methods should be adopted based on comprehensive science.

4.2 *Why N_2-Fixing Bacteria are a Good Model Organism Group*

In a natural soil ecosystem, biological N_2-fixation – the conversion of atmospheric nitrogen gas (N_2) to a biologically usable form (i.e., ammonia, NH_3) – is the primary source of fixed nitrogen for plants and animals, making it a critical process in terrestrial and aquatic food webs, and a key component of the global nitrogen cycle (Campbell 1993, Zehr *et al.* 2003, Bürgmann *et al.* 2004). There is also increased interest in exploiting biological N_2-fixation, as part of an effort to increase the sustainability of agricultural practices around the world (Brewin and Legocki 1996).

Biological N_2-fixation accounts for the production of over $2x10^{13}$ g N yr^{-1} globally, while lightning, the other source of fixed nitrogen (as nitrate), supplies 10^{12} to 10^{13} g N yr^{-1} (Raymond *et al.* 2004). The biological ability to fix N_2 is exclusive to a select group of prokaryotes spread across bacterial and archael domains, with both anaerobic and aerobic metabolisms. This includes numerous soil bacterial genera (e.g., *Rhizobium*, *Bradyrhizobium*, *Frankia*, *Azotobacter*, *Beijerinckia*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Clostridium* sp.), as well as cyanobacteria (Beauchamp and Hume 1997). This phylogenetic and physiological diversity makes it impossible to select for the entire community of N_2-fixing organisms on a single selective media (Poly *et al.* 2001). The biological N_2-fixation reaction is catalyzed by the nitrogenase enzyme complex (Raymond *et al.* 2004), and is extremely metabolically-expensive, requiring 16 ATP molecules and 8 reducing equivalents per N_2 molecule reduced (Campbell 1993, Martinez-Romero 2000). The nitrogenase complex is comprised of five structural genes (nif H, D, K, E, and N) which code for two component proteins (Raymond *et al.* 2004). The nifH gene codes for one of the two component proteins, the dinitrogenase reductase γ_2 homodimer (Raymond *et al.* 2004). The N_2-fixation reaction is inhibited by O_2; however aerobic bacteria are able to carry out the reaction by physically sequestering the nitrogenase enzyme from O_2 (e.g., in cyanobacteria) (Prescott *et al.* 1996) or by only fixing nitrogen under anaerobic conditions (Hopkins 1999).

In a soil environment, N_2-fixing bacteria are either free-living (generally referred to as diazotrophs) and live in loose association with roots of legumes and non-legumes, or inhabit root nodules of legume plant species, in a symbiotic relationship (generally termed rhizobia) (Beauchamp and Hume 1997). In most agricultural systems, the majority of N_2-fixation is provided by rhizobia (e.g., *Rhizobium* and *Bradyrhizobium* sp.) (Beauchamp and Hume 1997); however, less is known about the contribution of free-living diazotrophs, which can be significant in some environments (Brewin and Legocki 1996, Bürgmann *et al.* 2003). Although the significance of free-living diazotrophs as a source of fixed nitrogen is generally considered minor and variable compared to rhizobia, biomass turnover (through cellular disintegration and mineralization) provides a longer-term source of fixed nitrogen (Chan *et al.* 1994, Bürgmann *et al.* 2005), and there are other potential benefits to crops including production of plant growth-promoting phytohormones or competition with pathogens (Bürgmann *et al.* 2005). Agricultural soils which have high C:N ratios are a major exception, as the favourable growth conditions (with labile C to provide energy and reducing sources) promote diazotrophic activity (Chan *et al.*

1994). This is important to note for the experiment, as the differences in N_2-fixing community structure and activity among unamended and amended soils will most likely be related to the amount of organic material present.

The only other method of creating biologically-usable forms of nitrogen is carried out through the Haber-Bosch ammonia synthesis process, which is extremely expensive and energy intensive (Mauseth 1995); current methods of manufacturing inorganic fertilizers requires production of hydrogen gas and high temperature and pressure which is equivalent to approximately 1% of the global annual energy supply (Smith 2002). In order to limit dependence on synthetic fertilizers, the process of crop rotation with leguminous crops has been used by farmers for many years. Given the immense importance of the role of N_2-fixing bacteria in the global nitrogen cycle (contributing approximately 50% of the total N input into agricultural soils, globally (Smith 2002), it is apparent that the potential for a loss of N_2-fixing ability in an agricultural soil would have serious ramifications for farmers, potentially making them reliant on synthetic nitrogen fertilizers.

In terms of their use as a model organism group for assessment of the effects of biosolids land-application, N_2-fixers are a good candidate for diversity analysis, as the nitrogenase enzyme complex as a whole has been highly conserved through evolution and shows a high degree of correlation to sequences derived from 16S-ribosomal subunit evaluation (Zehr *et al.* 2003). Although relatively small degrees of lateral gene transfer have been observed, nifH is still considered to be an appropriate tool for determining the presence of potentially active N_2-fixing phylotypes (Bürgmann *et al.* 2005), and has been used to determine the relative diversity of uncultivated N_2-fixing bacteria in termite gut, sediment, soil, estuarine, salt marsh and oligotrophic oceanic samples (Zehr *et al.* 2003). As of 2002, there were 1500 sequences of nifH listed in GenBank (most from environmental samples), making it one of the largest non-ribosomal gene datasets in existence for un-cultivated organisms (Zehr *et al.* 2003). By amplifying the nifH gene, it is possible to simultaneously get a positive confirmation of N_2-fixing bacterial presence in soils (a confirmation of potential activity), as well as a measure of community structure. O'Connor *et al.* (2005) specifically suggested there has been insufficient study of ecological effects of biosolids (and other residuals) land-application on the entire soil

ecosystem (including indigenous soil microbes, invertebrates and wildlife), and that effects on specific biological processes (such as nitrogen-fixation) requires further study.

Although N_2-fixing bacteria have been the focus of numerous biosolids land-application studies, this thesis experiment is novel; although it is well known that soil organisms in general have a great impact on soil fertility, the linkage between the biodiversity of the soil ecosystem, and soil organism function and process has not yet been completely elucidated (Emmerling *et al.* 2002). This relationship is often termed the "structure-function" or "biodiversity-function" relationship, and although poorly understood, is thought to be of great importance in maintaining many environmental functions (Tiedje *et al.* 1999, Griffiths *et al.* 2001, Crecchio *et al.* 2004), and determining the true ecological effects of environmental perturbations (Crecchio *et al.* 2004), including soil amendment in agricultural settings. Of the numerous European studies on the effects of municipal biosolids on N_2-fixing bacteria, only two studies looked at the effects on the structure-function relationship of N_2-fixing bacteria, but for a single symbiotic species (*Rhizobium leguminosarum* biovar. *trifolii*) (Giller *et al.* 1989, Hirsch *et al.* 1993). This research will determine the effects of land application on the structure-function relationship of the entire community of free-living diazotrophic N_2-fixing bacteria, which have not been commonly used to determine the effects of municipal biosolids land-application.

4.3 Rationale for Test Methods Used

The test methods used in this thesis cover a wide range of disciplines in order to gain a comprehensive understanding of the ecological effects of land-applying municipal biosolids on soil bacteria. Using multiple techniques to cover-off deficiencies in individual methods is necessary, as there is no single method that can be used to fully describe a soil microbial community (Kirk *et al.* 2004, Winding *et al.* 2005).

N_2-fixing activity was measured as consumption of $^{15}N_2$ relative to argon as an internal standard through GC/MS. The commonly employed method of quantifying N_2-fixation *in situ* based on acetylene-reduction to ethylene is indirect, and was not chosen based on the level of criticism regarding its ecological relevance (Giller 1987). In addition, acetylene itself can inhibit other bacterial processes involved in the N cycle (e.g., nitrification), which can create confounding effects on test results (Sloth *et al.* 1992). Use of $^{15}N_2$ has become more common as an analytical

technique, and there are variations on how it can be used (Danso *et al.* 1993). Cellular respiration was included as an ancillary, non-specific measure of microbial activity following soil amendment.

N_2-fixing community structure was evaluated using molecular techniques, including PCR to amplify an N_2-fixing structural gene (nifH) which is the standard target for detection and identification of potential diazotrophic species in environmental samples (Bürgmann *et al.* 2004). Culturing techniques are not feasible for diazotrophs because of the physiological diversity of the species (Poly *et al.* 2001). It is important to note that the presence of the nifH gene represents the potential for active N_2-fixation, as the process (including transcription of nifH) is tightly regulated and may depend on environmental conditions (Bürgmann *et al.* 2003, Zehr *et al.* 2003). If the purpose of the experiment was to use a molecular marker alone to indicate active N_2-fixing bacterial presence, detection of nifH mRNA (through reverse transcription) would be necessary; however, this experiment employs direct measurement of the uptake of $^{15}N_2$ as a real-time indicator of N_2-fixing activity, with the use of nifH strictly a measure of N_2-fixing community structure. The chosen group of possible universal nifH primers contain a high degree of sequence degeneracy, which is required to achieve amplification of the extensive range of diazotroph phylotypes which may be present in each treatment (Widmer *et al.* 1999). For example, the degenerate nifH-universal primer set designed by Widmer *et al.* (1999) detected a wide range of N_2-fixing genera in forest soils and litter including *Rhizobium*, *Simorhizobium*, *Azospirillum*, *Bradyrhizobium*, *Azorhizobium*, *Herbaspirillum*, *Thiobacillus* as well as cyanobacteria.

Analysis of the structure of N_2-fixing bacterial communities was conducted using Denaturing Gradient Gel Electrophoresis (DGGE) of nifH. DGGE is a highly appropriate method for determining small changes in genetic sequences, and under some circumstances, can provide resolution at the species level (Kirk *et al.* 2004). DGGE with nifH has been employed successfully for ecological studies of soil diazotrophs (Demba Diallo *et al.* 2004, Bürgmann *et al.* 2005), although it is important to note that many N_2-fixing species have multiple copies of the nifH gene (Zehr *et al.* 2003, Raymond *et al.* 2004), meaning the presence of one band will not necessarily correspond to the presence of one species (i.e., one species may have multiple bands on a DGGE gel) (Demba Diallo *et al.* 2004).

General bacterial community structure was also considered, and was measured on Biolog EcoPlates™. This technique is not specific for N_2-fixation, but provides an additional description of the differences in microbial communities among treatments at community-level resolution (Kirk *et al.* 2004). Biolog substrate utilization has been specifically suggested as a means of determining effects of biosolids land-application on microbe diversity (O'Connor *et al.* 2005) and the EcoPlate™ and other Biolog plates have been widely used as a measure of functional diversity in environmental samples (Zak *et al.* 1994, Garland 1997, Preston-Mafham *et al.* 2002).

Chemical analysis was conducted (by external laboratories) to provide additional description of the soil environments in each treatment (which could correlate to microbial activity), and to determine if any resulting ecological effects associated with biosolids land application could be correlated to levels of particular contaminants (i.e., metals, anions, or PPCPs).

To summarize: the research objectives were to determine the effects of land-applying municipal biosolids on nitrogen-fixing bacteria (structure and function). Additional measures of general microbial ecology (i.e., cellular respiration, community-level physiological profile) were used to more fully describe the changes in the overall microbial community. Chemical analysis of biosolids and biosolids- and manure-amended soils, in addition to the un-amended reference soil, was also included to provide an indication of the potential stressors or causative agents of any observed changes in microbial ecology, in addition to the potential effects of viable bacteria in the biosolids on the indigenous soil microbial community.

5 Experimental Methods

5.1 Soil Collection

Reference test soil was obtained from a private residential property in Oro, ON on February 22, 2006 (Figure 2). Sampling was conducted in the winter in order to achieve a sufficient duration for pre-test soil conditioning. The site has been owned by the current residents for five years, and has been under pasture with a mixture of grasses and alfalfa for over 10 years. Cattle (5-15) have been grazing on the site for the past five years. The soil collection site was located approximately 150 m from the Sturgeon River. Existing snow cover at a depth of 60 cm was removed, and soil was collected to a depth of approximately 20 cm using clean shovels. A total volume of 60 L soil was removed and stored in plastic bags in two 30 L coolers. Grasses were present on the soil surface and live earthworms were observed in the soil at the time of sampling.

Figure 2: Collection of experimental soil in Oro, ON on February 22, 2006. Soil was collected from a pasture area with active cattle grazing (visible in background).

5.2 Experimental Set-up

5.2.1 Soil Conditioning

The soil was sieved through Nos. 8 and 6 Fisher Scientific sieves (Ø 2.36 mm and 3.35 mm, respectively). Large root masses (> 1-3 mm diameter) were removed, but smaller roots were retained. Once a sufficient volume of soil was sieved, the entire bulk sample was homogenized, and approximately 800 mL of soil was aliquoted into 15 1-L Ziploc® brand storage containers. Each storage container had been previously perforated with 14 drainage holes and fitted with clean plastic window screening to maintain the flow of water and reduce blockage of the drainage holes. After drainage holes were inserted, containers and mesh were rinsed with 5% hydrochloric acid and tap water (tap water was used for watering). The outside of each container was covered with aluminum foil to maintain dark conditions below the soil surface. On March 7, 2006, each pot was planted with two legume species, Ladino clover (*Trifolium repens* L.), and alfalfa (*Medicago sativa* L.), and a common forage grass, timothy (*Phleum pratense* L.). Pre-planting was conducted to pre-condition the test soils prior to initiation of the experiment, and confirm the quality of the reference soils as a suitable material supporting plant growth. The end of a sterilized flame loop was used to disperse the seeds to a depth of 8 cm, and soil was lightly packed. Test containers were maintained in a laboratory under fluorescent lights on a 12:12 light:dark cycle. Two days after sowing there were germinated alfalfa visible in all containers.

The watering regime was based on maintenance of a minimum soil moisture level. Soil moisture during soil conditioning, and during the duration of the experiment, was measured using a Lincoln Soil Meter hygrometer (Lincoln Irrigation Inc., Lincoln NE). Once the soil in each individual container reached a reading of '4' or lower (equivalent to 40% saturation), the container was watered with 100 mL of tap water. Soil moisture was checked an average of 3 times per week.

Containers were maintained under laboratory conditions for 4.5 months prior to test initiation on July 26, 2006. Robust growth of alfalfa and timothy was achieved in all test containers; however, clover did not germinate. Immediately prior to test initiation all above-ground plant material was removed at the soil surface and weighed, with root systems retained in the soil.

5.2.2 Experimental Design

The laboratory experiment was conducted using a complete randomized design with four treatments and five replicates. The four treatments included reference, un-amended agricultural soils, organic manure-amended soil, biosolids-amended soil, and biosolids only. The reference soil (reference treatment) consisted of pre-conditioned soil from Oro, ON, which provided an experimental reference to describe the unaltered status of the indigenous soil microbial community at each test interval. Organic manure-amended soil (soil+manure treatment) consisting of pre-conditioned soils amended with organic manure and was used to differentiate potential effects of the toxicity of metals and/or organic pollutants of biosolids from the stimulatory effects of organic material enrichment on the indigenous soil microbial community. The organic manure was Green Earth Premium Compost from Nu-Gro IP Inc., Brantford ON, containing 62% organic matter with pH 7.5, and moisture maximum of 51%. The biosolids-amended soil (soil+biosolids treatment) consisted of pre-conditioned soils amended with biosolids from the Kitchener WWTP (collected on July 13, 2006). No further information on biosolids sample or time of collection was available. The biosolids treatment consisted of Kitchener WWTP biosolids alone, and was included as a positive control to determine any inhibitory effects on indigenous soil microbial community composition or N_2-fixation rates based on contaminants or viable bacteria contained in biosolids (if these bacteria were able to out-compete indigenous bacteria, and affect community function).

Experimental units (individual test containers) were assigned to treatments randomly and containers were placed on the laboratory bench at random (to account for variations in laboratory light and temperature conditions) (Figure 3). Six test intervals were assessed over the three-month test duration as follows: day 0, which was used as a baseline measurement, in order to establish homogeneity among experimental units prior to soil amendment; day 1, which was used to determine the immediate, short-term changes to the soil equilibrium immediately following soil amendment; week 1; week 2; week 6, the half-way point for the experiment; and month 3 (test termination). On day 0, pre-test measurements were taken on all treatments (the reference agricultural soils used for reference, soil+biosolids, soil+manure and biosolids), and on day 1, soils in the soil+biosolids and soil+manure containers were incorporated with the pre-determined amount of biosolids or manure and all treatments were re-seeded with Ladino clover, alfalfa and timothy. The containers were re-planted in order to replicate agricultural field conditions, and

potentially allow analysis of free-living rhizobia associated with the clover and alfalfa rhizosphere.

Figure 3: Experimental test set-up at day 1. Positions of test containers were randomly assigned. Treatment labels are as follows: blue = reference; green = manure-amended soil; yellow = biosolids-amended soil; red = biosolids.

5.2.3 Determination of Soil Amendment Rates

Prior to addition of the two soil amendment treatments, the average moisture content and organic material content of the biosolids and manure were determined for 5 replicate samples. Approximately 10 g wet material was added to ceramic crucibles (pre-combusted and weighed). The biosolids and manure were then dried in a radiant heat oven (Imperial II, Lab-Line Instruments, Inc., Melrose Park, IL) at 105°C, until dry (16 hours for biosolids, 4 hours for manure). Once dry, the crucibles were weighed to determine moisture content. The materials were combusted for 4 hours in a muffle furnace (Thermolyne 30400, Barnstead/Thermolyne Corp., Dubuque, IA) at 550°C. The final weights were recorded and used to determine the organic material content. The average moisture content of the biosolids was 94.1% (5.9% solids) and 47.7% (52.3% solids) for the manure. The average organic material content was 60.2% in the biosolids, and 38.5% in the manure (dry weight).

The experimental soil amendment rate was set at 18 tonnes dry matter (DM) ha^{-1}, which is between the two maximum loading rates for biosolids application in Ontario (8 or 22 tonnes DM ha^{-1} every 5 years, depending on the number of exceedances of metal limits) (Ontario Ministry of

Environment and Ontario Ministry of Agriculture and Food 2004). Accounting for the differences in organic matter content of the two materials, 438 g biosolids (liquid), and 85 g manure was added to soil in the biosolids-amendment and manure-amendment test containers, respectively. To account for the difference in moisture content, an additional 412 mL of water was added to reference soil containers and 370 mL was added to manure-amended soil containers. Original data and detailed calculations are provided in Appendix B.

5.3 *Chemical Analyses*

Chemical analysis was conducted on bulk samples prior to test initiation (immediately following soil collection in February 2006), and on pooled samples from the five replicates of the three-month experimental test soils from all treatments (including biosolids-only) at test termination (October 2006). Analysis of metals, anions, nitrogen, pH and particle size was carried out by a commercial laboratory (AGAT Laboratories, Mississauga, ON), following analytical techniques accredited by the Standards Council of Canada (SCC) and Canadian Association for Environmental Analytical Laboratories (CAEAL). The metals and anions analyzed are included in Ontario Regulation 153 (the Ontario Records of Site Condition regulation associated with contaminated sites assessment). The entire list of parameters analyzed by AGAT is as follows:

- Metals (extractable): antimony, arsenic, barium, beryllium, cadmium, chromium, cobalt, copper, lead, molybdenum, nickel, selenium, silver, thallium, vanadium, and zinc;
- Anions: fluoride, chloride, bromide, phosphate, and sulfate;
- Nitrogen: nitrite (NO_2), nitrate (NO_3), total Kjeldahl nitrogen (TKN), organic nitrogen, and ammonia-N (NH_3-N);
- pH; and
- Particle size: distribution of particles as clay, sand and silt.

In January 2007, the pooled samples of the three-month test material from the biosolids, biosolids-amended soil and reference soil (frozen at -4°C since test-termination), plus a sample of the original liquid biosolids (stored at 4°C since collection and frozen immediately prior to shipping) were analyzed for 26 PPCPs by Dr. Alison Spongberg at the University of Toledo. Once received at the laboratory, samples were freeze-dried, then stored at -5°C before sequential ultrasonic extraction and PTFE syringe filtration, then evaporation and dilution with deep limestone bedrock groundwater. Extraction techniques followed Göbel *et al.* (2005) and Ternes

et al. (2005). Samples were analyzed for each PPCP compound on LC tandem MS using one of six chromatographic separation methods, and were quantified using seven point calibration curves of standards. The classes of PPCP and the compounds analysed (brand-names of common PPCPs are provided in parentheses) are as follows:

- Analgesics / acidic pharmaceuticals: acetaminophen (e.g., Tylenol®), salicylic acid (e.g., Aspirin®), and ibuprofen (e.g., Advil®, Motrin®);
- Antibiotics: ciprofloxacin, clindamycin, clarithromycin, sulfamethoxazole, sulfathiazole, sulfisoxazole, sulfamethizole, sulfamethazine, sulfadimethoxine, tetracycline, chlortetracycline-HCl, and vancomycin;
- Anti-epileptic / anti-depressant: carbamazepine;
- Anti-hyperglycemic / anti-diabetic: metformin;
- Benzothiapine: diltiazem;
- Histamine 2 blockers: cimetidine and ranitidine;
- Lipid-regulating agents: clofibric acid and gemfibrozil;
- Non-steroidal anti-inflammatory: diclofenac; and
- Stimulants: cotinine (e.g., cigarettes, etc.), caffeine (e.g., coffee, etc.), and paraxanthine (stimulant metabolite).

The LC/MS analyses were conducted on triplicate sub-samples, and concentrations were provided as the mean value ± standard deviation. Standard errors were calculated using the data provided.

5.4 N_2-fixation and Cellular Respiration (GC-MS)

A detailed test protocol for determining N_2-fixation and cellular respiration rates is provided in Appendix C. In summary, at each test interval, a modified 3 mL plastic syringe, cut to create an open barrel, was used to collect small core samples of approximately 5 g of material from randomly selected locations in each container (4 to 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 $^{15}N_2$ (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 material) was also spiked with $^{15}N_2$ to serve as an analytical reference.

Immediately following injection, the serum vials were analyzed on a GC/MS (AutoSystem XL, Perkin Elmer, Waltham, MA) in the Ryerson University Analytical Centre (RUAC). Gases were separated using a Supel-Q™ 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 50 µL of each sample was 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 (CO_2). At the next reading (6 hr), maximum absorbance readings were recorded for molecular mass 30 ($^{15}N_2$), 40 (Ar) and 44 (CO_2). At the final reading (48 hrs), maximum absorbance values were recorded molecular mass 30 ($^{15}N_2$) and 40 (Ar). These sample intervals were based on pilot-study incubations with this soil in which CO_2-production was linear over the 0 to 6 hour interval, and N_2 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 rates were based on changes in $^{15}N_2$:Ar ($^{15}N_2$ dilution) and CO_2:Ar (CO_2 production) through time, assuming Ar to be a conservative internal standard. The method employed was an adaptation of similar techniques used to measure denitrification in marine sediments (increase in N_2:Ar) (Kana *et al.* 1994, Cornwell *et al.* 1999, Laursen and Seitzinger 2002). The air blank sample was used to make any necessary corrections to measured gas ratios due to instrument drift. The $^{15}N_2$ and CO_2 concentrations in incubation vessels were then back-calculated from ratio measurements, based on atmospheric concentrations of Ar. The rate of analyte production or consumption per hour, per gram of soil was then determined and the rate of $^{15}N_2$ consumption was converted to a total of $^{15}N_2$+$^{14}N_2$ based on the initial ratio of $^{15}N_2$:$^{14}N_2$ in the incubation vials. The average rate of N_2-fixation and soil respiration for each treatment was determined at each test interval, and treatments were compared using a single-factor ANOVA ($\alpha = 0.05$) and a post-hoc test of means (Fisher's Least Significant Difference) in SAS 9.1.3 (SAS Institute Inc., Cary, NC). Average N_2-fixing activity and cellular respiration were plotted in Microsoft Excel.

5.5 *Community-level Physiological Profiling (Biolog EcoPlates™)*

The Biolog EcoPlate™ contains a mixture of five classes of carbon sources including two amines, six amino acids, seven carbohydrates, ten carboxylic acids, four polymers, and two

miscellaneous compounds (glucose-1-phosphate and D,L-α-gycerol phosphate). A more detailed test protocol used for community-level physiological profiling (CLPP) with Biolog EcoPlates™ (Biolog Inc., Hayward, CA) is provided in Appendix C.

Briefly, at each test interval, a 2.5 g sub-sample was taken from the homogenized 5 g bulk samples of each container, and transferred to a sterile 50 mL plastic centrifuge tube. An additional treatment blank was also prepared without test soil. Bacteria and other microbes 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 pellet was resuspended in 12 mL sterile saline solution (0.85% NaCl w/v), and a multi-channel pipettor was used to inoculate 100 µL of this suspension in individual wells of the Biolog EcoPlates™.

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) in the RUAC. The EcoPlate™ is designed to be read at 590 nm, however this lens was not available for the plate reader. Multiple readings were required to determine the optimal incubation period for all treatments. The run order followed was the same randomly-generated order as used for GC/MS analyses.

For each individual plate, the average optical density (OD) measurements were corrected for the average well colour development score (AWCD) of each plate, and calculated for each of the 31 carbon sources. The optimal inoculation duration was determined following Glimm *et al.* (1997). The AWCD-corrected OD values at 42 hours post-inoculation were then subjected to Factor analysis in SAS 9.1.3 (SAS Institute Inc., Cary, NC). Factor 1 and 2 scores were then analyzed using a single-factor ANOVA ($\alpha = 0.05$) with a post-hoc test (Fisher's Least Significant Difference) to determine differences among treatments in substrate utilization. Factor scores were plotted using SigmaPlot 10 (Systat Software Inc., San Jose, CA).

The loading scores for PC1 and 2 were also examined to determine if any particular group or groups of carbon sources accounted for a large proportion of the variability among treatments at each test interval (Appendix D).

5.6 *N_2-fixing Community Structure (PCR-DGGE of nif-H)*

5.6.1 DNA Extraction

The material remaining from the six test intervals was frozen at -80°C following collection, and a selection of samples from specific intervals were used to determine temporal changes in the structure of the N_2-fixing bacterial community. The test intervals and rationale for their use is as follows: day 0 was required as a pre-test baseline to establish that there was no clear underlying variability among soil treatments prior to soil amendment with biosolids or manure; week 2 was chosen because it was expected to be an adequate interval following soil amendment to observe shifts in microbial communities and N_2-fixing activity appeared similar between day 7 and week 2 (this also corresponded to the period when plants began to exhibit phytotoxicity in response to biosolids amendment); week 6 was chosen because of shifts in EcoPlates™ (biosolids-amended soils were distinct from all other treatments in carbon utilization pattern) and it was the half-way point of experiment; and month 3 (test termination) was chosen because there was no significant difference among all soil treatments in the EcoPlates™, and among all treatments for N_2-fixation (GC/MS data). This period also provides an indication of the longer-term shifts in community structure following soil amendment.

Beginning on October 31, 2006, bacterial DNA was extracted from the frozen samples using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA). This commercially-available product was chosen based on its superior level of performance in a comparative study of DNA extraction methods for soil samples (Dore 2002). The DNA extraction was conducted as per the product protocol, which involves bead-beating for sample homogenization, with mechanical and chemical cell lysis. The resulting 100 µL volume of extracted bacterial DNA in sterile elution buffer (10 mM Tris) was then frozen at -80°C prior to amplification with polymerase chain reaction (PCR).

5.6.2 nifH Amplification

Over a five-month period, starting in January 2007, PCR of the nifH gene was attempted using numerous published primer sets (Table 5).

Table 5: Primers attempted for nifH amplification.

nifH Primer	Sequence (5'→3')	Target Length (bp)	Source
nifH-F nifH-R	AAA GGY GGW ATC GGY AAR TCC ACC AC TTG TTS GCS GCR TAC ATS GCC ATC AT	457	Rosch *et al.* 2002
K07-F AMR-R	GCG TTC TAC GGT AAG GGC GGT ATC GGN AAR GCT ACT ACY TCG CCS GA	451	Rosch *et al.* 2002
nifH3 nifH4 nifH1 nifH2	ATR TTR TTN GCN GCR TA TTY TAY GGN AAR GGN GG GAY CCN AAR GCN GA AND GCC ATC ATY TCN CC	330 (nested)	Zehr and McReynolds 1989 and Zani *et al.* 2000 as combined by Izquierdo and Nüsslein 2006
nifH-univ For A nifH-univ For B nifH-univ R	GCI WTI TAY GGN AAR GGN GG GGI TGY GAY CCN AAV GCN GA GCR TAI ABN GCC ATC ATY TC	464 (outer) 371 (nested)	Widmer *et al.* 1999 as modified by Bürgmann *et al.* 2004

The concentrations of primer in the stock solutions were measured spectrophotometrically (GeneQuant Pro, Biochrom Ltd., Cambridge, UK), in order to determine the appropriate primer dilution rate for PCR. The appropriate PCR conditions and nifH primer pair was determined through a trial-and-error process using test samples (numerous week 2 and week 6 control and biosolids samples) and positive nifH control samples (*Anabaena* and *Nostoc* spp. from Carolina Biological Supply Co., Burlington NC).

PCR amplification was conducted in a GeneAmp 9600 Thermal Cycler (Perkin Elmer, Waltham, MA). PCR success was determined by running PCR products on 1.5% agarose gel (protein electrophoresis grade, Fisher BioReagents, Thermo Fisher Scientific, Waltham, MA), in 1x TAE buffer. Gels were run using 10 to 20 µL of PCR products stained with 2 µL 100x SYBR® Green I nucleic acid gel stain (Invitrogen Canada Inc., Burlington, ON) and 1 µL 6x blue/orange loading dye (Promega, Madison, WI). A 100 bp DNA ladder (Promega, Madison, WI) was also run on each gel (5 µL volume).

Optimization and troubleshooting was carried out and possible issues were identified, including the appropriate concentrations of $MgCl_2$, Bovine Serum Albumin (BSA) and DNA template. The primer set which was eventually chosen (nifH-universal) was from originally from Widmer *et al.* (1999) as modified by Bürgmann *et al.* (2004), who used a primer concentration of 2.0 µM, an annealing temperature of 56°C, $MgCl_2$ at 2.0 mM, BSA at 5 mg mL^{-1}, and 30 amplification cycles. The conditions used in this experiment for the first round of PCR were as follows: 10 µL

template (all treatments); 2 mM $MgCl_2$; 2 mg mL^{-1} BSA; 2 μM primers (nifH-universal F and R); 1.25 U Taq (0.25 μL volume used); and 50 μL PCR reaction volume. The PCR method was as follows: hold at 94°C for 5 minutes; 30 cycles of 94°C for 20 seconds, 56°C for 20 seconds, 72°C for 45 seconds; and hold at 72°C for 5 minutes for final elongation. Gels were visualized under UV light with a fixed CCV camera fitted with a SYBR® Green filter (BioDoc-It™ Imaging System and transilluminator table, UVP, Upland, CA), and gel images were saved digitally on compact flash and printed on a UVP Mitsubishi P93 printer.

Following initial PCR, nested PCR was carried out using the same nested primer set for nifH-universal from Widmer *et al.* (1999) as modified by Bürgmann *et al.* (2004), with an additional 40 bp GC clamp added to the forward primer for DGGE stabilization (Muyzer *et al.* 1993). The same nifH-universal R primer was used as for the outside PCR. The nested PCR reaction conditions were modified from Bürgmann *et al.* (2004). The conditions for the nested PCR were as follows: 2 μL template (directly from the first PCR products); 2 mM $MgCl_2$; 0.2 mg mL^{-1} BSA; 1 μM primers (nifH-universal internal F and R); 1.25 U Taq (0.25 μL volume used); and 50 μL PCR reaction volume. The PCR method was as follows: hold at 94°C for 5 minutes; 35 cycles of 94°C for 20 seconds, 53°C for 20 seconds, 72°C for 45 seconds; and hold at 72°C for 5 minutes for final elongation.

5.6.3 DGGE

Denaturing gradient gel electrophoresis (DGGE) was performed on nested nifH PCR products using the DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA). Nested PCR product (20 μL) was mixed with 4 μL 6x blue/orange loading dye (Promega, Madison, WI) in sterile 200 μL tubes, and loaded into each well. Each gel contained all five replicates of each treatment for one time interval. Initial DGGE conditions specific for nifH-universal were adapted from Bürgmann *et al.* (2005), including use of a 10% acrylamide/bis gel and a denaturing gradient of 35-60%. The run parameters (50 V for 15 minutes then 200 V for 5 hours at 60°C) were modified to 100 V for 17 hours at 60°C. After running two gels at this denaturing gradient, it was determined that a higher denaturant gradient (45-70%) was preferable, as it was observed that some bands appeared to run off the bottom of the gels. Detailed DGGE methodological information is provided in Appendix C.

Following electrophoresis, gels were soaked in a 1:10 000 solution of SYBR® Green I for 30 minutes, generally following the SYBR® Green I staining protocol from Molecular Probes, Inc. (2003). Gels were visualized under UV light with a fixed CCV camera fitted with a SYBR® Green filter (BioDoc-It™ Imaging System and transilluminator table, UVP, Upland, CA), and gel images were saved digitally on compact flash and printed on a UVP Mitsubishi P93 printer. Bands of interest were excised on a transilluminator table (2011 Macrovue, LKB Bromma, Sweden) using an ethanol-rinsed razor blade and placed in a sterile 500 μL tube, and the exact locations of excised bands were recorded on printed gel photos. Excised bands were then homogenized into 1 mm diameter pieces and covered with 100 μL autoclaved Millipore water for at least 5 hours at 4°C (some bands excised from multiple treatments were extracted into 200 μL autoclaved Millipore water). The DNA elutriate was then transferred to new 500 μL tubes, and the DNA concentration was determined spectrophotometrically.

Twenty of the excised bands were then sent for sequence analysis at the DNA Sequencing Facility at the Centre for Applied Genomics (TMDT-MaRS, Toronto, ON). Bands were chosen if they were unique to a treatment (or two treatments), or had a particularly high intensity, suggesting relatively high abundance in more than one treatment (but still recognizing the potential for template amplification bias).

Bands from DGGE gels were enumerated visually from gel photographs and the average nifH-universal genetic richness of each treatment was determined, and compared using a single-factor ANOVA ($\alpha = 0.05$) and a post-hoc test of means (Fisher's Least Significant Difference) in SAS 9.1.3 (SAS Institute Inc., Cary, NC). Average richness values were plotted in Microsoft Excel.

Analysis of diversity of the N_2-fixing community on DGGE gels was not conducted, as the loading density of samples in each gel was not standardized across treatments. To calculate abundance, band intensity would have to be used as a proxy for genetic variant abundance. While very intense bands may be inferred to represent variant abundance, and very faint bands may represent rare variants, measuring subtle differences in band intensity and relating it to differences in relative abundance would have introduced uncertainty, given the potential for template bias (particularly when using degenerate primers).

6 Results

Although the effects of biosolids application on crop yield was not originally included as a major component of this study, it is important to note that at two weeks post-amendment, the alfalfa and timothy in biosolids-amended soil treatments began to exhibit wilting and desiccation (Figure 4). These effects were not observed in the other soil treatments. Soon after, the vegetation on the biosolids-amended soils began to die. The biosolids-only treatment had minimal timothy germination, with no alfalfa present at any point of the experiment, while the reference and manure-amended soils showed abundant growth of timothy and alfalfa, in similar density. Ladino clover did not successfully germinate in any treatment. In addition to the apparent phytotoxicity in biosolids-amended soil, at approximately 2 weeks post-amendment, a blue-green coloured microbial mat began to form on the surface of the biosolids-amended soil and biosolids treatments.

Figure 4: Photo of test containers taken under experimental conditions at two weeks post-amendment. Clockwise, from top left: biosolids, manure-amended soil, biosolids-amended soil, and reference. Note the presence of timothy in the primarily liquid biosolids, and the apparent acute phytotoxicity in the biosolids-amended soil treatment.

Following the three-month test duration, alfalfa and timothy densities in the reference and manure-amended soils were similar and covered approximately 70% of test container soil surface area (Figure 5). All plants in the biosolids-amended soils had died shortly after the photo taken in

Figure 4. Because of the initial high moisture content of the biosolids treatment (94% liquid), there was relatively little material remaining by test termination (Figure 5). Although the hygrometer readings from the biosolids-amended soils were routinely well above '4' (or 40% saturation), the soil surface appeared desiccated throughout most of the experimental duration, and the texture was more dense and hard-packed than the other soil treatments. As a function of the greater plant biomass in the reference and manure-amended soils, and the associated uptake by roots, watering was required at a higher frequency than in the biosolids-amended soil and biosolids treatments to maintain the minimal moisture standard.

Figure 5: Photos of test containers at test termination (3 months post-amendment). Clockwise, from top left: reference, manure-amended soil, biosolids, and biosolids-amended soil.

6.1 Soil Chemistry

Metals analysis of pre-test soils did not find any exceedances of MOE soil standards for agricultural use (potable groundwater condition), however it was noted that chloride, copper and zinc levels were elevated (6.73 $\mu g\ g^{-1}$, 16.1 $\mu g\ g^{-1}$, and 34.7 $\mu g\ g^{-1}$, respectively), presumably due to the past use of municipal water for irrigation. Analysis of post-test biosolids determined an exceedance of the MOE/OMAFRA 'more restrictive' biosolids land application standard for copper (1200 $\mu g\ g^{-1}$ compared to 760 $\mu g\ g^{-1}$; Table 6). This would restrict land application of

these biosolids to a maximum application rate of 8 tonnes ha^{-1} 5 $years^{-1}$. Other metals (arsenic, barium, chromium, lead and zinc) were elevated in biosolids and biosolids-amended soils compared to reference and manure-amended soils. One exception was nickel, which was higher in reference soils than the two amended soils. Among the N forms, nitrate and nitrite were high in biosolids-amended soil (324 $\mu g\ g^{-1}$ and 10.6 $\mu g\ g^{-1}$), and exceeded MOE soil standards for agricultural use (potable groundwater condition) (Ontario Ministry of Environment 2007). Total Kjeldahl N, organic N and ammonia as N were also higher than in reference and manure-amended soil. In addition, biosolids-amended soil had much higher concentrations of sulfate (659 $\mu g\ g^{-1}$) and chloride (388 $\mu g\ g^{-1}$) compared to reference and manure-amended soils (74 and 252 $\mu g\ g^{-1}$, and 81.4 and 124 $\mu g\ g^{-1}$, reference and manure-amended soil sulfate and chloride concentrations, respectively). In terms of soil structure, the three soil treatments had almost identical composition of sand, silt and clay, and loam texture. The pH was similar in reference and biosolids-amended soil (6.3 and 6.42, respectively), but higher in manure-amended soil (7.29).

Table 6: Chemical analysis results from analysis of test material at test-termination (3 months post-amendment). Analyses were conducted by AGAT laboratories.

Parameter	MOE/OMAF Biosolids Standard - more restrictive [1]	MOE/OMAF Biosolids Standard - less restrictive [2]	Biosolids	MOE Table 2b Soil Standards (Ag or other use)[3]	Soil+biosolids	Soil+manure	Reference
Metals (extractable)							
Antimony	-	-	<1.6	15	<1.6	<1.6	<1.6
Arsenic	75	170	3.7	11	4.2	3.8	4.1
Barium	-	-	660	680	52.1	31.3	32.8
Beryllium	-	-	<0.4	8	<0.4	<0.4	<0.4
Cadmium	20	34	1.5	17	<0.4	<0.4	<0.4
Chromium	1060	2800	67.2	340 (10)	11.8	9.1	10.8
Cobalt	150	340	3.6	50	4.2	4	4.2
Copper	760	1700	1200	180	60.6	25	32
Lead	500	1100	38.5	140	8.2	6.9	7.2
Mercury	5	11	NA	0.13	NA	NA	NA
Molybdenum	20	94	9	8.2	0.5	<0.5	<0.5
Nickel	180	420	38.6	130	2.3	<0.6	10.3
Selenium	14	34	4.2	1	<0.8	<0.8	<0.8
Silver	-	-	12.6	16	0.6	<0.4	<0.4
Thallium	-	-	<0.4	1.8	<0.4	<0.4	<0.4
Vanadium	-	-	8	86	20	19.1	20
Zinc	1850	4200	793	340	71.5	44.3	42.8
Anions							
Bromide	-	-	<1.00	-	<0.10	<0.10	<0.10
Chloride	-	-	7480	2200	388	124	81.4
Fluoride	-	-	<1.00	-	<0.10	<0.10	<0.10
Phosphate	-	-	<10.0	-	<0.20	<0.20	<0.20
Sulfate	-	-	5560	-	659	252	74
Nitrogen							
Total Kjeldahl Nitrogen	-	-	66 800	-	4270	3030	3110
Organic Nitrogen	-	-	65 000	-	3910	2810	2950
Ammonia as N	-	-	1780	-	362	216	158
Nitrate (NO_3)	-	-	77.6	90	324	47.3	61.2
Nitrite (NO_2)	-	-	54	9	10.6	0.28	<0.10
General							
% Sand	-	-	NA	-	44	46	47
% Silt	-	-	NA	-	46	43	42
% Clay	-	-	NA	-	10	11	11
Soil Texture	-	-	NA	-	Loam	Loam	Loam
pH 2:1 Water:Soil Extraction	-	-	6.84	-	6.42	7.29	6.3

Footnotes:

Units are all $\mu g\ g^{-1}$ except pH

NA = Not Analyzed

biosolids samples had higher detection limits for some parameters based on the presence of high chloride

: indicates an exceedance of a Biosolids Land-Application, or Soil Standard

[1] MOE/OMAF, 2004; for biosolids applied at a rate up to 22 tonnes ha^{-1} 5 yrs^{-1}.

[2] Biosolids applied at a rate up to 8 tonnes ha^{-1} 5 yrs^{-1}.

[3] MOE, 2007. Soil, Ground Water and Sediment Standards for Use Under Part XV.1 of the Environmental Protection Act
TABLE 2b: Full Depth Generic Site Condition Standards in a Potable Ground Water Condition - Medium and Fine Textured Soils

The PPCP analysis showed numerous compounds were below detection limits in all treatments (Table 7). There were relatively high levels of three common analgesics detected in all samples, including reference soils. The highest level of any compound was for ibuprofen (e.g., Advil®). The highest average ibuprofen concentration was in biosolids (3.51 $\mu g\ g^{-1}$), followed by liquid biosolids (2.24 $\mu g\ g^{-1}$) and biosolids-amended soil (1.64 $\mu g\ g^{-1}$), with ibuprofen also detected in reference soil ($7.64x10^{-1}\ \mu g\ g^{-1}$). Salicylic acid (e.g., Aspirin®) was highest in the liquid biosolids ($6.49x10^{-1}\ \mu g\ g^{-1}$), biosolids ($5.55x10^{-1}\ \mu g\ g^{-1}$), and biosolids-amended soil ($5.28x10^{-1}\ \mu g\ g^{-1}$), and was at a similar level in the reference soil ($2.67x10^{-1}\ \mu g\ g^{-1}$). Acetaminophen (e.g., Tylenol®) was highest in the biosolids ($4.54x10^{-1}\ \mu g\ g^{-1}$), biosolids-amended soil ($3.72x10^{-1}\ \mu g\ g^{-1}$) and liquid biosolids ($2.87x10^{-1}\ \mu g\ g^{-1}$), and was also reasonably high in reference soil ($2.59x10^{-1}\ \mu g\ g^{-1}$). Of the other compounds, carbamazepine (an antiepileptic / antidepressant), clofibric acid (a lipid-regulating agent) and diclofenac (a non-steroidal anti-inflammatory) were at notable concentrations in all treatments (except carbamazepine, which was below detection limits in reference soils).

Table 7: Average PPCP concentrations (n =3) in test material at test-termination (3 months post-amendment), plus a sample of the same liquid biosolids used for the experiment, and stored at 4°C since collection. Analyses were conducted by Alison Spongberg at the University of Toledo.

Parameter	Liquid Biosolids	S.E.	Biosolids	S.E.	Soil + biosolids	S.E.	Reference	S.E.
Analgesic / acidic pharmaceuticals								
acetaminophen	2.87E-01	1.57E-01	4.54E-01	6.81E-03	3.72E-01	1.34E-01	2.59E-01	1.32E-01
ibuprofen	2.24	8.44E-02	3.51	2.31E-01	1.64	2.74E-02	7.64E-01	3.83E-01
salicylic acid	6.49E-01	4.77E-02	5.55E-01	1.74E-02	5.28E-01	3.49E-02	2.67E-01	1.22E-01
Antibiotics								
chlortetracycline-HCl	BDL		BDL		1.17E-02	1.01E-03	2.03E-02	4.37E-03
ciprofloxacin	1.73E-02	4.03E-03	1.59E-02	1.77E-03	5.50E-03	1.21E-03	2.01E-02	1.51E-02
clarithromycin	6.13E-03	1.57E-03	2.90E-03	1.15E-04	7.09E-04	2.56E-04	2.51E-03	1.65E-03
clindamycin	3.01E-02	2.14E-02	6.29E-03	2.90E-04	1.10E-04	6.56E-05	1.90E-04	1.31E-04
sulfamethazine	BDL		3.22E-03	1.86E-04	BDL		3.27E-03	4.67E-04
sulfamethizole	BDL		3.38E-03	8.25E-04	BDL		2.25E-03	5.29E-04
sulfamethoxazole	BDL		BDL		BDL		BDL	
sulfathiazole	BDL		BDL		BDL		BDL	
sulfisoxazole	BDL		BDL		BDL		BDL	
tetracycline	BDL		9.90E-03	2.80E-03	BDL		BDL	
vancomycin	BDL		BDL		BDL		BDL	
sulfadimethoxine	1.70E-03	1.83E-04	4.54E-03	1.82E-03	BDL		3.28E-03	8.53E-04
Antiepileptic / Antidepressents								
carbamazepine	6.19E-01	4.99E-02	8.30E-01	9.25E-02	9.52E-03	7.42E-04	BDL	
Antihyperglycemic / Antidiabetics								
metformin	BDL		BDL		3.42E-02	4.25E-03	BDL	
Benzothiapine								
diltiazem	1.37E-03	3.95E-04	4.91E-04	9.33E-05	BDL		BDL	
Histamine 2 blockers								
cimetidine	BDL		BDL		BDL		BDL	
ranitidine	BDL		BDL		BDL		BDL	
Lipid-regulating agents								
clofibric acid	1.57E-01	4.93E-03	1.70E-01	3.36E-03	1.44E-01	1.04E-02	7.14E-02	3.57E-02
gemfibrozil	6.88E-03	1.62E-03	6.67E-03	1.68E-04	BDL		BDL	
Non-steroidal Anti-inflammatory								
diclofenac	2.17E-01	3.05E-03	2.17E-01	1.35E-02	1.87E-01	1.54E-02	7.88E-02	3.94E-02
Stimulant								
caffeine	3.32E-02	3.68E-03	3.05E-02	2.06E-03	1.74E-02	9.09E-04	2.43E-02	1.14E-03
cotinine	5.23E-03	6.12E-04	BDL		BDL		BDL	
paraxanthine (metabolite)	BDL		BDL		BDL		BDL	

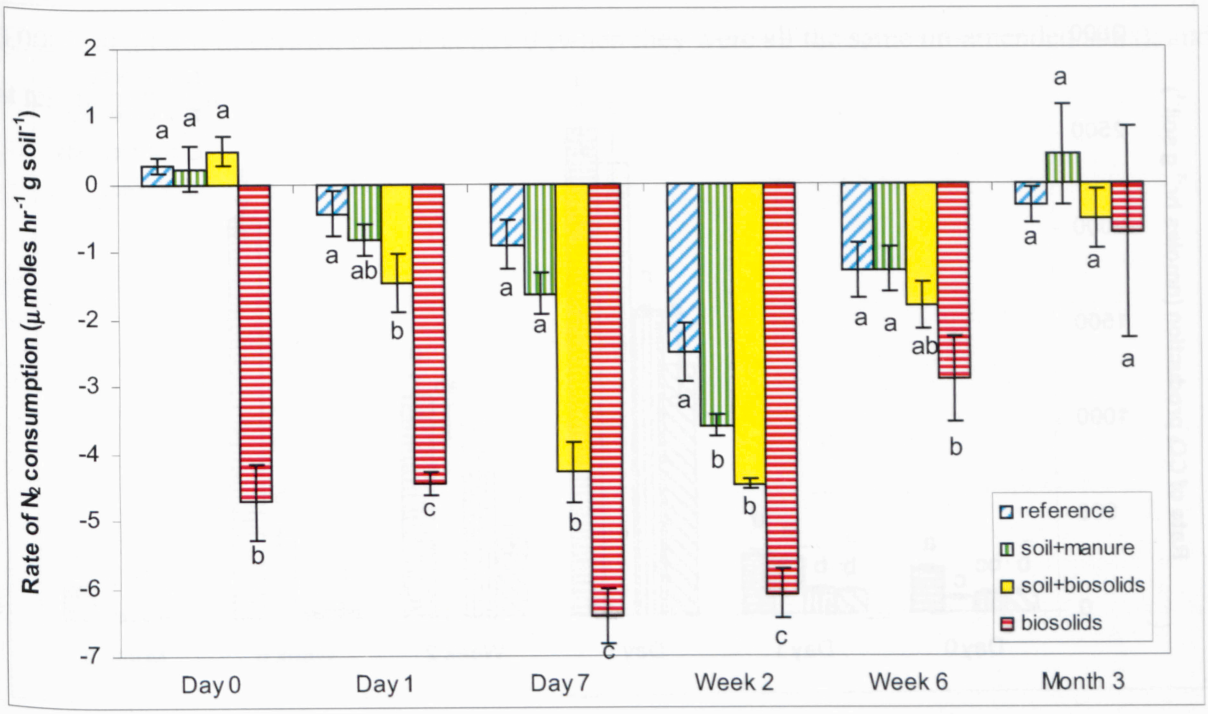
Footnotes:
all concentrations are in µg/g dry weight, from the average of three sub-samples
BDL stands for delow detection limit

6.2 N_2-fixation and General Activity

N_2-fixation over the three-month period showed an increase in most treatments up to weeks 1 and 2, except in biosolids, which were higher at day 0 than day 1 (Figure 6). All treatments showed a decline in activity following week 2. The rates of fixation in control and manure-amended soils were often similar, and lower than in biosolids-amended soil and biosolids treatments. The rate of N_2-fixation in biosolids-amended soil was often lower than in biosolids at most time periods, with the rate of N_2-fixing activity significantly higher in biosolids than all other treatments at all time intervals up to, and including week 6 (except biosolids-amended soil

at week 6). Differences among control, manure-amended, and biosolids-amended soils decreased over time, with no significant difference among the three soil treatments after six weeks, and no significant difference among all treatments at month 3 (test termination) ($p = 0.82$).

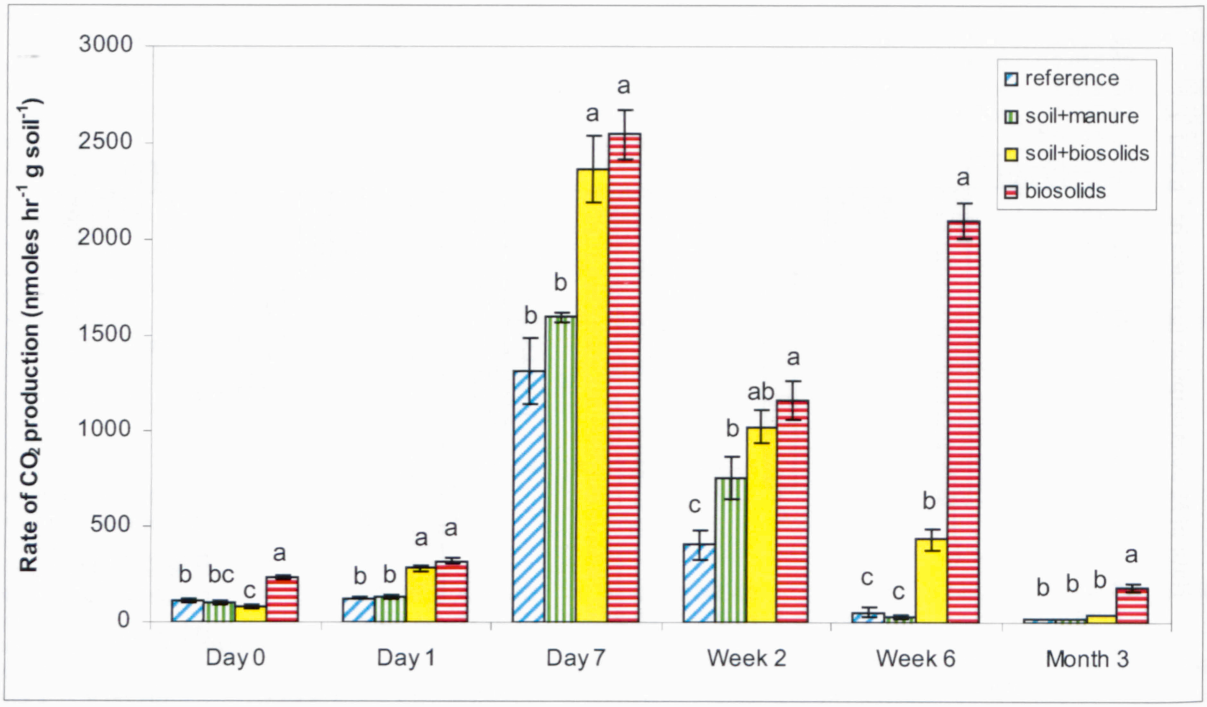
Figure 6: N_2-fixation in all treatments at all test intervals (n = 5; standard error bars shown). For each test interval, N_2-fixation rates among treatments were compared by single-factor ANOVA ($\alpha = 0.05$) and post-hoc LSD t-test; significantly different values are indicated by different letter.



General cellular respiration activity showed a similar pattern to N_2-fixation over the experiment duration (i.e., similar groupings, and level of activity at each test interval) (Figure 7). Respiration increased greatly between day 1 and day 7, and then decreased from day 7 on in most treatments, until test termination at month 3, except in the biosolids, which maintained much higher respiration than the other three treatments at week 6. At most time points activity in biosolids-only and biosolids-amended soils was significantly elevated relative to reference and manure-amended soils, but effects were largely temporary. When comparing all four treatments at test termination, post-hoc tests determined that respiration in the biosolids remained significantly higher than the three soil treatments ($p < 0.0001$), with no significant difference among the three soil treatments. When only comparing mean respiration among the three soil treatments, post-hoc

tests determined there was some enhanced respiration remaining in the biosolids-amended soils relative to reference and manure-amended soils at test termination ($p < 0.0001$).

Figure 7: Cellular respiration in all treatments at all test intervals (n = 5; standard error bars shown). For each test interval, respiration rates among treatments were compared by single-factor ANOVA ($\alpha = 0.05$) and post-hoc LSD t-test; significantly different values are indicated by different letter.



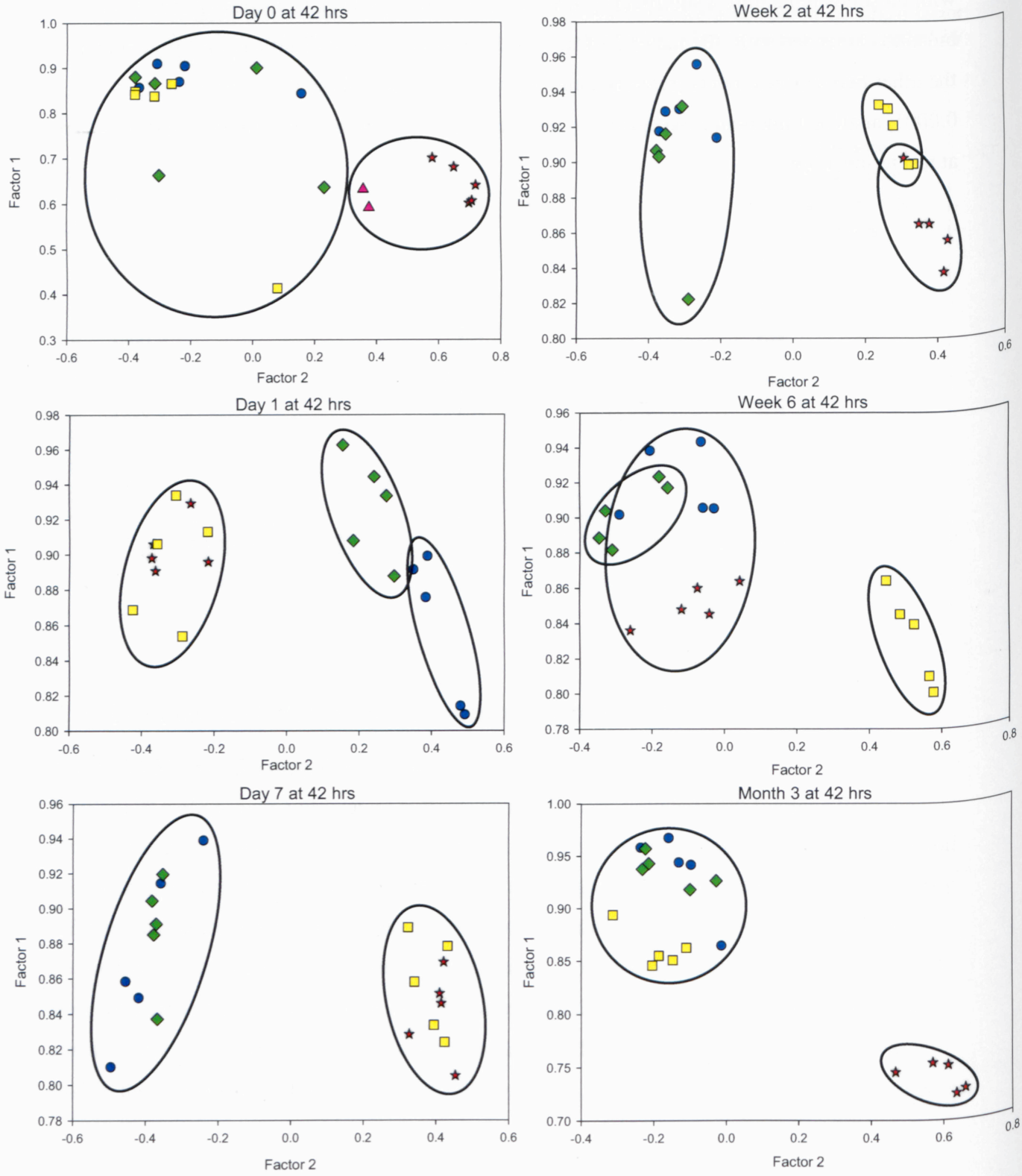
6.3 *General and N_2-fixing Community Structure*

6.3.1 CLPP on Biolog EcoPlates™

EcoPlate™ carbon source utilization patterns generally corresponded with findings from the activity measures. At day 0, all soil treatments (the manure-amended and biosolids-amended soil prior to amendment, and the unamended reference soil) grouped together, and were significantly different than the organic manure and biosolids used to amend the soil ($p < 0.0001$) (Figure 8). Following soil amendment, the reference and manure-amended soils could be clearly discerned from biosolids-amended soils and biosolids, which remained as a group up to two weeks post-amendment (where they formed two distinct groups). Following this test interval, there was a shift in the composition of communities in all treatments, with the reference soil and biosolids

grouping, and significantly different from the manure-amended soil and the biosolids-amended soil, which formed two separate groups ($p < 0.0001$). By test termination (3 months), there was no significant difference between manure and biosolids-amended soils and the reference soils, with biosolids remaining a significantly different group ($p < 0.0001$). Considering strictly the biosolids-amended soils, the factor 2 values for this treatment were significantly different than the other two soil treatments (reference and manure-amended soil) at all test intervals ($p < 0.0001$, at all test intervals), except at day 0 (when they were all the same un-amended soils), and at test termination.

Figure 8: Factor 1 and 2 scores from EcoPlate™ average AWCD-corrected OD. Ellipses indicate groupings of Factor 2 scores (α = 0.05). Biosolids are represented by red stars, reference are blue circles, biosolids-amended soils are yellow squares, manure-amended soils are green diamonds, and manure (only analyzed at day 0) are pink triangles.



With regards to principle component loading scores, carbohydrates and carboxylic acids frequently accounted for a high percentage of the total variability at most test intervals. One carboxylic acid (2-hydroxy-benzoil acid) and two carbohydrates (D-cellobiose and α-D lactose) frequently accounted for the largest proportion of variability among treatments at each test interval. In the last two test intervals (week 6 and month 3), two amino acids (L-asparagine and L-arginine) began to account for the highest level of variability among treatments. The most important carbon sources accounting for the most variability at PC1 and PC2 at each test interval are shown in Table 8.

Table 8: Highest positive and negative PC 1 and PC 2 scores of carbon sources in Biolog EcoPlates™ accounting for variability among treatments at all six test intervals.

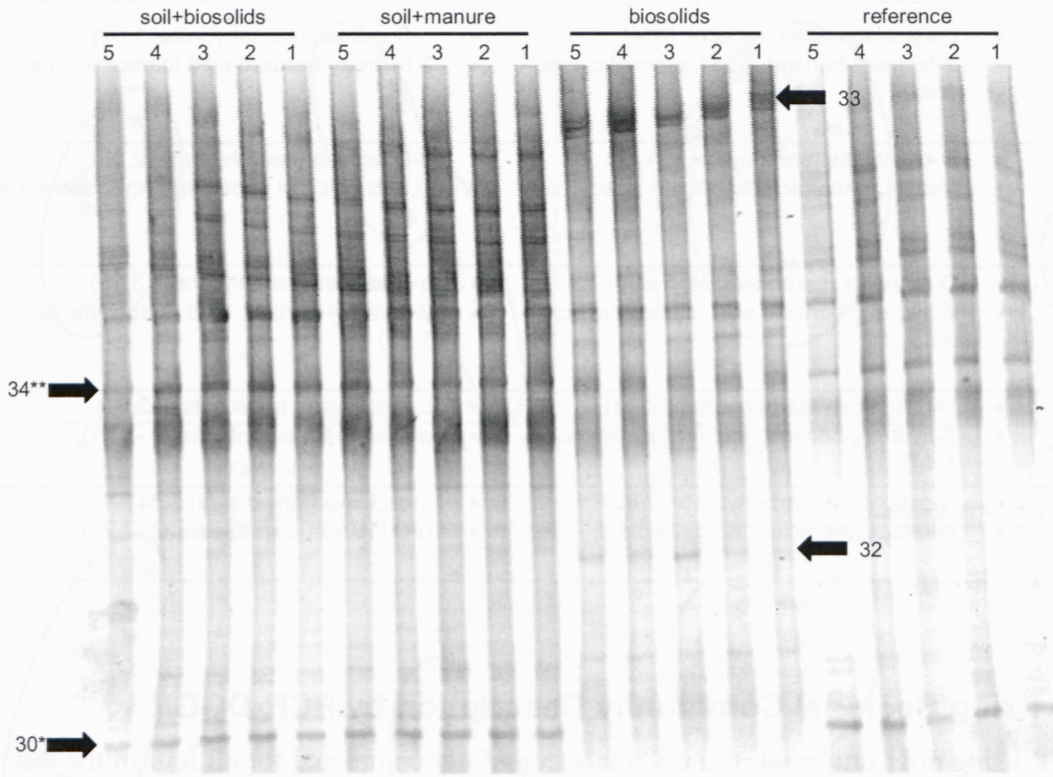
Test Interval	PC 1	PC 2
Day 0	• Tween 40 (polymer; 9.62) • 2-hydroxy-benzoic acid (carboxylic acid; -4.99)	• N-acetyl-D-glucosamine (carbohydrate; 10.58) • γ-hydroxy-butyric acid (carboxylic acid; -3.4361)
Day 1	• L-asparagine (amino acid; 5.54) • 2-hydroxy-benzoic acid (carboxylic acid; -3.96)	• α-D lactose (carbohydrate; 1.69) • 2-hydroxy-benzoic acid (carboxylic acid; -1.36)
Week 1	• D-cellobiose (carbohydrate; 4.87) • glycyl-L-glutamic acid (amino acid; -3.50)	• α-D lactose (carbohydrate; 2.08) • D,L- α-glycerol phosphate (miscellaneous; -1.14)
Week 2	• D-mannitol (carbohydrate; 5.39) • 2-hydroxy-benzoic acid (carboxylic acid; -3.95)	• α-D lactose (carbohydrate; 3.15) • 2-hydroxy-benzoic acid (carboxylic acid; -1.34)
Week 6	• D-cellobiose (carbohydrate; 7.38) • 2-hydroxy-benzoic acid (carboxylic acid; -4.44)	• L-asparagine (amino acid; 3.21) • α-D lactose (carbohydrate; -3.21)
Month 3	• D-cellobiose (carbohydrate; 7.82) • α-ketobutyric acid (carboxylic acid; -4.39)	• L-arginine (amino acid; 5.54) • α-D lactose (carbohydrate; -3.21)

6.3.2 N_2-fixing Bacterial Community Description by PCR-DGGE

Nested PCR using nifH-universal primers was successfully completed for all four of the test intervals chosen for molecular analysis. Visual analysis of DGGE nifH-universal patterns and comparison among treatments was challenging due to the non-uniformity of denaturant gradient. For some treatments it was difficult to determine if bands present were the same as bands in

another treatment, as the gradients were not perfectly horizontal. However, the degree of similarity in banding patterns within treatments was generally high at most time intervals, inferring that differences between treatments were not simply due to stochasticity. Generally speaking, the patterns for nifH-universal (i.e., the N_2-fixing community structure) among the three soil treatments at day 0 (pre-test) were similar, and distinct from biosolids-only patterns (Figure 9). At this point the soil had not been amended, and the soil communities should have been identical. Although there were some bands present in all four treatments, including biosolids (e.g., band #34), there were two bands in the lower section of the gel (band #30, and an un-labelled band above band #32) present in the three soil treatments, but not the biosolids treatment. Conversely, bands #32 and #33 were only present in the biosolids treatment.

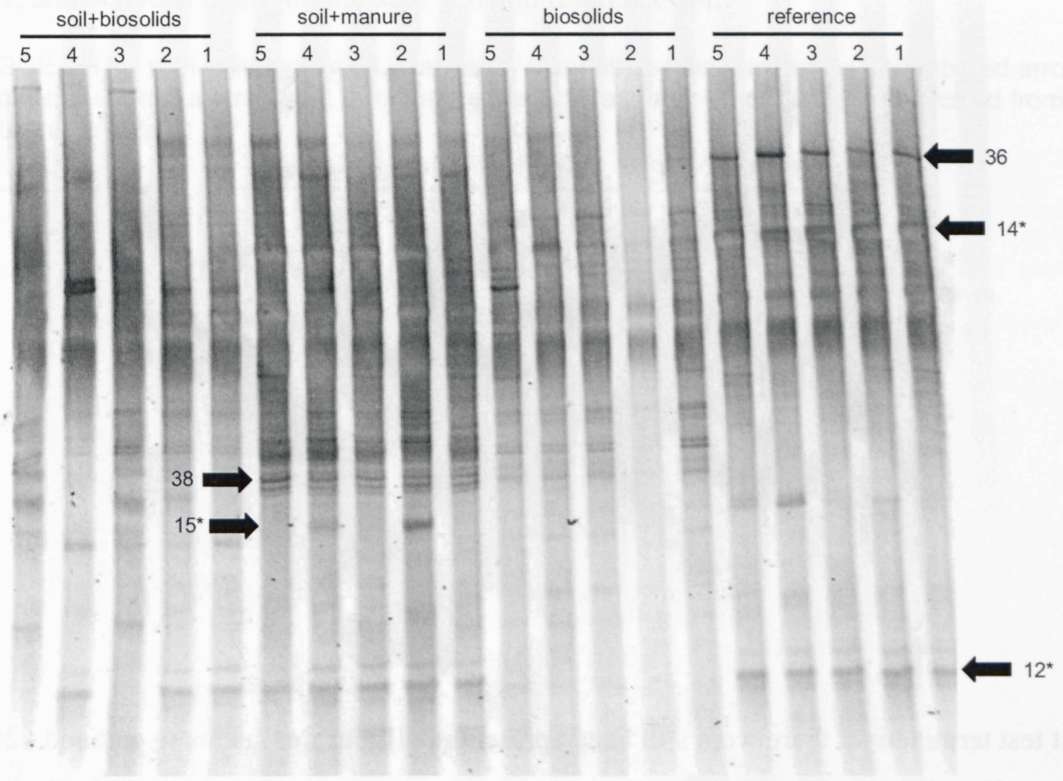
Figure 9: DGGE gel for nifH-universal in all treatments at day 0 (pre-test). Numbered arrows indicate bands of interest that were sequenced; the single asterisk indicates a band excised from the three soil treatments (excluding biosolids-only), and the double asterisk indicates a band excised from all four treatments.



At week 2, there were still some bands present across all three soil treatments, but absent in the biosolids treatment (e.g., bands #12, #15 and #36; Figure 10). However, the overall nifH-

universal banding patterns in the three soil treatments were not as similar as in day 0 (Figure 9). Some bands did appear to be present across all four treatments, including band #14 and the un-marked region of blurred bands below it. In general, the number and intensity of bands in the manure-amended soil treatment appeared higher than all other treatments, especially in the region of band #38.

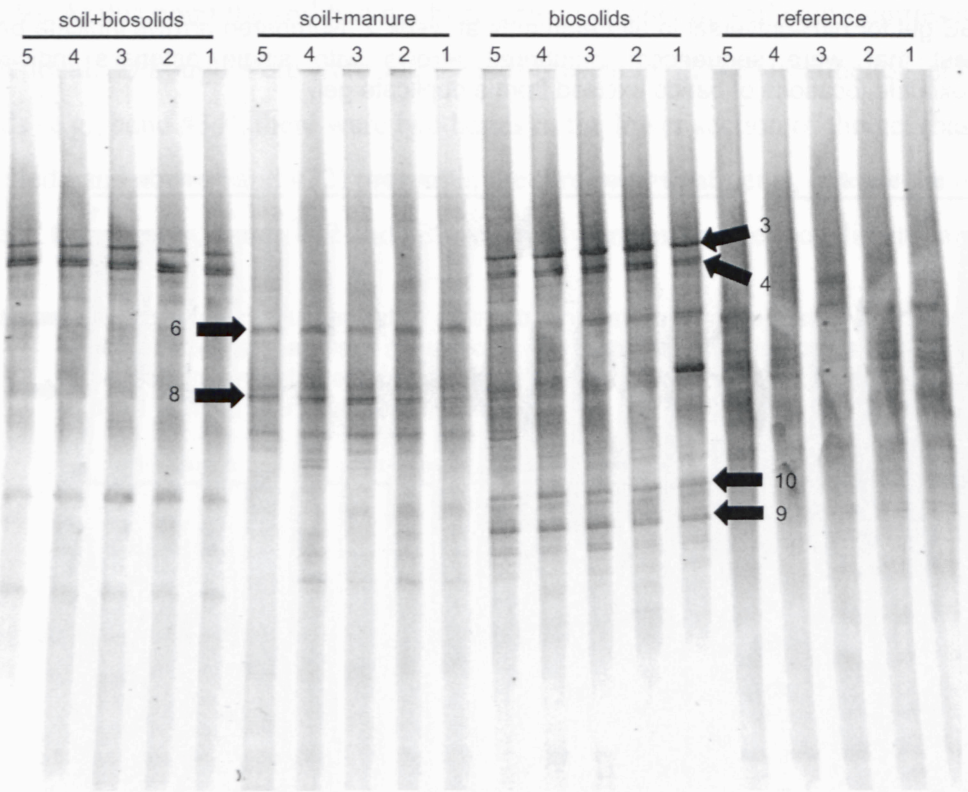
Figure 10: DGGE gel for nifH-universal in all treatments at week 2. Numbered arrows indicate bands of interest that were sequenced; numbered arrows with single asterisks indicate the approximate locations of bands excised from a duplicate gel.



At week 6, the total number of nifH-universal bands (i.e., N_2-fixing community richness) in all treatments was lower, and the appearance of the biosolids-amended soil and biosolids treatment was obviously divergent from the pattern of manure-amended soil and reference soil (Figure 11). In particular, bands #3 and #4 were intense, distinct bands present in the two biosolids treatments, but absent in manure-amended and reference soil. Although there were obvious similarities between the biosolids-amended soil and biosolids treatments, the overall patterns of the two treatments were not identical; there were bands present in biosolids that were absent in

biosolids-amended soils (e.g., bands #9 and possibly #10). Bands #6 and #8 were distinct, bright bands in manure-amended soil and were possibly present in both biosolids and reference soils, but were absent or very indistinct in biosolids-amended soil.

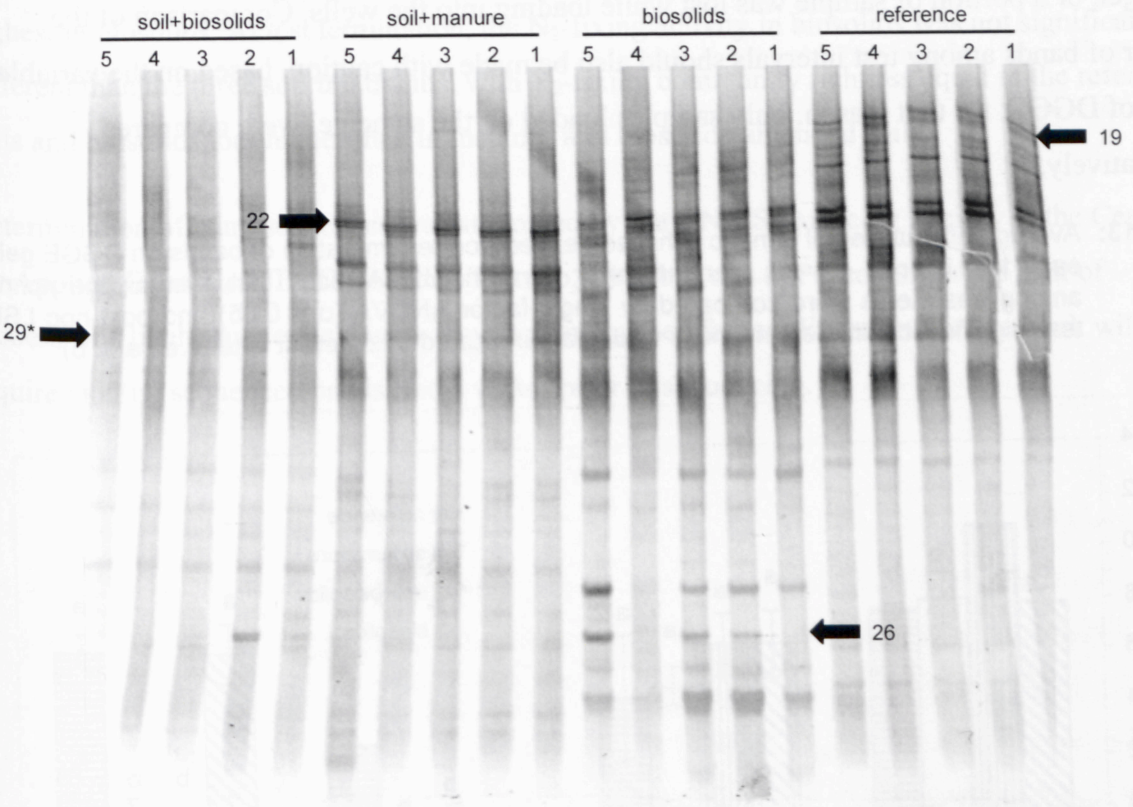
Figure 11: DGGE gel for nifH-universal in all treatments at week 6. Numbered arrows indicate bands of interest that were sequenced.



At test termination, there were still bands present in all four treatments (e.g., band #29 and the blur of bands immediately below it); however, the community composition of reference soils appeared to have diverged from the other treatments more than at any other test interval, particularly with the presence of band #19, and the band immediately below it which did not appear to be present in any other treatments (Figure 12). Manure-amended soil replicate #3 may also contain band #19, though it is indistinct. In general, reference and manure-amended soils appear most similar, especially near band #22 which appears absent in biosolids-amended soil, and is possibly absent in biosolids (the orientation of the denaturing gradient is particularly non-uniform at this section of the gel). Although similar, the two treatments were not wholly identical, as mentioned by the significant divergence at band #19. There were sections of the

biosolids and biosolids-amended soil treatments that were identical (i.e., band #26 which was present in biosolids and biosolids-amended soil but absent in manure-amended and reference soil), however as with other test intervals, the two treatments were not wholly identical, with numerous dark bands in the lower half of the gel only present in biosolids (e.g., the blurred section below band #26), and some bands only present in biosolids-amended soil (e.g., the faint row of bands present in biosolids- and manure-amended soil located 2 bands above the location of band #26). In general, each treatment appeared to have unique components relative to all other treatments, although there were more obvious similarities between biosolids and biosolids-amended soils, and between manure-amended soil and reference soil.

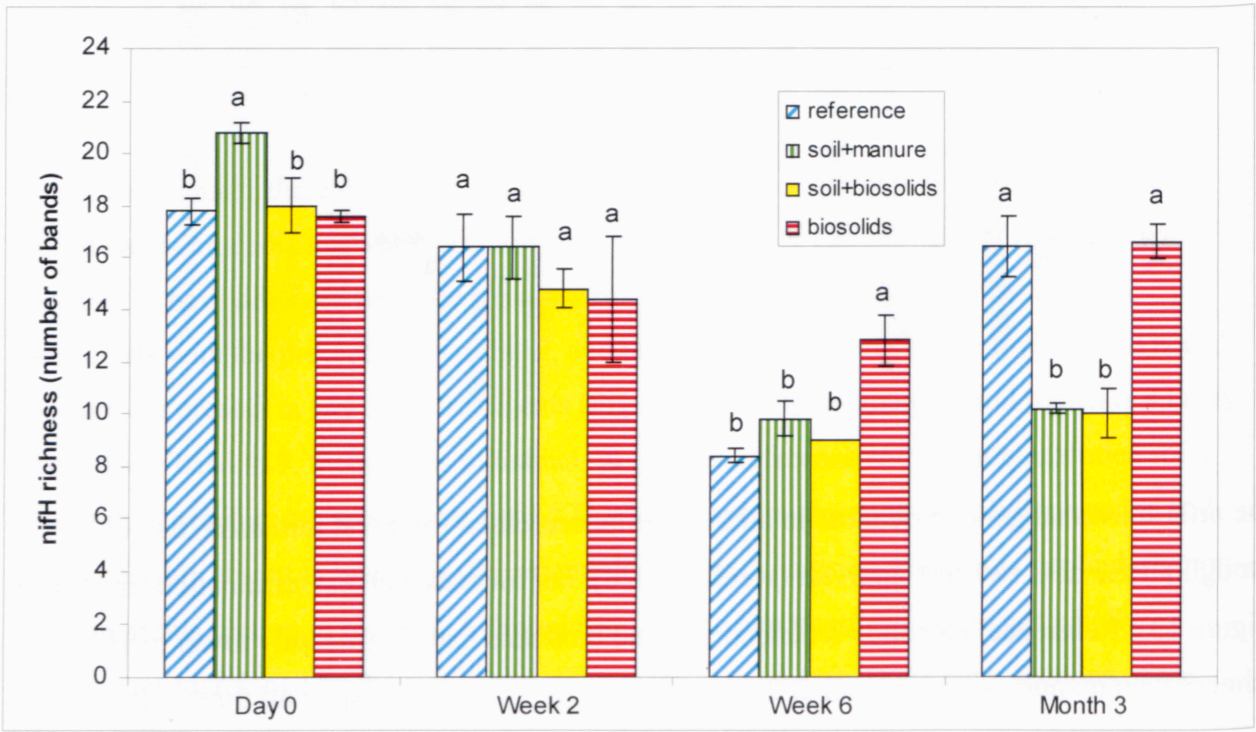
Figure 12: DGGE gel for nifH-universal in all treatments at month 3 (test termination). Numbered arrows indicate bands of interest that were sequenced; the asterisk indicates a band excised from all four treatments.



The nifH-universal genetic richness in all treatments decreased from test initiation (day 0) throughout the next two test intervals (week 2 and 6), but increased at test termination (month 3) (Figure 13). Reference, biosolids-amended soil and biosolids had significantly lower nifH richness than manure-amended soil at day 0 (pre-test) ($p = 0.0065$), despite all three soil

amendment treatments containing the same un-amended reference soil at that time interval. At week 2, there was no significant difference in richness among treatments ($p = 0.7074$). Richness in biosolids was significantly higher than the three soil treatments at week 6 ($p = 0.0004$), and at test termination the richness in biosolids and reference soils was significantly higher than the two soil amendment treatments (manure and biosolids) ($p < 0.0001$), despite the biosolids treatment being extremely desiccated and a fraction of the initial volume. Because of the somewhat subjective nature of method used to enumerate bands (i.e., visual counts), and the variability in resolution of bands within different treatments and different locations on the gel, small differences between the average richness of treatments at a time interval should be interpreted carefully; it is possible that there were more bands present in some treatments, but the camera was not optimally focused, the SYBR® Green stain was not uniformly absorbed throughout the entire gel, or a portion of sample was lost while loading into the wells. Comparison of the number of bands among test intervals should also be made with caution, based on the variable nature of DGGE; for that reason, only samples loaded on the same gel were compared quantitatively.

Figure 13: Average nifH-universal genetic richness derived from enumeration of bands on DGGE gels of each test interval (n = 5; standard error bars shown). At each test interval, the richness among treatments were compared by single-factor ANOVA ($\alpha = 0.05$) and post-hoc LSD t-test; significantly different richness values are indicated by different letter (i.e., a or b).



When considering the structure-function relationship of the N_2-fixing community, at some points in time it appears there were a relatively small number of N_2-fixing species in the biosolids and biosolids-amended soil treatments responsible for N_2-fixing activity. At day 0, N_2-fixing activity in the biosolids treatment was much greater than in the unamended soils (all soil treatments were identical at that point); however, the nifH-universal richness in the biosolids treatment was not significantly different than two of the three soil treatments. At week 2, N_2-fixing activity was also significantly higher in biosolids than the three soil treatments and slightly higher in the biosolids-amended soil compared to manure-amended soil and reference soil; N_2-fixing community richness was not correspondingly high in the two biosolids treatments at week 2, and was slightly lower than the other soil treatments. At week 6, N_2-fixing community richness was significantly higher in biosolids than all three soil treatments and N_2-fixing activity was similarly highest in biosolids. At test termination, the N_2-fixing activity in biosolids was not significantly different than the three soil treatments, with N_2-fixing community richness equal in the reference soils and biosolids, but higher than in manure- and biosolids-amended soils.

Determination of band sequences was attempted by the DNA Sequencing Facility at the Centre for Applied Genomics (TMDT-MaRS, Toronto, ON), but was not successful. The lack of success was likely due to the degeneracy in primers used for amplification. Future work will require cloning sequenced bands into a vector prior to sequencing.

7 Discussion

The Kitchener WWTP biosolids contained levels of commonly measured metals that were well within the range of values reported for other jurisdictions in Table 4, and also included the same high concentration of copper, seen in biosolids from Toronto, the Greater Vancouver Regional District, and New York State. The effect of biosolids metals, including copper, on soil organisms has been well-studied, with high copper in biosolids associated with toxicity or bioaccumulation in bacteria (Bååth *et al.* 1998, Sandaa *et al.* 1999), ciliates (Forge *et al.* 1993), spiders (Larsen *et al.* 1994), nematodes (Georgieva *et al.* 2002) and earthworms (Kruse and Barrett 1985), although many of these employed metal-spiking prior to soil amendment. In addition, two anions (chloride and sulphate) were relatively high in biosolids-amended soils, and although they are not regulated for biosolids land application, they can be toxic in high doses (Eaton 1942, Bright and Addison 2002). An EC_{50} value for chloride as low as 301 $\mu g\ g^{-1}$ was found in a study assessing springtail (*F. candida*) reproductive effects in field soils treated with NaCl (Bright and Addison 2002). The Kitchener biosolids-amended soils had 388 $\mu g\ g^{-1}$ chloride, which is in the same range, implying that level of chloride in soil amended with these biosolids may be of ecological concern.

In terms of the results for PPCP analyses, because of the lack of biosolids effects studies which focus on PPCP, it is only possible to compare the concentrations of PPCPs in Kitchener WWTP biosolids to other reported values. Harrison *et al.* (2006) conducted a survey of numerous organic contaminants from biosolids around the world. They found concentrations of analgesics (in dry weight) for acetaminophen ranging from $6x10^{-7}$ to 4.54 $\mu g\ g^{-1}$, ibuprofen between $6x10^{-6}$ to 3.99 $\mu g\ g^{-1}$, and salicylic acid between $2x10^{-6}$ and 13.74 $\mu g\ g^{-1}$. Biosolids from the Kitchener WWTP stored for the 3-month test duration had acetaminophen toward the higher end of that range ($2.87x10^{-1}$ $\mu g\ g^{-1}$), ibuprofen at the highest end of that range (3.51 $\mu g\ g^{-1}$), and salicylic acid in the middle of that range ($6.49x10^{-1}$ $\mu g\ g^{-1}$). Harrison *et al.* (2006) also found gemfibrozil was below detection up to 1.192 $\mu g\ g^{-1}$, and the antibiotic ciprofloxacin between $5x10^{-2}$ and 4.8 $\mu g\ g^{-1}$. Kitchener WWTP liquid biosolids had $6.88x10^{-3}$ $\mu g\ g^{-1}$ gemfibrozil (in the middle of the range), and $1.73x10^{-2}$ $\mu g\ g^{-1}$ ciprofloxacin (near the bottom of the range). Kinney *et al.* (2007) analyzed biosolids from nine US WWTPs, and found a range of carbon-normalized concentrations of carbamazepine between 15 to 1200 $\mu g\ kg^{-1}$ organic C (with a median

concentration of 68 μg kg^{-1} organic C). The concentration of carbamazepine in Kitchener WWTP liquid biosolids was 6.19 $x10^{-1}$ μg g^{-1}, or 619 μg kg^{-1}, not corrected for organic carbon. Without more toxicity data for these compounds, it is not possible to determine if the levels of PPCPs in Kitchener biosolids would be ecologically significant in the receiving environment (i.e., agricultural soil).

The phytotoxicity exhibited in the biosolids-amended soils may have been caused by the extremely high levels of chloride and sulphate, 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. As noted in Section 3, soil moisture has a large influence on the uptake of biosolids contaminants by soil organisms. In this experiment, soil moisture was not constant throughout the test duration, and may have affected the results. On the other hand, the texture, organic matter, and % clay (which all affect the cation exchange capacity, a common measure of metal bioavailability), was similar among the biosolids- and manure-amended soils among treatments, and there was abundant plant growth in the manure-amended soils, indicating physical aspects of the biosolids-amended soils may not have been the primary cause for phytotoxicity.

Other than the obvious phytotoxic effect in biosolids-amended soils (which may have been simply drought stress), most of the test methods used in this experiment, i.e., measurement of N_2-fixing activity and cellular respiration and CLPP with Biolog EcoPlates™, found evidence of a only a temporary shift in bacterial community activity following land-application of municipal biosolids, namely an increase in N_2-fixing activity and cellular respiration in biosolids and biosolids-amended soils relative to reference soil. Many of the biosolids land-application studies reviewed found similar increases in general cellular respiration and other activity measures (e.g., enzyme activity) following application (Brendecke *et al.* 1993, Stamatiadis *et al.* 1999, Debosz *et al.* 2002, Sánchez-Monedero *et al.* 2004, Sheppard *et al.* 2005). Studies finding depressed microbial activity following biosolids application found these effects in biosolids spiked with

high levels of heavy metals like lead (Dar 1997), naturally-contaminated with heavy metals including copper, lead, molybdenum and zinc (Stamatiadis *et al.* 1999), or at application rates above 350 g kg^{-1} (Wong *et al.* 1998).

The findings of enhanced N_2-fixing activity in biosolids-amended soils in this experiment generally conflicts with most of the studies evaluating free-living and symbiotic N_2-fixation; most studies found decreased N_2-fixing activity (Brookes *et al.* 1986, Mårtensson and Witter 1990, Munn *et al.* 2001, Obbard and Jones 2001, Horswell *et al.* 2003, Broos *et al.* 2004), with only one study by Heckman *et al.* (1986) finding an obvious increase in N_2-fixing activity of soybean rhizobia (*B. japonicum*) in biosolids-amended soils, even at very high rates of biosolids amendment (up to 224 tonnes ha^{-1}). In this study, biosolids had been applied 10 years prior to analysis, and contained naturally-high concentrations of some heavy metals (e.g., 1329 mg kg^{-1} zinc, 13.4 mg kg^{-1} cadmium, and 360 mg kg^{-1} lead). An important difference between the findings from Heckman *et al.* (1986) and those finding inhibition of N_2-fixing activity is that many of them evaluated the effects of biosolids with high metals contamination (Horswell *et al.* 2003, Broos *et al.* 2004), evaluated N_2-fixation in long-term study sites with decades of biosolids application (Brookes *et al.* 1986, Mårtensson and Witter 1990, Obbard and Jones 2001), or used very high application rates (Munn *et al.* 2001).

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 (TIE) 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). Differences between manure and biosolids-amended soil may have also been a result of competition from bacterial species remaining in the biosolids following wastewater treatment, or changes in the nutrient (macro and micro) status of soil causing promotion of species adapted for specific nutrient requirements.

Marschner *et al.* (2003) conducted DGGE analysis with 16S primers and found two distinct microbial communities in biosolids and manure-amended soil, but these differences were not associated with changes in functional diversity (i.e., found evidence of functional redundancy).

Based on the much higher level of N_2-fixing activity and cellular respiration observed in the biosolids and also in biosolids-amended soil, it is likely there was an addition of bacteria and other microbes from biosolids occurring in biosolids-amended soil, and this has been partially confirmed by studies finding increased microbial biomass in soils following biosolids amendment (Dar 1997, Chander *et al.* 2001, Debosz *et al.* 2002, Sánchez-Monedero *et al.* 2004). Sastre (1996) and Marschner *et al.* (2003) have also suggested viable bacterial loading occurs following land-application, with associated shifts occurring in the soil microbial community. Contradictory findings of decreased general microbial biomass following biosolids amendment were found in studies of long-term biosolids application sites (Brookes and McGrath 1984), from biosolids spiked with copper (Bååth *et al.* 1998), cadmium, copper, lead and zinc (Sandaa *et al.* 1999), or zinc (Moffett *et al.* 2003). The findings of decreased biomass following metal-contaminated biosolids application confirms the toxicity of metals, but is less relevant if the metals are much more bioavailable, and at higher single-dose levels than would be typically applied in unadulterated biosolids.

In the absence of obvious changes in the ecosystem (i.e., large-scale deaths, or decreases in activity), determination of ecological effects in microbial organisms is complex, and there may not be straight-forward "positive" or "negative" effects associated with shifts in community structure (Kirk *et al.* 2004). In many cases there are multiple species capable of carrying out a single ecological function (functional redundancy within an ecological niche or guild), and the loss of a single species, while it may not greatly effect the overall function of the guild, may have effects on the resiliency of the community following large-scale disturbance (Kirk *et al.* 2004), and other cascading effects, potentially affecting predator-prey, and intra- and inter-specific competition relationships. Biosolids application or any agricultural activity, including cultivation, constitutes a physical, chemical and biological disturbance of the pre-existing conditions in soil, and provides the opportunity for r-selected colonizers to flourish (Smith 1996). Whether the resulting long-term changes in community structure are wholly positive or negative is not readily determined.

Although most biological test methods found temporary effects of biosolids application, the DGGE profiles of N_2-fixing bacterial community structure showed the possibility of a longer-term shift in community structure up to 3 months post-amendment (Figure 12). At test

termination, there were obvious bands in the reference samples that were not present in the biosolids-amended soils, or even in the manure-amended soils. Although it was not possible to confirm through sequence analysis, it is possible that cyanobacteria in the biosolids-amended soil may have accounted for the bulk of N_2-fixing activity. There was a blue-green microbial mat on the surface of the biosolids-amended soil at approximately 2 weeks post-amendment, at around the same time that the plants began to exhibit acute toxicity. There were only two studies on the effects of municipal biosolids land-application on the diversity of N_2-fixing bacteria; both were conducted on symbiotic rhizobia. Kinkle *et al.* (1987) found *B. japonicum* serogroups were similar in soybeans nodules in soils amended with 1, 56 and 112 tonnes ha^{-1} biosolids. Lakzian *et al.* (2002) found decreased richness in intergenic spacer region groups of *R. leguminosarum* biovar *viciae* (using RFLP analysis), but greater number and complexity of plasmid profiles in metal-spiked biosolids-amended soils. Numerous studies have evaluated the effects of biosolids land-application on the biomass or abundance of symbiotic rhizobia (most frequently *R. leguminosarum* biovar *trifolii*), and have generally found a decrease in biomass and/or abundance (Reddy *et al.* 1983, Mårtensson and Witter 1990, Chaudri *et al.* 1993, Lakzian *et al.* 2002, Horswell *et al.* 2003, Broos *et al.* 2004). One study on diazotrophs found cyanobacterial development was limited on biosolids-amended soils relative to manure-amended soils (Brookes *et al.* 1986). Some studies did find enhanced N_2-fixing rhizobia abundance following biosolids amendment (Kinkle *et al.* 1987, Obbard 2001, Obbard and Jones 2001), and two of these were in soils that had not been amended in over 10 years (Kinkle *et al.* 1987, Obbard 2001). Two studies on the structure-function of *R. leguminosarum* found N_2-fixation ability was absent in biosolids-amended soil, but present in manure-amended soil, and diversity in biosolids-amended soil was reduced relative to *R. leguminosarum* in manure-amended soil (Giller *et al.* 1989, Hirsch *et al.* 1993). These studies were conducted at the same long-term biosolids research plots (Woburn Experimental Farm), which had biosolids applied over a 20-year period starting in 1942 and high levels of cadmium, chromium, copper, lead and zinc. It is difficult to compare these effects on symbionts to free-living bacteria (as evaluated in this experiment), as there are complex interactions between the symbiotic rhizobia and host plant that affect their survival, and create very different growth requirements than for free-living diazotrophs.

Possible environmental conditions which could account for the changes in bacterial community include the level of organic material in test soils. Because of the metabolic expense of N_2-

fixation, free living diazotrophs are typically found in soils with high organic material (Campbell 1993). In our calculation of application rates for biosolids and manure (Appendix B), the level of organic material was normalized by ash-free dry weight, so differences between communities in manure- and biosolids-amended soils may not be a function of organic material content. Oxygen also has a large effect on diazotrophs; as the level of O_2 also decreases, more favourable N_2-fixing conditions are created (Chan *et al.* 1994). The availability of labile carbon sources and the level of O_2 in soils most likely decreased through the test duration as the easily metabolized carbon sources were consumed by all soil microbes (not just N_2-fixers), and microbial respiration occurred. Based on N_2-fixing activity, it appears the optimal conditions for N_2-fixation in biosolids-amended soils were reached in all treatments (except biosolids) at 2 weeks post-amendment (Figure 6). After that point, the metabolic expense of N_2-fixation overwhelmed the availability of organic carbon, and N_2-fixation was no longer possible at the initial high level. Demba Diallo *et al.* (2004) found the abundance of N_2-fixing bacteria was related to soil moisture, with higher abundance under *Acacia tortilis* ssp. *raddiana* and *Balanites aegyptiaca* in dryland Senegal soils during the rainy season.

In future research, longer-term studies are necessary to determine if the shift in N_2-fixing community structure in biosolids-amended soil remains through a single season, and in the years following amendment (biosolids in Ontario are applied on a 5-year rotation). Other researchers have suggested changes in community structure may appear before changes to soil function, and may serve as an early warning of toxicity, especially with regard to metals in biosolids (Giller *et al.* 1998, Witter *et al.* 2000). Studies on pathogen survival following biosolids amendment have found there is generally a period of up to one year before pathogen levels return to pre-amendment conditions (Edmonds 1976, Vasseur *et al.* 1996). Assuming many of the N_2-fixing species in biosolids-amended soil originated from the biosolids, these findings of a general die back of biosolids-originating bacteria have been confirmed, as the activity and general community measurements indicated biosolids and manure-amended soils returned to pre-amendment conditions in reference soils after three months. In terms of the structure of the N_2-fixing community in biosolids-amended soils, without sequence analysis it is not possible to determine if there were in fact fewer species filling the same ecological niche (N_2-fixation) in biosolids-amended soil, which may be of concern if decreased richness and total biodiversity creates a community that is less resilient to future change or perturbation (Kirk *et al.* 2004). It

may also be of concern if the post-amendment bacterial community is dominated by r-selected species, and contains fewer K-selected species, which have been shown to be more sensitive to heavy metals in a biosolids-amendment study on nematodes (Georgieva *et al.* 2002).

8 Conclusions

Applied scientific research is not conducted in a cultural vacuum. Political pressure greatly affected this experiment, and because of issues surrounding public perception of land-applying municipal biosolids, the intended source of biosolids for this experiment became unavailable. Managers of the Ashbridges Bay plant determined it was not politically favourable to allow ecological effects assessments to be conducted on their biosolids. This was a major setback, necessitating the use of a contingency biosolids source with much lower political and ecological importance (in terms of the potential scale of land to be affected by land-application policies). In North America there is a major emphasis placed on public education in order to influence public perception regarding the practice of municipal biosolids land application (Fitzhugh *et al.* 1994, Draman 1995, Hodson 1996). Education is critical to allow the public, especially land-owners of potential land-application sites, to make informed decisions; however, if education efforts minimize the risks associated with biosolids land application, and do not mention the number of studies that have been conducted on the ecological effects of the practice, they are incomplete. If promoted based on the potential cost savings compared to inorganic fertilization or manure, without sufficiently describing the level of contaminants and potential for accumulation in soil, the information is biased. According to the Communication and Public Consultation for Biosolids Management guide, biosolids communication and public consultation programs should adhere to the principles of trust, quality of information, fairness, and commitment, in order to build public relationships (Infraguide 2005).

Current initiatives to increase the "beneficial use" of municipal biosolids in Ontario should consider the potential for long-term ecological effects on soil organisms, which have been shown to occur in numerous studies described here. Unfortunately, the general loss of biodiversity of soil organisms in already heavily modified agricultural systems may not create enough concern to limit land application of municipal biosolids. Shifts in community structure are subtle, longer-term changes are less noticeable, and less often a major concern than large-scale deaths of more visible macroorganisms, such as earthworms. More often, human health effects, or economic losses must occur in order to stimulate changes to current practices, or to encourage funding for future study.

Holistic ecosystem-scale evaluations (at more than one trophic level) are a necessary component of a complete evaluation of ecological effects. Again, it is important to consider the interdependence of each trophic level. In this sense, there are no 'positive' or 'negative' effects, just perturbation of the system from the pre-existing steady-state conditions. Shifts in species composition may not directly alter the biological function of a specific trophic level (if there is functional redundancy); however, if predator-prey relationships are specific (i.e., a predator has a very-specialized prey item), cascading effects at other trophic levels may occur. When effects are propagated to other trophic levels, and occur directly at other trophic levels, there is the potential for much greater holistic changes to the soil ecosystem, and the potential for changes to soil quality, and ultimately, crop production becomes greater. The potential loss of biodiversity in soil ecosystems should constitute a rationale for use of the precautionary approach, and a limiting of, rather than expansion to, "beneficial use" of municipal biosolids before the full extent of impacts to terrestrial and aquatic ecosystems are identified. This is a sentiment that McBride *et al.* (1995) and Harrison *et al.* (1999) strongly advocate in the US.

This study was focused on expansion of research on one small, but agronomically important component of the soil ecosystem. It is unfortunate that the existing Ontario land-application standards for metals and pathogens do not reflect this type of biological effects data. In the UK, standards for zinc were specifically lowered based on evidence of toxic effects on microorganisms (Winder et al 1999). Because of the importance of soil organisms to soil fertility, it is critical for routine biological monitoring programs to be adopted in addition to existing chemical monitoring programs. This is required to ensure there is no ecological harm created when municipal biosolids are land-applied. Chemical monitoring programs alone are not sufficient, because it is not economically feasible to conduct chemical analyses on every potential biosolids contaminant, and the detection of a contaminant does not provide an indication of the bioavailability of the contaminant, the degradation products (especially for PPCPs and other complex organics), or the synergistic effects (Crouau *et al.* 2002).

One critical aspect of a successful biological monitoring program is the selection of appropriate group(s) of indicator organisms (i.e., bioindicators). In order to be effective, bioindicators must be present in sufficient numbers to yield meaningful data, and must have a community structure

that changes in response to the ecosystem stressors of interest (i.e., municipal biosolids amendment) (Golder-EVS 2006).

It is also advantageous to include multiple bioindicators, as individual assemblages will have unique physiological properties and responses to different types of stress (Golder-EVS 2006). European countries have already adopted numerous microbial bioindicators in soil monitoring programs, and the suggested minimum requirements for a comprehensive program include measures of microbial biomass, respiration, N mineralization, community diversity analysis (through CLPP, PLFA or PCR-DGGE), in addition to analysis of specific indicator species (Winding *et al.* 2005).

In Canada, one example of a federal-level biological assessment program with a regulatory framework that could be adopted in a biosolids land-application monitoring program is the environmental effects monitoring (EEM) programs for pulp and paper and metal mining effluent receiving environments. Site-specific evaluation of biosolids receiving environment effects was suggested by Munn *et al.* (2001) and Environment Canada promotes EEM as a concept that is applicable to numerous types of environmental assessment (both regulatory and non-regulatory) that can be used to "help determine the sustainability of human activities on ecosystem health" (Environment Canada 2005). The EEM program is rooted in the federal *Fisheries Act*, and focused on aquatic receiving environments, evaluating the effects of effluent release on fish, fish habitat and the use of fisheries resources through biological as well as chemical water quality monitoring. Terrestrial ecotoxicity tests for pulp mill and municipal biosolids land application have been developed at Ryerson University by Lynda McCarthy and Vadim Bostan (McCarthy *et al.* 2004, Bostan *et al.* 2005), and have involved bacteria, earthworms, and common crop species (e.g., *Brassica rapa*, *Phaseolus vulgaris*). They have also conducted aquatic tests to evaluate the potential effects of biosolids run-off to surficial waterways, and these have involved duckweed (*Lemna*), algae (*Pseudokirchneriella*), other common toxicity test organisms (e.g., *Daphnia magna* and *Hyalella azteca*) (McCarthy *et al.* 2004, Bostan *et al.* 2005). The advantages of these tests are that they are typically shorter in duration (up to 28 days), do not require specialized equipment, involve more simple analytical techniques than those employed here (i.e., visual estimations of % survival), and are more suited to routine monitoring programs which may be carried out by personnel with a limited technical expertise. Additionally, ciliates (e.g.,

Colpoda steinii) and springtails constitute potential bioindicators, as they represent two additional trophic levels, and have already been used in numerous studies reviewed here (Forge *et al.* 1993, Campbell *et al.* 1997, Cole *et al.* 2001, Crouau *et al.* 2002, Petersen *et al.* 2003, Scott-Fordsmand and Krogh 2004). By incorporating biological assessment into routine monitoring programs municipalities will be better able to build public trust, and possibly, greater acceptance of the practice of land-applying municipal biosolids, if they are able to demonstrate a lack of long-term ecological effects. If it cannot be demonstrated that biosolids do not cause ecological effects when land-applied, an alternative management option must be considered.

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Appendix A – Summary of Literature Review Findings on the State-Of-Knowledge of the Ecological Effects of Land-Applying Municipal Biosolids on Agricultural Lands

Appendix A – Summary of Literature Review Findings on the State-Of-Knowledge of the Ecological Effects of Land-Applying Municipal Biosolids on Agricultural Lands

Group	Biosolids Type	Biosolids Contaminants of Concern[†††††††]	Study Location	Soil Characteristics	Impacts	Reference
General Microbes						
Microbes (bacterial, actinomycete, and denitrifyer abundance; respiration; and enzyme activity: dehydrogenase, urease, invertase, cellulase, amylase, and protease)	Municipal sewage (2 sources: Greater Chicago Calumet and Southwest WWTP; heated and anaerobically-digested biosolids)	Heavy metals (in biosolids-amended soils, range of average concentrations at 3 rates of application, as HCl extractable metals mg/kg): • Cd 6.9-16.6 • Cr 17.7-60.7 • Cu 29.9-70.3 • Fe 803.4-1694.9 • Mn 618.5-792.6 • Ni 6.5-10.8 • Pb 36.8-70.4 • Zn 131.5-333.7	USA - Ohio?	Post-amendment (including control): • Blount type silt-loam • pH 6.4-7.4 • Total accumulative biosolids application rates: 0, 92, 185, 369 dry tonnes ha^{-1} applied over 6 year period • Planted with corn	• Elevated biomass and protease, amylase and dehydrogenase activity • Shift in bacterial community structure with more denitrifying bacteria and less N_2-fixing bacteria (*Azotobacter*) following amendment	Varanka *et al.* 1976
Non-specific bacteria (bacterial biomass, respiration)	Municipal sewage (West London; anaerobically digested and lagoon-dried; used long-term biosolids test site soils)	Heavy metals (in biosolids-amended soils; as total and EDTA extractable in mg/kg): • Cd 8.6 / 4.7 • Cu 102 / 57 • Ni 27 / 7 • Zn 289 / 139	England	Long-term biosolids test site (post-amendment): • Woburn Experimental Farm • Sandy-loam • Biosolids applied at 8.2 or 16.4 tonnes OM ha^{-1} a^{-1} between 1942-1961, (>20 years since last application) • pH 6.8 • CEC 22.5 meq/100 g • Organic C 1.97% • Total N 0.17%	• Biomass in biosolids-amended soil was lower than in manure-amended and inorganic fertilized soil, 20 years post-amendment	Brookes and McGrath 1984

[†††††††] The contaminants of concern listed here may not be the only causative agent, they are the contaminants that were measured, reported and in some cases, a selection of contaminants that were significantly elevated

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Bacteria (used isolation techniques for 2 types of communities: total heterotrophic aerobic community and the Cd-resistant community)	Sewage (2 types; sources and treatments not specified)	Heavy metals (in long-term test sites, range from 4 sites as total metals mg/kg): • Cd 1.2-120 • Cu 41-589 • Pb 31-482 • Zn 109-1900 In short-term test sites (as total metals mg/kg) • Cd 1.4 • Cu 37 • Pb 69 • Zn 192	England (laboratory experiment)	2 types of sites used, long-term and short-term (post-amendment) (in Thames Valley): Long-term • Loam • pH 5.5-6.5 • Sludge applied from 1920-1960s Short-term • Loam • pH 5.1 • Sludge applied in 1982	• Cd-resistant bacterial communities contained no Gr+ species • Adaptation to Cd occurred • No effect on total communities	Barkay *et al.* 1985
Non-specific microbes (viable heterotrophic plate counts for bacteria, actinomycetes and fungi; acridine orange direct-counts; dehydrogenase and respiration)	Municipal sewage (City of Tucson; liquid, anaerobically-digested biosolids)	Heavy metals (in biosolids, range for 4 years, as mg/kg dry weight): • Ag 2.67-63 • Cd 7.33-14.5 • Cr 32-94.5 • Cu 568-957 • Ni 26.0-50.8 • Pb 89.3-221 • Zn 800-1590	USA - Arizona	Marana Agricultural Center (in amended and control plots): • Sonoran desert • Pima clay loam soil • Biosolids applied for 4 years at 2 cumulative loading rates: 8 tons ha^{-1} and 24 tons ha^{-1} • CEC 249-270 mmol (+)/kg • Total Organic C 4.5-5.94 g/kg • Total N 747-820 mg/kg • pH 7.7 • Planted with upland cotton (*Gossypium hirsutum* L.)	• Increased dehydrogenase activity and initial CO_2 production following amendment • No effect on microbial abundance • No correlation between microbial activity and cotton yield	Brendecke *et al.* 1993

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Non-specific bacteria (aerobic bacteria and fungi abundance, enzyme activity: urease, phosphatase, and glucosidase)	2 types of sewage sludge (Industrial and urban; source and treatment not specified)	None specified	Spain	Post-amendment soil (including control): • Clay sandy-loam • Organic matter 0.95-3.82% • N 0.08-0.36% • pH 6.89-8.26 • Biosolids applied annually for 8 years at 2 rates: 50 and 100 tons ha^{-1}	• Biosolids introduced microorganisms into soil and increased microbial activity (urease, phosphatase and glucosidase) relative to inorganic fertilized and un-amended control	Sastre *et al.* 1996
Non-specific bacteria (biomass C and N-mineralization rate)	Municipal sewage (Okhla, New Delhi; treatment not specified), biosolids-amended soils spiked with lead salts	Lead spiked biosolids at 3 rates (in biosolids, as mg/kg): • Pb 100 / 250 / 500	India (laboratory experiment)	Pre-amendment, 3 soil types used: • Organic C 0.47%, Sandy loam • Organic C 1.61%, Loam • Organic C 0.72%, Clay loam • Biosolids added at 0.75% DW	• No significant impacts found in most treatments, although decreased microbial biomass, C and N mineralization at very high Pb levels • Higher microbial biomass in biosolids-amended soils • Spiked with Pb salts –higher bioavailability	Dar 1997
Bacteria (used DNA probes for numerous bacterial target groups and also measured non-specific indicators biomass C, respiration rate, Biolog plates and community tolerance)	Municipal sewage (various sources; used long-term biosolids test sites)	Heavy metals (in high rate amended biosolids, as mg/kg): • Cu 8000 • Ni 4000 • Zn 16 000 Heavy metals (in long-term test soils, range from both sites as mg/kg): • Cd 0.22-0.85 • Cr 16-81 • Cu 18-282 • Ni 12-89 • Zn 66-359	England	2 long-term biosolids test sites (post-amendment): Lee Valley • Silty-loam • Test plot since 1968 • pH 6.5 • CEC 22.5 meq/100 g • Organic matter 4.9% Luddington • Sandy-loam • Long-term site, duration not given • pH 5.8 • CEC 11.5 meq/100 g • Organic matter 1.8%	• Significant changes to bacterial community structure found using metal tolerance, PLFA measures (no differences using Biolog plates) • Microbial biomass C and respiration significantly lower in high Cu plot relative to uncontaminated biosolids-amended plots • Used metals spiking - higher bioavailability	Bååth *et al.* 1998

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Non-specific bacteria (CO_2 production, N and C mineralization and PO_3 production rate)	Municipal sewage (Taipo STP anaerobically digested biosolids)	Heavy metals (in biosolids, as total metal mg/kg): • Cd 1.84 • Cu 870 • Mn 337 • Ni 59 • Pb 146 • Zn 1448	Hong Kong (laboratory experiment)	Pre-amendment: • Sandy soil • pH 7.03 • EC 0.081 dS/m • Total N 789 mg/kg • Organic C 1.22 mg/g • Soil had higher Mn and Pb than biosolids • Biosolids applied at 0, 25, 50, 150 and 350 g kg^{-1}	• Increased respiration rates, N mineralization and PO_3 production at most rates of amendment • Decreased C mineralization • Decreased rates at 350 g kg^{-1} rate of amendment	Wong *et al.* 1998
Bacteria (used DNA probes for numerous target groups)	Source not provided – used long-term biosolids test sites, some spiked with Cd, Cu, Ni and Zn to reach the European Union guideline limits	Heavy metals (in biosolids-amended soils, as un-spiked and spiked biosolids amended soils in mg/dry kg): • Cd 0.6 / 2.7 • Cu 21 / 94 • Ni 11 / 32 • Zn 102 / 359	Germany	Pre-existing biosolids test sites (Braunschweig; range for low and high metal biosolids-amended sites): • Sandy-silt • Planted with spring rapes • pH 5.3-6.8 • Organic C 1.07-1.51% • Low metal sites amended with 100 m^3 ha^{-1} yr^{-1}; high metal amended with 300 m^3 ha^{-1} yr^{-1} from 1980-1991	• Significant effects on bacterial community structure and lower number of bacterial genomes • Used metal-spiking - higher bioavailability	Sandaa *et al.* 1999

Group	Biosolids Type	Biosolids Contaminants of Concern††††††††	Study Location	Soil Characteristics	Impacts	Reference
Non-specific bacteria (soil respiration rates, biomass N, N-mineralization rate, nitrification rate and available N)	Municipal sewage (NE Lincoln STP anaerobically digested biosolids)	Heavy metals (in biosolids, as mg/kg): • Cu 1163 • Cr 43.4 • Ni 65.5 • Pb 222 • Zn 2292 All are below US EPA exceptional quality guidelines	USA-Nebraska	2 types of biosolids test sites (range for all biosolids-amended sites; post-amendment): • Silty-clay loam • Planted with wheat • Biosolids application for up to 18 years (3 year rotations), or single application in same year • pH 5.96-6.79 • EC 0.12-0.30 dS/m • Total available N 9.1-19.5 mg/g • C:N ratio 6.0-10.1	• No significant negative impacts to bacterial processes noted – found increased respiration, biomass N, N mineralization and nitrification	Stamatiadis *et al.* 1999
Non-specific bacteria (biomass C, respiration rate, SIR, PLFAs and community tolerance)	Sewage (source not provided – used long-term biosolids test sites)	Same as in Sandaa *et al.* 1999	Germany	Same as in Sandaa *et al.* 1999	• Significant changes in bacterial communities (PLFA profile) • Enhanced respiration rate and biomass in low-metal biosolids-amended soils • Used metal spiking but to fall within EU guidelines	Witter *et al.* 2000

Group	Biosolids Type	Biosolids Contaminants of Concern††††††††	Study Location	Soil Characteristics	Impacts	Reference
Arbuscular mycorrhizal fungi (*Glomus mosseae*, *G. intraradices*, *Gigaspora rosea*)	Sewage (Institute of Hydrological Research, Nancy, France; treatment not specified) three types used: un-spiked, spiked with heavy metals and spiked with organic pollutants	Heavy metals (in biosolids, as un-spiked and spiked in mg/dry kg): • Cd <0.05 / 19.3 • Cu 425 / 1309 • Ni 30 / 189 • Zn 1230 / 3316 Organic pollutants (in spiked biosolids, as mg/dry kg): • BAP 36.4 • Fluoranthene 374 • Phenanthrene 188 • Pyrene 50.4	France (laboratory experiment)	• Quartz sand (1.6-2.5 mm dia) • Mixed as 5% fresh weight biosolids • Planted with *Medicago truncatula*	• Un-spiked and PAH and heavy metal spiked biosolids associated with reduction in fungal colonization • Used high rate of metal spiking - higher bioavailability	Jacquot *et al.* 2000
Bacteria (used DNA probes for numerous target groups)	Sewage (source not provided – used long-term biosolids test sites)	Same as in Sandaa *et al.* 1999	Germany	Same as in Sandaa *et al.* 1999	• No significant impacts on community structure of the culturable fraction of bacterial community, but significant shifts in the non-culturable community • Used metal spiking but to fall within EU guidelines - higher bioavailability	Sandaa *et al.* 2001
Non-specific bacteria (biomass C and qCO_2) and fungi (ergosterol as biomass indicator)	Sewage (source not specified; 2 different sites)	Heavy metals (in biosolids-amended soils, as average for 7 test sites; as total and EDTA extractable in mg/dry kg): • Cu 41 / 23 • Pb 46 / 27 • Zn 227 / 104	Germany	3 sites same as Sandaa *et al.* 1999 (Braunschweig), 4 sites from Gottingen: • pH 6.8-7.4 • CEC 136-263 $\mu mol_c/g$ • Total C 1.3-5.6 % • Total C:N 9.0-11.2	• Found shift in fungi:bacteria ratio and effects on other measures with increasing metals input	Chander *et al.* 2001

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Non-specific bacteria (biomass C and N, PLFA profiles, DFA hydrolysis and β-glucosidase activity, and CO_2 production)	Municipal sewage (WWTP outside Copenhagen, anaerobically digested)	Heavy metals (in biosolids, as mg/dry kg) • Cd 2.4 • Cr 32.5 • Cu 360 • Hg 3.5 Organic pollutants (as mg/dry kg) • LAS 4300 • PAH 7.1 • NPE 49 • DEHP 52	Denmark	Askov Experimental Station, pre-amendment: • Sandy loam • pH 6.8 • CEC 10 meq 100 g^{-1} • Total C 1.6% • Total N 0.14% • Biosolids added at 4.2 tonnes DM ha^{-1}	• Short-term positive changes in soil dynamics following biosolids-amendment	Debosz *et al.* 2002
Ammonia-oxidizing bacteria	Municipal sewage (source not specified; anaerobically digested)	None specifically mentioned	Scotland	Rigg Foot field site, control plots: • Acidic brown forest soils (also described as upland grassland soils) • pH 4.9 • Organic C 23.8% • NH_3 0.70 µmol/g moist soil • Kjeldahl-N 4.27 mmol/g dry soil • Biosolids applied at 10 l m^{-2} and 20 l m^{-2}	• Significant increase in AOB activity and shifts in community structure	Gray *et al.* 2003
Microbes (PLFA analysis, 16S and general eukaryotic PCR/DGGE, enzyme activity: urease, xylanase, protease, alkaline phosphomonoesterase, arylsulfatase	Sewage sludge (source not specified; treatment not specified)	None specified	Germany	Re-claimed mine site (in sludge-amended soil): • Re-claimed loess soil • Experiment established in 1969 • Organic C 0.82% • Total N 0.109% • C:N ratio 7.5 • Sludge applied at rate of 7.6 tonnes ha^{-1} yr^{-1} • Crop rotation with beets, wheat, rye, barley and potato	• Total bacteria PLFAs, Gr+ bacteria, and the ratios of Gr+:Gr- and bacteria:fungi were higher in the sludge-amended soils • PCR-DGGE showed bacterial communities in sludge-amended soil were different than in other amended soils • No relationship between eukaryotic community structure and function	Marschner *et al.* 2003

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Bacteria (16S cloning, determination of operational taxonomic units (OTU))	Municipal sewage (source not specified; spiked with Zn)	Heavy metals (in reference and Zn-spiked biosolids-amended soil, as dry mg/kg): • Cd 0.3 / 0.9 • Cu 13 / 22 • Ni 7 / 6 • Zn 57 / 400	England	ADAS Gleadthorpe Research Center (post-amendment): • Sandy loam • 9% clay • pH 5.7-6.2 • Org C 1.36-1.4% • Planted at various times with barley, Italian ryegrass, sugar beet, white clover, peas • Biosolids applied at a rate of 100 tonnes ha^{-1} (dry solids) in 1982 and 1986	• Biomass decreased in Zn-spiked and unaltered biosolids-amended soil • OTU richness and evenness in Zn-spiked biosolids lower than in unaltered biosolids-amended soil	Moffett *et al.* 2003
Non-specific bacteria (biomass C and qCO_2)	Municipal sewage (source not specified; primary aerobic) with various degrees of composting treatment	Heavy metals (in pre-composted biosolids, as mg/kg): • Cd 0.3 • Cu 17 • Cr 12 • Ni 6 • Pb 13 • Zn 79 All are under current European guideline limits	Spain	Pre-amendment: • Silty-loam • Agricultural semiarid soils • pH 7.8 • CEC 11.9 meq/g • EC 0.28 dS/m • Total N 1.1 g/kg • Org matter 17.2 g/kg • Higher concentrations of some heavy metals than biosolids (i.e., Cu, Mn, Cd)	• Biosolids application caused increased bacterial biomass and activity, no real significant negative impacts found	Sanchez-Monedero *et al.* 2004

Group	Biosolids Type	Biosolids Contaminants of Concern[†††††††]	Study Location	Soil Characteristics	Impacts	Reference
Non-specific bacteria and fungi (changes in CH_4 and CO_2 gas)	Sewage (source not specified; anaerobically digested)	Heavy metals (in pre-composted biosolids, as mg/kg): • Cu 19 • Cr 12 • Ni 8 • Pb 26 • Zn 30 All are under current European guideline limits	Scotland (laboratory experiment)	Rigg Foot site (same as Grey *et al.* 2003); post-amendment: • Grassland system • pH 4.84 • Org matter 15.3% • Total N 0.45% • Total C 6.51% • C:N 14.49 • Some sites had lime treatment	• Biosolids application caused increased bacterial activity, no significant negative impacts found	Sheppard *et al.* 2005
N_2-Fixing Bacteria						
Bacteria (*Rhizobium japonicum*)	Municipal sewage (Winston-Salem NC WWTP; sand dried)	Heavy metals (in biosolids; in mg/kg): • Cd 14 • Cu 496 • Fe 8250 • Mn 323 • Ni 200 • Zn 1020	USA-North Carolina (laboratory experiment)	2 soil types used, both mixed as final sludge:soil ratios of 13:1, 9:1 and 5:1: Mecklenburg clay • pH 7.1 • CEC 16.89 meq/100 g Enon sandy loam • pH 6.35 • CEC 8.45 meq/100 g	• Biosolids applied at 5:1 associated with reduced survival of *B. japonicum*	Reddy *et al.* 1983
Cyanobacteria	Same as Brookes and McGrath 1984	Same as Brookes and McGrath 1984	England (laboratory experiment)	Same as Brookes and McGrath 1984	• Significant impact on Cyanobacteria abundance and on N_2 fixation – last biosolids application over 20 years prior, still altered soil community structure	Brookes *et al.* 1986

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Bacteria (un-specified rhizobia symbiotic with soybean)	Sewage (source not specified; anaerobically-digested and heat-treated)	Heavy metals (range in high and low pH soils and 3 rates of biosolids application in biosolids-amended soil; in mg/kg): • Cd 0.15-0.59 • Cu 5.1-7.8 • Mn 20-53 • Ni 1.0-5.1 • Zn 38-120	USA-Maryland (laboratory experiment)	Soil collected from USDA Beltsville Agricultural Research Center (in all treatments post-amendment, including control): • Fine sandy loam • pH 6.1-7.1 • Single biosolids application in 1976 at 4 rates: 0, 56, 112 and 224 tons ha^{-1} • soils kept at low or high pH with $CaCO_3$ • planted with soybeans (*Glycine max*)	• Nodulation and N_2-fixing activity in soybean symbionts significantly higher in biosolids-amended soil compared to un-amended reference soil	Heckman *et al.* 1986
Bacteria (un-specified rhizobia symbiotic with soybean)	Same as Heckman *et al.* 1986	Same as Heckman *et al.* 1986	USA-Maryland (laboratory experiment)	2 sites, Beltsville (same as Heckman *et al.* 1986) Fairland (post amendment): • Sandy loam • Single biosolids application in 1975 at 3 rates: 0, 56 and 112 tons ha^{-1}	• N_2-fixing activity enhanced at Beltsville, but was decreased with increasing biosolids application rates at Fairland	Heckman *et al.* 1987
Bacteria (*Bradyrhizobium japonicum*)	Same as Heckman *et al.* 1986	Same as Heckman *et al.* 1986	USA-Maryland (laboratory experiment)	Same as Heckman *et al.* 1986	• *B. japonicum* serogroups similar in soils amended with 1, 56 and 112 tons ha^{-1} biosolids • Abundance increased with increasing biosolids application	Kinkle *et al.* 1987
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Same as Brookes and McGrath 1984	Same as Brookes and McGrath 1984	England	Same as Brookes and McGrath 1984	• Significant effects on community structure (lack of diversity) and on N_2 fixation (nodules ineffective)	Giller *et al.* 1989

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Bacteria (cyanobacteria, general diazotrophs and *Rhizobium leguminosarum* biovar *trifolii*)	Municipal sewage (Uppsala sewage treatment works; treatment not specified)	Heavy metals (as total amount applied from 1956-1988; in kg/ha): • Cd 1.2 • Cu 290 • Cr 14 • Hg 1.3 • Ni 7.0 • Pb 31 • Zn 427	Sweden	Ultuna, Uppsala long-term test site (in control and biosolids-amended plots, post-amendment): • Clay soil • pH 5.3-6.2 • %C 1.2-2.7 • %N 0.16-0.28 • C:N ratio 7.42-9.45 • Biosolids applied from 1956 to 1988	• Cyanobacterial growth delayed and N_2-fixation rate 100 times lower, *R. leguminosarum* bv *trifolii* abundance lower and nodulation delayed in biosolids-amended soil • No effect on number of nodules or N-content of plants, and minimal impact on diazotrophic N_2-fixation (only observed after glucose addition)	Mårtensson and Witter 1990
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Municipal sewage (source not specified; anaerobically digested) for 1st year, subsequently, similar biosolids with less metals and spiked Cd, Cr, Cu, Ni, Pb and Zn salts were used for a total of 10 yrs biosolids application	Heavy metals (in biosolids-amended soils, as range for all 4 types of test sites in arable soil and ex-woodland sites; as total in mg/dry kg): • Cd 0.39-4.17 • Cr 16-111 • Cu 16-120 • Ni 6-37 • Pb 33-128 • Zn 82-441 Exceedances of German limits for Cd, Cu, Cr, Pb and Zn and UK limits for Cd, Cu and Zn	Germany	2 long-term biosolids test sites since 1980 (13 yrs prior to study) white clover absent for over 20 yrs; (post-amendment) – Braunschweig but different than reported by Sandaa et al 1999: Site 1 • Silty-loam • old arable soil • pH 6-7 • CEC 7.3 meq/100 g Site 2 • Silty-loam • ex-woodland soil • pH 5.1-6.0 • CEC 9.6 meq/100 g	• Significant impact on rhizobia abundance in non-spiked and spiked biosolids-amended soils	Chaudri *et al.* 1993
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Same as Brookes and McGrath 1984	Same as Brookes and McGrath 1984	England	Same as Brookes and McGrath 1984	• Significant reduction in N_2-fixing ability and shift in rhizobia community in biosolids-amended soils	Hirsch et al 1993

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Municipal sewage (five sources; treatment not specified)	Heavy metals (range in five sources, as mg/kg): • Cd 2-32 • Cr 54-488 • Cu 439-1274 • Hg <2-27 • Ni 20-162 • Pb 81-303 • Zn 512-2669	Australia	Applied five different biosolids samples to one site, and applied one biosolids sample to six sites, range for all six sites, post-amendment: • pH 4.2-6.4 • Total C 7.2-62.8 g/kg • CEC 1.8-37.1 cmol(+)/kg • Biosolids applied once at 3 rates: 0, 60 and 240 tonnes DW ha^{-1}	• Effects of biosolids amendment were highly site-specific, with clover grown in some soils showing reductions in average shoot weight and N_2-fixation • Suggest N_2-fixing effectiveness depends most on soil type, then biosolids type and application level - site-specific evaluations of biosolids effects are required	Munn *et al.* 2001
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Same as Bååth *et al.* 1998	Same as Bååth *et al.* 1998	England	Luddington site same as used in Bååth *et al.* 1998	• N_2-fixation was lower in biosolids-amended soils than reference soils • Spiking of biosolids with metals further reduced N_2-fixation • Rhizobia abundance lower in metal-spiked biosolids-amended soils, but elevated in non-spiked biosolids-amended soils	Obbard and Jones 2001
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Same as Chaudri *et al.* 1993	Same as Chaudri *et al.* 1993	Singapore (soil from Germany)	Same as Chaudri *et al.* 1993	• Significant reduction on rhizobia abundance and activity and N_2 fixation in high-metal biosolids-amended soils, enhancement in low-metal biosolids-amended soils	Obbard 2001

Group	Biosolids Type	Biosolids Contaminants of Concern††††††††	Study Location	Soil Characteristics	Impacts	Reference
Bacteria (*Rhizobium leguminosarum* biovar *vicia*)	Same as Chaudri *et al.* 1993	Same as Chaudri *et al.* 1993	Germany	Same as Chaudri *et al.* 1993	• Decreased overall abundance and diversity in intergenic spacer region groups of *R. leguminosarum* bv *viciae*, but greater number and complexity of plasmid profiles in high metal-spiked biosolids-amended soils compared to un-amended controls	Lakzian *et al.* 2002
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Municipal sewage sludge (source not specified; heavy metal contaminated raw sludge used)	Heavy metals (in biosolids; mg/kg): • Cd 3-10 • Cu 400-570 • Cr 180-320 • Ni 190-270 • Pb 64-130 • Zn 1620-2400	New Zealand	• pH 6.0 • Total C 5.9% • Total N 0.48% • Biosolids applied from 1991-1994 • Total of 9300 wet tonnes applied (8-9 tonnes N ha^{-1})	• Confounding effects – difficult to determine biosolids-based effects – did see some lower abundance and biosensor activity in biosolids plots relative to control	Horswell *et al.* 2003
Non-specific rhizobia and *Rhizobium leguminosarum* bv *trifolii*	Municipal sewage sludge (various sources; some anaerobically-digested, others not specified)	Elevated Cd and Ni in one sludge, Zn and Mn in another sludge, elevated Cd, Cu, Ni and Zn from a third sludge	Belgium (laboratory study, soil from numerous European countries used)	Soils from France and England (range for all sites): • pH 5.2-7.5 • CEC cmol/kg 2.5-29.3 • Total C 0.9-6% • Biosolids applied at various rates and durations, up to 100 tonnes DM ha^{-1} 2 yr^{-1}, and for up to 10 years	• Effects on N_2-fixing ability in white clover apparent at low abundance of *R. leguminosarum* bv *trifolii* (log MPN < 3) • metal toxicity resulted in reduction of N_2-fixation at one site	Broos *et al.* 2004

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Bacteria (*Rhizobium leguminosarum* biovar *trifolii*)	Same as Broos *et al.* 2004	Same as Broos *et al.* 2004	Same as Broos *et al.* 2004	Soils selected from same sites as used by Broos *et al.* 2004	• *R. leguminosarum* bv *trifolii* abundance decreased with increasing Zn-contamination	Broos *et al.* 2005
Protists (Ciliates)						
Ciliate (*Colpoda steinii*)	Municipal sewage (Persley; treatment not specified) biosolids spiked with heavy metals	Heavy metals (in biosolids-amended soils, as EDTA extracted un-spiked and spiked to 2 x European standard, in mg/dry kg): • Cd 0.37 / 10.5 • Cr 0.54 / 8.7 • Cu 21.4 / 199 • Ni 2.0 / 98 • Pb 26.8 / 1259 • Zn 29.5 / 790	Scotland (used soil solutions in laboratory experiments)	Soils collected from the Macaulay Institute • Sandy loam • Biosolids added in 1988 (3 years prior) at 100 tonnes ha^{-1} • Planted with grass/clover mix	• Significant reduction in abundance in soil solutions from soils amended with biosolids spiked to 2 x European standard for Ni and Zn relative to un-amended control soils • Used metal spiking but to fall within EU guidelines, and double EU guidelines (higher bioavailability)	Forge *et al.* 1993
Ciliate (*C. steinii*)	Municipal sewage (various sources; used long-term biosolids test sites) Source not provided, biosolids spiked with one of Cu, Ni or Zn at a low and high rates of amendment	Heavy metals (in long-term test sites (range from both sites; mg/kg): • Cu 22-54 • Ni 16-32 • Zn 77-140	England (used soil solutions in laboratory experiment)	Same as Bååth *et al.* 1998	• Significant reduction in abundance in soil solutions from soils amended with biosolids having high and low-levels of Cu, Ni and Zn, but not in un-spiked biosolids-amended soils, relative to un-amended control soils	Campbell *et al.* 1997

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Arthropods						
Aphids (*Lipaphis erysimi*, *Myzus persicae* and *Brevicoryne brassicae*), flea beetles (*Phyllotreta cruciferae* and *P. striolata*), caterpillars (various late instar Lepidopteran species including: *Pieris rapae*, *Plutella xylostella*, *Diacrisia vierginica* and *Trichoplusia ni*)	Municipal sewage (mixture of 2 sources; Groton, NY and Marathon, NY; treatment not specified)	None specifically mentioned	USA – New York	Fox Research Farm • Silt loam soil • Planted with collard (*Brassica oleracea*) • Biosolids applied at a rate of 220 tonnes ha^{-1} (dry) • Biosolids applied in October 1983 and collards planted in May 1984	• After 14 weeks, flea beetle abundance was significantly higher on collards in biosolids-amended soils relative to un-amended control • After 14 weeks, alate aphid and Lepidopteran abundance was significantly lower on collards in biosolids-amended soils relative to un-amended control	Culliney and Pimentel, 1986
Web spiders (orb weavers, Araneidae) and ground spiders (wolf spiders, Lycosidae)	Municipal sewage (Milwaukee, WI Milorganite brand; anaerobically digested, heat dried commercially-available product)	Heavy metals (as loading rate, range for all plots in g ha^{-1} yr^{-1}): • Cd 177-529 • Cu 2867-3410 • Pb 2177-4238 • Zn 7758-11 478	USA-Ohio	Post-amendment: • Plots treated for 11 years, monthly, at a rate of 1792 kg ha^{-1} (1.792 tonnes ha^{-1}); total application of 8960 kg ha^{-1} yr^{-1}	• Significant bioaccumulation of Cd, Cu, Pb and Zn in spiders • Potential for movement up the foodchain	Larsen *et al.* 1994
Ground beetles (Carabidae)	Municipal sewage (Milorganite)	Same as Larsen *et al.* 1994	USA-Ohio	Same as Larsen *et al.* 1994	• Abundance, diversity and richness greater in biosolids plots • 2 species bioaccumulated Cd, Pb and Zn	Larsen *et al.* 1996
Springtails (Collembola, various species)	Municipal sewage (treatment not specified; 3 types used: uncontaminated, naturally Zn-rich and Cd-rich)	Heavy metals (in biosolids, as un-contaminated and metal-rich in mg/kg) • Cd 1.94 / 48.9 • Zn 725 / 6619 Biosolids-amended soil in mg/kg • Cd 0.33 / 2.2 • Zn 94.8 / 270	Scotland	SAC Auchinchruive • Well-drained sandy-clay loam overlying sandy-loam subsoil • Planted with Italian ryegrass Organic C 26 g kg^{-1} • Biosolids applied annually since 1994 (2 yrs prior to study) as de-watered cake	• Contaminated biosolids not associated with increased abundance, but uncontaminated biosolids were; • Species-specific effects (changes in community composition) in biosolids amended soils	Cole *et al.* 2001

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Springtails (*Folsomia candida*)	Municipal sewage (source not specified; digested, dehydrated and centrifuged)	None specified	France (laboratory experiment)	Synthetic soil • 70% quartz sand, 20% kaolinite clay, 10% peat • Biosolids application rates: 0, 12.5, 25 and 50%	• Reproductive effects LOEC 12.5%, EC50 > 50%	Crouau *et al.* 2002
Springtails and mites (numerous species)	Municipal sewage (source not specified; from two areas of the WWTP, high-contaminant sludge from the pre-settling tank-anaerobically-digested and dewatered, and the low-contaminated sludge from the aeration tank which was dewatered	Heavy metals and organic contaminants (in biosolids; range for high- and low-contaminated sludge, in mg/dry kg) • Cd 2.2 / 1.4 • Cr 30 / 18 • Cu 278 / 215 • Hg 2.6 / 0.9 • Ni 22 / 18 • Pb 106 / 68 • Zn 977 / 447 • PAHs 9.15 / 3.38 • DEHP 55 / 27 • NP 60 / 12.5 • LAS 2870 / 110	Denmark	Three-year field trial, planted with spring barley, oats and spring barley in 1998, 1999 and 2000, biosolids applied at 80% of expected crop N requirement: Askov Experimental Station • Same as Debosz *et al.* 2002 Lundgaard • Loamy sand • pH 6.3 • CEC 6.7 meq 100 g^{-1} • Total N 0.1%	• No effects on abundance of springtails and mites at Askov, but elevated abundance at Lundgaard in some treatments (high-contaminated biosolids, compost and manure-amended soils) • Species composition shifts; some species more abundant in high-contaminated biosolids-amended plots	Petersen *et al.* 2003
Springtails (*Folsomia fimetaria*)	Municipal sewage (Skaevinge rensningsanlaeg; unspiked, and spiked with NP)	Nonylphenol (in biosolids; as uncontaminated and the range spiked in mg/kg) • NP 15 / 25-200	Denmark (laboratory experiment)	• Sandy-loam • pH 6.5 • humus 3.0%, clay 10.6%, silt 11.8%, sand 74.6% • Biosolids added at a rate of 7 tonnes ha^{-1} (2.85 g sludge g^{-1} dry soil) • Biosolids pellet added at same concentration, but not homogenized	• Biosolids spiked with NP caused mortality and reduced reproduction • Biosolids spiked with NP and left as pellets also caused toxicity, but was less toxic	Scott-Fordsmand and Krogh, 2004

Group	Biosolids Type	Biosolids Contaminants of Concern[†††††††]	Study Location	Soil Characteristics	Impacts	Reference
Earthworms and Nematodes						
Earthworms (no species provided, used anecic worms)	Municipal sewage (source not specified)	Heavy metals (in sewage sludge, mg/kg dry weight): • Cd 44 • Cr 470 • Cu 950 • Pb 950 • Zn 3200	Switzerland	Worben • Cultivated farmland • Clayey mineral soil • Biosolids (95% water) applied annually at a rate of 20 tonnes ha^{-1} since 1976	• Significant bioaccumulation of Cd and Zn in earthworms from biosolids-amended soils, relative to control soils	Diercxsens *et al.* 1985
Earthworms (*Lumbricus rubellus*)	Municipal sewage (Milorganite – same as in Larsen *et al.* 1994 and 1996)	Heavy metals (in biosolids-amended soil, average of 4 sampling sessions; in µg g^{-1} dry weight): • Cd 1.29 • Cu 16.9 • Pb 23.09 • Zn 136.9	USA – Ohio	Same site and application rate as Larsen *et al.* 1994 and 1996, except only 4-year duration	• Significant bioaccumulation of Cd, Cu and Pb in earthworms in biosolids-amended soil relative to un-amended control	Kruse and Barrett, 1985
Earthworms *(L. terrestris)*	Municipal sewage (source not specified; 3 sources with different chemical precipitation processes: Calcium hydroxide, aluminum sulphate and ferric chloride)	Heavy metals (in biosolids-amended soils, as an input (kg ha^{-1}) in Ca-, Al- and Fe-biosolids: • Cd <1 / 3 / 1 • Cr 41 /138 / 22 • Pb 16 / 42 / 57 • Zn 94 / 385 / 84	Canada – Guelph, ON	Post-amendment • Elora Experimental Farm • Silty-loam • Biosolids applied from 1973-1980 at 200, 400, 800 and 1600 kg ha^{-1} yr^{-1} N (study conducted 1989-1990)	• Earthworm biomass significantly greater in biosolids-amended soil relative to un-amended control • Al-biosolids-amended plots were the only to have elevated Cd concentrations in earthworm tissue (up to 570 µg g^{-1} ashed weight)	Tomlin *et al.* 1993
Earthworms (numerous species)	Municipal sewage (source not specified; dried, anaerobically-digested de-watered biosolids, 28% solid)	None mentioned	Australia	Kentgrove South • 3 sites, with Alfisol or Inceptsol soil type • Planted with subterranean and white clover, cocksfoot, phalaris, perennial ryegrass and ryecorn • Biosolids applied once in 1992 at a rate of 30, 60 and 120 tonnes ha^{-1} (study conducted 7 years later)	• Increased abundance and biomass in all biosolids-amended soils relative to un-amended control • Shifts in species composition • Caged bioassays found no significant effects	Baker *et al.* 2002

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Nematodes (numerous species (community-level assessment)	Municipal sewage (source not specified, used 3 types: uncontaminated, spiked with Cu, Ni, Zn, Zn+Cu and Zn+Ni)	Heavy metals (in biosolids; as cumulative concentrations in soil, range for each spiking treatment, as mg/kg) • Cu 173.6-476.9 • Ni 19.1-44.2 • Zn 161.1-596.7 • Zn+Ni 75.2-141.7 / 12.3-27.0 • Zn+Cu 108.9-493.2 / 67.6-301.5	England	Same as Moffett *et al.* 2003	• Found shift in community structure to "stressed communities" 8 years post-biosolids amendment	Georgieva *et al.* 2002
Earthworms (multiple species)	Municipal sewage (various sources; treatment not specified)	Organic pollutants (range in biosolids-amended soils; as pg/dry g) • PBDEs 29-840 000 • PCBs 450-3800 • PCDD 16-7900 • PCDF 14-340	Sweden	Four test sites (post-amendment); Igelosa and Petersborg: • Medium/light clays • Organic matter 4.7-5.4% and 2.6-2.8% • Biosolids applied every 4 years from 1981-1997 at 1 or 3 tonnes (dry) ha^{-1} Lamna • Slightly clay soils • Organic matter 3.3-3.7% • Biosolids applied 1998 at 2.3 tonnes (dry) ha^{-1} Bjorketorp • Organic matter 4.9-5.7% • Planted with clover • Biosolids applied 1978-1982 at 25 tonnes (dry) ha^{-1}	• BCAFs in decreasing order were: ortho-PCBs ~ PBDEs > non-ortho-PCBs > 2,3,7,8-PCDD/Fs	Matscheko *et al.* 2002

Group	Biosolids Type	Biosolids Contaminants of Concern††††††††	Study Location	Soil Characteristics	Impacts	Reference
Earthworms *(L. rubellus)*	Municipal sewage (Milorganite – same as in Larsen *et al.* 1994 and 1996)	Heavy metals (in biosolids amended soils; measured from 1978-1993, as mg kg^{-1}): • Cd 1.3-2.7 • Cu 16.9-36.0 • Pb 23.1-48.0 • Zn 81.0-140.5	USA – Ohio	Same as Larsen *et al.* 1994	• Genotypic and allele shifts in biosolids-amended soil populations	Peles *et al.* 2003
Earthworms *(L. terrestris)*	Municipal sewage (Ankara Wastewater Treatment Plants; anaerobically-digested)	Heavy metals (in biosolids and biosolids-amended soil, as total metals in mg kg^{-1}): • Cd 6.3 / 1.1 • Cu 214.5 / 33.7 • Cr 135.2 / 26.8 • Pb 180.4 / 38.6 • Ni 75.8 / 46.5 • Zn 435.9 / 59.1	Turkey (laboratory study)	Soil collected from Ondokuz Mayis University Agricultural Faculty • Under cultivation for 15 years • Biosolids applied at 0, 25, 50, 100, 200, 300 and 400 g kg^{-1}	• Earthworm DHA and ASA enzyme activity decreased in biosolids-amended soils (ASA only at rates >100 g kg^{-1}) relative to un-amended control soil • Earthworm UA and APA enzyme activity significantly increased in biosolids-amended soil relative to un-amended control soil	Kizikaya and Hepşen, 2004

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Earthworms (*Eisenia foetida*), and nematodes (*Caenorhabditis elegans*)	Municipal sewage (19 large municipalities, treatment not specified)	Heavy metals (range for biosolids, control and biosolids-amended soil, in mg kg^{-1}): • As <0.1-30 • Cd 0.35-40 • Cu 9.8-585.9 • Cr 13.65-560 • Fe 164.71-41 776 • Hg <0.1 (all) • Pb 0.7-170 • Mn 170-2072 • Mo 0.35-15.75 • Ni 12.95-130.55 • Se <0.1-50 • Zn 49-1042.7	USA (laboratory study)	Soils collected from multiple sites, 7 sites with short- and long-term biosolids amendment used for toxicity testing; range for control and biosolids-amended sites: • pH 4.7-7.8 • Organic matter 2.8-10.5 % • Conductivity 0.39-4.65 dS m^{-1}	• Earthworm bioassays found significant reduction in growth in two sites and significant increase in cocoon production in two of the long-term application sites. • Nematode bioassays found mortality in two long-term sites and one short-term application site	Banks *et al.* 2006
Multiple Trophic Levels						
Winter wheat (*Triticum aestivum* cv. *Brigadier*), grain aphids (*Sitobion avenae*), beetles (*Bembidion lampros*)	Municipal sewage (source not specified; anaerobically-digested)	Heavy metals (in biosolids-amended soil at 10 and 15 tonnes ha^{-1} application; as mg kg^{-1}): • Zn 31.1 / 30.7	England	East Lulworth, Dorset • Sandy loam • pH 4.5-4.6 • Total C 3.1-3.5% • Biosolids applied twice at rates: 0, 5, 7.5, 10, 15, and 20 tonnes ha^{-1} (DW); only 0, 10 and 15 tonnes ha^{-1} (DW) plots used for this study	• Zn bioaccumulated in all 3 trophic levels • Biomagnification might occur; however, Zn in each trophic level only significantly different among biosolids treatments in 2 of 4 winter wheat harvests, and not in aphids or ladybirds	Winder *et al.* 1999

Group	Biosolids Type	Biosolids Contaminants of Concern†††††††	Study Location	Soil Characteristics	Impacts	Reference
Spring wheat *T. aestivum* cv. *Alexander*), aphids (*S. avenae*), ladybirds (*Coccinella septempunctata*)	Same as Winder *et al.* 1999	Heavy metals (in biosolids-amended soil at 10 and 30 tonnes ha^{-1} application; as mg kg^{-1}): • Cd 0.169 / 0.284 • Zn 49.0 / 62.1	England (laboratory study)	Same as Winder *et al.* 1999 • 0, 10 and 30 tonnes ha^{-1} (DW) biosolids-amended plots used for this study	• Zn bioaccumulated in wheat and aphids, not ladybirds • Cd did not bioaccumulate in any trophic level (not significantly different among treatments)	Green *et al.* 2003

Appendix B – Calculation of Soil Amendment Rates

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Sample	crucible - empty	crucible+wet biosolids	weight wet biosolids	crucible+105°C oven dried biosolids	weight dry biosolids	% solids	crucible+550°C oven dried biosolids	weight 550°C dry biosolids	% organic matter
1	17.8423	28.1824	10.3401	18.4763	0.6340	6.1315	18.0916	0.2493	60.6782
2	18.9347	28.3084	9.3737	19.5090	0.5743	6.1267	19.1559	0.2212	61.4835
3	18.6450	28.3971	9.7521	19.2066	0.5616	5.7588	18.8742	0.2292	59.1880
4	18.1814	28.4062	10.2248	18.7695	0.5881	5.7517	18.4192	0.2378	59.5647
5	18.1239	28.0618	9.9379	18.7011	0.5772	5.8081	18.3540	0.2301	60.1351
average:			9.9257		0.5870	5.9153		0.2335	60.2099
					% liquid:	94.085			

for experiment - want equivalent to 18 dry tonnes biosolids ha^{-1}
Area of 1 L Ziploc container is 12 x 12 cm (144 cm^2)
convert tonnes to grams: 18 tonnes x 1 000 000 grams/1 tonne = $1.8x10^7$ grams
convert hectares to cms: 1 hectare x 100 000 000 cm^2/1 hectare = $1x10^8$ cm^2

for container with area of 144 cm^2, use: $1.8x10^7$ grams/$1x10^8$ cm^2 = XX grams/144 cm^2
xx grams = 25.92 grams biosolids -- dry weight

convert dry to wet weight: use ratio wet : dry of 9.926 : 0.587
9.926/0.587 = xx/25.92
xx grams wet weight = 438.30 grams Weight of biosolids added to each container

reference container - need to water with 412 mL - account for water in biosolids (94% of 438.3 grams)

Sample	crucible - empty	crucible+wet manure	weight wet manure	crucible+105°C oven dried manure	weight dry manure	% solids	crucible+550°C oven dried manure	weight 550°C dry manure	% organic matter
1	15.8138	25.9478	10.1340	20.7460	4.9322	48.6698	18.9145	3.1007	37.1335
2	17.9148	28.0250	10.1102	22.6354	4.7206	46.6915	20.8385	2.9237	38.0651
3	18.1063	28.4221	10.3158	22.9887	4.8824	47.3293	21.0355	2.9292	40.0049
4	18.0851	28.0852	10.0001	22.8660	4.7809	47.8085	20.9952	2.9101	39.1307
5	18.0915	28.1330	10.0415	22.9101	4.8186	47.9869	21.0622	2.9707	38.3493
average			10.1203		4.8269	47.6972		2.9669	38.5367
					% liquid:	52.3028			

org. matter in biosolids / manure: 1.5624046

for experiment - want to also use equivalent of 18 dry tonnes per hectare

still want 25.92 grams dry weight manure

convert dry to wet weight: use rato wet : dry 10.120 : 4.827
10.120/4.827 = xx/25.92
xx grams wet weight = 54.34 grams
accounting for lower organic matter content than biosolids -- 54.34 x 1.56 = 84.77 grams Weight of manure added to each container

to account for moisture in manure = 84.77 x .52 = 44 mL water
SO -- add 412 mL minus 44 mL - to account for difference between biosolids and manure moisture content
`= 368 ~ 370 mL water added to manure-amended containers

Appendix C – Detailed Experimental Protocols for GC/MS, Biolog EcoPlates and DGGE

Appendix C – Detailed Experimental Protocols for GC/MS, Biolog EcoPlates and DGGE

GC/MS (N_2-fixation and cellular respiration)

Part A: Vial preparation

1. Weigh out 2.0 g soil sample of each treatment (x 20 total) on analytical balance using weighing paper and transfer into clean (sterile) 20 mL glass vial (Wheaton 20 mL serum glass vials) pre-labelled with colour-coded laboratory tape as follows:
   - green = soil+manure 1-5
   - blue = control 1-5
   - red = biosolids 1-5
   - yellow = soil+biosolids 1-5

   Immediately after weighing out, seal vial with rubber septum stopper.

2. Inject 100 µL of $^{15}N_2$ (Scotty Bottle – from Scott Analytical Services) into each vial (x 20). Also inject 100 µL of $^{15}N_2$ into an empty vial – this will be the air blank. Fill a small balloon directly from the Scotty Bottle spigot and pierce the balloon wall with the syringe to obtain the gas. Place in Falcon 6-well tissue culture plates (used old ones from Bostan lab) according to randomly generated plate reading order as follows (used excel command "=rand()*(20-1)+1)":

run order	treatment number	treatment ID
1	3	control 3
2	6	soil+biosolids 1
3	13	soil+manure 3
4	18	biosolids 3
5	17	biosolids 2
6	12	soil+manure 2
7	9	soil+biosolids 4
8	2	control 2
9	14	soil+manure 4
10	20	biosolids 5
11	10	soil+biosolids 5
12	15	soil+manure 5
13	1	control 1
14	19	biosolids 4
15	11	soil+manure 1
16	8	soil+biosolids 3
17	5	control 5
18	-	blank
19	16	biosolids 1
20	7	soil+biosolids 2
21	4	control 4

Part B: GC/MS analyses

1. RUAC lab – immediately after spiking with $^{15}N_2$, take 0 hr reading on GC/MS (AutoSystem XL Gas Chromatograph with a Perkin Elmer TurboMass Mass Spectrometer). Go to TurboMass 4.1 software (password protected) – will have "Leigh" program with the run parameters already set: run time = 1.0 min, flow rate = 0.5 mL/min, injection temp = 80°C, oven temp = 35 °C (isothermal), split off, scan TIC from 10-50 m/z

2. In the software - highlight the identifiers for all samples to be analyzed (will be 21 each time – the 20 test vials plus the 1 air blank) – select "run" and wait for equilibration and pre-run to be completed.

3. Inject each sample manually with a glass 100 µL gastight syringe – before each injection, draw sample of ambient air, release into air. Insert into rubber septum of first vial, draw up 100 µL sample, release into air. Re-insert into rubber septum and draw up to ~90 µL, pump back into vial, repeat (total of 2 "pumps"), then draw up to 80 µL then back down to 50 µL. Wait until pre-run is at 0.1 sec and place syringe vertically over injector port. Wait until GC control panel and computer screen say "Ready". Place syringe completely vertical and immediately above injector port. Use left hand to guide needle straight down into injector port, and hold syringe barrel with right hand. As soon as barrel hits injector port plate, depress plunger and pull out quickly, and quickly hit "RUN" button with left hand.

4. After run has completed, select the chromatogram, select "mass" and enter 30. Read off maximum peak heights for parameters of interest at that reading interval (i.e., at 30 ($^{15}N_2$), 40 (Ar) and 44 (CO_2)). Make sure to de-select the stopwatch icon so the real time empty chromatogram for the subsequent run isn't on the screen.

5. Take plates out of incubator at next defined reading interval – 6 hrs for CO_2 analysis, 48 hrs for $^{15}N_2$ analysis (separate datasheets for CO_2 and N_2) – repeat steps 2-4.

Part C: Data analysis

1. **Convert measured signal value to be analyte signal strength relative to Argon:** Convert recorded value of analyte signal strength (m/z) to measured 'relative to argon' value ($^{15}N_2$:Ar ratio, O_2:Ar ratio, CO_2:Ar ratio) for air blanks (for each test interval; at each time interval of 0hr, 6hr and 48hr) and all individual analyte measurements

   $$\text{analyte signal relative to argon} = \frac{\text{recorded analyte signal strength (as m/z)}}{\text{Ar signal strength (as m/z)}}$$

   e.g., for CO_2 in the day 0 air blank, at 0 hr: $\frac{3.26 \times 10^5 \text{ m/z}}{1.18 \times 10^6 \text{ m/z}} = \mathbf{0.28}$

   e.g., for CO_2 in the day 0 biosolids #1 sample, at 0 hr: $\frac{4.42 \times 10^6 \text{ m/z}}{1.26 \times 10^6 \text{ m/z}} = \mathbf{3.51}$

2. **Determine 'true' $^{15}N_2$:Ar ratio, O_2:Ar ratio, CO_2:Ar ratio that should exist in the samples:** Use known average concentrations of each molecule in atmospheric air (Pidwirny

2007). By determining what the true ratio should be in atmospheric air, this can be used to determine the 'drift' or provide a measure of how inaccurate the measured ratio is from the GC/MS used in this experiment. The 'true' value is used to correct the 'measured' value from each air blank used at each test interval (for 0hr, 1 day, 7 days, 2 weeks, 6 weeks and 3 months) – only used 1 air blank per test interval (6 times total).

Ar in atmosphere is 0.93% (Pidwirny 2007), and average atmospheric conditions are such that a 25.8 mL vial (24.13 mL after subtracting the volume taken up by the soil) should have 0.93% Ar (0.224 mL which equals 9.17×10^{-6} moles).

I know I injected 100 µL of $^{15}N_2$ into the vial, so there has to be 4.087×10^{-6} moles of $^{15}N_2$ in the vial (1 mole / 24.47 L = x mole / 0.0001 L)

Average atmospheric conditions are such that a 25.8 mL vial (24.13 mL after subtracting the volume taken up by the soil) should have 20.95% O_2 (5.055 mL which equals 2.067×10^{-4} moles), and 0.0360% CO_2 (0.008687 mL which equals 3.55×10^{-7} moles) (Pidwirny 2007).

- for $^{15}N_2$ - injected 4.087×10^{-6} moles $^{15}N_2$, and air has 9.17×10^{-6} moles Ar

  $^{15}N_2$ 'true ratio' = $\frac{4.087 \times 10^{-6} \text{ moles } ^{15}N_2}{9.17 \times 10^{-6} \text{ moles Ar}} = \mathbf{0.446}$

- for O_2 - known O_2 is 2.067×10^{-4} moles and air has 9.17×10^{-6} moles Ar

  O_2 'true ratio' = $\frac{2.067 \times 10^{-4} \text{ moles } O_2}{9.17 \times 10^{-6} \text{ moles Ar}} = \mathbf{22.541}$

- for CO_2 - known CO_2 is 3.55×10^{-7} moles and air has 9.17×10^{-6} moles Ar

  CO_2 'true ratio' = $\frac{3.55 \times 10^{-7} \text{ moles } CO_2}{9.17 \times 10^{-6} \text{ moles Ar}} = \mathbf{0.039}$

3. **Determine correction factor for measured ratio (instrument measurement error):** Now have 'measured' $^{15}N_2$:Ar ratio, O_2:Ar ratio, and CO_2:Ar ratio and know what the 'true' ratios should be. A correction factor for the GC/MS can be calculated from these values as follows:

   measured ratio x correction factor = true ratio or

   $$\frac{\text{true } ^{15}N_2\text{:Ar ratio}}{\text{measured } ^{15}N_2\text{:Ar ratio}} = \text{correction factor for } ^{15}N_2$$

   e.g., correction factor for CO_2 in the day 0 air blank, at 0 hr: $\frac{0.039}{0.28} = \mathbf{0.140}$

   So for each air blank, it is necessary to divide the true ratios by the measured ratios to determine the correction factors for each analyte ($^{15}N_2$, O_2, CO_2).

4. **Calculate the corrected signal strength ratio values for each analyte:** use the correction factors from previous step to determine the corrected signal strength:

   correction factor x analyte signal relative to argon = true ratio-corrected analyte signal strength

   e.g., corrected signal strength value for CO_2 in the day 0 biosolids #1 sample, at 0 hr:
   3.51 x 0.140 = **0.491**

5. **Determine corrected analyte quantity based on what true quantity of Ar should be in the vial:** Calculate the analyte quantity in the vial relative to what the actual or 'true' quantity of argon is in the vial using the known quantity of Ar which should be in the vial ($9.17x10^{-6}$ moles Ar). The true ratio of each analyte (relative to Ar) is known, and if the 'true' amount of Ar in the vial is known, the 'true' amount of analyte can be calculated by cross-multiplying as follows:

   $$\text{corrected signal strength ratio} = \frac{\text{analyte}}{\text{Ar}} = \frac{\text{unknown analyte quantity (x moles)}}{\text{'true' Ar quantity } (9.17x10^{-6} \text{ moles})}$$

   corrected signal strength ratio x 'true' Ar quantity = unknown analyte quantity

   e.g., corrected value for CO_2 in the day 0 biosolids #1 sample, at 0 hr:
   0.491 x $9.17x10^{-6}$ moles Ar = **$4.51x10^{-6}$ moles CO_2**

6. **Determine the difference in analyte quantity over the analyte-specific test period (to be used to calculate the rate of change in analyte quantity):** Difference determined for CO_2 over a 6-hour duration (from 0 to 6 hrs post-injection), difference determined for $^{15}N_2$ and O_2 over a 42-hour duration (from 6 to 48 hrs post-injection) as:
   final analyte quantity – initial analyte quantity = difference in analyte quantity

   e.g., difference in CO_2 in the day 0 biosolids #1 sample from 0hrs post-injection to 6 hrs post-injection:
   $7.25x10^{-6}$ moles CO_2 - $4.51x10^{-6}$ moles CO_2 = $2.75x10^{-6}$ moles CO_2

7. **Calculate rate of analyte production (or consumption) per hour per gram soil**: Divide the difference in analyte quantity by the analyte-specific test period (6 or 42 hours), and by the quantity of soil (2 g in each vial) as follows:

   $$\frac{\text{difference in analyte quantity}}{\text{6 (or 42 hrs) x 2 g}} = \text{rate of production (or consumption) } hr^{-1} \text{ g soil}^{-1}$$

   e.g., rate of CO_2 production in the day 0 biosolids #1 sample:

   $$\frac{2.75x10^{-6} \text{ moles } CO_2}{\text{6 hrs x 2 g soil}} = 2.29x10^{-7} \text{ moles } CO_2 \text{ } hr^{-1} \text{ g soil}^{-1}$$

8. **Convert $^{15}N_2$ to $^{14}N_2$+$^{15}N_2$:** assumptions are that I injected 100 µL of $^{15}N_2$, so there has to be $4.087x10^{-6}$ moles of $^{15}N_2$ in the vial (1 mole / 24.47 L = x mole / 0.0001 L). Atmospheric conditions are such that a 25.8 mL vial (24.13 mL headspace after subtracting volume of soil) should have 78.08% $^{14}N_2$ (Pidwirny 2007), which equals 18.84 mL $^{14}N_2$, and $7.70x10^{-4}$ moles $^{14}N_2$ (1 mole / 24.47 L = x mole / 18.84 mL). Assume at all times, there is a ratio of $^{15}N_2$:$^{14}N_2$ = $4.087x10^{-6}$:$7.70x10^{-4}$ = 0.00531. The inverse is $^{14}N_2$:$^{15}N_2$ =188.40, so the calculated concentration of $^{15}N_2$ is converted to $^{14}N_2$ by multiplying the $^{15}N_2$ concentration by 188.4, and the total concentration of $^{14}N_2$+$^{15}N_2$ is obtained by adding the two concentrations together.

Biolog EcoPlates

Part A: Plate preparation

1. Take 4 or 5 3 mL modified plastic syringe core samples of soil from each treatment container (x 20)

2. Place in pre-labelled whirl-pak baggie and homogenize – labelled with:
   - Date / time sampled
   - Treatment ID (control 1-5, soil+biosolids 1-5, soil+manure 1-5, biosolids 1-5)
   - Time ID (day 0, day 1, week 1, week 2, week 6 and month 3)

→ the remaining soil not used for BIOLOG EcoPlates is used for nifH (nitrogenase gene) PCR-DGGE study component – the MoBio PowerSoil™ DNA extraction kit requires 0.25g

3. Weigh out 2.5 g soil sample of each treatment on analytical balance and place in new (sterile) 50 mL plastic centrifuge tube – labelled with treatment ID

4. To each of the 20 centrifuge tubes (and the additional tube which will be a blank – making 21 total):
   - Add 25 mL millipore water using dedicated 25 mL graduated cylinder),
   - Add a small drop Tween 80 (Victorio *et al.* 1996 – used 0.01% Tween 80 v/v) (measure using plastic disposable pipet) and
   - Add 25 µL sodium pyrophosphate (Victorio *et al.* 1996 – used 0.01% sodium pyrophosphate v/v) to each centrifuge tube (this is the extraction step – these two substances are "de-flocculating agents")

5. Secure lid, up-end each centrifuge tube to completely mix soil into liquid phase (so it does not adhere to bottom of centrifuge tube) then place on Vortex Genie for ~ 10 seconds or until it appears to be completely mixed.

6. Place weighed and balanced centrifuge tubes into tabletop centrifuge – model HN-S International Equipment Co. (originally in Lab 305, but not used, so we were allowed to move it into the Bostan lab) set for 1000 rpm for 5 minutes
   - Can only do 8 samples a time in tabletop centrifuge so need to do as 3 batches (8, 8, 5 +1 water balance tube)

7. Transfer 10 mL of supernatant (note colour / turbidity) into clean, autoclaved 50 mL round bottom centrifuge tube – pre-labelled as treatment ID

8. Add 10 mL phosphate buffer to tube (to wash bacterial pellet) using 10 mL dedicated phosphate buffer syringe with aliquots taken from clean, dedicated beaker

9. Seal tube with parafilm and place on Vortex Genie for ~10 seconds until it appears the sample is completely mixed

10. Take rack of all 21 centrifuge tubes into room 311 and use Sorvall RC 5C Plus centrifuge with rotor SS34 (Code 5) set at 10 000 rpm and 10 minutes – balance and weigh centrifuge tubes to ensure opposite tubes are balanced with top loading scale on bench opposite centrifuges.
    - Can only do 8 samples a time in centrifuge so need to do as 3 batches – can use 2 centrifuges at a time, so each round will take 20 minutes (do 8 and 8 then 5 +1 water balance)

11. After centrifuged, remove supernatant with dedicated, clean 10 mL plastic pipette and add another 10 mL of phosphate buffer to re-wash the bacterial pellet

12. Repeat steps 7-10 two more times (3 washes total). Note successive size / appearance of bacterial pellet in each step. Total duration of extraction/washing takes 1 hour – 20 minutes per step x 3 steps

13. After 3rd wash step, once the supernatant is removed, add 12 mL 0.85% NaCl saline to the tubes, seal with parafilm and vortex on Vortex Genie for 10 seconds.

14. Place sample into clean multi-channel pipettor reservoir – these are disposable – use 1 reservoir per treatment (5 total – 1 per treatment and 1 for method blank)

15. Use 8 channel multi-channel pipettor from Dr. Foster's lab to load a single BIOLOG EcoPlate with 100 µL sample into each well

16. Place plate (with lid on) into incubator in the Bostan lab (Sanyo Incubator) set at 25° C and NO light. Stack plates according to randomly generated plate reading order used for GC/MS.

Part B: Plate reading

6. RUAC lab – take plates out of incubator at first defined reading interval – keep in correct run order and place in Rubbermaid tote

7. Turn on plate reader – Thermo Labsystems MultiSkan Ascent and computer – go to saved session (sessions can be created individually) called "Leigh.sed" – saved on C:\ASCSW26\Leigh\ – this has BIOLOG EcoPlate well information, thesis contact information, the run order and automatic commands saved

8. press "START" – software will automatically put up message to load first plate (control 3) – will automatically scan at 570 nm (Ryerson does not currently have a 590 nm filter) and then open door and request second plate (soil+biosolids 1) press "continue" and door will close and second plate will be read – will cycle through all plates until finished all 21

9. to SAVE – save ALWAYS as a session in each day's folder – automatically saves all sheets – BUT – in order to use data in Excel on other computers without the Ascent software, must manually save each sheet into the day's folder as the treatment ID (so will have 21 files with each treatment, plus the 4 session files in each day's folder)

a. save on C drive
b. PRINT each sheet as hardcopy back up after EACH session / day

DGGE

Part A: Casting the gel

1. The DGGE gel denaturant solutions used are shown below. All solutions (except the 25x TAE buffer) came with the Bio-Rad DCode Electrophoresis Reagent Kit for DGGE/CDGE:

Reagent	45% denaturant solution	70% denaturant solution
40% acrylamide	25 mL	25 mL
25x TAE buffer	4 mL	4 mL
Formamide	18 mL	28 mL
Urea	18.9 g	25.2 g
dH2O	Fill to 100 mL	Fill to 100 mL

2. Because I used 10% acrylamide/bis gels of 16 x 16 cm size and 1 mm thickness, 16 mL of each denaturant solution was required per gel. Immediately before casting, 150 µL of 10% ammonium persulfate and 15 µL of TEMED (of 99% purity), which creates a final concentration slightly below 0.09% for both reagents, was added to each denaturant solution. The concentration of ammonium persulfate and TEMED allowed approximately 20 minutes before total polymerization in a pre-test trial.

3. A 25-well comb was used for each gel, to facilitate loading all 5 replicates of the 4 treatments for each time interval. A 1 mm and 0.75 mm comb was used; however, use of the 0.75 mm comb in a 1 mm gel is not recommended for future DGGE work, as the remaining gel on the outside of the wells (created by having a comb which was 0.25 mm smaller) created striations of acrylamide which trapped air pockets and made it difficult to flush out the wells.

Part B: Loading and running the gels

1. Before loading gels, wells were flushed to remove any remaining denaturant or unpolymerized gel material, and the 1x TAE running buffer was pre-heated to 60°C for approximately 2 hours.

2. For each sample, 20 µL of the nested PCR product and 4 µL of 6x loading dye were pipetted into 200 µL tubes, mixed in the pipette, then loaded into each well using specialized narrow DGGE gel pipette tips.

3. Gels were run for 17 hours at 100 V and 60°C (equivalent to 1700 V-hours). For each run, two gels containing all treatments from an entire test time interval were run (day 0 and month 3 were run together, and 2 weeks and 6 weeks were run together).

Part C: Visualizing the gels

1. Following DGGE, gels were transferred by hand to clean acrylamide sheets and placed in a plastic tote. A solution of 1:10 000 SYBR® Green I dye was poured over the gel and the tote was covered in aluminum foil for 30 minutes. The SYBR® Green stain solution was re-used, and stored at 4°C in aluminum foil-wrapped 1 L glass bottles. Because of the photo-instability of the dye, the solution was refreshed with 10 µL of stock 10 000x SYBR® Green I dye before soaking each gel.

2. Gels were visualized in the Wolfaart laboratory using their BioDoc-It™ Imaging System and transilluminator table (UVP, Upland, CA) fitted with a special SYBR® green lens. Gel images were saved digitally on compact flash and printed on a UVP Mitsubishi P93 printer.

3. Bands of interest were excised using ethanol-rinsed razor blades and excised gel material was placed in a sterile 500 µL tube. Gels were homogenized into 1 mm diameter pieces and then soaked in 100 µL sterile Millipore water. Samples were sent to the DNA Sequencing Facility at the Centre for Applied Genomics (TMDT-MaRS, Toronto, ON) for sequencing.

Appendix D – Principal Component Loading Scores for Biolog EcoPlates™

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Carbon class	Carbon source	day 0	day 0	day 1	day 1	week 1	week 1	week 2	week 2	week 6	week 6	month 3	month 3
		Prin1	Prin2	Prin1	Prin2	Prin1	Prin2	Prin1	Prin2	Prin1	Prin2	Prin1	Prin2
amine	phenylethyl amine	-3.32411	-0.879	0.49957	0.66225	-0.61834	0.78081	-0.99832	-0.185	-1.86889	1.25333	-1.88568	1.46214
amine	putrescine	1.96685	-1.1424	1.41249	-0.50195	-0.13549	-0.10618	0.24905	-0.04378	-0.78231	1.9788	-0.28586	1.75174
amino acid	L-arginine	-0.90979	0.7988	1.3203	0.87037	1.79971	0.92861	2.12832	-0.00294	-1.02363	2.13742	0.25502	2.40873
amino acid	L-asparagine	6.26597	-0.6183	5.53761	-0.91841	4.34212	0.68332	4.99003	0.15374	3.19656	3.21456	4.29965	1.62636
amino acid	L-phenylalanine	-3.4623	0.103	-2.86782	-0.24826	-2.67459	-0.94558	-3.60446	-0.91376	-3.02476	0.10503	-3.88839	-0.39114
amino acid	L-serine	6.32107	-1.2507	2.5054	-0.24593	3.02251	-0.22337	2.06199	0.40266	0.55914	2.10447	0.42311	2.18038
amino acid	L-threonine	-4.42045	-0.616	-3.26361	-0.14823	-3.34814	-0.18907	-3.56514	-0.20486	-4.09734	-0.83211	-4.17169	-0.4584
amino acid	glycyl-L-glutamic acid	-3.80658	-0.1829	-3.29064	-0.86645	-3.49743	-0.86586	-3.3502	-0.3366	-3.62884	-0.66614	-3.8659	-0.52665
carbohydrate	β-methyl-D-glucoside	-0.90304	2.8911	1.77368	0.25414	3.50062	-0.63867	3.30892	-0.23499	4.39516	-0.83313	4.6346	-0.94328
carbohydrate	D-xylose	-3.83994	-0.0997	-1.13158	1.43944	-0.79467	1.16984	-0.04265	0.84387	0.78465	-1.29961	0.34955	-2.25707
carbohydrate	i-erythritol	-3.87121	-0.3659	-3.08903	0.28622	-3.15332	-0.28711	-3.46869	-0.87958	-3.76742	-0.91732	-3.81108	-0.67244
carbohydrate	D-mannitol	5.73196	-1.0984	2.41869	0.07222	4.81746	-0.06949	5.3931	-0.55936	7.27252	-0.20002	7.53561	-1.25971
carbohydrate	N-acetyl-D-glucosamine	7.79587	10.575	2.75662	-0.20187	3.7293	-0.30613	3.69219	-0.10424	5.5633	-1.06567	6.12975	-0.8148
carbohydrate	D-cellobiose	-2.14563	1.7396	1.81021	1.28662	4.87093	-0.44047	4.78006	-0.50437	7.38428	-0.09977	7.8187	0.05915
carbohydrate	α-D lactose	-4.17016	-0.2971	-1.55683	1.6947	-2.41076	2.07742	-1.51369	3.15073	1.16527	-3.21413	-0.36747	-3.23723
carboxylic acid	D-galactonic acid γ-lactone	6.66822	-0.6274	0.73942	0.46085	2.12159	-0.12172	2.67093	-0.40065	3.84437	-1.1421	4.4294	-0.58249
carboxylic acid	pyruvic acid methyl ester	1.73138	1.2066	0.94118	-0.57952	1.91546	-0.52636	2.37894	-0.59462	2.26335	0.72046	2.65988	1.28551
carboxylic acid	D-galacturonic acid	7.45828	-2.4361	1.75402	-0.52038	2.41689	-0.4655	2.64745	-0.35858	4.04607	-1.20725	4.55495	-0.49144
carboxylic acid	2-hydroxy-benzoic acid	-4.99234	-0.6155	-3.96294	-1.35903	-3.35172	-1.13292	-3.95047	-1.33924	-4.43767	-0.78101	-4.09948	-0.20026
carboxylic acid	4-hydroxy-benzoic acid	-2.40989	-0.1786	2.31173	0.21743	-0.00039	0.83672	-0.20855	1.02701	-1.23116	1.46269	-1.32384	1.15049
carboxylic acid	γ-hydroxy-butyric acid	1.51694	-3.4361	1.54784	-0.59321	0.27707	0.84122	-0.87098	0.70327	-1.58611	0.875	-1.63687	0.92742
carboxylic acid	D-glucosaminic acid	-3.70936	-0.0392	-3.17882	-0.21043	-3.18545	-0.03544	-3.35703	0.20214	-3.91969	-0.92313	-3.86634	-0.56086
carboxylic acid	itaconic acid	-2.92368	-0.4423	-2.52535	-0.55087	-2.48387	-0.76054	-3.06929	-0.676	-2.33012	-0.70603	-2.841	-0.66054
carboxylic acid	α-ketobutyric acid	-4.48763	-0.597	-3.28134	0.01015	-3.4521	-0.18034	-3.82576	-0.22531	-4.35581	-0.80236	-4.39098	-0.50774
carboxylic acid	D-malic acid	-2.34092	2.0722	2.25505	-0.52347	-0.36318	0.20322	0.70716	0.11561	-0.38788	0.59368	-1.03776	1.04602
miscellaneous	glucose-1-phosphate	-2.80801	0.9166	-2.08962	0.77065	0.48826	-0.29008	0.87368	-0.62278	1.89727	-1.02119	1.5885	-0.77052
miscellaneous	D,L-α-glycerol phosphate	-2.19434	-0.8265	-2.55141	-0.96595	-2.43758	-1.13361	-2.85803	-0.48929	-2.51502	-0.90886	-2.56756	-0.54825
polymer	tween 40	9.62033	-2.3447	0.68877	-0.18057	1.19905	-0.45618	0.62621	-0.06841	-0.04718	1.20675	-0.03644	0.97963
polymer	tween 80	7.38729	-2.3943	1.15515	-0.37197	1.34554	-0.51196	0.78159	-0.33355	0.09163	1.29368	0.30252	1.12836
polymer	α-cyclodextrin	-3.59898	-0.0257	-1.20442	1.01548	-1.83849	0.6839	-1.92562	0.64702	-2.11313	-0.2584	-2.81458	-0.3943
polymer	glycogen	-2.14577	0.211	2.56567	-0.05405	-2.10101	1.4815	-0.68074	1.83187	-1.34658	-0.06767	-2.09035	-0.72882