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This thesis is dedicated to my grandparents:
Jack and Violet Savery

Abstract

Pulp and paper mills generate biosolids as a by-product of their effluent treatment systems. These biosolids show excellent potential as a soil conditioner. However, the detection of high levels of *E. coli* (10^2 to 10^5 CFU/gdw), in the absence of any significant fecal loading, has caused concern among effluent treatment system operators, land applicators and regulators. This research examines the sources and ecology of *E. coli* strains comprising the population of *E. coli* in the biosolids of an eastern Ontario pulp and paper mill by applying the molecular microbial source-tracking tool repetitive sequence-based polymerase chain reaction. Confirmed *E. coli* were successfully isolated from 2 forested sites, treated mill process feed water, storm effluent, mill fibres, wood chips, primary and secondary clarifier effluents and sludges, and biosolids. While laboratory isolates could be accurately distinguished from forest and industrial isolates, cluster and jackknife analyses were incapable of differentiating reliably among isolates from the forest, on-site inputs, effluent treatment system and biosolids (average rates of correct classification values ranged from 43.9% to 72.9%). No input could be excluded as a source of *E. coli* to the effluent treatment system. Many fingerprint types were unique to the primary and secondary clarifiers; however, only a subset of the fingerprint types recovered from the primary and secondary clarifiers were detected in the biosolids. This suggests that the *E. coli* strains found in mill biosolids were those able to survive the hot, desiccating conditions of dewatering. Highly similar fingerprint types (> 80% similarity) were recovered repeatedly over an 8-month period from the effluent treatment system (primary and secondary clarifiers) and biosolids. These results are consistent with growth within the effluent treatment system and biosolids, rather than fecal loading. Over a 3-year period the pulp and paper mill effluent treatment

system underwent large-scale changes, including a 50% reduction in effluent volume upon the elimination of on-site pulping activities. New fingerprint types were detected in the biosolids following the restructuring, and the Shannon index of diversity increased. Disruption of established operating conditions could have opened niches, allowing new strains to colonize the mill effluent treatment system successfully.

Résumé

Les usines de pâtes et papiers produisent des biosolides comme produits dérivés de leurs systèmes de traitement des effluents. Ces biosolides présentent un excellent potentiel pour la fertilisation du sol. Cependant, la détection de niveaux élevés de *E. coli* (10^2 à 10^5 CFU/gdw), en absence d'un apport de matières fécales, préoccupe les opérateurs de systèmes de traitement des effluents, les agriculteurs et les autorités gouvernementales.

Cette recherche examine les sources et l'écologie des souches de *E. coli* présentes dans les biosolides d'une usine de pâtes et papiers de l'Est de l'Ontario, en utilisant l'outil moléculaire de localisation de source microbienne, le Rep-PCR. *E. coli* ont été identifiés et isolés à partir de deux sites boisés, d'eau provenant du système de traitement des eaux usées de l'usine, d'eau pluviale, de fibres d'usine, de copeaux de bois, des effluents et des boues provenant des décanteurs primaires et secondaires, et finalement, des biosolides. Alors que les isolats de laboratoire ont pu être séparés précisément des isolats provenant de la forêt et de l'usine, les analyses typologiques et du jackknife étaient incapables de différencier de façon fiable les isolats provenant de la forêt, de l'usine, du système de traitement des effluents et des biosolides (valeurs ARCC de 43,9 à 72,95%). Aucun apport au système de traitement des effluents n'a pu être exclu comme source potentielle de *E. coli*. Plusieurs types d'empreintes génomiques de *E. coli* étaient uniques aux décanteurs primaires et secondaires; cependant, seulement certains types d'empreintes retrouvés dans les décanteurs primaires et secondaires ont été détectés dans les biosolides. Ceci suggère que les souches de *E. coli* localisées dans les biosolides de l'usine étaient celles qui ont survécu les conditions très chaudes et asséchantes du processus d'assèchement des boues. Des types d'empreintes très similaires (> 80% de similarité) ont été retrouvés à plusieurs reprises dans

le système de traitement des effluents (décanteurs primaires et secondaires) et des biosolides durant une période de huit mois. Ces résultats sont conséquents de la croissance bactérienne à l'intérieur du système de traitement des effluents et des biosolides, plutôt que par des apports fécaux. Pendant une période de 3 ans, le système de traitement des effluents de l'usine de pâtes et papiers a subi des changements majeurs incluant une réduction de 50% du volume des effluents lors de l'élimination des activités de dépulpage sur le site. Des nouveaux types d'empreintes ont été détectées dans les biosolides suivant la restructuration, et l'index de diversité de Shannon a augmenté. La perturbation des conditions existantes dans les opérations a pu faire en sorte que de nouvelles niches sont devenues disponibles, ce qui a permis à des nouvelles souches de coloniser le système de traitement des effluents de l'usine.

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List of Abbreviations

µg	microgram(s)
µL	microlitre(s)
µm	micrometre(s)
µS	microSiemens
°C	degrees Celsius
AAF/I	aggregative adherence fimbriae I
AFLP	amplified fragment length polymorphism
AOX	adsorbable organic halides
APHA	American Public Health Association
AP-PCR	arbitrarily primed PCR
ARA	antimicrobial resistance profiling
ARCC	average rate of correct classification
AST	activated sludge treatment
ATCC	American Type Culture Collection
Bfp	bundle forming pilus
BOD	biological oxygen demand
bp	base pair(s)
CFA/I	colonization factor I
CFA/II	colonization factor II
CFU	colony forming unit(s)
CFU/gdw	colony forming units(s) per gram dry weight
CFU/mL	colony forming unit(s) per millilitre
CFU/100mL	colony forming units(s) per 100 millilitres
C:N	carbon to nitrogen ratio
CNFI	cytotoxic necrotizing factor I
COD	chemical oxygen demand
DAEC	diffusely-adherent <i>E. coli</i>
d	days
DNA	deoxyribonucleic acid
eae	gene for intimin
EAEC	enteroaggregative <i>E. coli</i>
EAST1	gene for low molecular weight heat stable toxin
ECF	elementary chlorine free
EHEC	enterohemolytic <i>E. coli</i>
Ehx	EHEC hemolysin
EIEC	enteroinvasive <i>E. coli</i>
EPEC	enteropathogenic <i>E. coli</i>
ERIC PCR	enterobacterial repetitive intergenic consensus
ETEC	enterotoxigenic <i>E. coli</i>
g	gram(s)
gdw	gram dry weight
h	hour(s)
hly	α-hemolysin

HUS	hemolytic uremic syndrome
Ipa	invasion plasmid antigen
kb	kilobase(s)
L	litre(s)
LT	heat labile toxin
m	metre(s)
M	molar
m ³	metres cubed
MAR	multiple antibiotic resistance
MISA	municipal industrial strategy for abatement
mg	milligram(s)
mg/mL	milligram(s) per millilitre
mL	millilitre(s)
mM	millimolar
MPN	most probable number
mRNA	messenger ribonucleic acid
MUG	4-methylumbelliferyl- β -glucuronide
MW	molecular weight
NMEC	neonatal meningitis <i>E. coli</i>
ONPG	2-nitrophenyl- β -D-galactopyranoside
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
RAPD	random amplified polymorphic DNA
RAS	recycled activated sludge
REP	repetitive extragenic palindromic
Rep-PCR	repetitive sequence-based PCR
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SD	standard deviation
Sta	heat stable toxin I
Stb	heat stable toxin II
STEC	shiga-toxin producing <i>E. coli</i>
Stx 1	shiga toxin (or shiga-like toxin) I (also see VT)
Stx 2	shiga toxin (or shiga-like toxin) II (also see VT)
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCF	totally chlorine free
TSS	total suspended solids
USEPA	United States Environmental Protection Agency
uidA	gene for β -D-glucuronidase
UPEC	uropathogenic <i>E. coli</i>
UV	ultraviolet
VBNC	viable but nonculturable
VT	verocytotoxin, see Stx 1 and Stx 2
VTEC	verocytotoxic <i>E. coli</i> , see STEC
WAS	waste activated sludge

Introduction

Pulp and paper mills use effluent treatment systems to ensure that their wastewaters meet regulatory standards; biosolids are a necessary by-product of this process.

Traditionally, biosolids are disposed of via landfilling or incineration. However, a more environmentally and economically feasible option may be to apply biosolids to silvicultural or agricultural lands. However, the presence of high levels of *E. coli* and other bacteria typically used as indicators of fecal contamination are a cause for concern.

Previous research has suggested that pulp and paper mill *E. coli*, and other bacterial indicators, are not simple transients in pulp and paper mill effluent treatment systems. These microorganisms seem to grow continuously within established niches in mill effluent treatment systems. This means that their presence should not be considered a reliable indicator of fecal contamination, especially in the absence of any known fecal input.

This project focuses on 3 remaining research questions:

- 1) What are the sources of biosolids-isolated strains of *E. coli*?
- 2) What is the ecology of *E. coli* within the mill?
- 3) What effect does significantly altering the composition of mill effluents have on pulp and paper mill effluent treatment system and biosolids *E. coli* populations?

Repetitive sequence-based PCR (Rep-PCR), a method of microbial source tracking capable of generating highly discriminatory genetic fingerprints, was used to differentiate between strains of *E. coli*, and thus address the research questions.

The pathogenicity of the *E. coli* strains examined in this study were evaluated in a complementary study (Appendix 1).

Chapter 1 Literature Review

Pulp and paper mills produce biosolids as a necessary by-product of their effluent treatment system. These biosolids contain high levels of *E. coli*, coliforms, fecal coliforms and enteric streptococci, bacteria typically used as indicators of fecal contamination. This review discusses: 1a) pulp and paper mill biosolids production; 1b) biosolids composition and management; 1c) the microbial habitat provided by pulp and paper mills; 2a) indicators of fecal contamination; 2b) current indicators, with particular focus on *E. coli*; 3a) microbial source tracking (MST); 3b) MST methods currently in use.

1.1 Pulp and Paper Mills

1.1.1 Papermaking

Pulp and paper mills manufacture a wide variety of products, including: corrugated cardboard, newsprint, boxboard, coated paper, fine paper, and tissue. As a result, mills use different combinations of the available technologies in order to prepare the annually produced 300 000 000 tonnes of desired end product per year (119). Papermaking is a wood and water-based process. Wood is composed of simple sugars polymerized into long cellulose/hemicellulose fibers bound together by lignin (183).

The papermaking process (reviewed by (3), with additional information) involves 5 main steps:

- 1) Debarking and chipping: the processes of removing bark and grinding the logs into wood chips.
- 2-) Pulping reduces wood chips to cellulose-rich pulp.

Three methods of pulp production are in use: (i) mechanical pulping which passes wood chips through a grindstone, fibres are stripped and suspended in water; (ii) chemical pulping

which uses chemicals, in the presence of heat and pressure, to break down wood chips; and (iii) chemical thermo-mechanical pulping which relies on chemical treatment to partially soften wood chips which are then mechanically ground (183).

3-) Bleaching whitens brown pulp.

Traditionally, chlorine (Cl_2) was used as a bleaching agent. Because the resulting effluents were toxic (due to the production of chlorophenolics in the extracted lignin fragments), there was a shift away from the use of chlorine in the 1980s. Many mills are now either ECF (Elemental Chlorine Free) or TCF (Totally Chlorine Free). ECF mills use chlorine dioxide, while TCF mills use oxygen, ozone, alkali and hydrogen peroxide to whiten their pulp ((117) cited by (183)).

4-) Washing (or the alkali extraction stage) removes colour and bleaching agents using caustic soda (sodium hydroxide), or another alkaline agent.

5-) Papermaking involves applying a slurry of washed and bleached fibres to a wire cloth. The water is removed by gravity, compression, heat and suction. Filling agents (kaolin clay, titanium dioxide, or calcium carbonate) and sizing agents (usually rosin or starch) may be added to provide a smooth, opaque, white surface..

1.1.2 Pulp and Paper Mill Wastes

The wastes generated by this industry fall into 4 broad categories:

- 1-) Gaseous emissions, including: hydrogen sulfide, mercaptans, sulfur dioxide, nitrogen dioxide, carbon monoxide, and carbon dioxide (3; 73).
- 2-) Particulate matter: fly ash from coal fired power boilers and char from bark burners (3).
- 3-) Effluents (untreated): suspended solids (bark, dirt, wood fibers and pigment), dissolved colloidal organics (hemicellulose, sugars, sizing agents),

chromatophores (from lignin compounds), chlorinated compounds from bleaching (including phenols and dioxins), dissolved inorganics (sodium hydroxide, sodium sulfate) and thermal pollution of the receiving waters (3; 73).

4-) Solid wastes: bark, grit, mill wastes and sludges (3).

Since the 1980s, North American pulp and paper mills have reduced their waste products in all 4 categories by 80 to 90% (183). Some of the most significant improvements have been made in effluent treatment.

1.1.3 Pulp and Paper Mill Effluent

Pulp and paper mills consume large volumes of water, 10 to 300 m³ per tonne of paper produced, although water use in modern mills can be as little as 10 to 40 m³ of water per tonne of paper produced (118). There is a correspondingly high production of wastewater. The volume of wastewater produced by a mill depends on: the nature of the raw materials (hardwood, softwood), the final product being generated (i.e. corrugated cardboard vs. fine paper) and the extent of water recycling (3).

Many chemicals are dissolved or suspended in water during the pulping and papermaking process. In order to reduce both raw material costs and pollution, mills recover and recycle many of the chemicals present in wastewater. However, this water cannot be recycled indefinitely. Organic and inorganic compounds accumulate, corrosion occurs, bacterial loading increases, odours develop and ultimately there are deleterious effects on paper formation (183). Eventually, wastewaters are returned to receiving waters. Government regulations (both federal and provincial) specify that mill effluents must meet certain standards prior their return (141). Pulp and paper mill effluents require treatment to meet these standards.

Untreated effluents have the potential to cause considerable damage to receiving waters. Depending on the processes and technologies used, mill effluents may have high biochemical oxygen demand (BOD), high chemical oxygen demand (COD), high

concentrations of chlorinated compounds (adsorbable organic halides, AOX) and high concentrations of total suspended solids (TSS) (3).

BOD is a measure of the oxygen consumed as aerobic microorganisms break down organic compounds (4). COD is a measure of the oxygen used during the oxidation of chemicals (4). BOD and COD are usually expressed in terms of mg/L. Untreated effluents have been reported to have oxygen demands as high as 11 000 mg/L (183). Since natural waters are normally considered to be saturated with oxygen at 10 mg/L, the high demand for oxygen of wastewater effluents can cause damaging effects in the receiving waters, primarily by depriving downstream organisms of the oxygen they need to survive.

AOX compounds are produced during the bleaching stage of the papermaking process. For example, wood-based hydrocarbons, and some mill chemicals (i.e. defoamers, wood preservatives) when combined with halogens, can form halogenated organic compounds (3). Untreated mill effluents may contain: chlorinated lignosulfonic acids, chlorinated resin acids, chlorinated phenols, dioxins and furans (3). These chlorinated compounds are highly toxic, mutagenic, carcinogenic, lipophilic (fat soluble) and tend to bioaccumulate in the food chain (3). AOX compounds in untreated mill effluents, as reviewed by (183) and (134), have been demonstrated to cause a broad range of health effects in fish: liver damage, mortality in prolarvae, delayed sexual maturity, reduction in gonad size, alterations in reproduction and depression of secondary sex characteristics. Over the past decade, many mills have greatly reduced AOX compounds in their wastewaters. They have done so in two ways: by decreasing the production of AOX compounds in the bleaching process (i.e. by making the transition to ECF or TCF), and by removing AOX compounds from wastewater by secondary treatment (134).

High TSS concentrations in untreated effluents are due mainly to fibres, however, lignins, lignin derivatives, fatty acids, sulfur, sulfur derivatives, tannins and resin acids also contribute to the problem (3). These solids can block light in the water column, limiting plant growth and primary productivity, and altering the ecosystem of the receiving waters.

1.1.4 Pulp and Paper Mill Effluent Treatment

The effluent treatment process for the mill studied in this research project is shown in a simplified manner in Figure 1-1, and is representative of the general process.

River water is drawn in at the pump house and pre-treated so that is of a sufficiently high quality for in-mill processes; this involves screening and chlorination. The water is then sent where required within the mill: woodroom, kraft mill, paper mill or steam plant. The process waters (wastewaters from pulping, bleaching, and papermaking) and storm effluents are collected and combined in the mill sewers. Note that sanitary sewers are connected to the municipal wastewater system and are not part of the mill effluent stream. The mill wastewaters pass through the primary sump into the primary clarifier (a settling tank). Sludges (the settled particulates) are drawn out of the bottom of the primary clarifier, combined with the settled activated sludge biomass from the bottoms of the secondary clarifiers, and dewatered. The primary clarifier effluents pass into an air activated bioreactor where they undergo activated sludge treatment (AST). The bypass from the kraft mill adds additional highly toxic effluent to the bioreactor (the bypass wastewaters do not undergo primary clarification as they are low in suspended solids). The bioreactor contains a strongly aerobic water-activated sludge floc slurry with a very high microbial density, where rapid biological activity is harnessed to consume soluble organic compounds, greatly reducing BOD and COD and producing carbon dioxide and new biomass. Treated wastewaters flow into the secondary clarifiers where the activated sludge flocs settle out of the treated effluent. Most of this settled biomass is returned to the bioreactor to provide the aeration tank with the necessary density of biomass (recycled activated sludge, RAS). However, the biomass produces a 10-15% surplus on a daily basis. This surplus sludge is

waste activated sludge (WAS) and constitutes a disposal problem. The WAS is removed and combined with primary clarifier sludges, and this mixture is dewatered to form 'combined dewatered biosolids'. The clarified effluents from the secondary clarifier, now non-toxic with low suspended solids and low BOD, are returned to the receiving waters. As required by regulation, these treated effluents are tested for toxicity at the MISA (Municipal/Industrial Strategy for Abatement) sampling station. A more detailed description of the primary clarifier, activated bioreactor, secondary clarifiers and combined dewatered biosolids is provided below.

1.1.4.1 Primary Clarifier

The primary clarifier is a settling tank. Effluents are retained for 4 to 5 hours, allowing more than 80% of TSS to settle out (183); BOD and COD may be reduced by 70% and 30%, respectively (199). Primary sludge consists mainly of waste fibres (cellulose, hemicellulose, lignin, wood furnish, bark (93)) and lime.

1.1.4.2 Activated Sludge Treatment

AST relies on the activity of a biological community to remove organic compounds (and thus BOD) from the effluent. The biological community does this quite effectively, removing most of the BOD remaining after primary treatment (27).

AST systems are kept well oxygenated and well mixed. Either sodium hydroxide or sulfuric acid is used control the pH. Mill effluents have high carbon levels, but low nitrogen and phosphorus levels. As these nutrients can limit the growth of the AST biomass, nitrogen and phosphorus are added to yield a more productive BOD:N:P ratio of 100:5:1 (199).

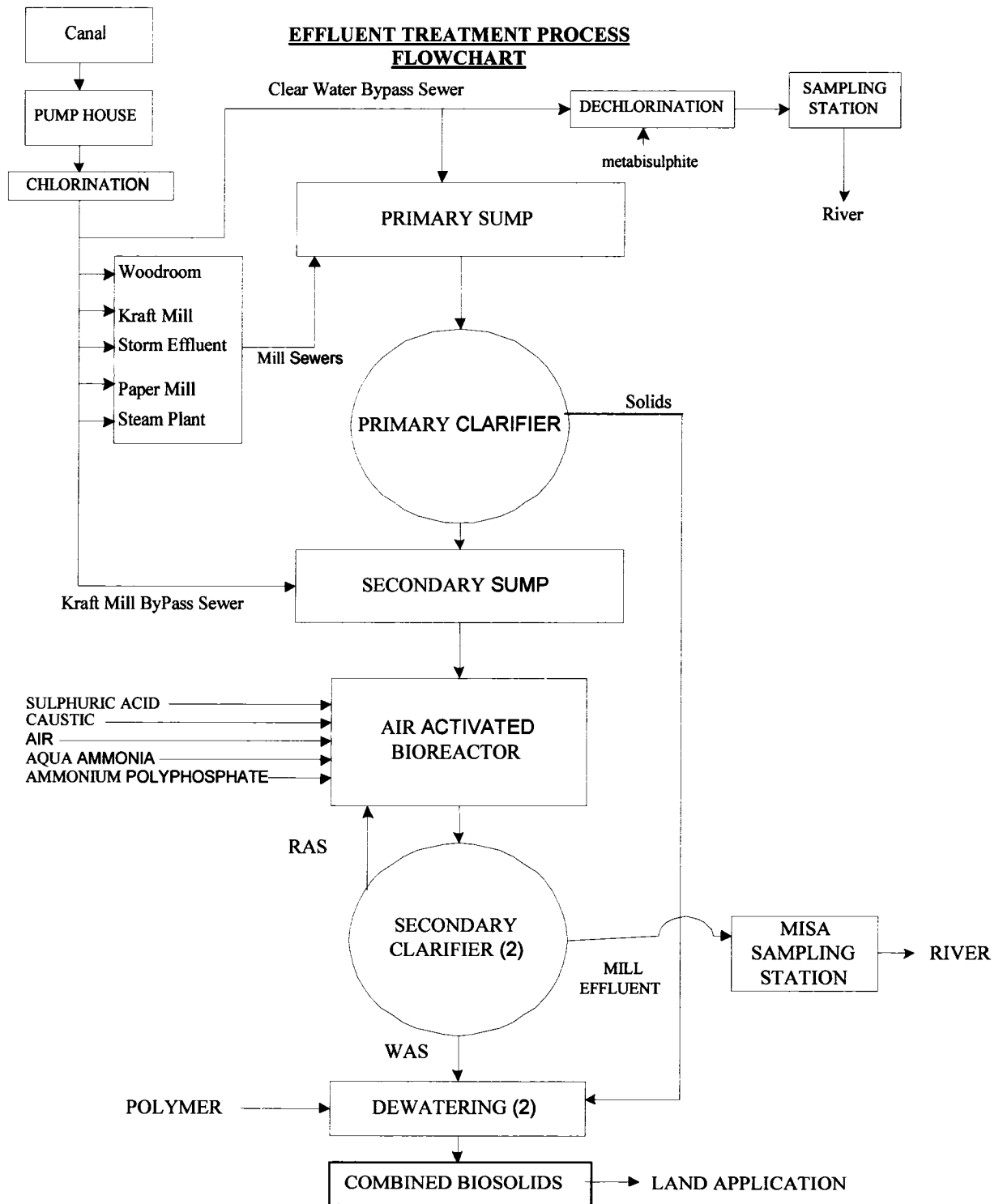


Figure 1-1: Schematic diagram of a pulp and paper mill effluent treatment system with primary clarification, activated sludge treatment (AST), and secondary clarification. MISA – Municipal/Industrial Strategy for Abatement; RAS – Recycled Activated Sludge; WAS – Waste Activated Sludge.

The concentrations of these chemicals must be carefully controlled. Excess nitrogen or phosphorus additions are an economic loss for the mill, and harmful to the environment as they raise the available N + P in the treated effluent causing eutrophication downstream (91).

AST systems require the maintenance of a quickly settling sludge. In an AS floc, filamentous floc-forming bacteria form a matrix to which a wide variety of other bacteria and non-living effluent particles adhere. Typically a floc will contain millions of viable cells of dozens to hundreds of different microbial strains. The active transport systems of the floc microbial community quickly take up nearly all dissolved nutrients in the effluent. In this manner, dissolved organic matter and effluent particulates are incorporated into the suspended biomass (183). AST systems are vulnerable to loss of efficiency from: sludge bulking (poor settling), due to excessive growth of filamentous bacteria; sludge foaming, due to excessive growth of certain foam-stabilizing bacteria; and poor settling due to excess free living cells or a loss/inactivity of key populations (170).

The biological details of AST microbiological communities remain, in many respects, poorly understood. Effluents typically take 9 to 10 hours to pass through the AST and into the secondary clarifiers.

1.1.4.3 Secondary Clarifiers

The secondary clarifier settling basins allow the biomass generated in the AST to settle over the 4 to 5 hour retention time. Secondary sludge is composed mainly of excess biomass, cellulose fibres and lignin (93). These sludges have a low fibre content and relatively high levels of nitrogen and phosphorous (151).

1.1.4.4 Combined Dewatered Biosolids

At the end of the effluent treatment process, non-toxic effluents are returned to receiving waters. Primary and secondary sludges are dewatered and combined to form combined dewatered biosolids.

In North America alone, over 1 090 000 tonnes of biosolids were produced in 2000; biosolid production is expected to increase over the next 50 years to global outputs between 4 300 000 to 5 400 000 tonnes per year (119).

Pulp and paper mills currently have 3 options when dealing with their biosolids: landfilling, incineration or land application (151). In North America an estimated 45% of biosolids are landfilled, 41-50% are incinerated and 5% are used for land application (119). A survey of Canadian pulp and paper mills in 1992 yielded similar responses: 54% landfilled, 30% incinerated, 17% land applied (23).

Landfilling is becoming increasingly costly as landfill space becomes less available and legislation becomes more restrictive (151). Landfill conditions are not conducive to rapid biodegradation, so biosolids break down very slowly (151). High transportation costs are also an issue (90).

Incineration is another a costly option. Biosolids are dewatered, but still have a high moisture content. Biosolids must undergo significantly more dewatering to be efficiently incinerated, and even then produce little heat (119). Furthermore, there is a high capital cost involved for the mill as the biosolids must be run through a fluidized bed system rather than a simple grate boiler (119). Mabee and Roy (119) suggest that as air emission regulations become increasingly strict, it may become uneconomical for mills to burn their biosolids. The savings in heating costs would be offset by costs of obtaining permits or paying air emission fines.

The third option is to find a use for biosolids. Proposed useful applications include: agricultural mulch, forest fertilizer, liming agent, soil buffering agent, construction material, or even animal feed (27; 93).

Reports regarding the efficacy of pulp and paper mill biosolids as a soil conditioner are mixed and have been summarized in

Table 1-1. Primary biosolids are a good long term source of organic carbon. However, immediately following application, plant yields may be depressed due to immobilization of nitrogen (15; 110; 190) or increased salt levels (15; 53). Higher yields are obtained by using primary biosolids as a mulch or topdressing to prevent weed growth, and to insulate young plants from extreme temperatures (115). More immediate positive effects are seen with secondary or combined biosolids. In general, combined dewatered biosolids have been demonstrated to: increase organic matter, increase available nutrients, reduce erosion, improve soil structure, increase pH (good for acidic soils), and improve water-holding capacity (27; 120; 160; 194). The biosolids produced by each mill are unique in terms of their chemical and biological composition (146) (due to different fibre sources, specific processes and chemicals used). The efficacy of biosolids as a soil conditioner also depends on: soil type, soil pH, soil moisture content, crop type and original soil nutrient content (27).

Land applied materials have the potential to contaminate soils, vegetation and runoff (which can in turn affect surface and ground waters). The environmental safety of land applying pulp and paper mill biosolids has been questioned, particularly in regards to heavy metals, AOX compounds and pathogens (151). However, most pulp and paper mill biosolids have relatively low heavy metal concentrations, comparable to those found in cattle manure, and typically an order of magnitude lower than those found in municipal biosolids (15; 151). Biosolids generated by ECF or TCF mills are reported to have low to non-detectable levels of the AOX compound TCDD and are not predicted to pose a significant risk to terrestrial animals (15; 131). Although high levels of the bacteria typically used as indicators of fecal contamination, including *E. coli*, have been found in pulp and paper mill biosolids and effluent treatment systems (63; 128; 139), no pathogens have been detected in pulp and paper mill biosolids to date (63; 122; 139; Appendix 1)

Table 1-1: An overview of studies examining the effects of various types and applications of paper industry biosolids (pulp mill, paper mill or pulp and paper mill) on plant yields.

Biosolid Type and Application	Effect on Plant Yield	Study
Primary	Decrease	oats, <i>Avena sativa</i> L.(45) ¹ ; maize and wheat(35) ¹ ; tomato, <i>Lycopersicon esculentum</i> L., cucumber, <i>Cucumis sativus</i> L., peppers, <i>Capsicum annuum</i> L.(155) ² (38) ² ; wheat, <i>Triticum aestivum</i> L.(191) ¹ ; lettuce, <i>Lactuca sativa</i> L.(192) ¹ ; yellow lupines, <i>Lupinus luteus</i> L.(190); clover, <i>Trifolium subterraneum</i> (53); tomato, <i>Lyc. Esculentum</i> L., cress, <i>Lepidium sativum</i> L., radish, <i>Raphanus sativus</i> L.(110)
Primary Year 2 of application	No Decrease	Yellow lupines, <i>Lup. Luteus</i> L.(190); common bermudagrass, <i>Cyanodon dactylon</i> L.(54)
Primary Incorporated	Decrease	(28;29) ¹ ; hybrid poplar, <i>Populus deltoides</i> x <i>P. trichocarpa</i> (86) ¹
Primary Incorporated	Increase	(79) ¹ ; (28) ¹ ; (164) ¹
Primary Top Dressing	No Decrease	wheat, <i>T. aestivum</i> L.(29) ¹
Primary Top Dressing	Increase	Hybrid poplar, <i>P. deltoides</i> x <i>P. trichocarpa</i> (85); hybrid poplar, <i>P. deltoides</i> x <i>P. trichocarpa</i> (86) ¹ ; hybrid poplar(115)
Secondary	Increase	tomato, <i>Lyc. Esculentum</i> L., cucumber, <i>Cuc. sativus</i> L., peppers, <i>Cap. Annuum</i> L.(155) ² (38) ² ; Hybrid poplar, <i>P. deltoides</i> x <i>P. trichocarpa</i> (86) ¹ ; wheat, <i>T. aestivum</i> L.(189) ¹
Combined	No Decrease	4 deciduous ornamental shrubs(37) ¹ ; 4 nursery species(15); lodgepole pine(120); pole-sized northern hardwood forest(55)
Combined	Increase	red pine, <i>Pinus resinosa</i> (105) ¹ ; corn, <i>Zea mays</i> , wheat, <i>Trit. sp.</i> , soya beans, <i>Glycine max</i> (14) ² ; grapes, <i>Vitis sp.</i> (38) ² ; lettuce, <i>Lat. Sativa</i> L.(26) ¹ ; 4 deciduous ornamental shrubs (37) ¹ ; bluegrass, <i>Poa pratensis</i> (206) ¹ ; 4 nursery species(15); brome grass, white spruce(120); wheat and grass(150); brome grass, white spruce, lodgepole pine(121); 10 different plant species(146)

¹ studies cited in (27)

² studies cited in (15)

1.1.4.5 Pulp and Paper Mills as a Microbial Habitat

In-mill conditions are conducive to microbial growth. Pulp and paper mills are open to contamination from air, water and raw materials (100). Temperatures are typically between 30 to 60⁰C, while the pH is within 4.5 to 9.0 (112). Furthermore, mill waters are rich in nutrient sources including: cellulose, hemicellulose, starches and wood resins (100; 111; 186).

Mill bacteria are not considered to be problematic unless they form biofilms, produce enzymes that hasten spoilage, generate foul odours, produce pigments, or pose a health concern (113; 177). In order to prevent excess growth in certain key components (i.e. the paper machine and its white water loop), most mills treat key points in the water system with degradable biocides. These are mostly proprietary commercial products designed to have short half-lives once they are added to water so that they are not detrimental to the performance of the downstream biotreatment system.

In the 1950s, mills were concerned with the discolouration of finished paper by bacteria (particularly pink slimes) (186). Studies were initiated to understand which bacteria were responsible, and the industry dealt with the problem by using what are now recognized as toxic biocides (186). Since that time, mills have changed their processes from acidic to alkaline or neutral, begun using gentler biocides and new chemicals for drainage, retention and depositions, increased the speed of paper machines, increased the use of recycled fibres, and made an effort to reuse or recycle mill waters (144). This has led to the production of in-mill waters with more concentrated nutrient salts and degradable carbon levels (186). Some mills have reinitiated studies to examine the microbial communities in modern pulp and paper mills.

These mill studies have detected bacteria (in the order of 10^5 to 10^8 CFU/mL) in the raw materials used to make paper (187). For example: starches support *Bacillus circulans*, *Cellulomonas flavigena* and *Pantoea agglomerans*; ground wood carries *Bac. coagulans*, and *Burkholderia cepacia*; and pulped broke supports *Burk. cepacia*, *Klebsiella pneumoniae* and *R. pickettii* (187).

Once in the mill, some of these bacteria colonize the paper machines, forming slimes (which may or may not be pigmented). More than 19 genera have been identified in these slimes (101; 112; 144; 186; 187).

Other bacteria colonize the warm, nutrient-rich in-mill waters. In-mill waters have been reported to contain: *Alcaligenes piedchaudii*, *Burk. cepacia*, *K. pneumoniae*, *R. pickettii*, *Sphingomonas capsulata* and other *Sphingomonas sp.* (187); *Bac. cereus*, *Bac. simplex*, *Brevibacillus sp.*, *Paenibacillus sp.*, *Amycolatopsis fastidiosa*, *Hydrogenophaga sp.* and *Pseudoxanthomonas sp.* (177). Warm in-mill waters tend to select for mesophilic and thermophilic bacteria. It should come as little surprise that an estimated 1 to 5% of mill bacteria are coliforms (44). This still amounts to substantial numbers of coliforms living within mill systems: 10^3 to 10^7 coliforms/100mL effluent, 10^6 to 10^8 coliforms/gdw biosolids (62). The 'coliform community' of an Ontario pulp and paper in mill system was composed of: 63% *Klebsiella sp.*, 21% *Enterobacter sp.*, 9% *E. coli* and 6% *Citrobacter freundii* (62). The more exclusive 'fecal coliform community' of a pulp and paper mill is typically dominated by thermotolerant *K. pneumoniae*. Reports on the percentage *E. coli* present in the pulp and paper mill 'fecal coliform community' vary: 2.1 to 21.3% *E. coli* (128); 25 to 78% *E. coli* (139); approximately 20% *E. coli* (62).

Finished paper products have been through a hot, drying process. Most bacteria isolated from end-products are spore formers, dominated by *Bacillus*, *Brevibacillus* and *Paenibacillus* (187).

From start to finish, pulp and paper mills offer a broad range of habitats to diverse communities of microorganisms. However, these communities remain poorly characterized and understood. Many studies examining the microbiology of pulp and paper mills report that they have isolated a bacterial species from a novel habitat, or isolated a novel species or genus. For example: only 86% of 390 aerobic strains from papermaking machines and raw materials could be classified to genus and species (187); of 177 mill isolates, at least 10 isolates were thought to represent new genera (177); of over 100 isolates collected from a Canadian mill, 30% were unidentifiable (44); of 197 mesophilic aerobic isolates, 40% were suggested to belong to novel species (178).

1.2 Bacterial Indicators

1.3.1 Indicator Bacteria

“An indicator is an organism or substance whose presence and concentration signals the occurrence of another entity in a matrix under examination” (180).

Over a century ago, microbiologists realized that the contagions responsible for causing disease could be spread by water. Many serious illnesses, including typhoid, cholera, dysentery and gastroenteritis use water as a reservoir and/or vector of transmission (reviewed in (7)). To prevent the spread of these diseases, the sanitary quality of drinking and recreational waters is monitored closely. However, monitoring the water supply for every type of waterborne pathogen would be impractical, expensive and time-consuming (108). A list, by no means exhaustive, of potentially pathogenic waterborne organisms includes (4):

1. Bacterial pathogens: *Salmonella sp.*, *Shigella sp.*, *Vibrio sp.*, *E. coli* (certain strains), *Pseudomonas aeruginosa*, *Campylobacter sp.*
2. Viral pathogens: Coxsackie A and B, Adenovirus 3 and 4, Hepatitis A, Norwalk, Rotavirus, Poliovirus, Echovirus, Reovirus and Enterovirus
3. Eukaryotic pathogens (yeasts and protozoans): *Cryptosporidium parvum*, *Giardia lamblia*, *Candida albicans*, *Acanthamoeba sp.*, *Naegleria sp.*

The source of these pathogenic bacteria is often fecal matter. Although human fecal contamination is associated with the highest incidences of waterborne disease, the feces of other animals are also known to carry microorganisms pathogenic to humans. For example, wild birds are vectors for *Campylobacter spp.*, *Salmonella spp.*, *Listeria spp.* (156) and *E. coli* O157:H7 (196). *E. coli* O157:H7 have also been isolated from the feces of deer (57; 135), and goats and sheep, but their main reservoir is in cattle (reviewed by (39; 147)). Cattle feces are also associated with *Cryptosporidium*, while poultry litter may carry *Campylobacter*, and *Salmonella* is found in a range of warm- and cold-blooded animal hosts.

The desirable qualities of an ideal indicator of fecal contamination have been reviewed by (108; 180). Ideally, indicator bacteria should always:

1. be present in feces (of humans, and possibly other animals)
2. be present in higher concentrations than pathogens, and be present at detectable levels even when pathogens are not
3. vary proportionally with pathogens (i.e. more indicator bacteria should represent a greater concentration of pathogens)
4. fail to multiply outside of the host
5. survive outside the host environment for longer time periods than pathogens
6. be more resistant to unfavourable environmental conditions (i.e. pH, temperature, low nutrient conditions, disinfectant) than pathogens

7. be detectable using simple, standard methods that yield easy to interpret, characteristic, reproducible results.

To test for fecal contamination of drinking and recreational waters, certain groups of bacteria associated with fecal matter have been identified and used as indicators. This review will focus on the most commonly used indicators of fecal contamination: coliforms, fecal coliforms, *E. coli* and fecal streptococci – and briefly discuss some other microorganisms and substances currently under evaluation.

1.3.2 Coliform Bacteria

Coliform bacteria are one of the main indicators used to determine the suitability of water for domestic and industrial use (4). The density of coliforms is considered to be proportional to the degree of fecal contamination and therefore representative of the sanitary quality of water (4).

Coliform bacteria are members of the family *Enterobacteriaceae*. In total, 83 species spanning 19 genera are formally recognized *Enterobacteriaceae* (103). This family of Gram negative, non-spore forming, facultatively anaerobic, rod-shaped bacteria are able to ferment D-glucose to produce acid and often gas (103). Coliforms are a subset of this group that are also able to ferment lactose, with gas and acid production within 48 hours at 35⁰C (4). More recently, coliforms have been defined as those bacteria capable of cleaving the artificial substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) to yield a coloured product (108). This group of bacteria includes the genera: *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Rahnella*, *Serratia*, and *Yersinia* (103; 103).

Mammals eliminate an estimated 2×10^9 coliforms per day per capita ((71) cited by (180)). Although coliform bacteria are strongly associated with the mammalian gastrointestinal tract, they are not limited to this environment. These widely distributed organisms are known to use diverse metabolic pathways, and are able to exploit a broad range of habitats (103). Strains of non-intestinal origin have been reported from a variety of

environments, including: soil (24; 49; 172), bark (49), healthy wood (10; 12; 128), plants (22; 49; 66), pristine tropical waters (17), pristine stream sediments (25; 84), insects (66), biofilms (107; 125; 188), and pulp and paper mills (12; 31; 58; 63; 64; 139; 139; 182; 186). Coliforms of non-intestinal sources are further reviewed by (5) and (108).

Causing further confusion, coliforms of non-intestinal origin often show a greater ability to survive in the aquatic environment than coliforms of intestinal origin (4).

These findings clearly show that coliforms are not a specific enough indicator to be used to assess the sanitary quality of water under all circumstances.

1.3.3 Fecal Coliform Bacteria

Fecal coliforms are a subset of the coliform group; they have all the same characteristics as coliform bacteria and are also able to ferment lactose with gas and acid production within 24 hours at 44.5°C (4). The selection of thermotolerant coliforms increases test specificity, leading to fewer false-positives. However, there are published cases, reviewed by (5) and (108), of 'fecal coliforms' of non-intestinal origin, including: soil (24; 49; 172), bark (49), healthy wood (12; 128), plants (66), pristine tropical waters (17), pristine stream sediments (25; 84), insects (66), and pulp and paper mills (12; 63; 64). There is increasing agreement that fecal coliforms do not always represent fecal contamination and that situations exist where these microorganisms do not constitute an appropriate indicator (4).

1.3.4 *E. coli*

The most widely used and accepted indicator of fecal contamination is *E. coli*. These bacteria are present in concentrations of 10⁶ to 10⁸ cells/g feces (80). The constant warm temperatures and high nutrient concentrations of the gastrointestinal tract are ideal for

E. coli growth (52). When eliminated from the gastrointestinal tract, *E. coli* find themselves in less hospitable environments which may include such growth limiting conditions as: low nutrient concentrations, osmotic stress, predation, and large variations in pH and temperature (203).

E. coli are fecal coliforms that undergo certain characteristic reactions. The IMViC tests (a battery of biochemical tests commonly used to differentiate between enterobacterial genera and species) yield the following results for typical *E. coli* (106):

1. Indole positive: converts tryptophan to indole
2. Methyl red positive: produces acidic waste products as glucose is fermented
3. Voges-Proskauer negative: fails to produce acetylmethylcarbinol during glucose fermentation
4. Citrate negative: cannot use citrate as a sole carbon source

More recently, *E. coli* have been defined by their ability to cleave the artificial substrate 4-methylumbelliferyl- β -D-glucuronide (MUG), through the activity of the enzyme β -D-glucuronidase, to a fluorescent product when grown in EC-MUG medium at a temperature of 44.5⁰C within 24 hours (4). However, the prevalence, activity and specificity of the β -D-glucuronidase enzyme may not yet be fully understood (36; 56; 99). This uncertainty has contributed to increasing interest in developing molecular methods for detecting *E. coli* based on the presence of the β -D-glucuronidase gene (13; 51), and assays for β -D-glucuronidase enzyme activity (67-69).

E. coli are associated with the gastrointestinal tracts of warm-blooded animals. These bacteria play a key role in: promoting a rapid and healthy immune response in their host, synthesizing Vitamins K and B, excluding pathogens from the system, helping to maintain peristalsis and the integrity of the host intestinal mucosa (reviewed by (16)). While the majority of *E. coli* are harmless commensals, some strains are recognized as contagions responsible for human illness. There are several distinct groups of pathogenic *E. coli*; these

have been identified and described briefly in

Table 1.2.

Commensal *E. coli* strains typically lack the specialized virulence factors that are present in pathogenic strains (47). Pathogenic strains are difficult to distinguish from nonpathogenic strains using traditional culture-based microbiological methods (136). Pathogenicity is increasingly being identified through the detection of genes encoding specific virulence factors including: toxins, adherence systems, iron acquisition systems and capsule antigens (78; 102; 104; 133; 197).

Although *E. coli* is strongly associated with fecal contamination there are many studies showing the presence of high levels of *E. coli* in situations where no known fecal contamination has occurred, especially under conditions offering relatively constant warm temperatures, including: tropical waters (17; 24; 25; 48; 172), thermally altered reservoirs (74), and industrial effluent systems (63; 128; 139). The elevated *E. coli* levels detected in these studies are generally attributed to growth in the environment. Strains of non-intestinal origin have also been reported from: soil (24; 172), healthy wood (12; 128), plants (66), pristine stream sediments (25; 84), and insects (66).

The survival paradigm for *E. coli* is 1 day in water, 1.5 days in sediment and 3 days in soil (203). Close examination reveals that this is seldom the case. *E. coli* have been reported to survive for: more than 60 days in sterile river water and sterile artificial sea water (20), up to 16 days in a lake water microcosm (21), more than 200 days in sterile soil (185), and more than 70 days in manure (59). After application of manure to natural soil, *E. coli* were detected for: more than 28 days (142), 60 days (8), up to 64 days (40), and more than 120 days (137).

E. coli may improve their chances of surviving outside the intestinal niche by: growing within plant vascular systems (173), multiplying within protozoan vacuoles (11), or possibly by entering a viable-but-non-culturable state (VBNC) (203).

Table 1.2: *E. coli* pathotypes and their characteristics: primary habitat, clinical manifestation, persons or animals most likely to be affected, virulence factors and mode(s) of transmission(96; 136).

<i>E. coli</i> Pathotypes	Description
Commensal <i>E. coli</i>	- Found in mucous layer of mammalian colon - Produces vitamins, prevents pathogen growth, develops infant immune system - Transmitted through fecal contamination of food or water
ETEC Enterotoxigenic <i>E. coli</i>	- Colonizes mucous layer of small intestine - Watery diarrhea, low grade fever, vomiting, nausea, abdominal cramps - Childhood/traveler's diarrhea in developing world; diarrhea in young animals - Heat Labile Toxin (LTI and LTII^a), Heat Stable Toxin (STa and STb^a), Low-MW heat stable toxin (EAST1), Colonization factor antigen (CFA/I and /II) - Transmitted through fecal contamination of food or water
EPEC Enteropathogenic <i>E. coli</i>	- Attaches to epithelial cells of villus tips in the small intestine - Profuse watery diarrhea, vomiting, fever - Major cause of infant diarrhea in the developing world, most adults are immune - Low-MW heat stable toxin (EAST1), Intimin (eae), Bundle-forming pilus (Bfp) - Fecal-oral transmission
STEC Shiga toxin-producing <i>E. coli</i> i.e. O157:H7	- Colonizes the colon and travels through bloodstream to kidneys - Uncomplicated diarrhea, hemorrhagic colitis, hemolytic uremic syndrome - More common in developed countries; young and old are most severely affected - Shiga toxin I (Stx1), Shiga toxin II (Stx2), Low-MW heat stable toxin (EAST1), EHEC hemolysin (Ehx)^a, α-hemolysin (hly), Intimin (eae) - Transmitted through fecal contamination of food or water (cattle are main reservoir), or person-to-person; low infectious dose (less than 100 cells)
EIEC Enteroinvasive <i>E. coli</i>	- Invades and destroys epithelial cells of small and large intestine - Watery diarrhea, dysentery - Found mainly in tropics - Invasion plasmid antigen (Ipa), Aerobactin - Transmitted through fecal contamination of food or water, or person-to-person
EAEC Enteraggregative <i>E. coli</i>	- Adhere to Hep-2 cells (intestine) and each other in 'stacked brick configuration' - Watery or mucoid diarrhea, low grade fever - Adults and children in developing countries are affected - Low-MW heat stable toxin (EAST1), Aggregative adherence fimbriae (AAF/I) - Fecal-oral transmission
DAEC Diffusely-adherent <i>E. coli</i>	- Colonizes intestinal mucosa, specifically HEp-2 cells, in a monolayer - Chronic diarrhea for 14 days or longer - Affects children of less than 1 year - Virulence factors are still poorly understood - Fecal-oral transmission
UPEC Uropathogenic <i>E. coli</i>	- Invade and replicate within uroepithelial cells - Urinary tract infections, asymptomatic bacteriuria, cystitis, pyelonephritis - Occurs in women more frequently than men α-hemolysin (hly), Cytotoxic necrotizing factor (CNFI), Aerobactin, F1C fimbriae; S fimbriae; P fimbriae, K1 capsule antigen; K5 capsule antigen - Strains isolated from the urine are identical to those isolated from the feces
NMEC Neonatal meningitis <i>E. coli</i>	- Infects membranes surrounding brain and spinal cord (meninges) or blood - Meningitis, sepsis - Affects infants - S fimbriae, Aerobactin, K1 capsule antigen - Transmitted by asymptomatic mothers or nursery staff to infants

^a isolated primarily from animals

It is clear that under some situations *E. coli* deviate from the requirements of an ideal indicator: they are not always associated with a fecal source; they demonstrate growth in non-intestinal environments (meaning that their concentration would no longer vary proportionally with the concentration of pathogens); and they may experience lengthy survival times outside the host. There is increasing awareness that *E. coli* may not always be an appropriate choice as an indicator of fecal contamination (4).

1.3.5 Fecal Streptococci

Fecal streptococci are another group of organisms that are frequently isolated from the feces of warm-blooded animals (16) and are currently in use as indicators of fecal contamination (4).

Fecal streptococci are Lancefield's Group D Streptococci (4). They include: *Streptococcus faecalis*, *Strep. faecium*, *Strep. avium*, *Strep. bovis*, *Strep. equinus* and *Strep. gallinarum* (4).

The ratio of fecal coliform bacteria to fecal streptococci was once used to differentiate between human and non-human sources. This test is no longer considered valid, as coliform bacteria and streptococci demonstrate different survival rates (4; 88).

Some research suggests that fecal streptococci may be fairly ubiquitous in the environment and not necessarily indicative of fecal contamination (reviewed by (5; 165; 180)). For example, these microorganisms have been successfully isolated from plants and insects (66).

Enterococci are a subgroup of fecal Streptococci, limited to *Strep. faecalis*, *Strep. faecium*, *Strep. gallinarum*, and *Strep. avium*. These bacteria are classified as Lancefield Group D Streptococci capable of growing in 6.5% saline with a pH of 9.6 at temperatures of both 10°C and 45°C (4). The concentration of enterococci is strongly correlated with the risk of contracting swimming-associated gastroenteritis (4). As a result, these

microorganisms are considered to be the best indicator of water quality for both fresh (limit of 33CFU/100mL) and marine (limit of 35CFU/100mL) recreational waters (4).

1.3.6 Other Potential Indicators

There are approximately 10^{14} cells in the human gastrointestinal tract (52) - a diverse assemblage of an estimated 400 to 500 species (16). In theory, any one of these species has the potential to be an indicator of fecal contamination. In reality, many of these organisms have limited distributions, are present only in low numbers, are difficult to culture, do not survive well outside of the host, or are poorly characterized. Two microorganisms that have received some attention as potential indicators are *Clostridium* and *Bifidobacterium* (18). These organisms are present in high numbers in the intestine, with counts exceeding 10^{10} CFU/g feces (108). However, both organisms are obligate anaerobes, their survival outside the host is not well characterized, and more sensitive detection methods are required (165). *Clostridium* may be more indicative of the presence of viruses than other bacteria as they are able to form spores; while *Bifidobacterium* show limited survival outside of the host and may have an application indicating recent fecal contamination (165).

Enteric viruses are generally present small numbers, and are difficult to detect (165). Some other viruses associated with the gastrointestinal tract may be better candidates, for example, coliphages (viruses that infect *E. coli*) and bacteriophages of *Bacteroides fragilis* (6). These viruses seem to be host specific, and their concentration seems to correlate with enteric virus concentrations, however they are not always present in host feces and they remain poorly understood (165).

Still other methods are being developed to examine chemical indicators of fecal pollution. One of the most promising candidates is coprostanol – a waste product from the bacterial breakdown of cholesterol in the gastrointestinal tract (165).

1.3 Microbial Source Tracking

Microbial source tracking methods have been primarily developed in the clinical setting for: understanding the spread of disease, identifying the source of an outbreak, recognizing the sources of nosocomial infections, monitoring vaccination programs and detecting particularly virulent strains of a pathogen (143). More recently, these techniques have been applied to identify sources of fecal contamination to drinking and recreational waters. Once fecal sources have been identified, it becomes possible to: assess the risks associated with the fecal contamination; undertake strategies to reduce further contamination; and minimize the inconvenience, cost and concern caused by boil-water advisories, shellfish bans and beach closures (50).

A perfect microbial source tracking system will (reviewed by (176)):

1. Be able to type all isolates. The feature of interest should be present and typeable in all members of the species.
2. Yield reproducible data. Repeated typing events should consistently generate identical or similar results.
3. Produce stable results. Good source tracking methods use features that are continuously expressed by the isolate over time and generations.
4. Show an appropriate level of discriminatory power (the ability of the method to distinguish closely related strains from less closely related strains)

5. Demonstrate agreement with epidemiological data. The method should group strains in a manner that agrees with data from well-characterized outbreaks.
6. Be versatile (able to work with a broad range of different microorganisms).
7. Be practical (use available reagents and equipment).
8. Produce easily interpretable results.
9. Be cost-effective.
10. Provide results rapidly.

No single microbial source tracking system meets all of these criteria (75). Typically in an outbreak situation, isolates are screened using a 'quick and dirty method' while a complementary method is used for confirmation (176). Microbial source tracking methods mainly fall into phenotypic and genotypic categories. These are discussed in greater detail below, and some examples of the most common methods (and their successes and drawbacks) have been included.

1.4.1 Phenotypic Source Tracking

Phenotype refers to the physical traits expressed by an organism. These traits are encoded by genes, but their expression may be influenced by environmental factors. Bacterial phenotypic traits include: carbon source utilization, phage susceptibility, immunological markers and resistance to antibiotics.

Phenotypic markers tend to be less stable and reliable than genotypic markers due to the influence of the environment; they are also plagued by low typeability. However, these methods are some of the best-characterized and most entrenched methods of microbial source tracking. They are relatively inexpensive and continue to play a useful role.

1.4.1.1 Carbon Source Utilization

Bacteria use carbon as an energy source and to construct new biological molecules. Over time, bacteria have evolved different catabolic systems to exploit the carbon substrates that are available in the environments they inhabit. Carbon source utilization studies compare the abilities of different isolates to exploit a variety of carbon substrates (i.e. sugars, sugar alcohols, fatty acids, carbohydrates, nucleosides, etc.).

This technique is generally poorly discriminatory below the species level and is affected by phenotypic instability (165). Nonetheless, these methods are a cornerstone in microbiology. For example, coliforms are identified based on their ability to use lactose (4); *E. coli* are defined, in part, by their inability to use citrate as a carbon source (103); and *E. coli* O157:H7 are distinguished from other *E. coli* by their inability to ferment sorbitol (19).

Commercial carbon source utilization systems are available for use. A recent study found that *Enterococcus* isolates were correctly classified as human or non-human more than 90% of the time (76).

1.4.1.2 Phage Susceptibility

Prior to infecting a bacterial cell, bacteriophages must bind to specific receptors on bacterial cell surfaces. Different bacteria have different receptors, this means that while some bacteriophages will be able to bind, others will not. Bacteria can be differentiated based on which phages are able to cause infection. This method is reported to be more discriminatory than ribotyping (124), but less discriminatory than PFGE (154). Untypeability (143) and the necessity of having large banks of phages available limit the use of this method.

1.4.1.3 Immunological Methods

When bacterial cells invade a potential host, they are identified by antibodies which recognize antigens (key components of bacterial cellular structures) as foreign and trigger an appropriate response in the host's immune system to eliminate the invading cells. Serotyping uses the specific binding between antibody and antigen to type bacterial strains. *E. coli* are typed by their O, H and K antigens.

O antigens are somatic antigens; lipopolysaccharides embedded in the bacterial cell wall (80). O antigens are untypeable in rough colonies (80; 197).

H groups are identified via antigenic determinants on the bacterial flagella (80). H antigens are often recorded as untypeable when bacteria are sluggishly motile or non-motile (shown as NM or N-).

K antigens are capsular antigens. K antigens are no longer commonly used with *E. coli*, although they may come into play if the O group is untypeable, as capsules can mask O antigens (197).

Serotyping is tedious, expensive and typically only performed in large, regulated laboratories with the required banks of antisera (165). Since serotyping was developed in the clinical setting, environmental strains show an elevated rate of untypeability.

Certain serotypes are reproducibly associated with clinical disease, although the antigens themselves do not confer pathogenicity. This method has been in use in clinical settings for over 40 years and, despite its limitations, is likely to remain in use (1).

1.4.1.4 Multiple Antibiotic Resistance Typing (MAR)

MAR (or antibiotic resistance analysis, ARA) typing is based on the idea that humans and animals are exposed to different types and concentrations of antibiotics at

different frequencies. Over time, the selective pressures exerted by the presence of antibiotics lead to varying degrees of resistance among exposed bacteria (165).

For example, in a study examining *E. coli* isolated from Kenyan chickens and children (97), researchers were aware that the chickens were regularly exposed to tetracycline in drinking water and feed, whereas the children received ampicillin, co-trimoxazole and/or gentamicin intermittently. More than half (59.7%) of chicken isolates were resistant to tetracycline; while most (85.7%) of the *E. coli* isolated from children were multidrug resistant.

MAR typing involves: isolating the desired microorganism from a sample (of the matrix containing the pollution of unknown origin) as well as from possible sources, replica plating the isolates onto media with varying concentrations of different types of antibiotics, scoring the isolates according to their susceptibilities, and generating profiles which are analyzed using discriminate or cluster analysis (165).

MAR has many drawbacks. Firstly, antibiotic resistance is usually carried on plasmids (small, circular pieces of DNA, distinct from the bacterial chromosome). Plasmids are readily gained (if the selective pressure is present), but equally readily lost (once the selective pressure is gone). Changes in environmental conditions, lengthy storage times, and repeated sub-culturing can alter the MAR fingerprint generated by a given isolate (165). Secondly, this technique cannot be used to discriminate between isolates from sources that have not been exposed to antibiotics (i.e. isolates from the feces of wild animals, or environmental strains not associated with the intestine) (165). Thirdly, MAR requires the preparation of a library of profiles from isolates of known sources. There are many unknowns associated with the preparation of an MAR library, including: the appropriate

number of isolates per source, the stability of an MAR profile over time, and the geographic variability of MAR profiles. Fourthly, there are no set guidelines regarding: which bacteria should be analyzed (i.e. fecal coliforms, fecal streptococci or other), the number of antibiotics that should be employed, which antibiotic types, or in what concentrations.

MAR success is measured in terms of average rate of correct classification (ARCC). These values range from poor ARCCs of 46% to 78.4% ((81; 201; 202)) to more successful ARCC values of 84% to 95% (201; 202). MAR has also been used to distinguish between point and non-point sources in an estuary (148) and to determine the sources of fecal contamination in surface waters (98). One of MAR's success stories involves the correct identification of fecal loading to a rural stream (cattle), the implementation of measures to reduce fecal loading (i.e. restricting cattle access to stream), and the subsequent follow-up demonstrating a 94% reduction in the numbers of fecal coliforms (77).

1.4.2 Genotypic Source Tracking

Genotype refers to the genetic composition of an individual. Genotypic typing techniques offer several advantages over traditional phenotyping techniques: there is a higher rate of typeability (all bacteria have DNA), greater stability of markers (the environment has a more gradual impact), a broader range of discriminatory powers, and very fine-scale differentiation of closely related strains is possible. Genotypic methods include a wide variety of techniques: pulsed field gel electrophoresis, ribotyping, random amplified DNA, repetitive sequence-based PCR, and a number of other PCR-based restriction fragment length polymorphism or sequencing techniques.

1.4.2.1 Pulsed Field Gel Electrophoresis (PFGE)

PFGE has the highest discriminatory power and is the microbial source tracking gold standard (143). Although PFGE directly detects variation in less than 0.01% of the chromosome(176), the banding patterns detect a range of random genetic events, including: point mutations, insertions, and deletions (181). These events are detected as changes in band number or size.

In this method, a rare-cutting restriction enzyme is used to cut the chromosomal DNA into 30 or fewer fragments, ranging from 10 to 700 kb (176). Gel electrophoresis with a pulsed field (the orientation of the electric field is changed periodically) is used to clearly separate the large DNA fragments (176). The resulting PFGE banding patterns can then be compared.

This technique is versatile (it can be applied to any bacteria or yeast) (176), and straightforward, however, specialized equipment is required (143). Obtaining results by standard methods is time-consuming, typically taking 2 to 4 days (176), but rapid protocols are being developed (65). PFGE banding patterns have been shown to be relatively stable over extended periods of time and multiple subculturing events (89).

PFGE has been used: to clearly distinguish between sporadic *E. coli* O157:H7 strains and those associated with an outbreak and to confirm the source of an outbreak (92); and to epidemiologically group *E. coli* O157:H7 strains from multiple outbreak events in Ontario (154). However, a study designed to assess the diversity, persistence and possible sources of *E. coli* O157:H7 in a cattle range found that PFGE had difficulty grouping epidemiologically linked strains due to the high diversity of the isolates (159). PFGE works

well in outbreak situations where closely related isolates are being sought, but is not the best choice for situations where high diversity is expected (42; 78).

1.4.2.2. Ribotyping

Ribosomes are the organelles responsible for building proteins. As they play such an important role in the cell, the genes encoding these organelles are highly conserved, both within and between species (176). Ribotyping is an involved procedure: bacteria are isolated, cultured and identified; DNA is extracted, treated with restriction enzymes and subjected to gel electrophoresis; ribosome genes (rRNA genes) are detected using oligonucleotide probes in a Southern blotting procedure; the resulting profiles are analyzed using discriminant analysis or 100% similarity (32; 143).

While ribotyping produces stable, reproducible profiles (both within and between labs) and there are fully automated commercial kits available (176), the procedure is labour-intensive, costly and requires a source library (143). There is also a lack of consensus regarding the number and types of restriction enzymes to use, detection methods (colourimetric vs. radioactive Southern blotting), and data analysis (143; 176).

The discriminatory power of this technique varies, but is less than PFGE or Rep-PCR (143). Ribotyping offers the unique advantage of detecting and identifying bacterial species misidentified by culture-based techniques (78). Ribotyping also seems able to discriminate well between *E. coli* of human and non-human sources (32; 149). However, it is not discriminatory enough to usefully group *E. coli* O157:H7 isolates (9; 124).

1.4.2.3 Random Amplified Polymorphic DNA (RAPD)

RAPD (also known as arbitrary primed PCR, AP-PCR) involves allowing random, short primers (approximately 10 bp) to bind to chromosomal DNA at a low annealing temperature (143). PCR will amplify the regions between pairs of binding sites and generate DNA fragments of varying sizes. These fragments can then be resolved on an electrophoretic gel. The number and size of the resulting bands will vary depending on the bacterial strain (143).

This technique requires no prior sequence knowledge and can generate DNA fingerprints for a broad range of organisms, including bacteria, fungi and protozoans (176). RAPD is more discriminatory than ribotyping, but slightly less discriminatory than PFGE or Rep-PCR (143).

There are several drawbacks to this technique. Subtle changes in PCR conditions can lead to noticeable differences in results, leading to low reproducibility (130). Furthermore, neither primers nor PCR conditions are standardized as these are determined empirically (176).

Despite the problems with interlaboratory reproducibility, RAPD has been used to successfully differentiate between strains of bacteria in a number of studies: *E. coli* (34), porcine diarrheagenic *E. coli* (145), and *Xanthomonas fragariae* (152).

1.4.2.4 Repetitive Sequence-Based PCR (Rep-PCR)

Rep-PCR amplifies the regions between highly conserved repetitive elements in bacterial chromosomes, producing DNA fragments of different sizes (195). Thus far, 3 classes of repetitive bacterial DNA have been identified: REP, ERIC and BOX. These sequences are widely distributed among enteric bacteria (143; 195).

Repetitive extragenic palindromic (REP) elements are highly conserved 35 bp sequences found in non-coding parts of bacterial chromosomes (175). When transcribed to mRNA, these sequences have the ability to fold back on themselves forming a stable stem-loop structure. The stems of these structures are very highly conserved, while the loops show more variability (175). The function of these repetitive sequences remain poorly understood, however: the stem-loop structures have been shown to block mRNA degradatory enzymes, increasing the expression of downstream genes (138); DNA gyrase and DNA polymerase I have been demonstrated to bind specifically to REP sequences, suggesting a role in supercoiling bacterial DNA (72; 205); Shyamala *et al.* posit a role in chromosomal duplication events (168); others suggest that the widespread presence and highly homologous nature of REP sequences indicate that they are selfish DNA (87; 138). REP primers are short and contain deoxyinosines (a deoxynucleotide which can bind nonpreferentially to A, T, C or G) (114). Depending on the application, REP PCR may generate highly discriminatory complex patterns (195), or simpler patterns with a lower discriminatory level (165).

ERIC (enterobacterial repetitive intergenic consensus) sequences are 126 bp elements (143). Like REP sequences, they contain a highly conserved inverted central repeat (195). Some studies have found that ERIC primers yield more reproducible results than REP (114), while others report that ERIC primers may be more sensitive to suboptimal PCR conditions (i.e. contaminants) (165).

BOX sequences are mosaic repetitive elements composed of various combinations of boxA, boxB and boxC (with lengths of 59, 45, 50 bp respectively) (143). These sequences,

found in the non-coding region, form stem-loop structures (143) and can generate complex fingerprints.

Rep-PCR fingerprint patterns can be examined using a band-based or densitometric curve approach, the latter is considered to be less biased (2; 82).

Rep-PCR fingerprints are stable over time and repeated subculturing (83; 95; 166; 195). Reproducibility is high within a lab, but tends to be low between labs (83) (although similar groupings are achieved, the actual banding patterns cannot be directly compared). Culture technique, DNA extraction method and concentration of template DNA have not been shown to affect reproducibility (83), although choice of thermocycler does (2). Rep-PCR is described as having a discriminatory power slightly less than PFGE (143).

Differentiation between species or strains of many genera has been achieved with Rep-PCR, including: *Acinetobacter baumannii* (158; 171), *Bradyrhizobium japonicum* (94), *Citrobacter diversus* (204), *Clostridium difficile* (140), *E. coli* (78), *Rhizobium meliloti* (43), *Xanthomonas fragariae* (152), *Bartonella sp.* (161), *Legionella sp.* (70), *Streptomyces sp.* (162) and fungi *Drechslera avenae* and *Stemphylium solani* (although in fungi Rep seemed to act more like RAPD) (129).

Rep-PCR has been used successfully with *E. coli* to: track pathogenic strains responsible for bovine mastitis (114), classify avian isolates (41), assess the diversity of strains in the Brazilian Arroio Feijo (48), distinguish between strains of human and animal origin with a high degree of confidence (33; 46), and confidently identify non-point fecal sources (166).

Rep-PCR is gaining a reputation as being an affordable, rapid, simple source-tracking system, that uses available equipment to deliver stable, reproducible results that offer a high degree of discriminatory power (143).

1.4.2.5 Amplified Fragment Length Polymorphism (AFLP)

AFLP involves: extracting and purifying DNA, cutting DNA with restriction enzymes, joining cut ends to radioactively or fluorescently labeled linkers, and running the products on an agarose gel (143). Since this technique targets random portions of the genome it is expected to be quite discriminatory (78). AFLP has been described as reproducible (143; 176), of unknown fingerprint stability (176), less time-consuming than PFGE (143), but more labour-intensive and costly than Rep-PCR (143).

AFLP has been used to successfully group strains by their pathogenicity (78) and host source (109).

1.4.2.6 PCR-based RFLP typing, and Sequencing

Polymerase chain reaction-based restriction fragment length polymorphism typing (PCR-based RFLP typing) involves amplifying a gene of interest, cutting it with restriction enzymes, running the DNA on an electrophoretic gel and examining the resulting profiles for differences in banding patterns (143). Primers can be designed to take advantage of conserved genetic regions flanking a more variable gene. One of the more popular variants of this technique examines the genes responsible for the features examined in phenotypic serotyping (i.e. flagellins, outer membrane proteins). Unlike phenotypic serotyping, no banks of antisera are required, the techniques and reagents are widely applicable and available, and untypeable serological phenotypes can be resolved (176). However, these kinds of PCR-based techniques offer moderate discrimination between strains as a single

gene is being examined (143). Horizontal gene transfer events or rapid genetic rearrangements can confuse results as only a small portion of the chromosome is examined (176).

Sequencing has the highest discriminatory power. It is sensitive, accurate, reproducible and results can be easily compared between laboratories. Historically, the specialized equipment, reagent cost, and time-consuming nature of the method meant sequencing was impractical for use as a microbial source tracking tool (176). However, new sequencer technologies, high-throughput system development and reductions in equipment and reagent cost mean that sequencing has the potential to become a practical microbial source tracking technique (176) and has already become a gold standard for viral typing (143).

Chapter 2: Sources of *E. coli* to Pulp and Paper Biosolids

2.1 Introduction

Papermaking consumes enormous volumes of water, and generates equally large volumes of wastewater. These wastewaters must be treated to reduce biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS) and adsorbable organic halides (AOX compounds) which are damaging to the receiving waters (3). The resulting biosolids are a byproduct of this treatment process: a combination of the solids removed during primary clarification (wood fibres and particles, bark, calcium carbonate, clay, ash particles and mill process chemicals) and the surplus microbial floc biomass from the activated sludge (secondary biotreatment) aeration tank (93). Traditional disposal methods include landfilling and incineration; however, combined dewatered biosolids have excellent potential as a soil conditioner. Pulp and paper mill biosolids generally have low levels of toxic metals, excellent water holding capacity, and C:N:P ratios that are favourable for plant growth (27). However, the presence of high levels of *E. coli* and other indicators of fecal contamination are cause for concern for mill workers, policy makers and the public.

The occurrence of these microorganisms is widespread, even in the absence of any known fecal input (i.e. cases where mill sewage was treated off-site) (12; 31; 58; 62; 63; 128; 139). If these ‘fecal indicator’ bacteria do not come from any detectable fecal source, where do they come from?

The high levels of *E. coli* in pulp and paper mill effluents and biosolids have generally been attributed to in-mill growth (128; 139), particularly within the log washer or wet debarker (128), the primary clarifier, and biofilms in pipes, tanks and reservoirs (63).

Some studies have suggested probable sources of inoculating *E. coli* (12; 63; 128), but this question has never been addressed directly using the ability of molecular techniques to distinguish clearly among *E. coli* strains (180).

This study searches for the inoculating source(s) *E. coli* in biosolids by applying the microbial source tracking tool, Repetitive sequence-based Polymerase Chain Reaction (Rep-PCR), to a collection of confirmed *E. coli* isolates and generating highly discriminatory molecular isolate fingerprints from both biosolids and potential sources. Rep-PCR has been used in epidemiological studies (114; 41) and to track fecal contamination (33; 46; 48; 166), but has never before been applied to an industrial situation.

An improved understanding of the sources of pulp and paper mill *E. coli* will help to:

- 1) Assess the microbial risks associated with using pulp and paper mill biosolids as a soil conditioner. Do *E. coli* in biosolids come from ‘high risk’ (fecal) or ‘low risk’ (nonfecal) sources?
- 2) Provide mills with the information needed for effective and efficient treatment (i.e. pre-treatment of key waste streams vs. post-production biosolids treatment).
- 3) Assist regulatory bodies in the development of new microbiological standards appropriate for pulp and paper biosolids.

2.2 Materials and Methods

2.2.1 Sampling Sites

In order to compare biosolids-isolated *E. coli* to *E. coli* isolated from an environmental (non-fecal) source, two forested sites were sampled on November 5, 2004. Both sites had

undergone recent cuts, and the harvested logs were sent to the mill being studied in this investigation.

The first site was a 16 year old hybrid poplar plantation (*Populus sp.*) located in South Glengarry (eastern Ontario, Canada). The site had recently experienced a strip cut. Three sample sets (located 10 m from each other) of leaf litter and soil were collected from the cut portion of the site. Single samples of soil from machinery treads, and decayed wood were also collected. Two samples (located 10 m from each other) of healthy wood, leaf litter and soil were collected from the uncut portion of the site.

The second site was an approximately 40 year old hardwood forest located in North Glengarry (eastern Ontario, Canada). Tree species included: red maple (*Acer rubrum*), sugar maple (*A. saccharum*), yellow birch (*Betula alleghaniensis*), black cherry (*Prunus serotina*) and white ash (*Fraxinus americana*). The site had recently experienced a selective cut. Three sample sets (located a minimum of 10 m from each other) of soil, leaf litter, and healthy wood (4 samples, 1 of each of: yellow birch, black cherry, red maple and white ash), and single samples of bark and decayed wood were sampled from the cut portion of the site. Three sample sets (located a minimum of 10 m from each other) of soil, leaf litter and healthy wood (4 samples, same species as above), and single samples of bark and decayed wood, were collected from the uncut portion of the site. Sampling sites in the uncut region were selected to include trees that had been marked for future harvest.

A total of 19 sets of samples were collected on a biweekly basis between May 2004 and February 2005 from an Ontario hardwood kraft pulp and paper mill using primary clarification (mechanical fibre and particle settling), and an activated sludge biotreatment system (to purify mill wastewater). Mill water and wastewater systems are diagrammed in

detail in Figure 1-1. Mill sanitary wastewaters were collected separately and processed through the municipal water treatment facility. Each sample set consisted of combined dewatered biosolids and three inputs to the effluent treatment system: chlorinated mill process feed water, mill fibre (cleaner rejects imported from a corrugated cardboard manufacturer, used to promote efficient screwpress dewatering), and wood chips/dust. Storm effluent samples were collected on four occasions. Samples were also collected from locations in the effluent treatment system: primary clarifier effluents and fibre rejects; effluents (supernatants), recycled activated sludges (RAS) and waste activated sludges (WAS) from both secondary clarifiers.

2.2.2 Sampling Procedures

Liquid and solid grab samples were collected and stored on ice for transport to the St. Lawrence River Institute of Environmental Sciences (SLRIES), located in Cornwall, Ontario. Samples were stored at 4°C. All processing occurred within 24 hrs of sample collection.

Soil samples were collected to a depth of 5 cm using a 3.5 cm diameter sterile polyethylene tube (Corning) as a corer. Leaf litter, bark and decayed wood samples were collected with a gloved hand and packed in sterile WhirlPak bags (Nasco). Healthy wood was sampled by using a sterile increment borer to remove a core from the tree at breast height. Healthy wood cores were also stored in sterile WhirlPak bags (Nasco).

Liquid grab samples were collected in sterile 250 mL polypropylene bottles, with sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) as a preservative (Systems Plus). Primary and secondary clarifier effluent grab samples were collected as they passed out of the clarifiers. Storm effluent was sampled as it passed from a free-flowing sewer into the primary sump during

wet weather events. The remaining liquid grab samples (mill feed water, primary sludge, RAS and WAS) were collected from pipes or hoses which were flushed for a minimum of 30 seconds prior to sample collection.

Mill fibres were stockpiled in the yard and periodically shoveled into the primary sump. Samples were collected from below the surface of the pile. Wood chips and dust were collected from debris that had settled next to the chipper conveyor belt. Biosolids in this mill were continuously produced by screw presses, stored outdoors and shipped off-site every 3 hours. Samples were collected from the surface of the outdoor pile. Solid grab samples were collected in sterile Whirl-Pak polyethylene bags (Nasco).

2.2.3 *E. coli* Isolation

Feed water samples (100 mL) underwent membrane filtration (0.45 µm filter composed of mixed cellulose esters, Millipore); all other liquid samples were spread directly (or with the appropriate dilution in phosphate buffer (4)) onto Differential Coliform Media (DCM) plates (SLRIES). Solid samples were weighed, diluted in phosphate buffer (4), shaken for 1 min. and spread onto DCM plates (SLRIES). A minimum of 4 filters or 4 spread plates were prepared for each sample. Plates were incubated for 24 hrs +/- 2 hrs at 35⁰C. Blue colonies (β-D-glucuronidase positive) were streaked onto either MacConkey Agar without salt (Difco) or Eosin Methylene Blue Agar (BBL, Accumedica). Plates were incubated overnight at 35⁰C, and strong lactose fermenters (pink on MacConkey; black or gold on EMB) were streaked onto Luria Bertani (LB) agar (Fisher BioTech). Plates were incubated overnight at 35⁰C.

2.2.4 *E. coli* Confirmation

Putative *E. coli* isolates were confirmed using the classic IMViC (indole, methyl red, Voges-Proskauer, Citrate) and MUG (4-methyl-umbelliferyl-D-glucuronide) tests. Isolated colonies on LB agar were used to inoculate: 1) trypticase (BBL); 2) MR-VP broth (Accumedia); 3) Simmon's Citrate (EM Science); and 4) nutrient agar supplemented with MUG (Difco). Isolates were considered *E. coli* if they: 1) were able to produce indole from tryptophan; 2) were able to produce acidic products when grown in MR-VP broth; 3) were unable to use citrate as a sole carbon source; and 4) were able to convert MUG to a fluorescent product. Isolates deviating by more than 1 test result were discarded. Tests were prepared, performed and interpreted as described in (106).

2.2.5 *E. coli* Preservation

E. coli isolates were grown overnight in LB broth (Fisher BioTech). Equal parts broth culture and sterile 80% glycerol were combined and stored at -20°C. As needed, strains were resuscitated by incubation overnight at 35°C on LB agar. Strains were then streaked on EMB (BBL, Accumedia) and incubated overnight at 35°C. A single lactose-fermenting colony was selected and streaked onto LB agar (Fisher BioTech) and grown overnight at 35°C.

2.2.6 DNA Extraction

E. coli template DNA was prepared using a whole cell extraction method. An isolated colony was suspended in 100µL of sterile DNase/RNase free water (Gibco) and agitated.

2.2.7 PCR Protocol

DNA fingerprints were generated using the REP primers (repetitive enterobacterial palindromic sequences) (REP1R and REP2), reactions, and thermocycler protocols described by (157) modified to use 2 μ L of template DNA with 23 μ L of PCR reaction mixture. All isolates were extracted, amplified and visualized in separate duplicate runs. Each PCR run included a negative control (DNase/RNase free water), and a positive control (*E. coli* K12). PCRs were performed on an Eppendorf Mastercycler epGradient thermocycler.

2.2.8 Detection of Amplified DNA

PCR products were visualized using gel electrophoresis. Amplified DNA was run on 1.5% agarose gel (110V; 2.5 hrs), stained with ethidium bromide and viewed using an AphaDigiDoc RT Gel Documentation System. A 1kb ladder (Promega) was used as a size standard. Four ladders were run per 26 well gel.

2.2.9 Analysis

Data were analyzed using GelComparII v. 4.5 (Applied Maths). Isolates that failed to amplify or did not generate reproducible fingerprints were excluded from further analysis. Fragments between 250 and 10 000 bp were analyzed. Fingerprint similarity was calculated using cosine correlation (a densitometric curve-based method) with an optimization setting of 0.41. Similarity was used to infer the genetic relatedness of 2 or more fingerprints. For the purposes of this study, fingerprints having a similarity of: <60% were considered to be unrelated (127); >85% were visually identical and considered to be closely related; >85% and collected from the same sample matrix and sampling event were considered clonal (82). Dendrograms organized fingerprint similarity data to reveal

patterns in genetic relatedness. Clusters were defined as groups of fingerprints sharing a similarity of >85%. Dendrograms were constructed using the Unweighted Pair Group Method using Arithmetic averages (UPGMA). Jackknife analysis involved manually assigning fingerprints to their sample type. Each fingerprint, in turn, was then treated as an unknown and the maximum similarity coefficient was used to reassign the fingerprint to the sample type to which it showed the greatest similarity. The percent of fingerprints of a given sample type being correctly reassigned to that sample type during jackknife analysis was referred to as the Rate of Correct Classification (RCC). Average rates of correct classification (ARCC) were calculated as a weighted average of RCCs for all sample types examined in a given scenario. Misassignments (or misclassifications) referred to the percent of fingerprints of a given sample type that were reassigned to a particular incorrect sample type. Clones were removed prior to performing jackknife calculations to avoid artificially inflating RCC values (suggested by (82)).

To allow comparisons between the various inputs, effluent treatment system and biosolids *E. coli* populations, basic ecological parameters were calculated: strain richness (S), Shannon Diversity Index (H), and strain evenness (E). Strain richness was defined as the number of unique fingerprint types identified in a sample type. The Shannon Diversity Index measured of the order (or disorder) within a system by taking both richness and abundance into account. It was calculated using $H = -\sum((P_i)/\ln[P_i])$. Strain evenness compared the abundance of the various strains detected within each population and was calculated using $E = H/\ln S$. Larger values (approaching 1) signified an even abundance of diverse strains, while values approaching 0 suggested the dominance of a single strain.

2.3 Results

2.3.1 *E. coli* Detection

Very few *E. coli* were successfully isolated from the forested sites. No *E. coli* were detected in healthy wood, decaying wood, or bark samples (n=15). Low concentrations were recovered from soil from heavy machinery tracks from the poplar plantation and soil and leaf litter from the hardwood forest (Figure 2-1).

All on-site inputs yielded *E. coli* (Figure 2-2). Storm effluents were sampled 4 times, and were always found to contain high *E. coli* levels (10^3 to 10^5 CFU/100mL). Low *E. coli* levels (less than 10 CFU/100mL) were detected in the treated mill process feed waters at 1 of 19 (5%) of sampling events. *E. coli* were isolated 84% of the time from mill fibre, in numbers comparable to those found in pulp and paper mill biosolids. *E. coli* were detected in wood chips and dust at 47% of sampling events, and when detected were present in concentrations of 10^2 to 10^4 CFU/gdw.

E. coli were detected throughout the effluent treatment system. Within the primary clarifier, *E. coli* were detected in 53% of samplings, and were present in concentrations between 10^1 and 10^4 CFU/100mL (Figure 2-3). *E. coli* were routinely isolated (92% of samplings) from the secondary clarifiers; concentrations as high as 10^3 to 10^5 CFU/100mL were often detected in activated sludge streams (RAS and WAS), while lower concentrations were detected less frequently in effluents (Figure 2-4).

E. coli were consistently isolated from pulp and paper mill biosolids; when detected, (95% of samplings) concentrations typically ranged between 10^2 to 10^5 CFU/gdw (Figure 2-5).

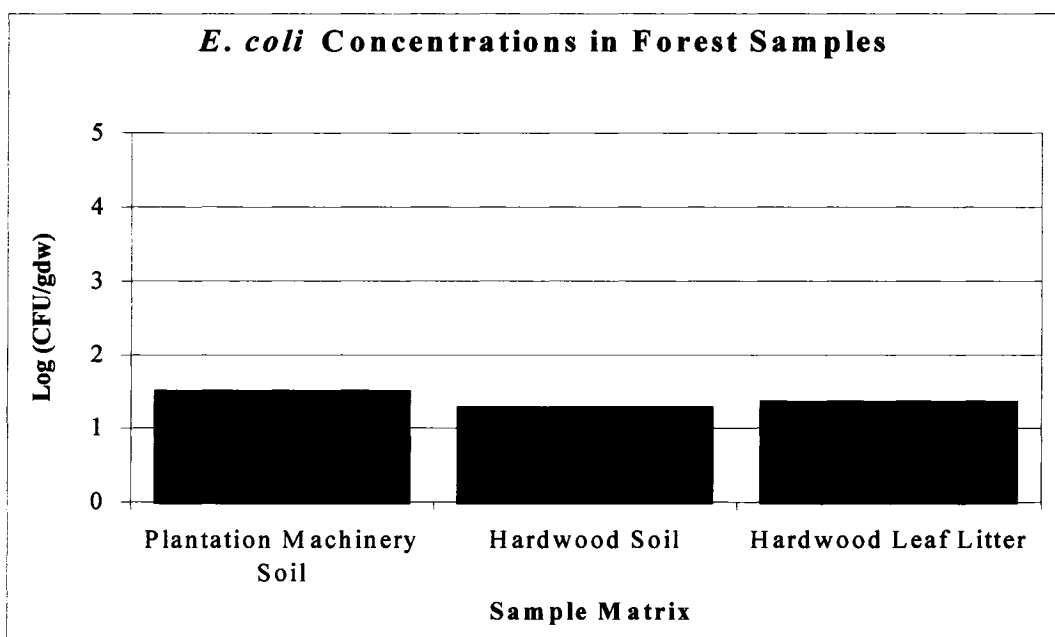


Figure 2-1: Log *E. coli* concentrations (CFU/gdw) in forest samples. Samples from healthy wood, decaying wood, soil, leaf litter and bark were collected from a poplar plantation and a hardwood forest in November, 2004. *E. coli* were only detected and confirmed in the three sample matrices shown above.

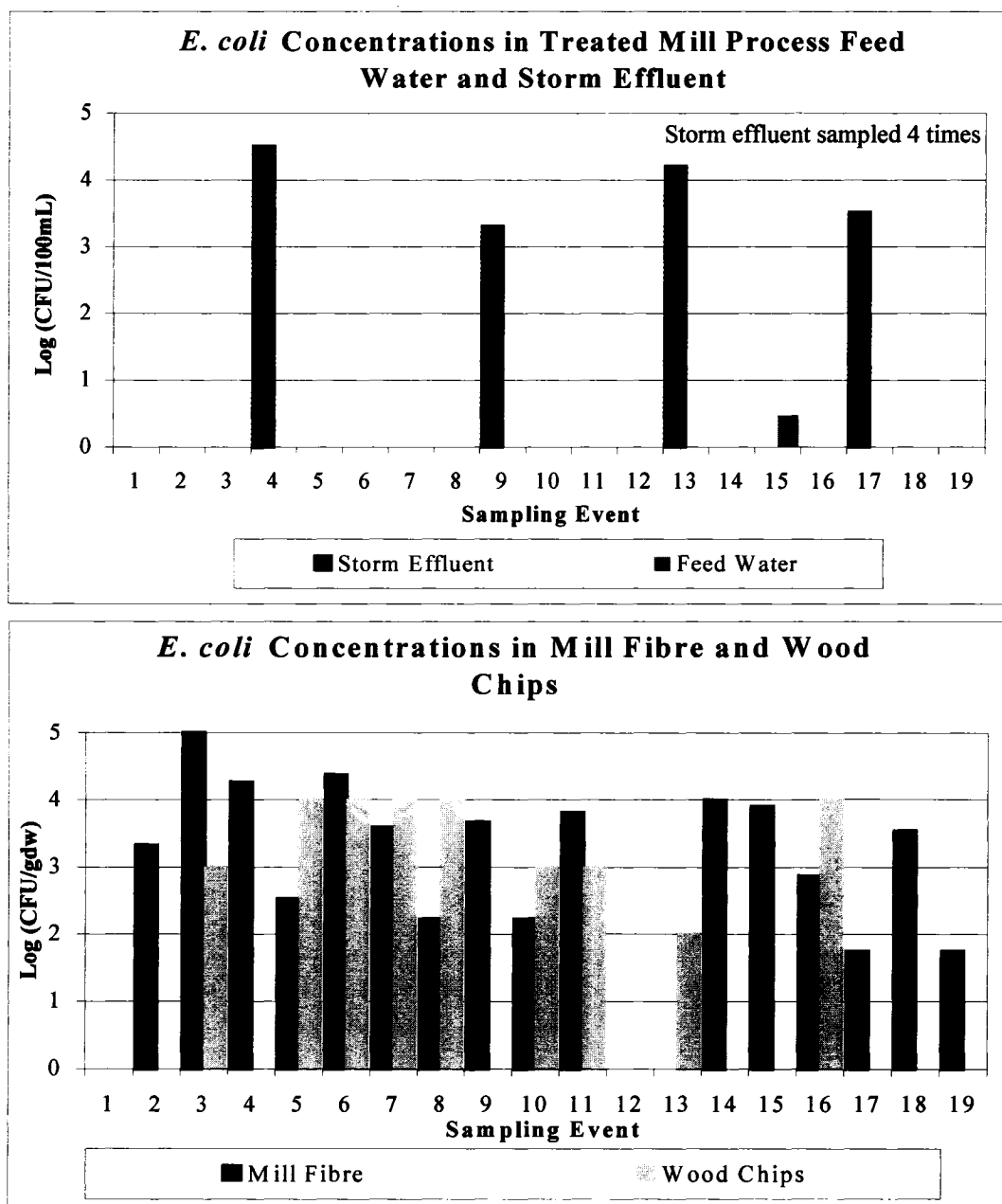


Figure 2-2: Log concentrations of *E. coli* detected in liquid (storm effluent, treated mill process feed water) (CFU/100mL) and solid (mill fibre, wood chips) (CFU/gdw) inputs to the effluent treatment system. Feed water, mill fibre and wood chips were sampled at 19 biweekly sampling events between May 2004 and February 2005. Storm effluents were sampled on only four occasions.

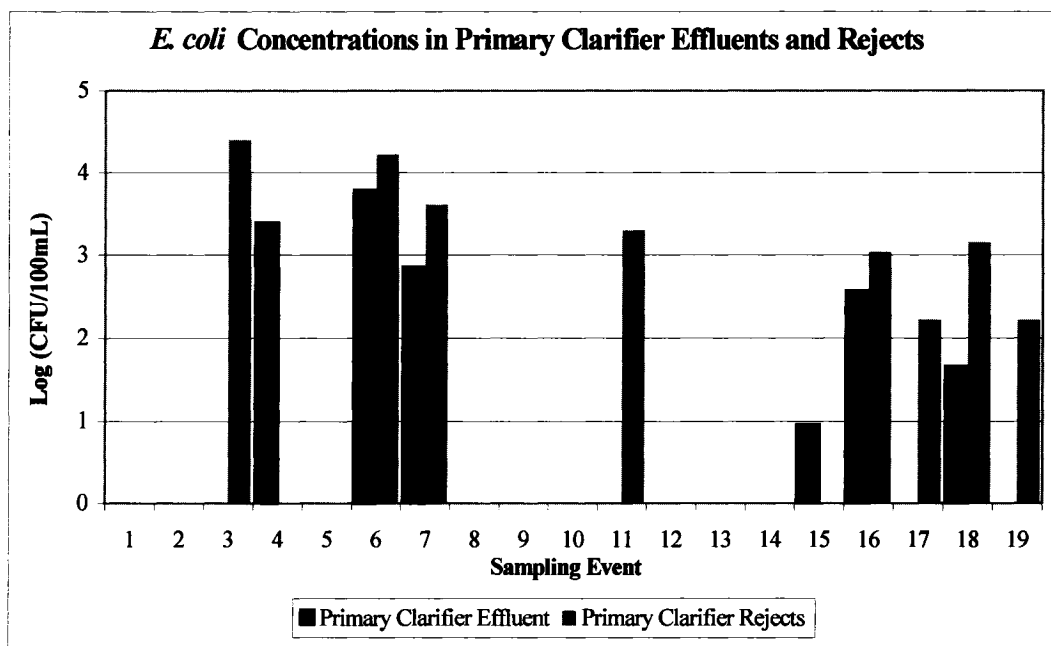


Figure 2-3: Log concentrations of *E. coli* (CFU/100mL) detected in pulp and paper mill primary clarifier effluents and rejects. The primary clarifier was sampled at 19 biweekly sampling events between May 2004 and February 2005.

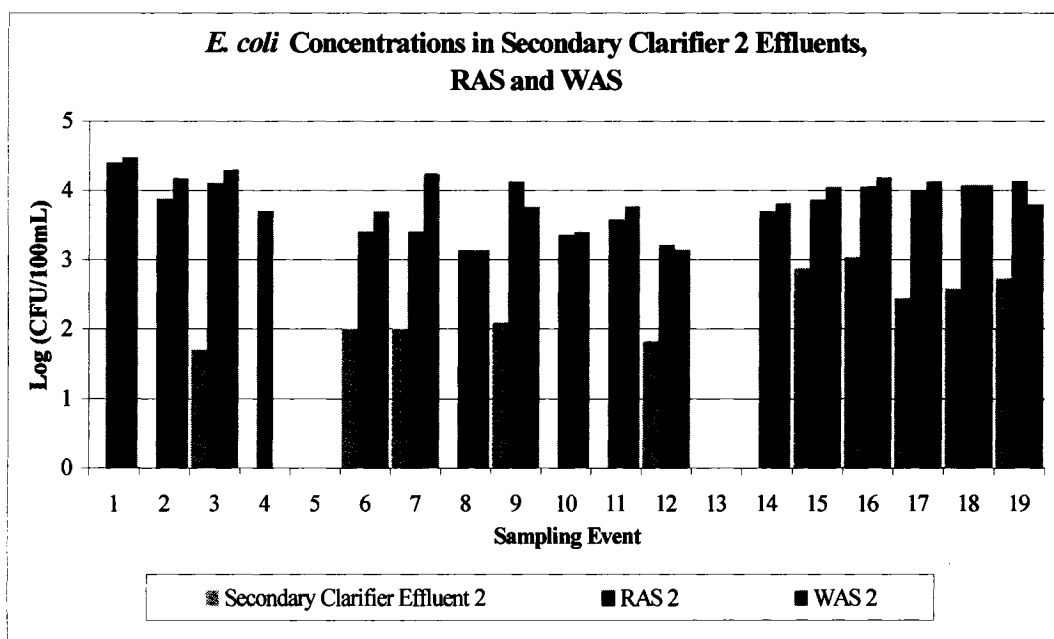
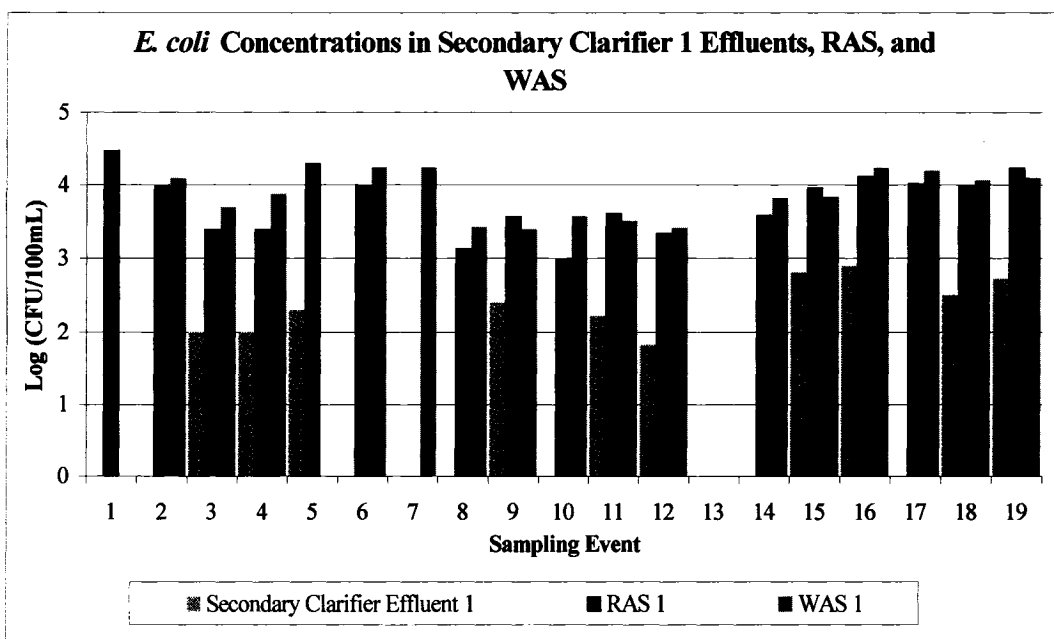


Figure 2-4: Log concentrations of *E. coli* (CFU/100mL) detected in pulp and paper mill secondary clarifier effluents, RAS and WAS. The secondary clarifiers were sampled at 19 biweekly sampling events between May 2004 and February 2005, (except secondary clarifier 1 effluents which were not collected at sampling event 17 as the access was frozen).

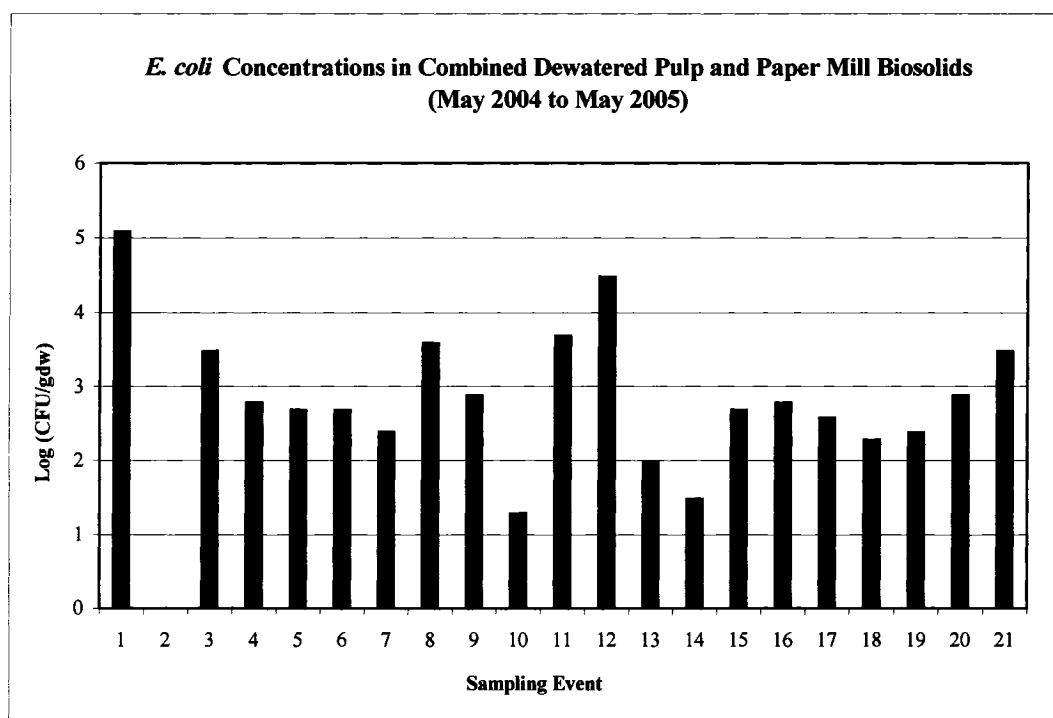


Figure 2-5: Log concentrations of *E. coli* (CFU/gdw) detected in combined dewatered pulp and paper mill biosolids. Biosolids were sampled at 19 biweekly sampling events between May 2004 and February 2005, excepting the final 2 sampling events (20 and 21) which were collected in May 2005.

2.3.2 Rep-PCR Fingerprints

Amplification of *E. coli* isolates with REP primers produced 1 to 11 fragments ranging in size from 200 to 10 000 base pairs (bp) (Figure 2-6). No bands were ever detected in a blank run; while laboratory strain K12 was always amplified, and consistently produced banding patterns with >90% similarity. Rep-PCR was not able to generate fingerprints for all isolates. A minimum of 3 amplification attempts were made for each isolate. Duplicates that did not generate fingerprints demonstrating greater than 90% similarity were excluded from further analyses.

A total of 99 distinct REP fingerprint patterns (less than 80% pairwise similarity) were detected among 330 isolates.

2.3.3 Strain Diversity Within a Sample Type

Pairwise comparisons were performed for each sample type. All sample types showed a range of similarity scores, from low (clearly distinct fingerprints) to high (indistinguishable fingerprints) (Table 2-1).

The percentage of isolates appearing in a cluster was calculated to provide some indication as to what fraction of the source group fingerprints could be attributed to bacterial growth. These values, and those describing strain richness, Shannon index of diversity, and strain evenness are shown in Table 2-1.

Some interesting patterns in fingerprint clustering were noted. Among storm effluent isolates, clusters occurred exclusively among isolates recovered from the same sampling event or subsequent sampling events. Mill fibre-isolated *E. coli* were mainly grouped into several large clusters, and the fingerprints had been isolated from many sampling events.

Wood chips showed little clustering, and clustering only occurred among isolates collected from the same sampling event. Effluent treatment system and biosolids clusters included fingerprints from numerous sampling events.

A jackknife analysis was performed for 8 sample types (forest, feed water, storm effluent, mill fibre, wood chips, primary clarifier, secondary clarifiers, and laboratory strain K12); a low ARCC value of 51.5% was obtained. Most sample types were misassigned to 2 to 5 other sample types. These misassignments are more fully described in Table 2-2 (results of this jackknife were virtually identical to those obtained for biosolids and 8 sample types). These results show that many fingerprints were not specific to a single sample type. Similarly low ARCC values were obtained under several alternative scenarios (i.e. excluding storm effluent fingerprints, separating secondary clarifier fingerprints into 2 groups, or combining primary and secondary clarifier fingerprints into a single group).

2.3.4 Comparing Sample Types

A composite dendrogram of all 9 sample types was constructed: forest, feed water, storm effluent, mill fibre, wood chips, primary clarifier, secondary clarifiers, biosolids and laboratory strain K12. The resulting dendrogram showed that even clusters with a high degree of similarity (greater than 80%) were rarely exclusive for any single sample type. Most clusters included fingerprints representing 2 to 5 sample types; however, some fingerprints from wood chips and primary and secondary clarifiers formed exclusive clusters.

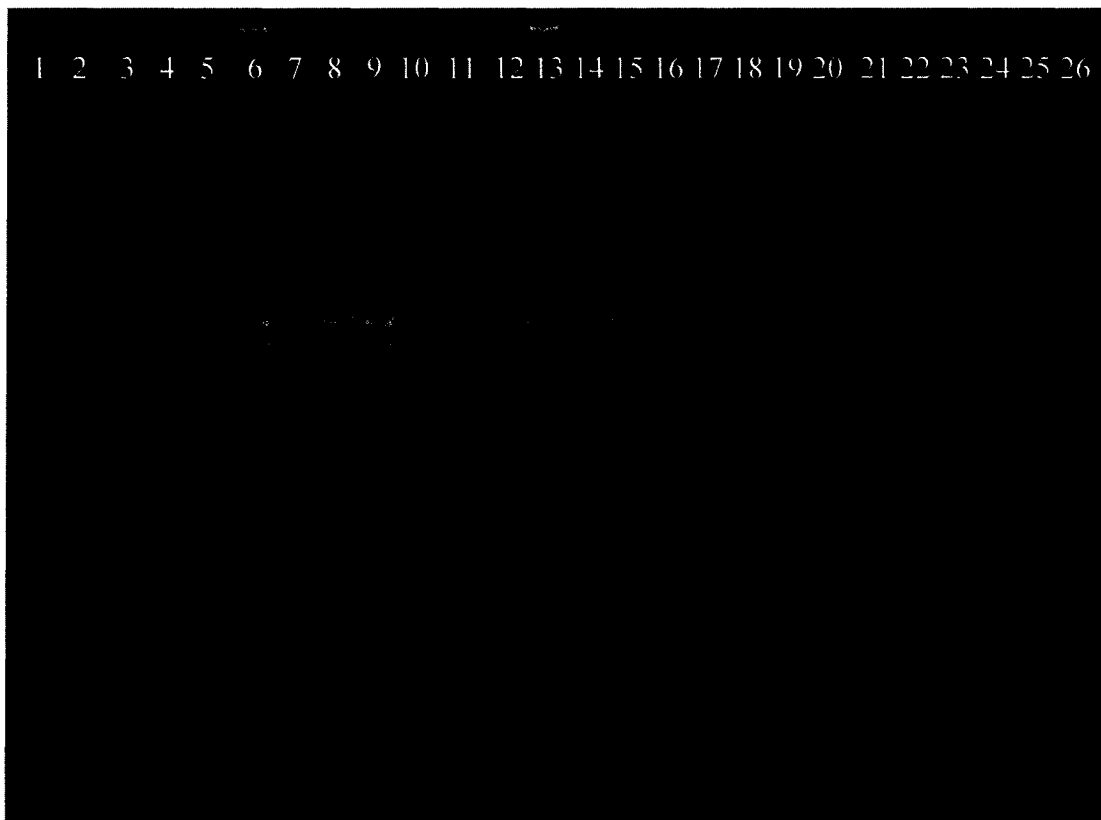


Figure 2-6: Rep-PCR fingerprints of biosolids-isolated *E. coli* generated by REP primers. Lanes 2 through 9, and 11 from sampling event 8; lanes 12 through 18, and 20 through 22 from sampling event 9; lane 23 from sampling event 10; and lanes 24 and 25 from sampling event 11. Lanes 1, 10, 20 and 26 contain a 1 kb molecular size marker.

Table 2-1: The number of amplified fingerprints, range of pairwise comparison scores (%), % clustered isolates, strain richness, Shannon index of diversity and strain evenness for each sample type. n – number of isolates; PCSR –Pairwise Comparison Score Range; S – strain richness; H – Shannon Index of Diversity; E – strain evenness; NA – not applicable.

Sample	N	PCSR (%)	Cluster (%)	S	H	E
Forest	2	5.6	NA	2	0.69	1.00
Feed Water	6	6.7 to 94.9	33	5	1.56	0.97
Storm Effluent	33	6.7 to 98.3	82	12	2.06	0.83
Mill Fibre	34	8.1 to 98.7	60	19	2.43	0.83
Wood Chips	20	8.1 to 98.3	30	14	2.39	0.91
Primary Clarifier	50	16.8 to 98.2	42	34	3.27	0.93
Secondary Clarifiers ¹	84	15.4 to 98.3	67	20	2.71	0.90
Biosolids	92	17.9 to 98.6	72	20	2.44	0.81
K12	2	NA	NA	1	NA	NA

¹Data was averaged for values generated for each secondary clarifier.

Jackknife analysis was used to assess the specificity of source groups, using the same 9 sample types as above. RCC values were calculated for each sample type and are shown in Table 2-2. The ARCC was 43.9%. Misclassifications were common, often fingerprints from a single sample type were misassigned to 2 to 6 other sample types (Table 2-2).

Even when sample types were divided into only 3 groups (biosolids, laboratory strain and 'sources') the ARCC increased only to 72.9%. Jackknife analysis using average similarity (as opposed to maximum similarity) also failed to increase the ARCC value.

ARCC values were low under every scenario examined, and jackknife analysis frequently misassigned isolates. This means that there is a low degree of specificity; fingerprints originating from a single sample type were often less similar to each other than to fingerprints from other sample types.

Table 2-2: The number of amplified fingerprints, and RCC values and associated misclassifications determined by jackknife analysis. n – number of isolates; RCC – Rate of Correct Classification; NA – not applicable.

Sample	N	RCC (%)	Misclassifications
Forest	2	0	• 50% biosolids • 50% secondary clarifier
Feed Water	6	0	• 40% storm effluents • 20% biosolids • 20% K12 • 20% secondary clarifier
Storm Effluent	33	26	• 32% biosolids • 26% storm effluents • 21% primary clarifier • 11% secondary clarifier • 5% mill fibre • 5% wood chips
Mill Fibre	34	25	• 21% biosolids • 21% secondary clarifier • 14% primary clarifier • 4% feed water • 7% storm effluents • 7% wood chips
Wood Chips	20	53	• 24% primary clarifier • 12% secondary clarifier • 6% biosolids • 6% storm effluents
Primary Clarifier	50	36	• 21% mill fibre • 17% biosolids • 13% secondary clarifier • 11% storm effluents • 2% wood chips
Secondary Clarifiers	84	62	• 18% biosolids • 7% storm effluents • 5% mill fibre • 5% primary clarifier • 1% wood chips • 1% forest
Biosolids	92	42	• 16% secondary clarifier • 10% storm effluent • 10% mill fibre • 8% primary clarifier • 6% feed water • 6% wood chips • 2% forest
K12	2	100	• 0%

2.4 Discussion

The *E. coli* concentration data presented in this study were obtained using spread plate or membrane filtration techniques. These methods probably underestimate the number of *E. coli* present (as they fail to separate cells bound together or to particles, and may stress cells through desiccation, excess pressure, or competition with interfering bacteria (4)).

This study focused on obtaining *E. coli* isolates from a broad range of sampling dates and sites, rather than collecting accurate bacterial enumeration data (as done by (63)).

Concentration data reported in this study are meant to: provide an estimate of minimum concentrations, compare concentrations between contributing sources (detecting large differences in concentration), and ensure that detected values are consistent with literature values. Although Figures 2-2 and 2-3 show that *E. coli* were frequently non-detectable in primary and secondary clarifier effluents and sludges, it seems doubtful that these microorganisms were not in fact present. The detection limit for these matrices seemed to be approximately 10^2 CFU/100mL. These matrices were challenging to work with due to: 1) high numbers of competing bacteria (in the test mill coliforms have been shown to outnumber *E. coli* 100 to 1000-fold (63)); 2) the matrices, particularly the sludges, contain high levels of particles; 3) mill effluents contain high levels of organics, which can limit the efficacy of the selective and differential media used.

Rep-PCR was able to generate reproducible fingerprints for most isolates tested. Rep-PCR successfully differentiated between laboratory strain K12 and all other isolates using a similarity cut-off of 80%. A large percentage of fingerprints showed greater than 85% similarity with other fingerprints; however, the presence of extensive genetic diversity within *E. coli* was also evident.

When all 9 sample types were compared, jackknife calculations yielded a low ARCC value of 43.9%. Often low ARCC values can be improved by reducing the number of groups (33). However, even when an ARCC value was calculated for biosolids vs. 'non-biosolids' (all other sample types pooled), it increased only to 72.9%.

Dendrogram results and low ARCC values are both indicative of a low degree of source specificity; this could be due to: 1) laboratory contamination; 2) insufficiently discriminatory method; 3) presence of relatively ubiquitous genetically similar strains of *E. coli*; or 4) on-site contact between sample types.

Laboratory contamination was unlikely to be the cause. Firstly, performing each reaction in duplicate (including the DNA extraction step) ensured that contamination events were identified; uncertain fingerprint patterns were excluded from analysis. Secondly, isolates collected at any given sampling event never came into contact with isolates collected more than 8 weeks later, and yet similar fingerprints were detected well beyond this timeline. Thirdly, Rep-PCR fingerprints were generated on 2 separate thermocyclers, from frozen stocks that had been revived on at least 2 separate occasions; both yielded similar fingerprint patterns, suggesting that frozen stocks were pure cultures.

Rep-PCR is considered to be one of the most discriminatory microbial source tracking (MST) techniques available, second only to pulsed field gel electrophoresis (PFGE) (143); however, PFGE has been shown to be unable to offer much more discrimination between strains of *E. coli* than Rep-PCR (127). Furthermore, the selection of Rep primers is largely empirical. This study used REP primers. Trial runs with ERIC and BOX primers were also performed. ERIC primers were unable to reliably differentiate between laboratory strain K12 and biosolids isolates, and reproducibility was noticeably lower than with REP

primers (data shown in Chapter 3). BOX primers were unable to discriminate between 5 biosolids isolates with distinct REP fingerprints (data not shown). However, BOX reactions were not completely optimized and the sample size was very small; the use of BOX primers should be examined in more detail.

E. coli show extensive genetic diversity, much of which seems to be strongly influenced by adaptation to the host (174). Perhaps only a genetically limited subset of *E. coli* have the ability to survive in nonintestinal niches. Or, perhaps nonintestinal *E. coli* have rid themselves of host-specific adaptations, reducing their typeability by conventional MST methods. These scenarios are speculative, and while possible, seem more complicated than required to explain the results.

Many of the sample types were in contact with other on-site sources. For example, mill fibre was added at the primary sump, so the isolation of similar strains from both mill fibre and the primary clarifier was predictable. On-site contact between sample types is addressed in more detail below for each sample type examined, and seems to account for much of the low source specificity.

2.4.1 Forest *E. coli* Isolates

Studies have detected coliform and fecal coliform bacteria on (49) or in (10) wood. Bagley, *et al.*'s work detected the coliforms *Klebsiella*, *Enterobacter* and *Citrobacter* within the sapwood and heartwood of redwoods; electron photomicrographs clearly showed the presence of high numbers of these bacteria within the redwood vascular system (10). The pattern of coliform detection led the authors to suggest that the coliforms may enter through the root system and travel into other tree tissues via the vascular system (10). Subsequent work has shown that *E. coli* O157:H7 can behave in a similar manner, as

demonstrated by survival and growth of these bacteria within lettuce plants (173) and bean sprouts (198).

In this work, no *E. coli* were detected in either healthy wood or decaying wood samples from the poplar plantation or hardwood forest, although other coliforms were frequently detected (data not shown). This could be in part an artifact of the sampling and culturing methods used (cutting wood with sterile scalpel, and agitating with buffer for 2 min). Other wood samplers have used more aggressive techniques, including hydraulic pressing (10), drilling (153) or blending (63).

Low concentrations of *E. coli* were detected in soil and leaf litter samples. Temperate soils typically have low levels of these microorganisms. Duncan and Razzell reported a range of fecal coliform values from non-detectable to only 2 MPN/100g soil (49), while other research has reported 14 CFU/gdw *E. coli* in 'clean' temperate soils (172). This is in marked contrast to contaminated soils which may support *E. coli* concentrations as high as 87 to 200 CFU/gdw (172), and tropical soils which typically have concentrations of 320 CFU/gdw (24).

No *E. coli* were successfully isolated from bark or decaying wood. Values published in literature suggest that microbial concentrations from bark are quite variable, ranging from less than 2 CFU/g to 240 CFU/g fecal coliforms (where lower concentrations were more typical) (49), to nondetectable levels of *E. coli* (128). These research findings suggest that *E. coli* and other fecal coliforms might be found in association with bark due to relatively recent fecal contamination.

Two unrelated isolates (<60% similarity) were amplified from the forest samples. One forest isolate showed low similarity with all other isolates (>80%), the other clustered with

fingerprints from wood chips, primary clarifier and biosolids. These results show that some *E. coli* from the forest are genetically similar to some *E. coli* from the mill; unfortunately, the low sample size does not allow the link between forest and mill *E. coli* to be made with confidence.

The ecological parameters examined are difficult to compare to those generated for other sample types due to the small sample size.

2.4.2 Feed water *E. coli* Isolates

As pulping and papermaking are water-based processes, mills consume enormous quantities of feed water on a daily basis. Feed water at the test mill is screened and chlorinated prior to use. However, despite treatment, coliforms were routinely isolated from these waters (data not shown), and *E. coli* were detected at a single sampling event. Coliforms and/or fecal coliforms are commonly detected in low numbers from pulp and paper mill feed waters (31; 63). The lone *E. coli* detection occurred during a mill shut down, when water demand had decreased significantly. Without constant flushing, chlorine residual in pipes and hoses may have decreased, resulting in greater bacterial growth (chlorine residuals lower than 0.2 mg/L are associated with increases in average bacterial densities in drinking water systems (107)). Bacterial cells could be present in feed water due to insufficient treatment, contamination, or shedding from biofilms. Gauthier and Archibald suggest that biofilms in pipes, tanks and machinery (where conditions permit) are a likely source of the coliforms, fecal coliforms and enterococci found in mill effluent streams (63). *E. coli*, including pathogenic strains (167), are known to be present in biofilms (126), even under low nutrient conditions (179) such as drinking water distribution systems (30; 107). Furthermore, the mill examined in this project was an

older mill. LeChevallier suggests that aging infrastructure, particularly in cases where iron pipe surfaces have corroded, contribute to biofilm formation and subsequent detections of 'regrowth' in drinking water systems (107)). The single occurrence of *E. coli* in mill feed water at low concentrations suggests that these bacteria are either rarely present, or present in extremely low concentrations (<10 CFU/100mL). Feed water containing low concentrations of *E. coli* from shedding biofilms may constitute a consistent source of bacteria to the mill effluent treatment system.

Only 6 feed water fingerprints were successfully isolated and amplified. Three unrelated (<60% similarity) fingerprint types were detected. The comparatively low proportion of possible clones (1 pair with >85% similarity) suggests that little growth occurred within feed water; this is consistent with the low temperatures detected. The ecological parameters are difficult to compare with those generated for other sample types due to the low sample size.

Jackknife analyses were unable to correctly assign the feed water isolates to their own group. This was in part an artifact of the small sample size. The dendrogram comparing biosolids to other sample types showed that half of the feed water isolates, including the clustered pair, were >80% similar to strains detected in biosolids (remaining fingerprint types did not resemble other fingerprints (<80% similarity)). If the isolates detected were due to shedding of in-mill biofilms, they might constitute a constant, dilute source of *E. coli* to the mill effluent treatment system. However, not all feed water strains seemed to be successful in making the transition from low nutrient, cold water systems to high nutrient, warm water systems. If the lone detection of feed water *E. coli* were instead due to

improper treatment of river water, the short term addition of these low levels of bacteria to the effluent treatment system would likely be undetectable.

2.4.3 Storm Effluents *E. coli* Isolates

Agricultural and urban storm effluents in Michigan yielded *E. coli* concentrations ranging from 4 to 4700 CFU/100mL (60). Urban storm effluent *E. coli* may come from pet or urban wildlife feces, sanitary sewer cross-connections, poor sanitation, sewer rodents, improper solid waste disposal, landwash or even regrowth within storm sewer sediments (123). *E. coli* concentrations detected in pulp and paper mill storm effluents are similar to those collected from urban storm effluents without sewage cross-connections (as concentrations did not exceed the 10^5 CFU/100mL mark (123)). Furthermore, dye tracer tests showed that test mill sanitary sewers were not connected to the effluent treatment system via cross-connection with a storm sewer or any other effluent stream (D. Craig, Domtar, pers. comm.), so it is assumed that human waste was not a contributing source of *E. coli*. On-site, visible sources of mill storm effluent *E. coli* included wildlife feces and leaching from on-site sample types (including bark, logs, wood chips, mill fibre, and biosolids). Landwash and regrowth within storm sewer sediments may have added to storm effluent concentrations.

Due to the nature of storm effluents, *E. coli* isolated from this sample type are expected to be genetically diverse. Fingerprints are expected to include: 1) types that resemble those recovered from other on-site sample types; 2) unique types from yard wastes that were not examined in detail; 3) highly similar types from sources of concentrated clonal strains or growth in pipe biofilms.

Storm effluents showed genetic diversity: 7 unrelated fingerprint types (< 60% similarity), or 12 distinct strains (< 80% similarity) were detected. However, both strain richness and Shannon index of diversity values were lower than for mill fibre and wood chips (sample types with comparable sample sizes). All 3 expectations were met. Firstly, jackknife analysis frequently misclassified storm effluent isolates as biosolids, primary clarifier, secondary clarifier, mill fibre or wood chips – all sample types which were identified as likely to contribute *E. coli* to storm effluents via leaching. Secondly, the dendrogram revealed that approximately a third of all storm effluent fingerprint types were unrelated (less than 60% similarity) to fingerprints from any other sample type. Thirdly, 82% of storm effluent isolates fell into clusters; most clusters were small (2-4 isolates), but one contained 13 isolates. Clustering was limited to isolates collected during the same, or subsequent, sampling events.

Urban storm effluents are known to carry indicators of fecal contamination, as well as pathogenic microorganisms (123), including *Salmonella*, ETEC or adenovirus (116). Although storm effluent discharges to the effluent treatment system are sporadic, these effluents contain high concentrations of *E. coli*. Storm effluents are also the sample type most likely to be carrying pathogenic microorganisms, as they are known to have come into contact with bird or mammal feces. Treatment of this waste stream prior to its addition at the primary sump would help to reduce the risk of pathogens entering the effluent treatment system; however, it seems unlikely that such pre-treatment would result in any significant decrease in the concentration of *E. coli* detected in biosolids.

2.4.4 Mill fibre *E. coli* Isolates

Mill fibres are cleaner rejects produced by a corrugated cardboard manufacturer. Raw materials included both new and recycled fibres. Mill fibre was trucked on-site where it was stockpiled and shoveled into the primary sump as required to increase the fibre content of effluents undergoing treatment, thus promoting efficient dewatering. *E. coli* concentrations in mill fibres may have increased during transportation and storage.

Ten unrelated fingerprint patterns (<60% similarity) were detected. Most mill fibre isolates (78%) clustered into just a few clonal groups (with >80% similarity). These clusters included isolates collected from multiple sampling events. Mill fibres were only correctly classified 25% of the time. Mill fibres were often misclassified as storm effluents, primary or secondary clarifier or biosolids. The presence of genetically similar strains in these different sample types is accounted for by on-site contact between these sample types: mill fibre is leached or sloughed into the storm effluent, and is deliberately added directly to the effluent treatment system. Similarities between mill fibre isolates and isolates from feed water and wood chips are more difficult to explain. Some sort of unrecognized on-site contact between these sample types may exist. It is also possible that Rep-PCR may have been less able to discriminate between certain isolates, and that further molecular analysis would result in greater discrimination between strains. The fibre sources at the corrugated cardboard manufacturing mill may introduce *E. coli* genetically similar to those detected at the study mill, or these fingerprints may represent a group of strains that are ubiquitous in industrial environments.

Interestingly, mill fibre and biosolids sample types had very similar values for strain richness, Shannon index of diversity and strain evenness, despite very different sample

sizes. Further work is needed to determine if these values are characteristic of this type of matrix.

2.4.5 Wood chips *E. coli* Isolates

Wood chips have most often been implicated as an important source of coliforms, fecal coliforms (12; 31; 63) and *E. coli* (12; 128) to pulp and paper mill effluents (and, in turn, biosolids). It has been posited that some of these bacteria may survive the pulping and bleaching process due to insufficient contact time or some kind of physical protection (12) or via entry to the system through storm effluents (63). The pulping and bleaching methods employed by the test mill are microcidal: Kraft pulping involves heating the pulp to 170°C in a concentrated, pressurized solution of sodium hydroxide and sodium sulfide for 4 hours; pulp bleaching involves repeated exposure to hot alkali, hydrogen peroxide and chlorine dioxide. It seems more likely that bacteria survive in the chip wash, storm run-off, or paper machine whitewater. Wood chip bacteria may also contribute to the formation of biofilms in the thousands of feet of wastewater piping.

E. coli may be present in wood chips at a range of concentrations: from nondetectable levels in stored chips, to concentrations in the order of 10⁴ CFU/100g in fresh poplar chips, and 10⁵ CFU/100g in fresh maple chips (128); and in wood chips and shavings from 50 to 13 300 MPN/gdw (12). Although *E. coli* are not always detected in wood (or waters in contact with wood), when detected, *E. coli* tend to be present in high concentrations. These *E. coli* may be due to residual fecal contamination (i.e. from bark, being dragged through the soil, during wood stockpiling), or environmental strains of *E. coli* existing in association with wood.

Wood chips demonstrated one of the highest RCC values (52.9%). Seventy-one percent of these isolates did not cluster out with any other sample types. The fingerprint types that were unique to wood chips may represent environmental strains unable to compete in the industrial habitat. Twelve unrelated (<60% similarity) fingerprints were recovered from wood chips samples. Wood chips also had strain richness and Shannon index of diversity values on the high end of calculated readings. The strain evenness value approached 1, suggesting little dominance by any single strain. These results are accounted for by the import of furnish from sites over a broad geographic area, and conditions that were not conducive to growth. The remaining wood chips fingerprint types were misclassified as storm effluents, primary and secondary clarifiers and biosolids. Wood chips come into contact with the effluent treatment system via used process waters and storm effluent.

2.4.6 Primary clarifier *E. coli* Isolates

The primary clarifier is the starting point for effluent treatment. Inputs to the primary clarifier include: all mill wastewaters (including streams from pulping, bleaching, and fine papermaking), storm effluents, and mill fibre. Solids settle, while clarified effluents undergo secondary treatment.

Primary clarifier effluents and rejects tend to contain high levels of coliforms, fecal coliforms and *E. coli*: 10^5 CFU total coliforms/100mL effluent (12); fecal coliform values from 10^3 to 10^4 CFU/100mL effluent and similarly high concentrations in the rejects (63); 10^4 to 10^5 CFU fecal coliforms/100mL effluent (31); *E. coli* concentrations of 10^5 to 10^7 CFU/100mL (139). These values, particularly those reported in (139) for *E. coli* concentrations, are higher than those detected in this study.

It would be expected that the variety of inputs would be reflected in the genetic diversity detected in the fingerprints recovered from the primary clarifier. Furthermore, previous work on *E. coli* from the test mill identified the presence of multiple *E. coli* serotypes (63). Gauthier and Archibald (63) also suggested that the primary clarifier was an important site of coliform growth within pulp and paper mills. If this is the case, some proportion of the fingerprints isolated from the primary clarifier should show evidence of clonality (i.e. include clusters of highly similar fingerprints). Primary clarifier isolates are expected to share some fingerprint types with secondary clarifier and biosolids sample types.

The primary clarifier isolates met all 3 of these expectations: 1) the *E. coli* population was genetically diverse; 17 unrelated (<60% similarity) fingerprint types were detected; this matrix had the highest strain richness and Shannon index of diversity values detected; many primary clarifier isolates closely resembled inputs to the primary clarifier (mill fibres, storm effluents, feed water or wood chips); 2) 42% of primary clarifier isolates were clustered in groups sharing >80% similarity and including isolates from at least 2 sampling events; 15% of these clusters were composed exclusively of primary clarifier fingerprints, results consistent with the continuous growth of a genetically diverse *E. coli* population within the primary clarifier; 3) many primary clarifier isolates were misclassified as secondary clarifier or biosolids.

2.4.7 Secondary clarifiers *E. coli* Isolates

AST involved adjusting effluent pH, and adding nutrients and biomass (RAS) to remove BOD from raw clarified effluent. The activated sludge floc biomass settled out of

the effluent/biomass slurry in the secondary clarifiers. Waste secondary clarifier sludges (WAS) were combined with primary sludges and dewatered to form biosolids.

Secondary clarifiers yielded concentrations of coliforms, fecal coliforms and *E. coli* similar to those reported from primary clarifiers: Caplenas *et al.* detected 10^6 CFU fecal coliforms/100mL effluent (31); Gauthier and Archibald detected values from 10^2 to 10^5 CFU/100mL coliforms and fecal coliforms in effluent (63); Niemi *et al.* found 10^5 to 10^6 CFU *E. coli*/100mL effluents and 10^6 CFU *E. coli*/100mL rejects (139); while Beauchamp *et al.* found 10^4 MPN coliforms/100mL effluent (12). Concentrations in the rejects were often an order of magnitude higher than in the effluents. Actual concentrations of *E. coli* detected were on the lower end of reported values.

The *E. coli* population in the secondary clarifiers should include *E. coli* from the primary clarifier (and thus inputs to the primary clarifier), and *E. coli* from the AST used to treat the raw effluent. Conditions in the secondary clarifiers may also be conducive to growth, so clonal isolates may be present. Since WAS constitutes 42% of biosolids, many secondary clarifier *E. coli* should resemble those detected in biosolids.

The secondary clarifier *E. coli* population was genetically diverse, but not as diverse as the primary clarifier, either in terms of strain richness or Shannon index of diversity; 11 unrelated (< 60% similarity) fingerprint types were detected. Many secondary clarifier isolates (64%) clustered closely (>80% similarity) with other secondary clarifier isolates, and each cluster contained isolates from at least 2 sampling events; 11% of secondary clarifier fingerprints formed exclusive clusters. These findings are consistent with growth of a diverse *E. coli* population within the secondary clarifier. Jackknife analysis was able to correctly assign secondary clarifier fingerprints to their source 61.8% of the time.

Secondary clarifier isolates were misclassified as primary clarifier, primary clarifier inputs, or biosolids.

2.4.8 Biosolids *E. coli* Isolates

Gauthier (62) reported a range of *E. coli* concentrations of 10^3 to 10^7 CFU/gdw from the combined biosolids of 7 pulp and paper mills in Ontario and Quebec, while Beauchamp *et al.* reported values ranging from non-detectable to 4500 MPN/gdw in the combined sludges of 2 Quebec pulp and paper mills (12). The concentration values obtained in this study fall within the range of values detected by other studies, but are lower than values detected by Gauthier at the same test mill. This disparity likely arises from 2 causes: 1) differences in technique (as discussed above); 2) the elimination of an on-site cardboard recycling effluent stream which could have led to real changes in the microbial ecology of mill effluents and biosolids (this will be further addressed in Chapter 3). However, Major, detected values of 10^2 to 10^3 CFU/gdw using the membrane filtration technique while the cardboard recycling effluent stream was being produced (122). This suggests that the differences were due more to technique than to changes within the mill between studies.

Rep-PCR is expected to detect genetic diversity among biosolids isolates, as other studies have demonstrated the presence of multiple serotypes from biosolids isolates collected from the test mill (63).

Four unrelated fingerprint types (<60% similarity) were detected in biosolids. Isolates producing fingerprints with a high degree of similarity were detected at the same and at different sampling events. Strain richness, Shannon index of diversity and strain evenness values were all lower for biosolids than for either the primary or secondary clarifier. These results are consistent with the survival of a subset of *E. coli* contributed by the primary and

secondary clarifiers during the dewatering process, and subsequent regrowth of these strains in the biosolids.

By jackknife analysis, 42% of biosolids fingerprints were most similar to other biosolids fingerprints, while the remaining 58% were most similar to fingerprints generated by source isolates. This means that it is possible that the inputs tested successfully contribute to biosolids *E. coli* populations.

2.4.9 Model of *E. coli* Sources

E. coli isolated from soil in a poplar plantation were genetically similar to strains isolated from wood chips, primary clarifier, secondary clarifier and biosolids. These results suggest that some proportion of *E. coli* in pulp and paper mill biosolids may come from the natural environment, although the origin of the forest *E. coli* remains unknown. The low sample size of forest *E. coli* (n=2) means that no link between forest and industrial environment could be established with certainty.

E. coli were isolated from all on-site sample types examined. Feed water, storm effluent, mill fibre and wood chips yielded strains that were genetically similar to strains detected in the effluent treatment system and biosolids (Figure 2-7). *E. coli* from feed water and wood chips were detected on a sporadic basis and less than a third of strains types could be considered possible contributing strains. Strains isolated from these sample types may be more competitive in low-nutrient or cooler temperature environments. Mill fibres contained the highest proportion of possible contributing strains. This could be due to 'preadaptation' to the industrial environment at the source mill. Alternatively, similar strains might be present in both mills as they used similar furnish and provided similar conditions. Storm effluent included strains similar to those isolated from all other sample

types, as well as unique strains that could have come from poorly characterized sample types (i.e. landwash, including wildlife feces). While concentrations in storm effluents were high, contributions to the effluent treatment system were sporadic; however, roughly 2/3 of all storm effluent isolates were similar to strains detected in the effluent treatment system. However, this value is misleading as the high proportion of seemingly contributing strains is more representative of the inputs common to both systems, and cross-contamination of storm effluents with biosolids runoff and leaching.

Primary clarifiers revealed the greatest genetic diversity, which reflected the number of inputs. Closely related isolates were detected at multiple sampling events. These results are consistent with the presence of a surviving and growing population within the primary clarifier, as suggested by (63). Secondary clarifier fingerprint types seemed to contribute much more successfully to the biosolids than the primary clarifier, even though biosolids are composed a greater proportion of primary rejects than WAS.

While the precise origins of biosolids *E. coli* remain uncertain, there were numerous ways for these bacteria to gain access to the effluent treatment system and biosolids. Once in the effluent treatment system, *E. coli* strains survived and multiplied in the warm, high nutrient wastewaters. Continued inputs of high levels of *E. coli* helped to maintain this population. Sporadic contributors were likely at a disadvantage compared with consistent sources, as they were less likely to successfully replace the established strains of the base population.

Inputs to Primary Clarifier

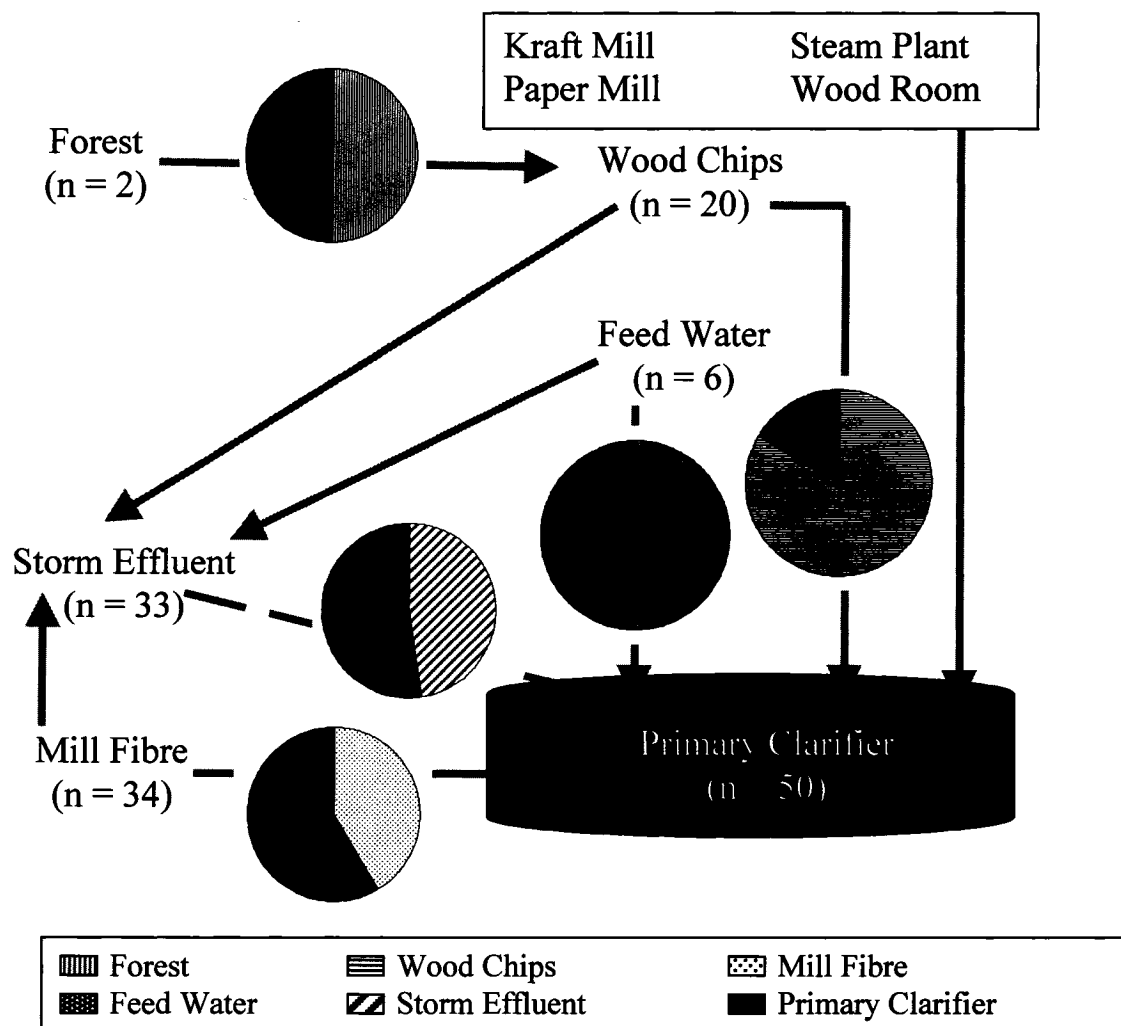


Figure 2-7: Diagram of the inputs to the primary clarifier showing known physical interactions between sample types. The dashed arrow line denotes the sporadic contribution of storm effluent. Pie charts illustrate the proportion of strains unique to a sample type to those detected in the sample type and having >80% similarity with strains detected in the primary clarifier.

Effluent Treatment System Contributions to Biosolids

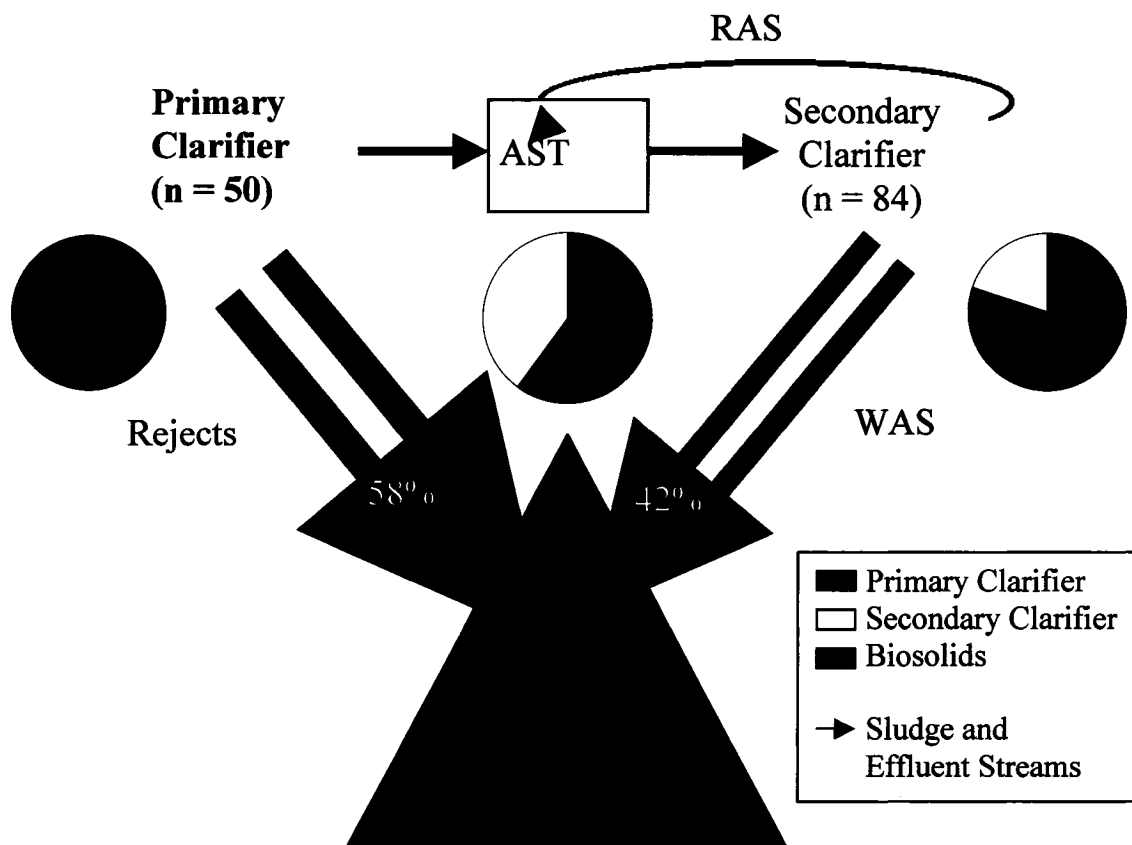


Figure 2-8: Diagram of the study mill effluent treatment system. Percent of possible contributing sources from each sludge stream were shown with pie charts (number of fingerprints detected in clarifier compared with the number of fingerprints in clarifier with >80% similarity to fingerprints detected in biosolids). AST – Activated Sludge Treatment; RAS – Recycled Activated Sludge; WAS – Waste Activated Sludge.

2.5 Conclusions

E. coli were detected in all inputs to the effluent treatment system, as well as throughout the effluent treatment system and resulting biosolids. Rep-PCR was able to detect intra-specific variation among *E. coli* isolates, and accurately distinguish industrial isolates from laboratory isolates. *E. coli* populations included both genetically dissimilar isolates (consistent with the presence of diverse strains) and genetically similar isolates (consistent with the occurrence of growth). Cluster and jackknife analyses were incapable of differentiating reliably among isolates from the inputs, effluent treatment system and biosolids. Low source specificity could be accounted for by on-site contact among populations.

This improved understanding of the sources of pulp and paper mill *E. coli* helps to address important management issues:

- 1) Most fingerprint types recovered from biosolids could be matched with fingerprint types isolated from elsewhere in the mill. This suggests that there was no large, unsampled input contributing to the *E. coli* population in biosolids. While some effluent treatment system inputs seemed to be low-risk environmental *E. coli* (i.e. treated mill process water, wood chips), other inputs came from higher risk sources (i.e. storm effluent, which likely included fecal *E. coli* from birds and mammals). Results showed that no inputs could be excluded as unable to contribute to *E. coli* populations in the effluent treatment system and biosolids.
- 2) Pre-treatment of any single input seems unlikely to result in a significant decrease in *E. coli* concentrations either in the effluent treatment system or biosolids. In fact, the mill is essentially reinoculated with *E. coli* through RAS return to the AST and through

storm effluent. To reduce the concentration of *E. coli* in biosolids, post-production treatment would be more beneficial than pre-treatment of any input or attempting to control in-mill populations. However, eliminating or pre-treating the storm effluent stream would likely significantly reduce the risk of pathogens entering the effluent treatment system.

3) Environmental *E. coli* contribute successfully to *E. coli* in the effluent treatment system and biosolids. Since an unknown (but almost certainly considerable) proportion of *E. coli* in the effluent treatment system and biosolids have been demonstrated to be of a nonfecal source, it is clear that it is inappropriate to use *E. coli* concentrations as a measure of fecal contamination or as a proxy for the presence of waterborne microbial pathogens.

Using only the presence of *E. coli* as an indicator of fecal contamination in biosolids is unreliable and misleading; high readings have not been associated with any significant fecal loading. Relying on inaccurate, inappropriate test results leads either to wasting biosolids which can be used as a productive soil conditioner or requiring unnecessary and costly treatments. Regulatory agencies need to work with pulp and paper mills to find more appropriate tools to assess the microbial safety of pulp and paper mill biosolids. Research efforts might choose to focus on developing more discriminatory assays using conventional indicators of fecal contamination (*E. coli*, coliforms or fecal coliforms); however a more fruitful approach might be the development of new, industry-specific bacterial or chemical indicators. Modern chemical and molecular technologies may soon make direct pathogen screening a feasible and economical option.

Chapter 3: Ecology of *Escherichia coli* Populations in Pulp and Paper Effluent Treatment Systems and Biosolids

3.1 Introduction

As necessary by-products of their effluent treatment systems, Canadian pulp and paper mills produce biosolids. These biosolids have good properties as a soil conditioner, however they contain high levels of enterococci, coliforms, fecal coliforms and *E. coli* (12; 62; 63). As these bacteria are typically used as indicators of fecal contamination, their presence is a cause for confusion and concern among regulatory bodies.

Indicators were primarily developed for use in protecting drinking water supplies from fecal contamination (a medium of transmission for many diseases). Since it is impractical to test directly for waterborne pathogens (108), using the more easily detectable indicators as a proxy is intended to act as an early-warning system.

There is increasing recognition that using standard indicators to assess the safety of pulp and paper mill biosolids is inappropriate (63; 180). The elevated concentrations of indicators in pulp and paper mill effluents and biosolids are generally assumed to be due to in-mill growth (12; 63; 128; 139). Moreover: many pulp and paper mill effluents and biosolids do not include any known fecal inputs (i.e. sewage) (63); pathogens have not been reported in association with pulp and paper mill effluents and biosolids (F. Archibald, pers. comm.); and exponential growth of mill-isolated *E. coli* in sterile mill effluents has been demonstrated (63; 122).

Despite increasing support for the existence of an endogenous in-mill community of continuously growing *E. coli*, the evidence accumulated to date is either circumstantial or

based on laboratory studies. Furthermore, the composition and dynamics of this population has not been characterized.

This study uses the highly discriminatory microbial source tracking tool Repetitive Sequence Based-PCR (Rep-PCR) to identify strain diversity and abundance of *E. coli* isolates from primary and secondary clarifiers and biosolids. In addition, the incidence of strain turnover will be documented.

If in-mill growth is responsible for the high levels of *E. coli* detected in biosolids, then multiple isolates yielding identical molecular fingerprints (clones) should be detected within a sample, and at multiple sampling events. However, if biosolids *E. coli* are present due mainly to external inputs, the opposite would be expected (i.e. detection of diverse molecular fingerprints (representing distinct strains) present within a sample, and low similarity between sampling events).

The resulting findings have important implications for the operation of pulp and paper mills as well as land application (agricultural and silvicultural) and for the government regulators of these activities.

3.2 Materials and Methods

3.2.1 Samples

A total of 19 sets of samples were collected on a biweekly basis between June 2004 and January 2005 from an Ontario hardwood Kraft pulp and paper mill using an activated sludge biotreatment system. The mill water and wastewater systems are diagrammed in Figure 1-1. This figure illustrates that mill sanitary wastewaters were processed through the municipal water treatment facility and were not part of on-site effluent treatment. Each

sample set consisted of: primary clarifier effluents and rejects; effluents, RAS and WAS from both secondary clarifiers; and combined dewatered biosolids.

3.2.2 Sampling Procedures

Liquid and solid grab samples were collected as described in Section 2.1.2.

3.2.3 *E. coli* Isolation

E. coli were isolated as described in Section 2.1.3.

3.2.4 *E. coli* Confirmation

Putative *E. coli* isolates were confirmed as described in Section 2.1.4.

3.2.5 *E. coli* Preservation

E. coli isolates were preserved as described in Section 2.1.5.

3.2.6 DNA Extraction

DNA was extracted via the whole-cell method described in Section 2.1.6.

3.2.7 PCR Protocol

E. coli isolates were amplified as in Section 2.1.7.

In addition to performing Rep-PCR with the Repetitive Enterobacterial Palindromic Sequences (REP primers), biosolids-isolated *E. coli* (n = 92) were amplified using Enterobacterial Repetitive Intergenic Consensus Sequences using the primers (ERIC1R and ERIC2). Reactions and thermocycler protocols were described by Rademaker and deBruijn (157) modified to use 2 μ L of template DNA with 23 μ L of PCR reaction mixture.

3.2.8 Detection of Amplified DNA

Gel electrophoresis was performed as in Section 2.1.8.

3.2.9 Analysis

Genetic fingerprints were analyzed as in Section 2.1.9.

Strain richness (S), the Shannon Diversity Index (H), and strain evenness (E) were calculated for isolates collected from the primary clarifier, secondary clarifier and biosolids. Strain richness was defined as the number of unique fingerprint types identified in a sample type. The Shannon Diversity Index measured of the order (or disorder) within a system by taking both richness and abundance into account. It was calculated using $H = -\sum((P_i)/\ln[P_i])$. Strain evenness compared the abundance of the various strains detected within each population and was calculated using $E = H/\ln S$. Larger values (approaching 1) signified an even abundance of diverse strains, while values approaching 0 suggested the dominance of a single strain.

The above values were calculated under 2 scenarios: 1) by using the total data set collected from each sample matrix; 2) by using data from sampling events where $n > 5$ and determining means and standard deviations for each sample matrix.

For analyses involving fingerprints generated using REP primers (biosolids, primary and secondary clarifiers), fingerprints were considered identical if they were more than 80% similar and showed no distinct band differences. ERIC generated fingerprints (biosolids) were considered identical if they were >95% similar and showed no distinct band differences. In all cases, distinct fingerprint types were given an identifying label. The letter denotes which super-cluster the fingerprint belongs to, the number the specific

cluster. These identifiers are specific to each matrix (i.e. fingerprint type A1 from the biosolids does not correspond to fingerprint type A1 in the primary clarifier).

3.2.10 Physical and Chemical Characteristics of Mill Effluents

Daily averages of temperature (degrees Celsius), pH, and conductivity (microSiemens) from May 1, 2004 to April 6, 2005 from mill primary and secondary effluent streams were provided by K. Jasim (Domtar, Inc.). Conditions in the clarifiers were based on measurements recorded from the Primary Clarifier Outflow (Bioreactor Cell 1 Inflow) and Secondary Clarifier Effluent Outflow (Mill Effluent Outflow).

3.3 Results

3.3.1 DNA fingerprint patterns

Both REP and ERIC primers were able to detect sub-specific variation in mill-isolated *E. coli*. REP primers generated fingerprints with 1 to 11 DNA fragments ranging in size from 200 to 10 000 bp (as shown in Figure 2-5). ERIC primers generated a similar range of bands, however, a much greater proportion of fingerprints generated 5 to 7 bands (Figure 3-1). Fragment size ranged from 150 bp to 6000 bp. No bands were ever detected in a REP or ERIC blank run, and the laboratory strain K12 always amplified and consistently produced identical banding patterns. Neither REP nor ERIC primers were able to generate fingerprints for all isolates. ERIC primers had a higher rate of non-amplification, and generating non-matching fingerprints than REP primers.

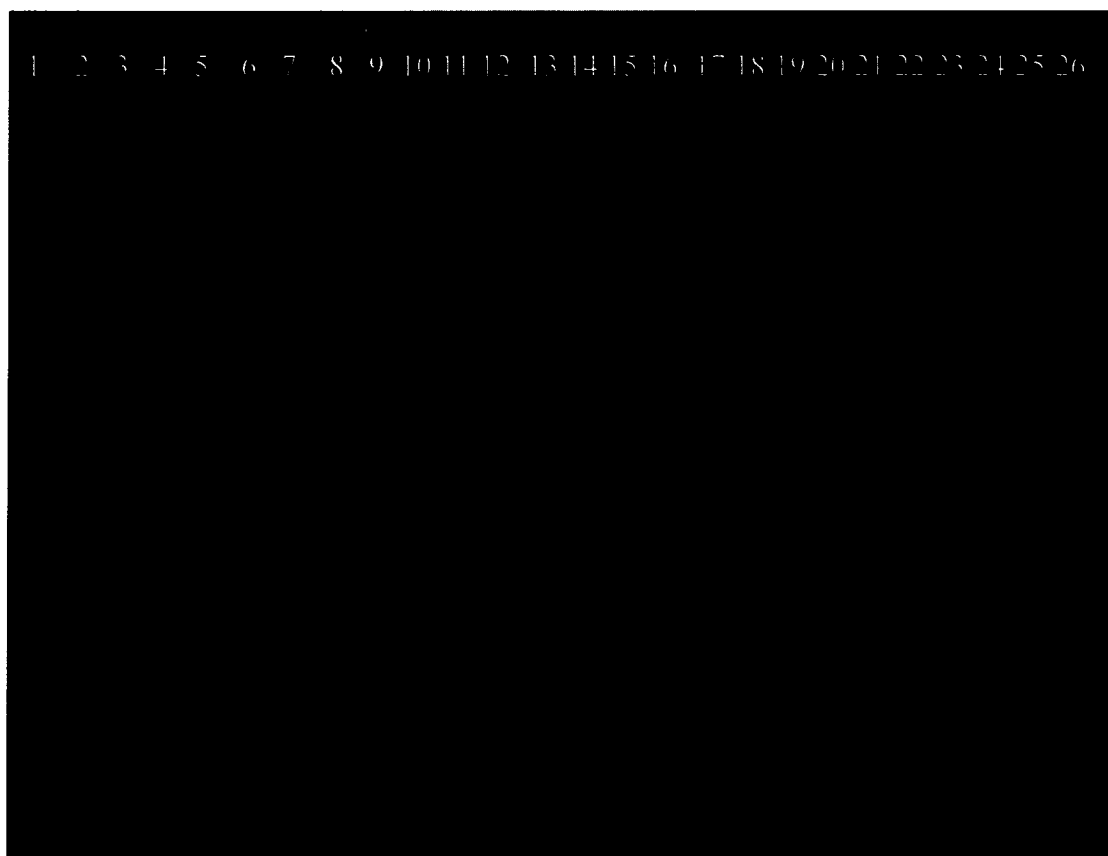


Figure 3-1: Rep-PCR fingerprints of biosolids-isolated *E. coli* generated by ERIC primers. Lane 2 contains the negative control (blank) and lane 3 contains the positive control (laboratory strain K12). Lanes 4 through 7 and 9, 11 and 12 contain fingerprints from sampling event 1; lane 10, sampling event 3; lane 5 and 16, sampling event 5; lane 15, sampling event 4; 14,17, 19 to 21; sampling event 6; lanes 22, 24 and 25, from sampling event 7; lane 23 from sampling event 8. Lanes 1, 8, 18 and 26 contain a 1 kb molecular size marker.

3.3.2 Primary Clarifier

The primary clarifier yielded the fewest isolates, but the greatest strain richness (Table 3-1) and the largest number of unique fingerprint types. Four fingerprint types were repeatedly detected in the primary clarifier (Figure 3-3).

3.3.3 Secondary Clarifier

Secondary clarifier *E. coli* ecology (in terms of strain richness, Shannon diversity and strain evenness) yielded values intermediate to those calculated for the primary clarifier and the biosolids (Table 3-1). Twelve fingerprint types were detected at multiple sampling events (Figure 3-4), 3 of these at 5 or more sampling events.

3.3.4 Biosolids

Biosolids-isolated *E. coli* were amplified using 2 sets of primers, REP and ERIC. These primer sets yielded distinct fingerprint patterns, however, the results drawn from each are compatible.

Using REP primers, each sampling event was found to include 0 to 2 unique fingerprint types. In 3 cases, a fingerprint type was detected at only 1 sampling event. The remaining 133 isolates were detected at between 2 and 12 separate sampling events. These results are illustrated in Figure 3-5. The dendrogram used to construct Figure 3-5 is shown in Figure 3-2.

Using ERIC primers, 0 to 4 unique fingerprint types were detected at each sampling event. Most fingerprint types were recovered from between 2 to 5 sampling events, as shown in Figure 3-6. ERIC primers consistently generated more fingerprint types, showed greater diversity, and greater strain evenness than REP primes (see Table 3-1).

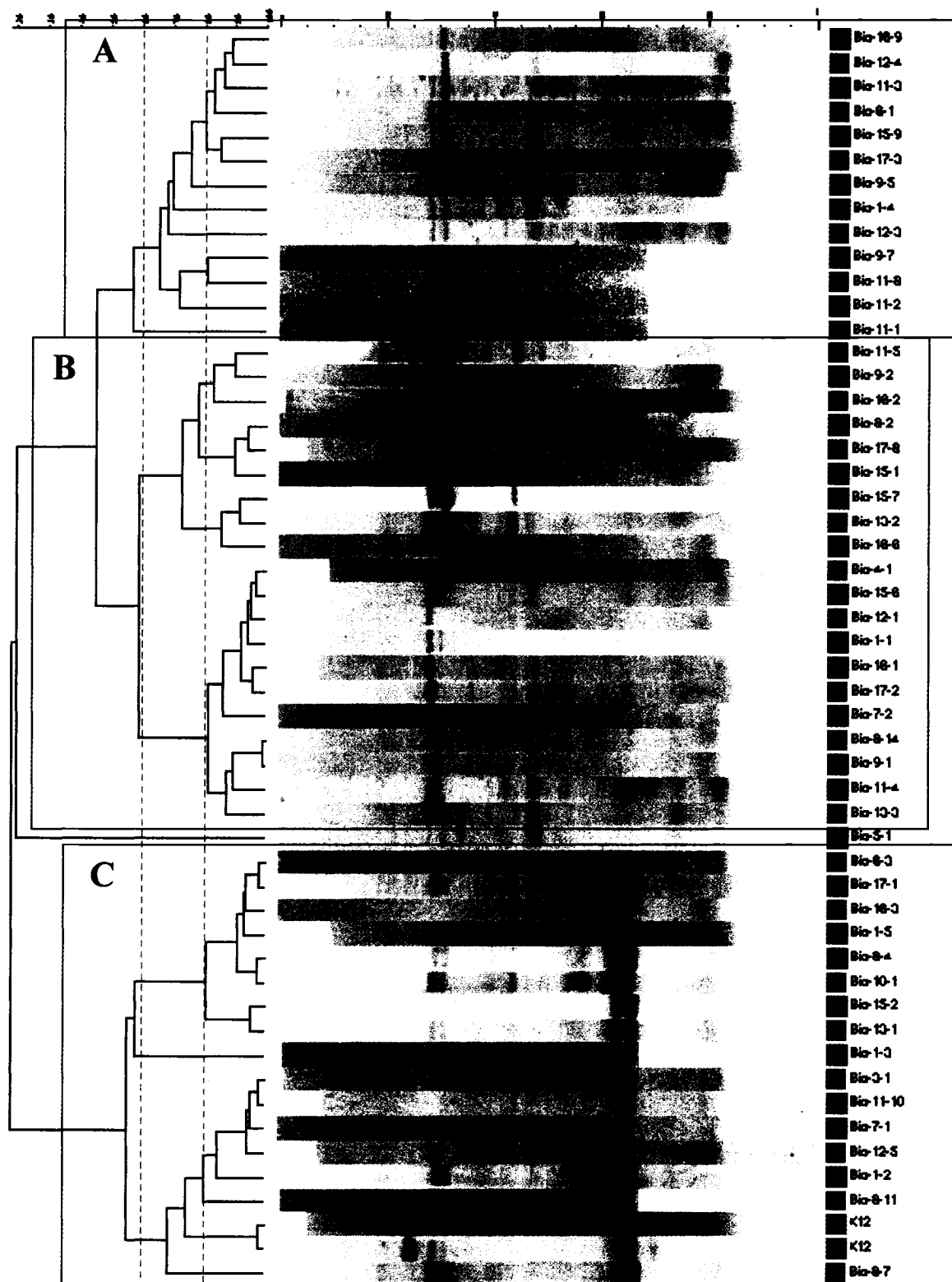


Figure 3-2: Dendrogram of biosolids isolates (shown in brown) (with clones excluded) (n=50) and the laboratory strain K12 (shown in red) (n=2). Super-clusters A, B, and C are outlined in blue; a single unclustered isolate is present between super-clusters B and C. Grey dashed lines show the 60 and 80% similarity cut-offs.

Table 3-1: Basic ecological parameters calculated for *E. coli* isolated from pulp and paper mill primary clarifier (REP primers), secondary clarifiers (REP primers) and biosolids (amplified with both REP and ERIC primers). Note that the top portion of the table (labeled TOTAL) refers to calculations made including all fingerprints for a given matrix, while the bottom portion of the table (labeled AVERAGED) refers to calculations made using data from sampling events where $n > 5$ and includes average values and standard deviations. n– sample size; S – strain richness, number of distinct fingerprints present in the sample matrix; E – strain evenness; H – Shannon Index of Diversity.

	Parameter	Primary Clarifier	Secondary Clarifier	Biosolids (with REP)	Biosolids (with ERIC)
TOTAL	n	50	83	91	84
	S	30	29	17	29
	E	0.89	0.91	0.82	0.92
	H	3.03	3.06	2.32	3.09
AVERAGED					
	n	10.0 +/- 5.9	6.0 +/- 0.6	5.6 +/- 1.9	5.5 +/- 2.0
	S	7.5 +/- 3.5	4.8 +/- 0.9	8.9 +/- 1.8	9.3 +/- 3.3
	E	0.96 +/- 0.02	0.94 +/- 0.11	0.88 +/- 0.07	0.95 +/- 0.04
	H	1.85 +/- 0.48	1.48 +/- 0.23	1.45 +/- 0.26	1.57 +/- 0.34

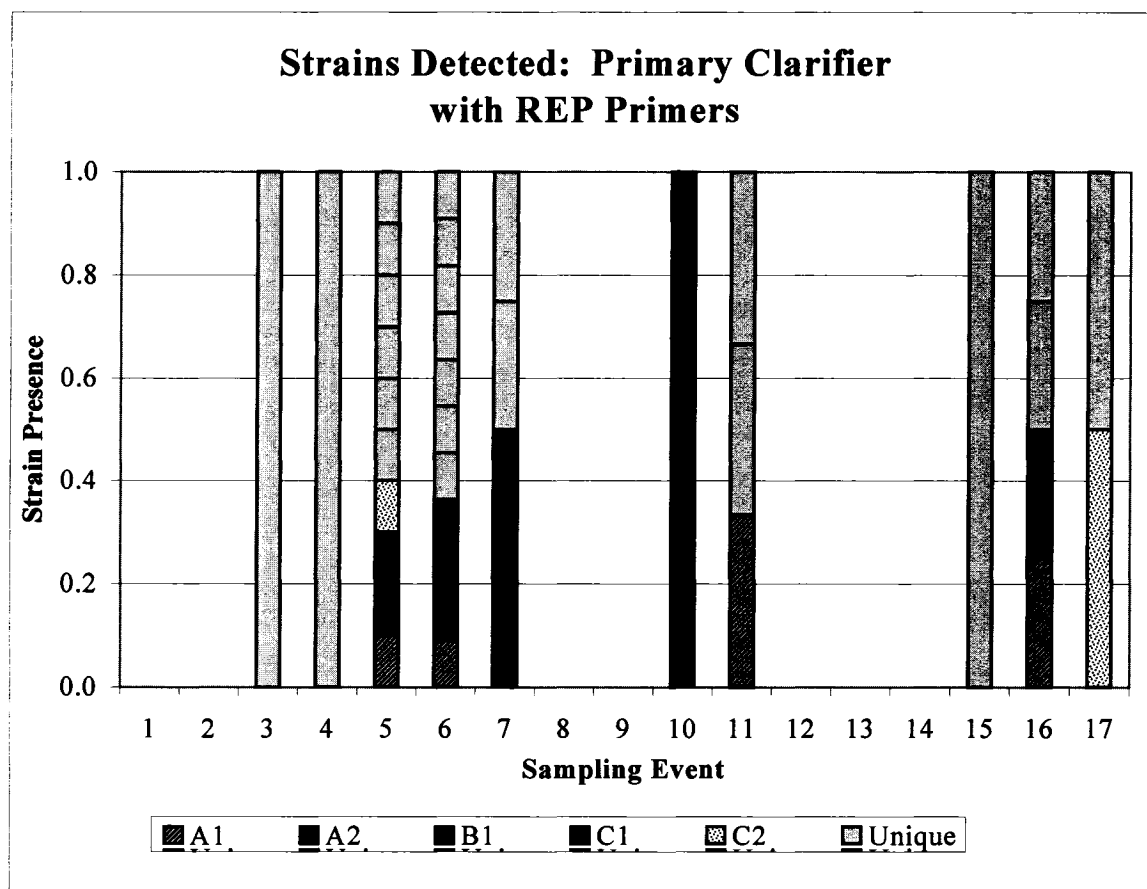


Figure 3-3: Rep-PCR fingerprints were generated for *E. coli* isolated from pulp and paper mill primary clarifier effluents and sludges using REP primers. Isolates sharing >80% similarity (cosine correlation) and visually identical fingerprint patterns were grouped into clusters. Fingerprints sharing >80% similarity but showing at least a 1 distinct band difference were grouped into separate clusters. The detection of these clusters was plotted for sampling events between June 2004 and January 2005. Clusters are marked as either present or absent in a particular sample, the graph does not indicate the proportion of any strain. Note that the yellow bars are used to denote the presence of fingerprint types that were detected only once.

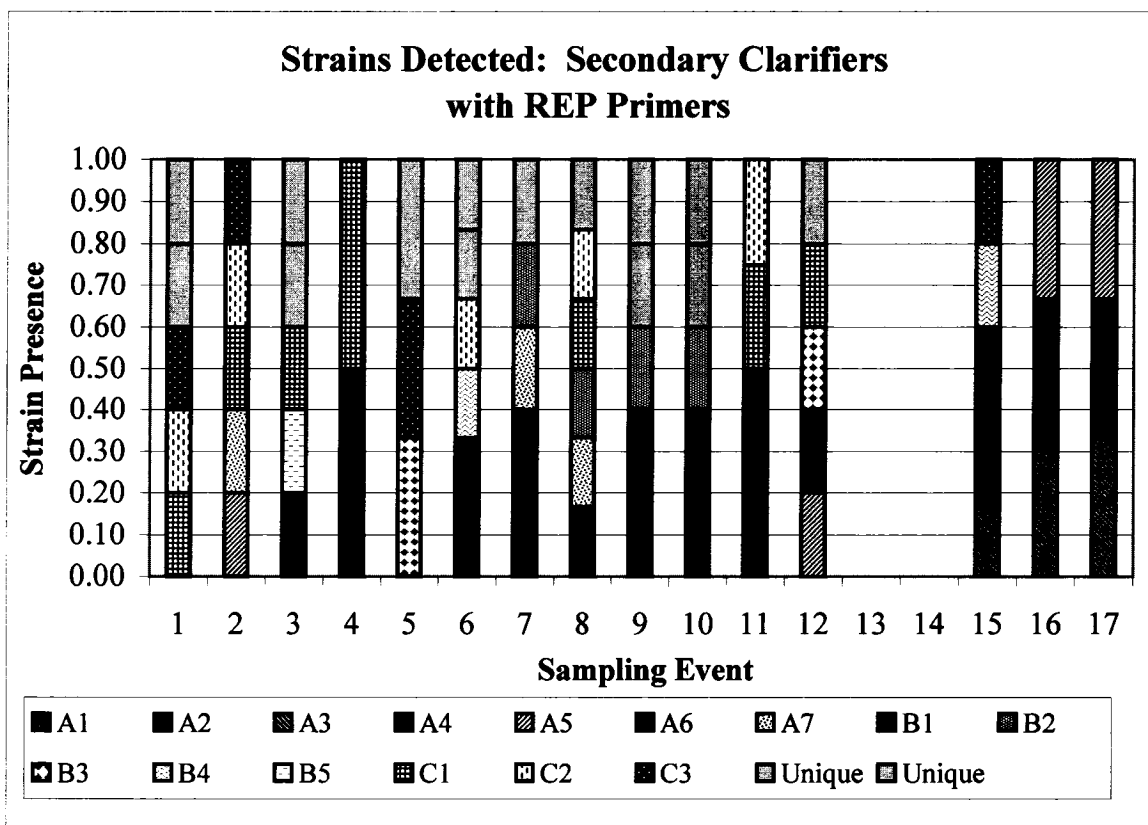


Figure 3-4: Rep-PCR fingerprints were generated for *E. coli* isolated from pulp and paper mill secondary clarifier effluents and sludges (isolates from the 2 clarifiers were pooled) using REP primers. Isolates sharing >80% similarity (cosine correlation) and visually identical fingerprint patterns were grouped into clusters. Fingerprints sharing >80% similarity but showing at least a 1 distinct band difference were grouped into separate clusters. The detection of these clusters was plotted for sampling events between June 2004 and January 2005. Clusters are marked as either present or absent in a particular sample, the graph does not indicate the proportion of any strain. Note that the yellow bars are used to denote the presence of fingerprint types that were detected only once.

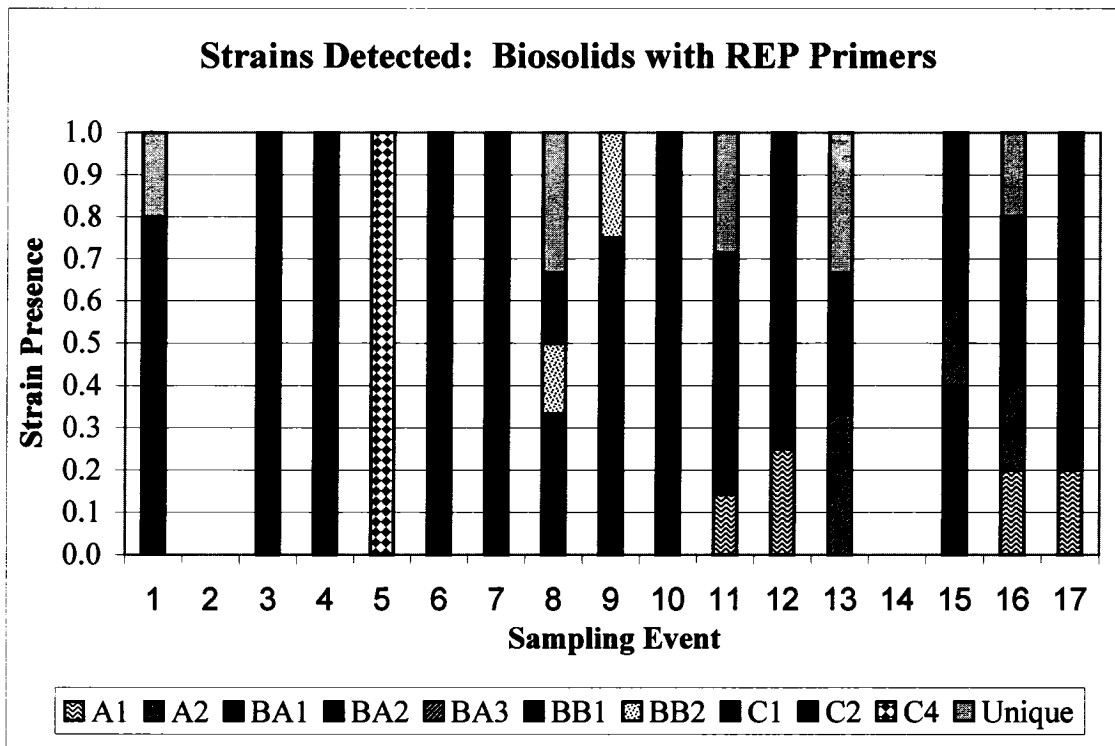


Figure 3-5: Rep-PCR fingerprints were generated for *E. coli* isolated from pulp and paper mill biosolids using REP primers. Isolates sharing >80% similarity (cosine correlation) and visually identical fingerprint patterns were grouped into clusters. Fingerprints sharing >80% similarity but showing at least a 1 distinct band difference were grouped into separate clusters. The detection of these clusters was plotted for sampling events between June 2004 and May 2005. Clusters are marked as either present or absent in a particular sample, the graph does not indicate the proportion of any strain. Note that the yellow bars are used to denote the presence of fingerprint types that were detected only once.

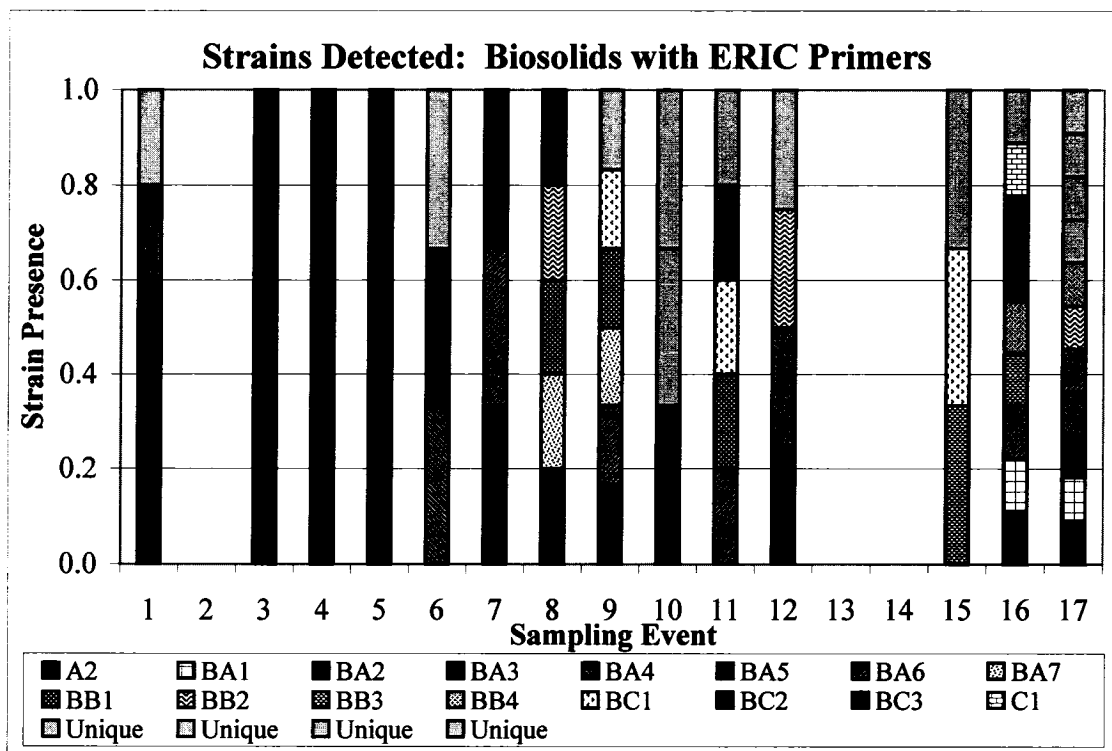


Figure 3-6: Rep-PCR fingerprints were generated for *E. coli* isolated from pulp and paper mill biosolids using ERIC primers. Isolates sharing >95% similarity (cosine correlation) and visually identical fingerprint patterns were grouped into clusters. Fingerprints sharing >95% similarity but showing at least a 1 distinct band difference were grouped into separate clusters. The detection of these clusters was plotted for sampling events between June 2004 and May 2005. Clusters are marked as either present or absent in a particular sample, the graph does not indicate the proportion of any strain. Note that the yellow bars are used to denote the presence of fingerprint types that were detected only once.

3.3.5 Physical and Chemical Characteristics of Mill Effluents

Primary and secondary effluents generally showed stable temperature, pH and conductivity (as illustrated in Figure 3-7 and Figure 3-8). Large declines in temperature and conductivity in late December were associated with a 2 week mill maintenance shutdown. Conditions subsequently stabilized until on-site pulping activities were eliminated in late January, 2005 – altering effluent composition significantly and decreasing effluent volume to less than half of its previous flow. At this point, conditions varied as the effluent treatment system as its operators gained familiarity with the new effluent conditions.

3.3.6 Stability of *E. coli* Populations in Biosolids

E. coli collected the summers of 2003, 2004, 2005 were pooled and analyzed and compared with isolates collected during the mill shut (shown in). Two fingerprint types were isolated during all 4 sampling events; a further 4 fingerprint types were isolated at multiple sampling events. Post-restructuring isolates have a higher strain richness and greater diversity than isolates collected at any of the other sampling events (Table 3-2).

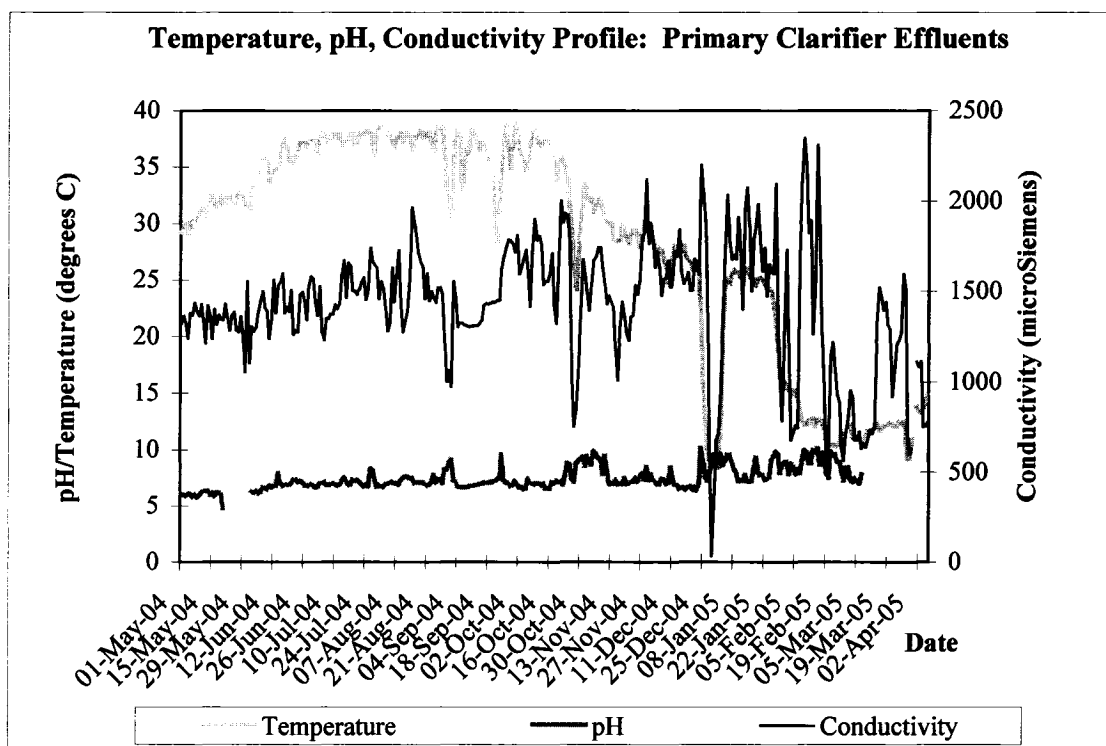


Figure 3-7: Daily average temperature, pH and conductivity measures for the primary clarifier effluent outflow (bioreactor cell 1 inflow) between May 1, 2004 and April 6, 2005.

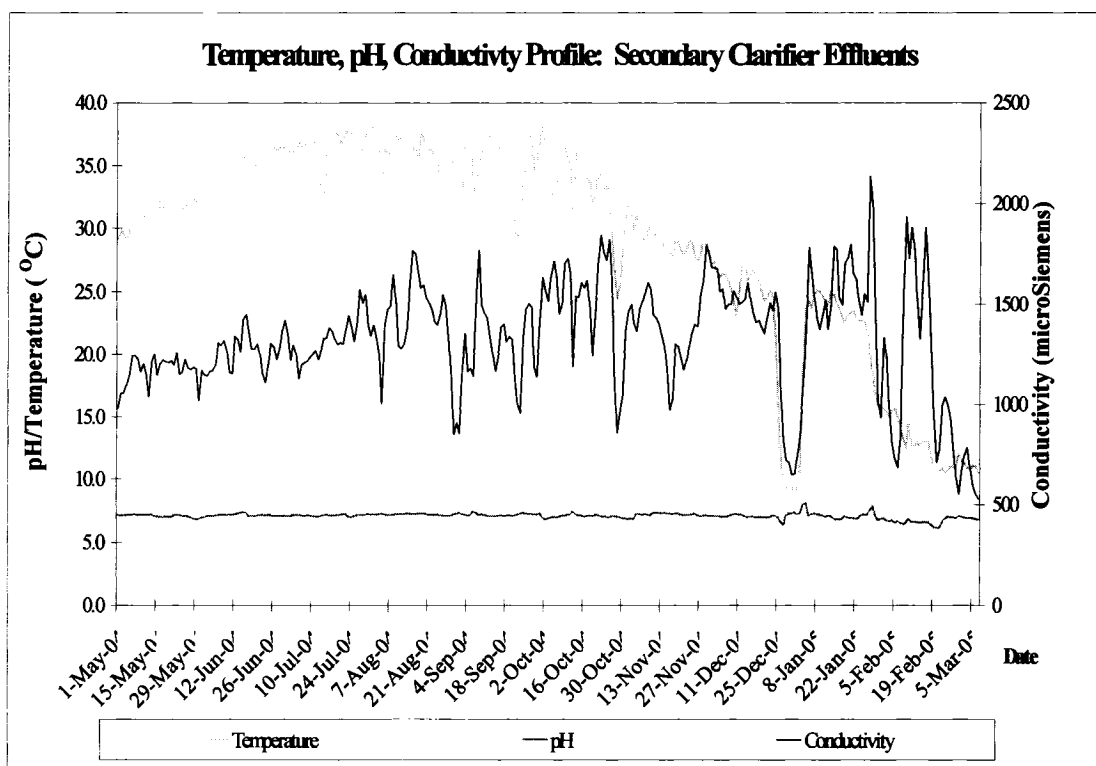


Figure 3-8: Daily average temperature, pH and conductivity measures for the secondary clarifier effluent outflow (mill effluent outflow) between May 1, 2004 and March 7, 2005.

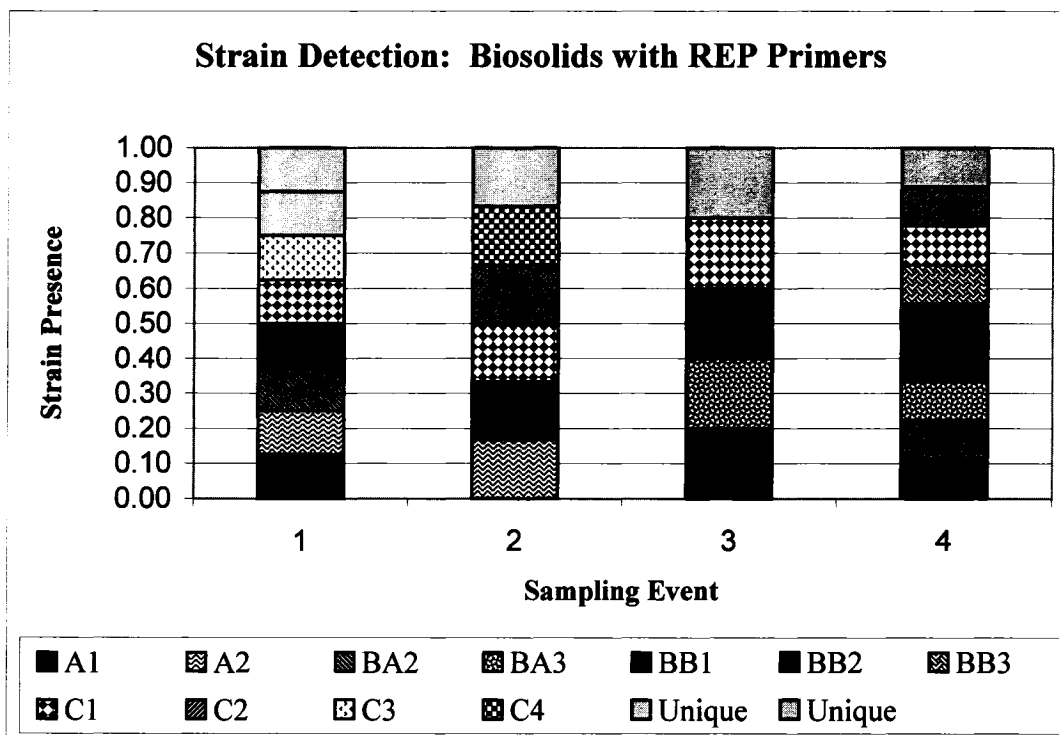


Figure 3-9: Rep-PCR fingerprints were generated for *E. coli* isolated from pulp and paper mill biosolids using REP primers. Isolates sharing >80% similarity (cosine correlation) and visually identical fingerprint patterns were grouped into clusters. Fingerprints sharing >80% similarity but showing at least a 1 distinct band difference were grouped into separate clusters. The detection of these clusters was plotted for sampling events between June to August 2003 (DSO), June to January 2004 and May 2005. Clusters are marked as either present or absent in a particular sample, the graph does not indicate the proportion of any strain. Note that the yellow bars are used to denote the presence of fingerprint types that were detected only once.

Table 3-2: Basic ecological parameters calculated for *E. coli* isolated from pulp and paper mill biosolids and amplified with REP primers. 2003 refers to isolates collected between May and August 2003; 2004, May to August 2004; DS 16, December 29, 2004 during a mill shut; and 2005, May 2005. N – sample size; S – strain richness, number of distinct fingerprints present in the sample matrix; E – strain evenness; H – Shannon Index of Diversity.

	2003	2004	DS 16	2005
N	22	18	10	20
S	8	6	5	9
E	0.83	0.90	0.91	0.92
H	1.72	1.62	1.47	2.03

3.4 Discussion

E. coli populations are found in the gastrointestinal tracts of warm-blooded animals. These populations are typically dominated by a single Rep-fingerprint type, with several non-dominant, distinct fingerprints detected in much lower concentrations (127). This pattern is quite distinct from what was detected in the effluent treatment system and biosolids of a pulp and paper mill. These industrial populations are not clearly dominated by any single Rep-fingerprint type. Rather, several distinct fingerprint types are detected at repeated sampling events over long periods of time.

3.4.1 Primary Clarifier

The primary clarifier is an area of particular interest as it was identified as an area of significant in-mill coliform and *E. coli* growth by Gauthier and Archibald (63). Unfortunately, the success rate isolating *E. coli* from primary clarifier effluents and rejects was low. Other researchers have had similar problems isolating *E. coli* from this sample matrix, and have suggested that these difficulties may be due to the presence of a high number of interfering microorganisms (122). Primary clarifier effluents and sludges are estimated to harbour 100 to 1000 times more coliforms than *E. coli* (63).

The primary clarifier is an area where all mill wastewaters are pooled and mill fibre is added. The chemical and physical characteristics of primary clarifier effluents are influenced by changes in the inputs. As a result, conditions in the primary clarifier are less stable than conditions in the secondary clarifiers.

Despite the variable conditions and the constant input of new strains of *E. coli*, several identical fingerprint types were detected at both the same and repeated sampling

events. This pattern of detection is suggestive of in-mill growth. A high number of unique strains, or strains detected only at a single sampling event, were also detected in this sample matrix. These results are suggestive of differential survival; many fingerprint types enter the primary clarifier, but only a core group of strains survives and multiplies, while other strains pass through the system, unable to compete. This model is supported by the jackknife analysis calculations in Chapter 2. A third (36.2%) of primary clarifier-isolated *E. coli* could be correctly reassigned to their source group. These isolates would constitute part of the core *E. coli* population.

Both total and averaged strain richness, strain evenness and Shannon index of diversity values were consistently high. These results are representative of a diverse *E. coli* population composed of many strains, with no single strain being particularly dominant.

3.4.2 Secondary Clarifier

The study site used 2 secondary clarifiers. However, the 2 clarifiers acted as a single functional unit: they received effluents that had undergone AST, contributed RAS to re-seed the AST, and contributed WAS to the biosolids. For the purposes of this study, isolates from both secondary clarifiers were pooled.

During AST, pH was adjusted, and nutrients were added. As a result, the physical and chemical conditions were more stable in the secondary clarifier than the primary clarifier.

While unique strains were detected in this secondary clarifier, they were detected less often and in fewer numbers at each sampling event than in the primary clarifier. The detection of identical fingerprint types both within a single sampling event and at multiple sampling events was consistent with in-mill growth. The repeated detection of identical

fingerprint types was more pronounced than in the primary clarifier; however this may have been due to the much larger sample size of secondary clarifier isolates. Many of the fingerprint types that were detected at multiple sampling events were distinct from those seen in the primary clarifier. These fingerprint types may have been added at the AST. As discussed in Chapter 2, 61.8% of secondary clarifier isolates could be correctly reassigned to their own source using jackknife analysis. Less than 10% of secondary clarifier isolates were mistaken for primary clarifier isolates. This suggests that the core *E. coli* populations in primary clarifier and secondary clarifier habitats are distinct from each other.

Ecological comparisons between the primary and secondary clarifiers were best examined using averaged values to reduce the effects of sample size on the calculated values. Strain richness was lower in the secondary clarifier than in the primary clarifier. Any single sample from the primary clarifier would contain more strains than would be detected in a similar sample from the secondary clarifier. High strain evenness values suggest that strain distribution was fairly (but not entirely) equal. Shannon index of diversity values were much lower in the secondary clarifier than the primary clarifier. This can be explained by the large number of *E. coli* inputs to the primary clarifier. Few of these strains survive primary clarification and competition against AST microbes to be detected in the secondary clarifiers.

3.4.3 Biosolids

Biosolids isolates were analyzed with 2 primer sets – REP and ERIC. Both primers detected similar patterns: the repeated detection of identical fingerprints at a single sampling event, and at multiple sampling events. These results suggest in-mill *E. coli* growth, and the presence of a fairly stable *E. coli* population in biosolids. The similar

results obtained using both primer sets suggests that the MST method employed offered a sufficiently high level of discrimination between strains.

ERIC primers were more descriptive, but more difficult to reproduce. They consistently yielded a greater number of fingerprint types than REP primers. This was unexpected, as REP primers contain deoxyinosines (bases which will bind to any other base, A, T, G, or C) and fingerprints are amplified using a lower annealing temperature (resulting in less primer specificity). Other researchers report variable result for these primers: ERIC was more reproducible than REP (114); ERIC was more sensitive to suboptimal conditions than REP (165); and ERIC and REP generated comparable, but not identical results (127).

Both total and averaged Shannon index of diversity values were lower in biosolids than in either the primary or the secondary clarifiers. This was not expected as biosolids were composed of both primary and secondary sludges and should contain fingerprint types from both of these matrices. However, an examination of the results shows that only a subset of the fingerprint types detected in each of these sample matrices actually contributes to the *E. coli* population in biosolids. It may be that clarifier *E. coli* populations are reduced by the mechanical pressure and hot, desiccating conditions characteristic of dewatering. Biosolids *E. coli* populations may be composed of strains that have survived these conditions; a subset of strains that are: more resistant to desiccation; more tolerant of hot temperatures; more resistant to mechanical stress; or more closely associated with large particles that shield them from adverse conditions. The high concentrations of *E. coli* in biosolids may be due to the regrowth of relatively few survivors. Regrowth of *E. coli* and

fecal coliforms has been recorded in manure (184; 185), composted sewage sludges (61; 193; 200) and pulp and paper mill biosolids (12).

3.4.4 Stability of *E. coli*

While mill conditions tended to remain relatively stable over time, the mill studied in this research project underwent several large-scale structural changes during the study period.

In 2003, the mill generated 125 000m³/d of wastewater which included effluents from: kraft (chemical sulfate) pulping, kraft bleaching (elemental chlorine free), fine papermaking, post-consumer boxboard fiber recycling and storm effluent. By 2004, the on-site post-consumer recycling facility had closed, and this waste stream was eliminated, reducing wastewater volume to 100 000m³/day. The change in effluent composition was such that there was difficulty achieving the required settling, particularly in the secondary clarifiers. Cleaner rejects from a mill producing corrugated cardboard from new and recycled fibres were shipped on-site and shoveled into the primary sump to increase the fibre content and improve the settling. In February of 2005, the mill eliminated all on-site pulping activities, this included: the woodyard, chipping, chip piles and pulping. Wastewater volume was further reduced to 50 000m³/d. Changes in effluent composition were visible (primary clarifier effluents changed from a dark, cloudy tea colour to a chalky white, and secondary clarifier effluents changed from pale tea colour to completely colourless).

On a much smaller scale, in December of 2004 there was a mill shut for maintenance. Effluent temperatures decreased, pH and conductivity were also affected. *E.*

coli were detected in the chlorinated feed water (perhaps due to decline in chlorine residual as the water was relatively stagnant in the hoses and pipes).

The changes in the physical and chemical composition of the effluents could affect the composition of the mill-*E. coli* population.

This question was examined by pooling biosolids isolates collected the summers of 2003, 2004 and 2005 and also comparing them to isolates collected from the mill shut (DS 16). The samples from the summer of 2003 and 2004 can be considered to represent the mill at a steady state as the effluent treatment systems were stable, and had been for some time. The samples collected from the summer of 2005 were collected in May, 2 months after the large scale restructuring occurred. The mill was still adjusting effluent treatment conditions, and the system was not stable.

An examination of Figure 3-8 shows that there are 2 fingerprint types that were detected under all 4 sets of operating conditions. It is interesting to note the presence of a new fingerprint type in the DS16 samples which was also detected in the 2005 samples, but had not been detected prior to that event. Furthermore, there is a greater number of fingerprint types in the biosolids in 2005, including a new fingerprint type, unseen prior to the restructuring but detected afterwards.

The post-restructuring *E. coli* population in the biosolids showed the highest level of diversity (by the Shannon Index of Diversity). The values for strain evenness were lowest for the 2003 samples, and slightly higher for 2004, DS16 and 2005. Strain evenness describes the similarity in abundance of different strains. This suggests that the mill-*E. coli* community is composed of several strains with similar abundances.

There are no firm guidelines on developing experimental and statistical designs to assess the diversity of a microbial population (132). In this case, strain evenness and the Shannon diversity index were calculated as in (169) and (163). The small population sizes (< 25) may mean that the results are skewed, and that certain fingerprint types were not detected, but were in fact present in the biosolids. Larger sample sizes would help to resolve this issue.

The apparent increase in strain diversity has important implications for the mill. Under normal operating conditions, effluent conditions are fairly constant and distinct *E. coli* populations seem to survive and multiply in the primary and secondary clarifiers and the biosolids. However, when the system was perturbed, new fingerprint types of *E. coli* had an opportunity to begin growing within the mill. In terms of the safety of the land application of biosolids, if mill-*E. coli* populations can be shown to be non-pathogenic, and the mill system has non-detectable levels of other microbial pathogens, it is likely to maintain this status quo and pathogenic microorganisms would find it difficult to establish a niche. However, when the system is disturbed, new strains of *E. coli* are able to colonize the mill, there could be no guarantee that new strains of *E. coli* or other pathogenic microorganisms would not be able to colonize the mill.

3.5 Conclusions

Rep-PCR was able to detect intra-specific genetic variation among *E. coli* isolated from the effluent treatment system and biosolids of a pulp and paper mill. ERIC primers tended to generate more complex banding patterns than REP primers, which may have increased the discriminatory power provided by the ERIC primer set. However, ERIC primers were less effective at generating reproducible fingerprints than REP primers.

E. coli with highly similar fingerprint patterns (>80% similarity) were isolated repeatedly over the 8-month study period from each environment examined. These findings are consistent with the presence of self-sustaining *E. coli* populations in the primary clarifier, the secondary clarifiers and the biosolids. These results provide further support for the existence of widespread in-mill *E. coli* growth throughout the effluent treatment system and in the biosolids. Growing *E. coli* do not accurately represent a threat of enteric disease or fecal contamination, and should not be used as an indicator of the microbial safety of pulp and paper mill biosolids.

E. coli populations in biosolids seem to be fairly stable, as some strains with very similar fingerprint types (>80% similarity) were repeatedly isolated from the biosolids over a three-year time period. These results suggest that under normal operating conditions, few new strains are able to successfully colonize the effluent treatment system and biosolids. Mill strains may be able to competitively exclude other strains of *E. coli*, including pathogenic strains, much like what occurs in the mammalian intestine. However, large-scale changes to the effluent treatment system do affect these in-mill populations, and may open new niches for colonization. Large-scale changes at the study mill resulted in the detection of new fingerprint types and an increase in Shannon index of diversity values more than 2 months after the changes began to be implemented.

The microbial ecology of pulp and paper mills remains poorly understood. Much work remains to improve our understanding of the structure and dynamics of the communities extent throughout the mill water and wastewater systems. The question of the survival of strains or genetic traits (i.e. pathogenicity factors, resistance factors) and the

competitiveness of pathogenic strains within the pulp and paper mill habitat remains unknown.

In established effluent treatment systems ‘normal mill flora’ seem to be stable and resistant to colonization by new strains. Results from this study are consistent with in-mill *E. coli* growth rather than fecal loading. Frequent monitoring of such *E. coli* populations does not seem to offer any improved understanding of the microbial risks posed by biosolids. Following large-scale alterations to the effluent treatment system, the *E. coli* populations may change; some post-alteration monitoring would be prudent to undertake at such times to ensure that no pathogenic strains have successfully colonized the system.

Chapter 4 Thesis Summary

4.1 Conclusions

Pulp and paper mill biosolids have demonstrated excellent potential as a soil conditioner. However, the presence of high levels of *E. coli* and other indicators of fecal contamination in the absence of any known fecal input has been a cause of confusion and concern. This research project was aimed at: 1) identifying the sources of *E. coli* detected in pulp and paper mill biosolids; 2) examining the ecology of biosolids *E. coli*; and 3) determining the effects of large-scale system changes on the biosolids *E. coli* population. The results presented in this thesis have led to an improved understanding of the populations of *E. coli* in pulp and paper mill effluent treatment systems and biosolids. This study provides useful information for mill operators, land applicators and regulators.

Concentration data and dendrograms described in Chapter 2 showed that all potential sources examined may contribute *E. coli* to the pulp and paper mill effluent treatment system and biosolids. The link between *E. coli* isolated from forested environments and industrial sites could not be established with confidence due to the small sample size. Treated mill process water, storm effluent, mill fibre, and wood chips all yielded *E. coli* that generated fingerprints that were highly similar to those detected in the effluent treatment system and biosolids.

In Chapter 3 highly similar fingerprint types were repeatedly recovered from the primary clarifier, the secondary clarifiers and the biosolids over an 8-month period. This data is consistent with the continuous growth of established *E. coli* populations in these

habitats. Diversity and strain richness tended to be greatest in the primary clarifier, where all wastewater streams were pooled. Secondary clarifiers were less diverse and had fewer unique strains, possibly due to greater stability within this habitat as conditions were less changeable and the only input was activated sludge treated effluents. Biosolids were expected to contain fingerprint types from both primary and secondary clarifiers. This was the case, however the diversity was much lower in the biosolids. Only a subset of primary and secondary clarifier strains are able to successfully contribute *E. coli* to pulp and paper mill biosolids. This may be due to differential survival during dewatering, and subsequent regrowth of the surviving strains.

Conditions in the effluent treatment system tend to be relatively stable in terms of temperature, pH and conductivity. However, there are seasonal variations in the conditions, which are exacerbated by mill activities (i.e. a mill shut in December allows temperatures in the clarifiers to decrease substantially). During the sampling period, the study mill was in transition; large scale changes were made to the effluent treatment system over a 3-year period. As discussed in Chapter 3, while many fingerprint types were detected both before and after the changes were made, diversity increased after the changes. New fingerprint types were detected in the biosolids both following the mill shut, and the large-scale changes.

4.2 Significance of Findings

These research findings provide important information for pulp and paper mill operators, land applicators and regulators.

Mill operators cannot effectively reduce the concentration of *E. coli* in their biosolids by pre-treating or eliminating any single waste stream. However, the risk of

pathogenic microorganisms gaining access to the system can be minimized by eliminating or pre-treating mill sewage and storm effluent waste streams.

Most fingerprint types detected in the biosolids shared a high degree of similarity with fingerprint types recovered from the effluent treatment system or inputs to the mill. This suggests that all major *E. coli* inputs were sampled. While the original sources of some inputs remain unclear (i.e. storm effluent, which may have come into contact with landwash or wildlife feces), many inputs contained *E. coli* that did not seem to be of a recent fecal source (i.e. treated mill process water, mill fibre). At least some part of biosolids *E. coli* does not seem to be of fecal origin. The most significant portion of biosolids *E. coli* seems to originate from the primary and secondary clarifiers, where *E. coli* seems to be continuously growing in established niches. As biosolids *E. coli* are demonstrably not (entirely) of a fecal source, and grow within the pulp and paper mill environment, it is inappropriate to use them as an indicator of the microbial safety of pulp and paper mill biosolids. Land spreading activities should not be restricted solely on the basis of high *E. coli* concentrations.

Pulp and paper mill biosolids should be reexamined following large-scale changes to the effluent treatment system. Large-scale changes alter the environments in the primary and secondary clarifiers, and upset the established microbial populations. At such times, there is an increased risk that pathogenic microorganisms might colonize the system.

4.2 Recommendations for Future Research

While this research supports earlier suggestions that *E. coli* is not an appropriate indicator of the microbial safety of pulp and paper mill biosolids, an appropriate indicator or test remains to be found. Future research could focus on making conventional *E. coli*,

coliform, fecal coliform or enterococci tests more discriminatory. A more beneficial route might be to examine new, industry-specific bacterial or chemical indicators. In the past, direct pathogen testing has not been feasible. However, new technologies may change this. For example, micro-arrays could be used to screen a single sample for a large number of different pathogenic microorganisms.

While *E. coli* do seem to be growing continuously in pulp and paper mill effluent treatment systems, little is known about the factors that affect their survival. The competitiveness of epidemiologically significant *E. coli* (i.e. pathogenic strains, or strains carrying antibiotic resistance factors) with established industrial strains merits further investigation.

References

- 1 Achtman, M. and G. Pluschke. 1986. Clonal analysis of descent and virulence among selected *Escherichia coli*. *Ann. Rev. Microbiol.* 40:185-210.
- 2 Albert, J. M., J. Munakata-Marr, L. Tenorio, and R. L. Siegrist. 2003. Statistical evaluation of bacterial source tracking data obtained by rep-PCR DNA fingerprinting of *Escherichia coli*. *Environ. Sci. Technol.* 37:4554-4560.
- 3 Ali, M. and T. R. Sreekrishnan. 2001. Aquatic toxicity from pulp and paper mill effluents: a review. *Adv. Environ. Res.* 5:175-196.
- 4 American Public Health Association. 1995. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, D.C.
- 5 Archibald, F. 2000. The presence of coliform bacteria in Canadian pulp and paper mill water systems - a cause for concern? *Water Qual. Res. J. Can.* 35:1-22.
- 6 Armon, R. and Y. Kott. 1995. Distribution comparison between coliphages and phages of anaerobic bacteria (*Bacteroides fragilis*) in water sources, and their reliability as fecal pollution indicators in drinking water. *Water Sci. Tech.* 31:215-222.
- 7 Ashbolt, N. J. 2004. Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology* 198:229-238.
- 8 Avery, L. M., P. Hill, K. Killham, and D. L. Jones. 2004. *Escherichia coli* O157 survival following the surface and sub-surface application of human pathogen contaminated organic waste to soil. *Soil Biol. Biochem.* 36:2101-2103.
- 9 Avery, S. M., E. Liebana, C.-A. Reid, M. J. Woodward, and S. Buncic. 2002. Combined use of two genetic fingerprinting methods, pulsed-field gel electrophoresis and ribotyping, for characterization of *Escherichia coli* O157 isolates from food animals, retail meats, and cases of human disease. *J. Clin. Microbiol.* 40:2806-2812.
- 10 Bagley, S. T., R. J. Seidler, H. W. Talbot Jr., and J. E. Morrow. 1978. Isolation of *Klebsiella* from within living wood. *Appl. Environ. Microbiol.* 36:178-185.
- 11 Barker, J., T. J. Humphrey, and M. W. R. Brown. 1999. Survival of *Escherichia coli* O157 in a soil protozoan: implications for disease. *FEMS Microbiol. Lett.* 173:291-295.
- 12 Beauchamp, C. J., Simao-Beaunoir, A.-M., Beaulieu, C., and Chalifour, F.-P. 2003. Coliform bacteria in paper sludges: where are they coming from? *Proceedings of 2nd Canadian Organic Residuals Recycling Conference*, April 24-25 2003; Penticton, B.C., Canada. p. 248-258.

- 13 Bej, A. K., J. L. DiCesare, L. Haff, and R. M. Atlas. 1991. Detection of *Escherichia coli* and *Shigella spp.* in water by using the polymerase chain reaction and gene probes for uid. *Appl. Environ. Microbiol.* 57:320-323.
- 14 Bellamy, K. L. 1989. Assessment of soil and water quality at a test agricultural site receiving Fraser Inc. sludge. ORTECH Rep. P. 88-48-5657/CI.
- 15 Bellamy, K. L., C. Chong, and R. A. Cline. 1995. Paper sludge utilization in agriculture and container nursery culture. *J. Environ. Qual.* 24:1074-1082.
- 16 Berg, R. D. 1996. The indigenous gastrointestinal microflora. *Trends Microbiol.* 4:430-436.
- 17 Bermudez, M. and T. C. Hazen. 1988. Phenotypic and genotypic comparison of *Escherichia coli* from pristine tropical waters. *Appl. Environ. Microbiol.* 54:979-983.
- 18 Bernhard, A. E. and K. G. Field. 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Appl. Environ. Microbiol.* 66:1587-1594.
- 19 Blood, R. M. and G. D. W. Curtis. 1995. Media for 'total' Enterobacteriaceae, coliforms and *Escherichia coli*. *Int. J. Food Microbiol.* 26:93-115.
- 20 Bogosian, G., L. E. Sammons, P. J. L. Morris, J. P. O'Neil, M. A. Heitkamp, and D. B. Weber. 1996. Death of *Escherichia coli* K12 strain W3110 in soil and water. *Appl. Environ. Microbiol.* 62:4114-4120.
- 21 Brettar, I. and M. G. Hofle. 1992. Influence of ecosystematic factors on survival of *Escherichia coli* after large-scale release into lake water mesocosms. *Appl. Environ. Microbiol.* 58:2201-2210.
- 22 Brown, C. and R. J. Seidler. 1973. Potential pathogens in the environment: *Klebsiella pneumoniae*, a taxonomic and ecological enigma. *Appl. Microbiol.* 25:900-904.
- 23 Bunnage, W. and S. Imada. 1996. Sludge characterization survey: how secondary fibre affects mill sludge. *Pulp and Paper Can.* 97:4-7.
- 24 Byappanahalli, M. N. and R. S. Fujioka. 1998. Evidence that tropical soil environment can support the growth of *Escherichia coli*. *Wat. Sci. Tech.* 38:171-174.
- 25 Byappanahalli, M., M. Fowler, D. Shively, and R. Whitman. 2003. Ubiquity and persistence of *Escherichia coli* in a midwestern coastal stream. *Appl. Environ. Microbiol.* 69:4549-4555.
- 26 Cabral, F. and E. Vasconcelos. 1993. The use as fertilizer of combined primary/secondary pulp-mill sludge. In *Optimization of plant nutrition. Edited by M.A.C. Frago and M.L. van Beusichem.* Kluwer Academic Publishers, Dordrecht. pp. 77-81.

- 27 Cabral, F., E. Vasconcelos, M. J. Goss, and C. M. d. s. Cordovil. 1998. The value, use, and environmental impacts of pulp-mill sludge additions to forest and agricultural lands in Europe. *Environ. Rev.* 6:55-64.
- 28 Cabral, F., E. Vasconcelos, and P. Monjardino. 1990. Biodegradabilidade no solo das lamas celulósicas. *In* Actas da 2^a Conferência Nacional da Qualidade do Ambiente, *Edited by* F. Santana, M.C.R. Santos, M.H. Costa, and D. Pereira. Universidade Nova de Lisboa, Lisboa. pp. E1-E6.
- 29 Cabral, F., E. Vasconcelos, and J. Q. Santos. 1990. Utilization of pulp-mill sludge as fertilizer to wheat crop (*Triticum aestivum* L.). *In* Proceedings of the First Congress of the European Society of Agronomy. *Edited by* A. Scaife. European Society of Agronomy, Paris, France. pp. 39-40.
- 30 Camper, A. K., W. L. Jones, and J. T. Hayes. 1996. Effect of growth conditions and substratum composition on the persistence of coliforms in mixed-population biofilms. *Appl. Environ. Microbiol.* 62:4014-4018.
- 31 Caplenas, N. R., M. S. Kanarek, and A. P. Dufour. 1981. Source and extent of *Klebsiella pneumoniae* in the paper industry. *Appl. Environ. Microbiol.* 42:779-785.
- 32 Carson, C. A., B. L. Shear, M. R. Ellersieck, and A. Asfaw. 2001. Identification of fecal *Escherichia coli* from humans and animals by ribotyping. *Appl. Environ. Microbiol.* 67:1503-1507.
- 33 Carson, C. A., B. L. Shear, M. R. Ellersieck, and J. D. Schnell. 2003. Comparison of ribotyping and repetitive extragenic palindromic-PCR for identification of fecal *Escherichia coli* from humans and animals. *Appl. Environ. Microbiol.* 69:1836-1839.
- 34 Cave, H., E. Bingen, J. Elion, and E. Denamur. 1994. Differentiation of *Escherichia coli* strains using randomly amplified polymorphic DNA analysis. *Res. Microbiol.* 145:141-150.
- 35 Cervato, A. and S. Silva. 1986. The use of industrial sludges in agriculture. I. Production of maize and wheat crops grown in succession. *Ann. Fac. Agrar. Univ. Cattol. Sacro Cuore* 26:165-187.
- 36 Chang, G. W., J. Brill, and R. Lum. 1989. Proportion of β -D-glucuronidase-negative *Escherichia coli* in human fecal samples. *Appl. Environ. Microbiol.* 55:335-339.
- 37 Chong, C. and R. A. Cline. 1993. Response of four ornamental shrubs to container substrate amended with two sources of raw paper mill sludge. *Hortscience* 28:807-809.
- 38 Cline, R. A. and C. Chong. 1991. Putting paper sludge to use in agriculture. *Highlights, Ontario Ministry of Agriculture and Food, Guelph* 14:16-18.
- 39 Coia, J. E. 1998. Clinical, microbiological and epidemiological aspects of *Escherichia*

coli O157 infection. FEMS Immunol. Med. Microbiol. 20:1-9.

- 40 Cote, C. and Quessy, S. 2003 Fate of human pathogens and indicator microorganisms in the soil after liquid hog manure spreading. Proceedings of 2nd Canadian Organic Residuals Recycling Conference. April 24-25 2003; Penticton, B.C., Canada. p. 323-330.
- 41 daSilveira, W. D., A. Ferreira, M. Lancellotti, I. A. G. C. D. Barbosa, D. S. Leite, A. F. P. deCastro, and M. Brocchi. 2002. Clonal relationships among avian *Escherichia coli* isolates determined by enterobacterial repetitive intergenic consenseus (ERIC)-PCR. Vet. Microbiol 89:323-328.
- 42 Davis, M. A., D. D. Hancock, T. E. Besser, and D. R. Call. 2003. Evaluation of pulsed-field gel electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli* O157:H7. J. Clin. Microbiol. 41:1843-1849.
- 43 DeBruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58:2180-2187.
- 44 Desjardins, E. and C. Beaulieu. 2003. Identification of bacteria contaminating a pulp and paper machine in a Canadian paper mill. J. Ind. Microbiol. Biotechnol. 30:141-145.
- 45 Dolar, S. G., J. R. Boyle, and D. R. Keeney. 1972. Paper mill sludge disposal on soils: effects on the yield and mineral nutrition of oats (*Avena sativa* L.). J. Environ. Qual. 1:405-409.
- 46 Dombek, P. E., L. K. Johnson, S. T. Zimmerley, and M. J. Sadowsky. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Appl. Environ. Microbiol. 66:2572-2577.
- 47 Donnenberg, M. S. 2000. Pathogenic strategies of enteric bacteria. Nature 406:768-774.
- 48 dos Anjos Borges, L. G., V. D. Vechia, and G. Corcao. 2003. Characterization and genetic diversity via REP-PCR of *Escherichia coli* isolates from polluted waters in southern Brazil. FEMS Microbiol. Ecol. 45:173-180.
- 49 Duncan, D. W. and W. E. Razzell. 1972. *Klebsiella* biotypes among coliforms isolated from forest environments and farm produce. Appl. Microbiol. 24:933-938.
- 50 Edge, T. and K. Schaefer. 2006. Microbial source tracking in aquatic ecosystems: state of the science and an assessment of needs. National Water Research Institute, Burlington, ON, Canada. 23 p.

- 51 Farnleitner, A. H., N. Kreuzinger, G. G. Kavka, S. Grillenberger, J. Rath, and R. L. Mach. 2000. Simultaneous detection and differentiation of *Escherichia coli* populations from environmental freshwaters by means of sequence variations in a fragment of the B-D-Glucuronidase gene. *Appl. Environ. Microbiol.* 66:1340-1346.
- 52 Farthing, M. J. 2004. Bugs and the gut: an unstable marriage. *Best Pract. Res. Clin. Gastroenterol.* 2:233-239.
- 53 Feagley, S. E., M. S. Valdez, and W. H. Hudnall. 1994. Papermill sludge, phosphorus, potassium, and lime effect on clover grown on a mine soil. *J. Environ. Qual.* 23:759-765.
- 54 Feagley, S. E., M. S. Valdez, and W. H. Hudnall. 1994. Bleached, primary papermill sludge effect on bermudagrass grown on a mine soil. *Soil Science.* 157:389-397.
- 55 Feldkirchner, D. C., C. Wang, S. T. Gower, E. L. Kruger, and J. Ferris. 2003. Effects of nutrient and paper mill biosolids amendments on the growth and nutrient status of hardwood forests. *For. Ecol. Manag.* 177:95-116.
- 56 Feng, P. C. S. and P. A. Hartman. 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli*. *Appl. Environ. Microbiol.* 43:1320-1329.
- 57 Fischer, J. R., T. Zhao, M. P. Doyle, M. R. Goldberg, C. A. Brown, C. T. Sewell, D. M. Kavanough, and C. D. Bauman. 2001. Experimental and field studies of *Escherichia coli* O157:H7 in white-tailed deer. *Appl. Environ. Microbiol.* 67:1218-1224.
- 58 Fortin, N., R. R. Fulthorpe, D. G. Allen, and C. W. Greer. 1998. Molecular analysis of bacterial isolates and total community DNA from kraft pulp mill effluent treatment systems. *Can. J. Microbiol.* 44:537-546.
- 59 Fukushima, H., K. Hoshina, and M. Gomyoda. 1999. Long-term survival of Shiga toxin-producing *Escherichia coli* O26, O111, and O157 in bovine feces. *Appl. Environ. Microbiol.* 65:5177-5181.
- 60 Gannon, J. J. and M. K. Busse. 1989. *E. coli* and enterococci levels in urban stormwater, river water and chlorinated treatment plant effluent. *Wat. Res.* 23:1167-1176.
- 61 Gantzer, C., P. Gaspard, L. Galvez, A. Huyard, N. Dumouthier, and J. Schwartzbrod. 2001. Monitoring of bacterial and parasitological contamination during various treatments of sludge. *Wat. Res.* 35:3763-3770.
- 62 Gauthier, F. 2000. Study of Coliform Bacteria in Canadian Pulp and Paper Mill Water Systems: Their Ecology and Utility as Health Hazard Indicators. Department of Natural Resources, McGill University, Montreal, Quebec.

- 63 Gauthier, F. and F. Archibald. 2001. The ecology of “fecal indicator” bacteria commonly found in pulp and paper mill water systems. *Wat. Res.* 35: 2207-2218.
- 64 Gauthier, F., J. D. Neufeld, B. T. Driscoll, and F. Archibald. 2000. Coliform bacteria and nitrogen fixation in pulp and paper mill effluent treatment systems. *Appl. Environ. Microbiol.* 66:5155-5160.
- 65 Gautom, R. K. 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other Gram-negative organisms in 1 day. *J. Clin. Microbiol.* 35 :2977-2980.
- 66 Geldreich, E. E., B. A. Kenner, and P. W. Kabler. 1964. Occurrence of coliforms, fecal coliforms, and streptococci on vegetation and insects. *Appl. Microbiol.* 12:63-69.
- 67 George, I., A. Anzil, and P. Servais. 2004. Quantifications of fecal coliform inputs to aquatic systems through soil leaching. *Wat. Res.* 38:611-618.
- 68 George, I., M. Petit, and P. Servais. 2000. Use of enzymatic methods for rapid enumeration of coliforms in freshwaters. *J. Appl. Microbiol.* 88:404-413.
- 69 George, I., P. Crop, and P. Servais. 2001. Use of β -D-galactosidase and β -D-glucuronidase activities for quantitative detection of total and fecal coliforms in wastewater. *Can. J. Microbiol.* 47:670-675.
- 70 Georghiou, P. R., A. M. Doggett, M. A. Kiehlhofner, J. E. Stout, D. A. Watson, J. R. Lupski, and R. J. Hamill. 1994. Molecular fingerprinting of *Legionella* species by repetitive element PCR. *J. Clin. Microbiol.* 32:2989-2994.
- 71 Gerba, C. P. 1987. Phage as indicators of fecal pollution, p. 197-209. *In* Wiley-Interscience, New York.
- 72 Gilson, E., D. Perrin, and M. Hofnung. 1990. DNA polymerase I and a protein complex bind specifically to *E. coli* palindromic unit highly repetitive DNA: implication for bacterial chromosome organization. *Nuc. Acids Res.* 18:3941-3952.
- 73 Gomes, J. F. P. 1999. Short notice on an environmental management system for application to pulp mill production. *J. Cleaner Prod.* 7:371-374.
- 74 Gorden, R. W. and C. B. Fliermans. 1977. Survival and viability of *Escherichia coli* in a thermally altered reservoir. *Wat. Res.* 12:343-352.
- 75 Griffith, J. F., S. B. Weisburg, and C. D. McGee. 2003. Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. *J. Water Health* 1:141-151.
- 76 Hagedorn, C., J. B. Crozier, K. A. Mentz, A. M. Booth, A. K. Graves, N. J. Nelson, and R. B. Reneau Jr. 2003. Carbon source utilization profiles as a method to identify sources of faecal pollution in water. *J. Appl. Microbiol.* 94:792-799.

- 77 Hagedorn, C., S. L. Robinson, J. R. Filtz, S. M. Grubbs, T. A. Angier, and R. B. Reneau Jr. 1999. Determining sources of fecal pollution in a rural Virginia watershed with antibiotic resistance patterns in fecal streptococci. *Appl. Environ. Microbiol.* 65:5522-5531.
- 78 Hahm, B.-W., Y. Maldonado, E. Schreiber, A. K. Bhunia, and C. H. Nakatsu. 2003. Subtyping of foodborne and environmental isolates of *Escherichia coli* by multiplex-PCR, rep-PCR, PFGE, ribotyping and AFLP. *J. Microbiol. Methods* 53:387-399.
- 79 Harrington, A. C. and D. DeBell. 1984. Effects of irrigation, pulp mill sludge and repeated coppicing on growth and yield of black cottonwood and red alder. *Can. J. For. Res.* 14:844-849.
- 80 Hartl, D. L. and D. E. Dykhuizen. 1984. The population genetics of *Escherichia coli*. *Ann. Rev. Genet.* 18:31-68.
- 81 Harwood, V. J., J. Whitlock, and V. Withington. 2000. Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. *Appl. Environ. Microbiol.* 66:3698-3704.
- 82 Hassan, W. M., S. Y. Wang, and R. D. Ellender. 2005. Methods to increase fidelity of repetitive extragenic palindromic PCR fingerprint-based bacterial source tracking efforts. *Appl. Environ. Microbiol.* 71:512-518.
- 83 Healy, M., J. Huong, T. Bittner, M. Lising, S. Frye, S. Raza, R. Schrock, J. Manry, A. Renwick, R. Nieto, C. Woods, Versalovic, and J. R. Lupski. 2005. Microbial DNA typing by automated repetitive-sequence-based PCR. *J. Clin. Microbiol.* 43:199-207.
- 84 Hendricks, C. W. and S. M. Morrison. 1967. Multiplication and growth of selected enteric bacteria in clear mountain stream water. *Wat. Res.* 1:567-576.
- 85 Henry, C. L. 1991. Nitrogen dynamics of pulp and paper sludge amendment to forest soils. *Wat. Sci. Tech.* 24:417-425.
- 86 Henry, C. L., D. W. Cole, T. M. Hinckley, and R. B. Harrison. 1993. The use of municipal and pulp and paper sludges to increase production in forestry. *J. Sustainable For.* 1:41-55.
- 87 Higgins, C. F., R. S. McLaren, and S. F. Newbury. 1988. Repetitive extragenic palindromic sequences, mRNA stability and gene expression: evolution by gene conversion? - a review. *Gene* 72:3-14.
- 88 Howell, J. M., M. S. Coyne, and P. L. Cornelius. 1996. Effect of sediment particle size and temperature on fecal bacteria mortality rates and the fecal coliform/fecal streptococci ratio. *J. Environ. Qual.* 25:1216-1220.

- 89 Iguchi, A., R. Osawa, J. Kawano, A. Shimizu, J. Terajima, and H. Watanabe. 2002. Effects of repeated subculturing and prolonged storage at room temperature of enterohemorrhagic *Escherichia coli* O157:H7 on pulsed-field gel electrophoresis profiles. *J. Clin. Microbiol.* 40:3079-3081.
- 90 Ishak, N. 1998. Ideal sludge management method hard to pinpoint. *Pulp Paper Can.* 99:37-37.
- 91 Jarvinen, R. 1997. Nitrogen in the effluent of the pulp and paper industry. *Wat. Sci. Tech.* 35:139-145.
- 92 Johnson, J. M., S. D. Weagant, K. C. Jinneman, and J. L. Bryant. 1995. Use of pulsed-field gel electrophoresis for epidemiological study of *Escherichia coli* O157:H7 during a food-borne outbreak. *Appl. Environ. Microbiol.* 61:2806-2808.
- 93 Jokela, J., J. Rintala, A. Oikari, O. Reinikainen, K. Mutka, and T. Nyronen. 1997. Aerobic composting and anaerobic digestion of pulp and paper mill sludges. *Wat. Sci. Tech.* 36:181-188.
- 94 Judd, A. K., M. Schneider, M. J. Sadowsky, and F. J. deBrujn. 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. *Appl. Environ. Microbiol.* 59:1702-1708.
- 95 Kang, H. P. and W. M. Dunne. 2003. Stability of repetitive-sequence PCR patterns with respect to culture age and subculture frequency. *J. Clin. Microbiol.* 41:2694-2696.
- 96 Kaper, J. B., J. P. Nataro, and H. L. T. Mobley. 2004. Pathogenic *Escherichia coli*. *Nature* 2:123-140.
- 97 Kariuki, S., C. Gilks, J. Kimari, A. Obanda, J. Muyodi, P. Waiyaki, and C. A. Hart. 1999. Genotype analysis of *Escherichia coli* strains isolated from children and chickens living in close contact. *Appl. Environ. Microbiol.* 65:472-476.
- 98 Kelsey, R. H., G. I. Scott, D. E. Porter, B. Thompson, and L. Webster. 2003. Using multiple antibiotic resistance and land use characteristics to determine sources of fecal coliform bacterial pollution. *Env. Mon. Assess.* 81:337-348.
- 99 Kilian, M. and P. Bulow. 1979. Rapid identification of Enterobacteriaceae. II. Use of β -D-glucuronidase detecting medium (PGUA agar) for the identification of *E. coli* in primary cultures of urine samples. *Acta Pathol. Microbiol. Scand. [B]* 87:271-276.
- 100 Klahre, J. and H.-C. Flemming. 2000. Monitoring of biofouling in papermill process waters. *Wat. Res.* 34:3657-3665.
- 101 Kolari, M., J. Nuutinen, F. A. Rainey, and M. S. Salkinoja-Salonen. 2003. Colored moderately thermophilic bacteria in paper-machine biofilms. *J. Ind. Microbiol. Biotechnol.* 30:225-238.

- 102 Kong, R. Y. C., C. L. SO, W. F. Law, and R. S. S. Wu. 1999. A sensitive and versatile multiplex PCR system for the rapid detection of Enterotoxigenic (ETEC), Enterohaemorrhagic (EHEC) and Enteropathogenic (EPEC) strains of *Escherichia coli*. Mar. Poll. Bull. 38:1207-1215.
- 103 Krieg, N. R. and J. G. Holt. 1984. Bergey's Manual of Systematic Microbiology. Williams & Wilkins, Baltimore/London.
- 104 Kuhnert, P., P. Boerlin, and J. Frey. 2000. Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment. FEMS Microbiology Reviews 24:107-117.
- 105 Lawrence, J. G. and H. Ochman. 1998. Molecular archeology of the *Escherichia coli* genome. Proc. Natl. Acad. Sci. USA 95:9413-9417.
- 106 Leboffe, M. J. and B. E. Pierce. 2002. Microbiology: Laboratory Theory and Application. Morton Publishing Company, Englewood, Colorado.
- 107 LeChevallier, M. W., N. J. Welch, and D. B. Smith. 1996. Full-scale studies of factors related to coliform regrowth in drinking water. Appl. Environ. Microbiol. 62:2201-2211.
- 108 Leclerc, H., D. A. A. Mossel, S. C. Edberg, and C. B. Struijk. 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. Ann. Rev. Microbiol. 55:201-234.
- 109 Leung, K. T., R. Mackereth, Y.-C. Tien, and E. Topp. 2004. A comparison of AFLP and ERIC-PCR analyses for discriminating *Escherichia coli* from cattle, pig and human sources. FEMS Microbiol. Ecol. 47:111-119.
- 110 Levy, J. S. and B. R. Taylor. 2003. Effects of pulp mill solids and three composts on early growth of tomatoes. Biores. Tech. 89:297-305.
- 111 Lindberg, L. E., B. K. Holmbom, O. M. Vaisanen, A. M. L. Weber, and M. S. Salkinaja-Salonen. 2001. Degradation of paper mill water components in laboratory tests with pure cultures of bacteria. Biodegradation 12:141-148.
- 112 Lindberg, L. E., B. R. Holmbom, O. M. Vaisanen, A. M. L. Weber, and M. S. Salkinoja-Salonen. 2001. Sugar composition of biofilms produced by paper mill bacteria. Appl. Microbiol. Biotechnol. 55:638-643.
- 113 Lindberg, L. E., S. M. Willfor, and B. R. Holmbom. 2004. Antibacterial effects of knotwood extractives on paper mill bacteria. J. Ind. Microbiol. Biotechnol. 31:137-147.

- 114 Lipman, L. J. A., A. deNijs, T.J.G.M. Lam, and G. Wim. 1995. Identification of *Escherichia coli* strains from cows clinical mastitis by serotyping by serotyping and DNA polymorphism patterns with REP and ERIC primers. *Vet. Microbiol.* 43:13-19.
- 115 Lo, A., A. Liard, and G. Velema. 1996. Use of primary sludge as a mulch in the forest plantations: can act as an effective weed suppressant for two years minimum. *Pulp Pap. Can.* 97:T473-476.
- 116 Loge, F. J., D. E. Thompson, and D. R. Call. 2002. PCR detection of specific pathogens in water: a risk-based analysis. *Environ. Sci. Tech.* 36:2754-2759.
- 117 Lovblad, R. 1999. Clean technology development in kraft pulping - Sodra Cell's strategy for minimizing environmental impacts of effluent discharges. *Environ. Prot. Bull.* 3-6.
- 118 Luonsi, A., P. Vuoriranta, and P. Hynninen. 1986. Reduction of pulp and paper industry effluent loading. *Wat. Sci. Tech.* 18:109-125.
- 119 Mabee, W. and D. N. Roy. 2003. Modeling the role of papermill sludge in the organic carbon cycle of paper products. *Environ. Rev.* 11:1-16.
- 120 Macyk, M. A. 1996. Research relative to land application of pulp mill wastes in Alberta. *Pulp Pap. Can.* 97:83-87.
- 121 Macyk, T. M. 1999. Land application of mechanical pulp mill sludges in Alberta: research and operational activities. *Pulp Pap. Can.* 100:166-169.
- 122 Major, J. 2003. Growth of mill-derived *Escherichia coli* in un-supplemented Domtar Cornwall Mill primary clarifier and blend tank effluent. Domtar, Inc. (Cornwall), Cornwall, Ontario.
- 123 Marsalek, J. and Q. Rochfort. 2004. Urban wet-weather flows: sources of fecal contamination impacting on recreational waters and threatening drinking-water sources. *J. Toxicol. Environ. Health Part A*, 67:1765-1777.
- 124 Martin, I. E., S. D. Tyler, K. D. Tyler, R. Khakhria, and W. M. Johnson. 1996. Evaluation of ribotyping as epidemiologic tool for typing *Escherichia coli* serogroup O157 isolates. *J. Clin. Microbiol.* 34:720-723.
- 125 Martin, R. S., W. H. Gates, R. S. Tobin, D. Grantham, P. Sumarah, P. Wolfe, and P. Forestall. 1982. Factors affecting coliform bacteria growth in distribution systems. *J. Am. Wat. Works Assoc.* 74:34-37.
- 126 McLean, J. C. and A. W. Decho. 2002. *Molecular Ecology of Biofilms*. Horizon Scientific Press, Wymondham, Norfolk, England.
- 127 McLellan, S. L., A. D. Daniels, and A. K. Salmore. 2003. Genetic characterization of *Escherichia coli* populations from host sources of fecal pollution by using DNA

- fingerprinting. Appl. Environ. Microbiol. 69:2587-2594.
- 128 Megraw, S. R. and M. O. Farkas. 1993. *Escherichia coli*: a potential source of native fecal coliforms in pulp and paper mill effluents. Pulp Paper Can. 94:39-41.
 - 129 Mehta, A., Y. R. Mehta, and Y. B. Rosato. 2002. ERIC- and REP-PCR amplify non-repetitive fragments from the genome of *Drechslera avenae* and *Stemphylium solani*. FEMS Microbiol. Lett. 211:51-55.
 - 130 Meunier, J.-R. and P. A. D. Grimont. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Res. Microbiol. 144:373-379.
 - 131 Meyn, O., M. Zeeman, M. J. Wise, and S. E. Keane. 1997. Terrestrial wildlife risk assessment for TCDD in land-applied pulp and paper mill sludge. Env. Toxicol. Chem. 16:1789-1801.
 - 132 Morris, C. E., M. Sardin, O. F.-K. P. Burge, N. Fromin, H. Girardin, M.-H. Guinebretiere, P. Lebaron, J. M. Thiery, and M. Troussellier. 2002. Microbial biodiversity: approaches to experimental design and hypothesis testing in primary scientific literature from 1976 to 1999. Microbiol. Mol. Biol. Rev. 66:592-616.
 - 133 Muhldorfer, I., G. Blurn, A. Donohue-Rolfe, H. Heier, T. Olschlager, H. Tshape, U. Wallner, and J. Hacker. 1996. Characterization of *Escherichia coli* strains isolated from environmental water habitats and from stool samples of healthy volunteers. Res. Microbiol. 147:625-635.
 - 134 Munkittrick, K. R., M. R. Servos, J. H. Carey, and G. J. Van Der Kraak. 1997. Environmental impacts of pulp and paper wastewater: evidence for a reduction in environmental effects at North American pulp mills since 1992. Wat. Sci. Tech. 35:329-338.
 - 135 Nagano, H., T. Hirochi, K. Fujita, Y. Wakamori, K. Takeshi, and S. Yano. 2004. Phenotypic and genotypic characterization of β -D-glucuronidase-positive Shiga toxin-producing *Escherichia coli* O157:H7 isolates from deer. J. Med. Microbiol. 53:1037-1043.
 - 136 Nataro, J. P. and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. 11:142-201.
 - 137 Natvig, E. E., S. C. Ingham, B. H. Ingham, L. R. Cooperband, and T. R. Roper. 2002. *Salmonella enterica* serovar Typhimurium and *Escherichia coli* contamination in soils with incorporated bovine manure. Appl. Environ. Microbiol. 68:2737-2744.
 - 138 Newbury, S. F., N. H. Smith, E. C. Robinson, I. D. Hiles, and C. F. Higgins. 1987. Stabilization of translationally active mRNA by prokaryotic REP sequences. Cell 48:297-310.
 - 139 Niemi, R. M. and A. Siitonen. 1987. Growth of *Escherichia coli* in a pulp and

- cardboard mill. Can. J. Microbiol. 33:541-545.
- 140 Northey, G., M. Gal, A. Rahmati, and J. S. Brazier. 2005. Subtyping of *Clostridium difficile* PCR ribotype 001 by REP-PCR and PFGE. J. Med. Microbiol. 54:543-547.
 - 141 Odendahl, S. and C. Russel. 1996. Environmental considerations for landfill development in the pulp and paper industry: a review of the issues and regulations. Pulp Pap. Can. 97:17-22.
 - 142 Ogden, I. D., D. R. Fenlon, A. J. A. Vinten, and D. Lewis. 2001. The fate of *Escherichia coli* O157 in soil and its potential to contaminate drinking water. Int. J. Food Microbiol. 66:111-117.
 - 143 Olive, M. D. and P. Bean. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. 37:1661-1669.
 - 144 Oppong, D., V. M. King, X. Zhou, and J. A. Bowen. 2000. Cultural and biochemical diversity of pink-pigmented bacteria isolated from paper mill slimes. J. Ind. Microbiol. Biotechnol. 25.
 - 145 Osek, J. 2000. Virulence factors and genetic relatedness of *Escherichia coli* strains isolated from pigs with post-weaning diarrhea. Vet. Microbiol. 71:211-222.
 - 146 Ou-Yang, W. and W.-S. Wu. 2002. Investigations of paper mill sludge as a component of container medium. Plant Path. Bull. 11:19-24.
 - 147 Park, S., R. W. Worobo, and R. A. Durst. 2001. *Escherichia coli* O157:H7 as an emerging foodborne pathogen: a literature review. Crit. Rev. Biotech. 21:27-48.
 - 148 Parveen, S., R. L. Murphree, L. Edmiston, C. W. Kaspar, K. M. Portier, and M. L. Tamplin. 1997. Association of multiple-antibiotic-resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. Appl. Environ. Microbiol. 63:2607-2612.
 - 149 Parveen, S., K. M. Portier, K. Robinson, L. Edmiston, and M. L. Tamplin. 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. Appl. Environ. Microbiol. 65:3142-3147.
 - 150 Philips, V. R., N. Kirkpatrick, I. M. Scotford, R. P. White, and R. G. O. Burton. 1997. The use of paper-mill sludges on agricultural land. Biores. Tech. 60:73-80.
 - 151 Pickell, J. and R. Wunderlich. 1995. Sludge disposal: current practices and future options. Pulp Pap. Can. 96:300-306.
 - 152 Pooler, M. R., D. F. Ritchie, and J. S. Hartung. 1996. Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the

identification of this phytopathogen. Appl. Environ. Microbiol. 62:3121-3127.

- 153 Prazmo, Z., J. Dutkiewicz, and G. Cholewa. 2000. Gram-negative bacteria associated with timber as a potential respiratory hazard for woodworkers. *Aerobiologica* 16:275-279.
- 154 Preston, M. A., W. Johnson, R. Khakhria, and A. Borczyk. 2000. Epidemiologic subtyping of *Escherichia coli* serogroup O157 strains isolated in Ontario by phage typing and pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 38:2366-2368.
- 155 Pridham, N. F. and R. A. Cline. 1988. Paper mill sludge disposal: completing the ecological cycle. *Pulp Pap. Can.* 89:173-175.
- 156 Quessy, S. and S. Messier. 1992. Prevalence of *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. in ring-billed gulls (*Larus delawarensis*). *J. Wildlife Dis.* 28:526-531.
- 157 Rademaker, J. L. W. and deBruijn F.J. 1997. Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In Caetano-Anolles, G., and P.M. Gresshoff (ed.), *DNA markers: protocols, applications and overviews*, J. Wiley and Sons, New York, NY. p. 151-171.
- 158 Reboli, A. C., E. D. Houston, J. S. Monteforte, C. A. Wood, and R. J. Hamill. 1994. Discrimination of epidemic and sporadic isolates of *Acinetobacter baumannii* by repetitive element PCR-mediated DNA fingerprinting. *J. Clin. Microbiol.* 32:2635-2640.
- 159 Renter, D. G., J. M. Sargeant, R. D. Oberst, and M. Samadpour. 2003. Diversity, frequency, and persistence of *Escherichia coli* O157 strains from cattle range environments. *Appl. Environ. Microbiol.* 69:542-547.
- 160 Riebel, P. 2001. Biosolids recycling: benefits include increased productivity. *Pulp Pap. Can.* 102:51-53.
- 161 Rodriguez-Barradas, R. J. Hamill, E. D. Houston, P. R. Georghiou, J. E. Clarridge, R. L. Regnery, and J. E. Koehler. 1995. Genomic fingerprinting of *Bartonella* species by repetitive element PCR for distinguishing species and isolates. *J. Clin. Microbiol.* 33:1089-1093.
- 162 Sadowsky, M. J., L. L. Kinkel, J. H. Bowers, and J. L. Schottel. 1996. Use of repetitive intergenic DNA sequences to classify pathogenic and disease-suppressive *Streptomyces* strains. *Appl. Environ. Microbiol.* 62:3489-3493.
- 163 Saikaly, P. E., P. G. Stroot, and D. B. Oerther. 2005. Use of 16S rRNA gene terminal restriction fragment analysis to assess the impact of solids retention time on the bacterial diversity of activated sludge. *Appl. Environ. Microbiol.* 71:5814-5822.
- 164 Santos, J. Q., E. Vasconcelos, and F. Cabral. 1990. Utilização das lamas celulósicas como fertilizante. In *Actas IV Encontro Nacional do Saneamento Básico. Edited by*

Associação portuguesa para estudos do saneamento básico (apesb), Porto. pp.386-391.

- 165 Scott, T. M., J. B. Rose, T. M. Jenkins, S. R. Farrah, and J. Lukasik. 2002. Microbial source tracking: current methodology and future directions. *Appl. Environ. Microbiol.* 68:5796-5803.
- 166 Seurinck, S., W. Verstraete, and S. D. Siciliano. 2003. Use of 16S-23S rRNA intergenic spacer region PCR and repetitive extragenic palindromic PCR analyses of *Escherichia coli* isolates to identify nonpoint fecal sources. *Appl. Environ. Microbiol.* 69:4942-4950.
- 167 Sherlock, O., R. M. Verborg, and Klemm P. 2005. The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* is self recognizing and induces bacterial aggregation and biofilm formation. *Infect. Immun.* 73:1954-1963.
- 168 Shyamala, V., E. Schneider, and G. F.-L. Ames. 1990. Tandem chromosomal duplications: role of REP sequences in the recombination event at the join-point. *EMBO J.* 9:939-946.
- 169 Smit, E., P. Leeftang, S. Gommans, J. van der Broek, S. van Mil, and K. Wernars. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Appl. Environ. Microbiol.* 67:2284-2291.
- 170 Smith, N. R., Z. Yu, and W. W. Mohn. 2003. Stability of the bacterial community in a pulp mill effluent treatment system during normal operation and a system shutdown. *Wat. Res.* 37:4873-4884.
- 171 Snelling, A. M., P. Gerner-Smidt, P. M. Hawkey, J. Heritage, P. Parnell, C. Porter, A. R. Bodenham, and T. Inglis. 1996. Validation of use of whole-cell repetitive extragenic palindromic sequence-based PCR (REP-PCR) for typing strains belonging to the *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* complex and application of the method to the investigation of a hospital outbreak. *J. Clin. Microbiol.*
- 172 Solo-Gabriele, H. M., M. A. Wolfert, T. R. Desmarais, and C. J. Palmer. 2000. Sources of *Escherichia coli* in a coastal subtropical environment. *Appl. Environ. Microbiol.* 66:230-237.
- 173 Solomon, E. B., S. Yaron, and K. R. Matthews. 2002. Transmission of *Escherichia coli* 0157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 68:397-400.
- 174 Souza, V., M. Rocha, A. Valera, and L. E. Eguiarte. 1999. Genetic structure of natural populations of *Escherichia coli* in wild hosts on different continents. *Appl. Environ. Microbiol.* 65:3373-3385.
- 175 Stern, M. J., G. F.-L. Ames, N. H. Smith, E. C. Robinson, and C. F. Higgins. 1984. Repetitive extragenic palindromic sequences: a major component of the bacterial

genome. *Cell* 37:1015-1026.

- 176 Struelens, M. J. 1998. Molecular epidemiologic typing systems of bacterial pathogens: current issues and perspectives. *Mol. Epidemiol.* 95:581-586.
- 177 Suihko, M.-L., H. Sinkko, L. Partanen, T. Mattila-Sandholm, M. Salkinoja-Salonen, and L. Raaska. 2004. Description of heterotrophic bacteria occurring in paper mills and paper products. *J. Appl. Microbiol.* 97:1228-1235.
- 178 Suihko, M.-L. and E. Stackebrandt. 2003. Identification of aerobic mesophilic bacilli isolated from board and paper products containing recycled fibres. *J. Appl. Microbiol.* 94:25-34.
- 179 Szewzyk, U., W. Manz, R. Amann, K.-H. Schleifer, and T.-A. Stenstrom. 1994. Growth and in situ detection of a pathogenic *Escherichia coli* in biofilms of a heterotrophic water-bacterium by use of 16S- and 23S-rRNA-directed fluorescent oligonucleotide probes. *FEMS Microbiol. Ecol.* 13:169-176.
- 180 Tamplin, M. L. 2003. The application and suitability of microbiological tests for fecal bacteria in pulp mill effluents: a review. *Water Qual. Res. J. Can.* 38:221-225.
- 181 Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33:2233-2239.
- 182 Thakur, I. S. 2004. Screening and identification of microbial strains for removal of colour and adsorbable organic halogens in pulp and paper mill effluent. *Process. Biochem.* 39:1693-1699.
- 183 Thompson, G., J. Swain, M. Kay, and C. F. Forster. 2001. The treatment of pulp and paper mill effluent: a review. *Biores. Tech.* 77:275-286.
- 184 Topp, E. and A. Scott. 2003. Persistence of pathogenic and indicator bacteria in agricultural soils. *Proceedings of 2nd Canadian Organic Residuals Recycling Conference, April 24-25 2003. Penticton, B.C., Canada.* p. 313-322
- 185 Unc, A. and M.J. Goss. 2003. Impact of manure properties on the survival of manure *Escherichia coli* in soils. *Proceedings of 2nd Canadian Organic Residuals Recycling Conference, April 24-25 2003. Penticton, B.C., Canada.* p. 331-346
- 186 Vaisanen, O. M., E.-L. Nurmiaho-Lassila, S. A. Marmo, and M. S. Salkinoja-Salonen. 1994. Structure and composition of biological slimes on paper and board machines. *Appl. Environ. Microbiol.* 60:641-653.
- 187 Vaisanen, O. M., A. Weber, A. Bennasar, F. A. Rainey, H. J. Busse, and M. S. Salkinoja-Salonen. 1998. Microbial communities of printing paper machines. *J. Appl. Microbiol.* 84:1069-1084.

- 188 van der Wende, E., W. G. Characklis, and D. B. Smith. 1989. Biofilms and bacterial drinking water quality. *Wat. Res.* 23:1313-1322.
- 189 Vasconcelos, E. and F. Cabral. 1992. Estudo comparativo da utilização como fertilizante de lamas celulósicas dos tratamentos primário e secundário. *In* Actas da Conferência Nacional da Qualidade do Ambiente. *Edited by* A. Rosa Pires, C. Pio, C. Boia, and F. Nogueira. Comissão Coordenadora da Região Centro (Coimbra), Averio. pp. 727-735.
- 190 Vasconcelos, E. and F. Cabral. 1993. Use and environmental implications of pulp-mill sludge as an organic fertilizer. *Env. Poll.* 80:159-162.
- 191 Vasconcelos, E., F. Cabral, and P. Monjardino. 1990. Impacto no solo da aplicação de lamas celulósicas. *Pedon.* 9:95-99.
- 192 Vasconcelos, E., F. Cabral, and J. Q. Santos. 1993. A utilização como fertilizante de lamas do tratamento de efluentes de fábricas de celulose. *In* Actas do Seminário Sobre Tratamento e Destino Final de Lamas de Águas Residuais. Laboratório Nacional de Engenharia Civil (LNEC), Lisboa. pp. 5.1-5.8.
- 193 Vasseur, L., C. Cloutier, A. Labelle, J. N. Duff, C. Beaulieu, and C. Ansseau. 1996. Responses of indicator bacteria to forest soil amended with municipal sewage sludge from aerated and non-aerated ponds. *Env. Poll.* 92:67-72.
- 194 Velema, G. 2004. Management and benefits of pulp and paper mill residuals at Domtar Cornwall. *Pulp Pap. Can.* 105:150-156.
- 195 Versalovic, J., T. Koeuth, and J. R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nuc. Acids Res.* 19:6823-6831.
- 196 Wallace, J. S., T. Cheasty, and K. Jones. 1997. Isolation of Verocytotoxin-producing *Escherichia coli* O157 from wild birds. *J. Appl. Microbiol.* 82:399-404.
- 197 Wang, L., B. Liu, Q. Kong, H. Steinruck, G. Krause, L. Beutin, and L. Feng. 2005. Molecular markers for detection of pathogenic *Escherichia coli* strains belonging to serogroups O138 and O139. *Vet. Microbiol.* 11:181-190.
- 198 Warriner, K., S. Spaniolas, C. Wright, and W. M. Waites. 2003. Internalization of bioluminescent *Escherichia coli* and *Salmonella* Montevideo in growing bean sprouts. *J. Appl. Microbiol.* 95:719-727.
- 199 Watson, S. B., J. Ridal, B. Zaitlin, and A. Lo. 2003. Odours from pulp mill effluent treatment ponds: the origin of significant levels of geosmin and 2-methylisoborneol (MIB). *Chemosphere.* 51:765-773.
- 200 Watt, M. and B. Cavers. Transient fecal coliform: regrowth or contamination? Proceedings of 2nd Canadian Organic Residuals Recycling Conference, April 24-25

2003. Penticton, B.C., Canada. p. 200-210.
- 201 Wiggins, B. A., R. W. Andrews, R. A. Conway, C. L. Coor, E. J. Dobratz, D. P. Dougherty, J. R. Eppard, S. R. Knupp, M. C. Limjoco, J. M. Mettenburg, J. M. Rinehardt, J. Sonsino, R. L. Torrijos, and M. E. Zimmerman. 1999. Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. *Appl. Environ. Microbiol.* 65:3483-3486.
- 202 Wiggins, B. A. 1996. Discriminant analysis of antibiotic resistance patterns in fecal streptococci, a method to differentiate human and animal sources of fecal pollution in natural waters. *Appl. Environ. Microbiol.* 62:3997-4002.
- 203 Winfield, M. D. and E. A. Groisman. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol.* 69:3687-3694.
- 204 Woods Jr., C. R., J. Versalovic, T. Koeuth, and J. R. Lupski. 1992. Analysis of relationships among isolates of *Citrobacter diversus* by using DNA fingerprints generated by repetitive sequence-based primers in the polymerase chain reaction. *Journal of Clinical Microbiology* 30:2921-2929.
- 205 Yang, Y. and G. F.-L. Ames. 1988. DNA gyrase binds to the family of prokaryotic repetitive extragenic palindromic sequences. *Proc. Natl. Acad. Sci.* 85:8850-8854.
- 206 Zhang, X., A. G. Campbell, and R. L. Mahler. 1993. Newsprint pulp and paper sludge as a soil additive/amendment for alfalfa and bluegrass: greenhouse study. *Commun. Soil Sci. Plant Anal.* 24:1371-1388.

Appendix 1

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