

Monitoring of waterways for evidence of faecal contamination from biosolids using DNA techniques

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Abstract

Increased nutrient levels in inland waterways have led to algal blooms and eutrophication in many agricultural regions. To ensure fertiliser inputs are managed more effectively, the source of contamination needs to be tracked and identified. Point sources could include inorganic fertilisers, livestock excreta, or more recently biosolids. The presence of faecal indicator microorganisms has been widely used to identify the presence of faeces, however, these methods cannot distinguish between human and animals samples. This study investigated PCR amplification as a molecular method to distinguish biosolids from livestock faeces of biosolids, cattle, sheep, poultry and kangaroo. This was achieved using published priming sequences and restriction site profiling of amplified DNA across the 16S rRNA gene of anaerobic gastrointestinal bacteria *Bacteroides spp* and *Bifidobacteria spp*. Preliminary investigation showed that of the three *Bacteroides spp* primer pairs investigated, two were useful for cow faecal material; though at lower annealing temperatures were also applicable to biosolids and sheep faecal material. The third primer pair was specific only for biosolids. All three primer pairs were unable to PCR-amplify *Bacteroides spp* sequences in faecal material of kangaroo. Of the three *Bifidobacteria spp* primer pairs, one was useful for sheep faecal material; though at lower annealing temperature was also applicable to biosolids and cow and kangaroo faecal material. The *Bifidobacterium angulatum* specific primer pair enabled the PCR detection of anaerobes only in biosolids and faecal material of kangaroo. The third, a *Bifidobacterium catenulatum* specific primer pair was suitable for faecal material of cow and at lower annealing temperatures was also applicable to the sample from sheep. Varying degrees of success were observed in faecal material from other animals. Generally, biosolids tested positive for *Bacteroides* and *Bifidobacteria* with all primers except for those specific for *B. angulatum*. For some primer sets, PCR amplification alone could not differentiate biosolids from other faecal samples. The serial dilution of water contaminated by a range of livestock excreta and biosolids is being examined further to enable the sensitivity of this method to be applied in the field.

Introduction

It is well established that nutrient enrichment of waterways with elevated levels of nitrogen (N) and phosphorus (P) from over-application of fertiliser products can lead to eutrophication and environmental problems (Batziaka *et al.* 2007; Pierzynski 2005; Grey & Henry 2002). Sources of nutrient contamination of waterways can include run-off by inorganic fertilisers or from the

grazing of livestock and wildlife (Sinton *et al.* 1998). It is unknown if an increase in nutrient levels in waterways surrounding biosolids application sites could indicate faecal contamination from biosolids. In Western Australia a total of 57,000 wet tonnes of biosolids products was applied onto agricultural land as fertiliser for the production of broad acre crops (Penney 2006), which resulted in a significant quantity of N and P being land-applied. Although there has been no evidence of pollution of waterways with nutrients arising from the land application of biosolids to date, it would be of benefit to develop a monitoring strategy in areas where biosolids are applied to ensure that there is no contamination. Biosolids are regulated near water bodies with buffer restrictions placed around these sensitive sites (DEP *et al.* 2002).

Often, the probable sources of faecal inputs (e.g., sewer outfalls, storm water drains, or run-off from grazed pasture) can be identified from geographical surveys. However, this approach can be unsatisfactory in some situations, particularly where it is important to quantify or apportion the inputs (Sinton *et al.*, 1998). For example, small rural communities such as in the Wongan Hills and Moora Districts, Western Australia, will frequently contain a wide variety of potential faecal sources, including grazing animals, animal containment areas, and small, scattered sewage treatment and disposal systems, often serving single households in addition to the more widespread use of inorganic fertilisers; all contributing to the overall nutrient loading. To distinguish the source of faecal contamination therefore, a distinction between the source of contamination needs to be established.

The aim of this study was to evaluate recombinant DNA techniques as investigated by Edge *et al.* (2007 & 2006) and Sinton *et al.* (1998) for their utility at differentiating biosolids from other animal faecal sources including the kangaroo (a marsupial), sheep and cattle (ruminants), chickens and horses (monogastric); with swine to be included in a further study. Surveyed published primer sequences were used to determine their applicability to samples from Moora in regional Western Australia.

Materials & Methods

Sampling location: Field monitoring will be conducted over the 2008 growing season on the Jameson Farm, Wongan Hills, Western Australia to monitor waterways for evidence of faecal and/or nitrate and phosphate contamination. As can be seen in Figure 1, the paddocks destined for the land application of biosolids are clearly identified, as are waterways and buffer zones.

Sample Collection:

i) Faecal Samples: Fresh faecal samples of livestock were as follows: cow (two dairy farms - Toodyay and Boyup Brook), swine (the Western Australian Pig Skills Centre Muresk), sheep (two sheep farms -York and Boyup Brook), kangaroo (York and Roo Gully Wildlife Sanctuary in Boyup Brook), chicken (Toodyay and Boyup Brook).

ii) Wastewater samples: Wastewater samples were collected from the Beenyup & Woodman Point WwTP's.

iii) Water samples will be collected from locations surrounding Perth, Western Australia including the Swan River, East Perth, Herdsman Lake, Bibra Lake, & Mundaring Dam.

Sterile containers were used for all wastewater and water samples.

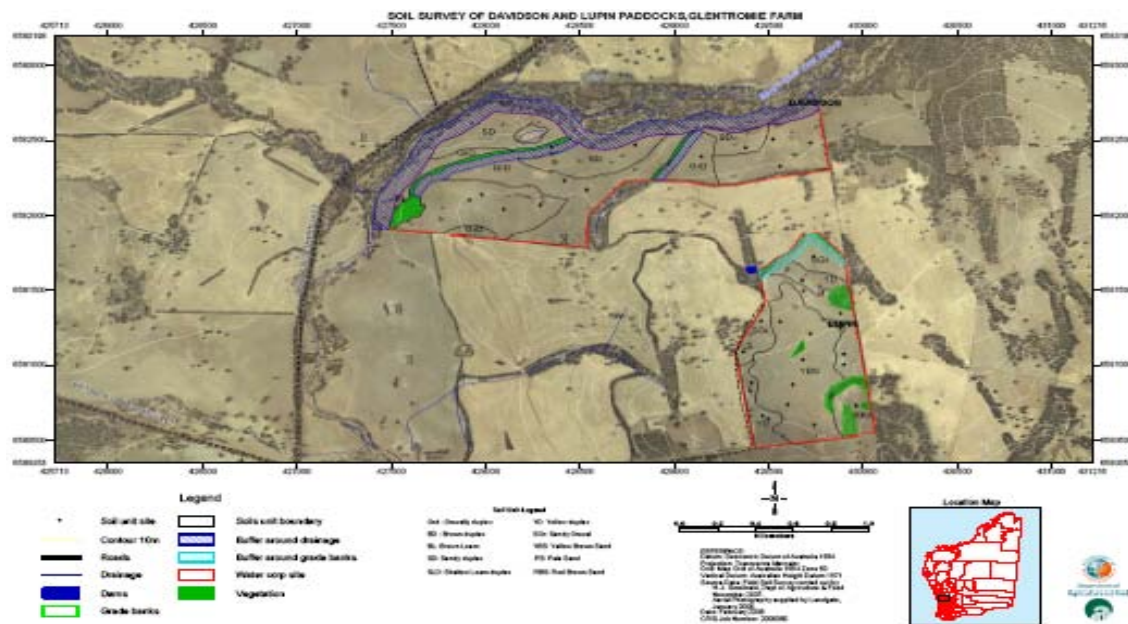


Figure 1. Typical biosolids application site located at Wongan Hills, Western Australia showing the biosolids land application site, waterways and buffer zones.

Isolation of DNA: Faecal samples: DNA was isolated from 200 mg of the various animal faecal samples using the QIA DNA Stool Kit, following the manufacturers protocol (QIAGEN 2004). Certified genomic DNA of reference bacteria from the ATCC (American Type Culture Collection) was used as controls and to confirm the specificity of primer pairs. They were; *Bacteroides fragilis* (*B. fragilis*, ATCC 25285), *Bacteroides vulgatus* (*B. vulgatus*, ATCC 8482), *Bifidobacterium adolescentis* (*B. adolescentis*, ATCC 15703), *Bifidobacterium infantis* (*B. infantis*, ATCC15697), *Escherichia coli* (*E. coli*, ATCC 10798) and *Staphylococcus aureus* (*S. aureus*, ATCC 10832).

PCR Amplification: Each PCR reaction contained 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl (pH 8.3), 0.25 μM primers, 250 μM deoxynucleoside triphosphates and 0.75 Units of Taq DNA polymerase. The amplification conditions were; 95°C for 2 min, then 35 cycles at 95°C for 15 s, 50°C for 30 s, and 72°C for 1 min and finally, 72°C for 10 min. PCR reagent blanks (i.e. without DNA) were run with each batch of amplifications. The specificity of each primer pair was also evaluated over an annealing temperature gradient of 45°C to 65°C across each DNA isolate.

Table 1: List of published primers used in this study.

Primer Pair	Faecal Indicator	Reference
Bac 32F/Bac 708R	<i>Bacteroides-Prevotella</i>	Bernhard and Field 2000b
BiANG1/BiANG2	<i>Bifidobacterium angulatum</i>	Matsuki 1999
BiCATg1/BiCATg2	<i>Bifidobacterium catenulatum</i>	Matsuki 1999

Primer Pair	Faecal Indicator	Reference
F1/F2	<i>Bacteroides-Prevotella</i>	Menaia 1998
G1/G2	<i>Bacteroides-Prevotella</i>	Menaia 1998
Im 26/Im 3	<i>Bifidobacterium genus</i>	Kaufmann 1997

Restriction enzyme digestion: Up to 10 µL of amplified DNA was digested with 5 Units of restriction enzyme in a final volume of 25 µL for 4 h at 37°C. A panel of twenty-four different restriction enzymes were screened for ability to provide informative restriction fragment patterns, which are diagnostic for each host. Digests were electrophoresed on 4% agarose MS (Roche Diagnostics) and restriction fragments were sized against a DNA molecular weight ladder VI (referred to as Lane D, in Figures 1 and 2). Molecular weights below 100 bp were not reported.

Results and Discussion

PCR amplification: It can be seen in Fig. 1, that DNA of approximately 950 bp was produced when DNA isolates were PCR-amplified with the F1/F2 *Bacteroides spp* primer pair of Menaia *et al.* (1998). Amplified DNA of the same size was detected in the ATCC reference standards *B. fragilis* and *B. vulgatus* (Lanes 1 and 2) and biosolids (Lane 8), and faecal material of cow and sheep (Lanes 7 and 10, respectively). None was detected in Bifidobacteria (*B. adolescentis* and *B. infantis*), *E. coli* and *S. aureus* (Lanes 3-6, respectively). The poor response in kangaroo faecal material (Lane 9) suggests that the F1/F2 primer pair was not suitable for marsupials. This was not surprising given that the majority of molecular work has been done on gastrointestinal anaerobes in animals other than kangaroo. In contrast, *B. adolescentis*, *B. infantis*, biosolids, and cow, sheep and kangaroo faecal material were positive with the Im 26/Im 3 *Bifidobacterium genus* primer pair except for the *Bacteroides spp* (*B. fragilis* and *B. vulgatus*), *E. coli* and *S. aureus* (data not shown). Overall, the ATCC reference standards helped establish the specificities of the primers.

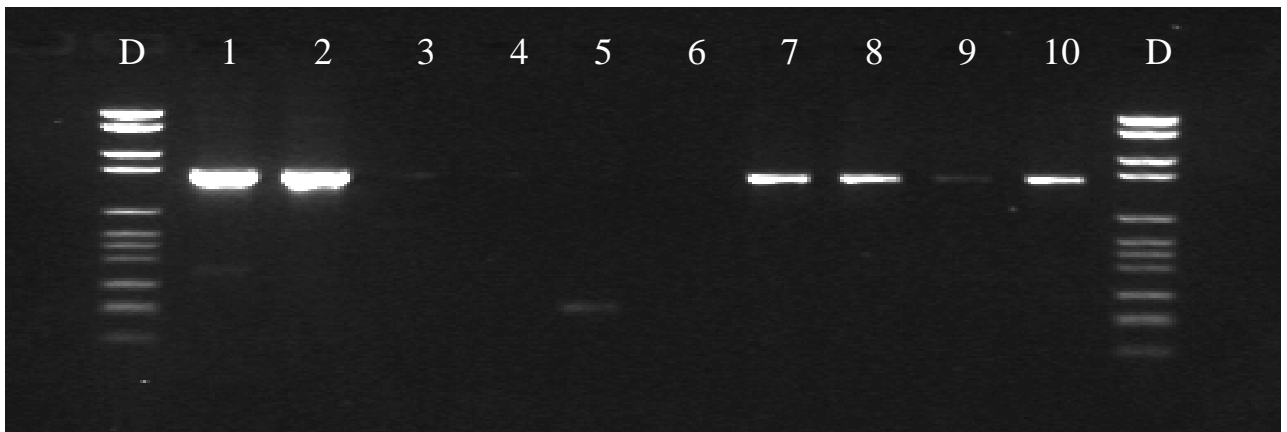


Figure 1. Specificity of PCR-amplification with F1/F2 *Bacteroides spp* primer pair. Lane 1: *B. fragilis*; Lane 2: *B. vulgatus*; Lane 3: *B. adolescentis*; Lane 4: *B. infantis*; Lane 5: *E. coli*; Lane 6: *S. aureus*; Lane 7: cow faecal material; Lane 8: biosolids; Lane 9: kangaroo faecal material; Lane 10: sheep faecal material; Lane D: DNA molecular weight ladder VI.

The results of PCR amplification with other primer pairs are summarised in Table 2. The DNA of approximately 700 base pairs (bp) was generated when DNA isolates were PCR-amplified with the Bac 32F/Bac 708R *Bacteroides spp* primer pair of Bernhard and Field (2000b) with the exception of kangaroo faecal material. Raising the annealing temperature to 61°C resulted in the detection of *Bacteroides spp* but only in cow faecal material. The same was observed for the F1/F2 primer pair of Menaia et al. (1998) but at an annealing temperature of 63°C. A positive response with the G1/G2 primer pair, also of Menaia et al. (1998), was recorded only in biosolids (650 bp), suggesting that these primers could be useful for detecting biosolids by PCR amplification alone.

Table 2. Detection of *Bacteroides spp* and *Bifidobacterium spp* in biosolids and in animal faecal material. Highlighted areas represent preferential amplification at the highest permissible annealing temperature.

Primer Pair	Biosolids	Cow	Sheep	Kangaroo
Bac 32F/Bac 708R	+	+	+	-
BiANG1/BiANG2	+	-	-	+
BiCATg1/BiCATg2	-	+	+	-
F1/F2	+	+	+	-
G1/G2	+	-	-	-
Im 26/Im 3	+	+	+	+

The Im 26/Im 3 *Bifidobacterium spp* primer pair of Kaufmann *et al.* (1997) detected anaerobes in all the samples. However, raising the annealing temperature to 61°C resulted in the detection of *Bifidobacteria* but only in sheep faecal material. Interestingly, 3 bands were evident in biosolids and this banding pattern was not affected by increases in the annealing temperature. It contrasted with the single band obtained from PCR amplification of biosolids with other primer pairs. The BiANG1/BiANG2 primer pair of Matsuki *et al.* (1999) was suitable only for biosolids and kangaroo faecal material. Indeed, it was the only primer pair that could distinguish biosolids (300 bp) from kangaroo faecal material (800 bp) by direct PCR amplification. The BiCATg1/BiCATg2 primer pair, also of Matsuki *et al.* (1999), was suitable only for cow and sheep faecal material (1 kbp) implying its usefulness for identifying *Bifidobacterium spp* in ruminants. The BiCATg1/BiCATg2 primer pair detected *Bifidobacteria* only in the faecal samples of cow and sheep. Raising the annealing temperature to 61°C resulted in the preferential amplification of DNA isolated from cow faecal material. Generally, and under conditions of lower stringency, the host identity of amplified DNA could not be distinguished by size alone. The only exception was with the BiANG1/BiANG2 primers that distinguished biosolids from kangaroo faecal material.

Restriction Enzyme Analysis:

Up to 24 restriction enzymes were screened for ability to produce the restriction fragment patterns, which enable the distinction of biosolids from other faecal sources. As can be seen in Fig. 2, restriction enzyme digestion of PCR-amplified DNA (with *Bacteroides spp* F1/F2 primer pair) produced an array of restriction fragment patterns, which distinguished biosolids from other faecal

samples. *Hpa II* reduced the 950 bp amplicon of biosolids into 4 restriction fragments of 150, 240, 270 and 290 bp (Lane 11). In contrast, the cow faecal material was distinguished by 3 fragments of 200, 270 and 360 bp (Lane 10). The sample of sheep faeces was characterised by 3 fragments of 250, 270 and 360 bp (Lane 12). Biosolids was also distinguished from other faecal material using *Ban II* (Lanes 1-3), *Dde I* (Lanes 4-6), *Hae III* (Lanes 7-9), *Rsa I* (Lanes 13-15) and *Taq I* (Lanes 16-18). Some partial digestion was observed but overall that did not affect the differentiation process. The utility of restriction enzymes was also empirically examined on amplified DNA of other primer pairs (data not shown); however, the F1/F2 PCR-amplified DNA offered the best resolution for distinguishing biosolids from other faecal sources.

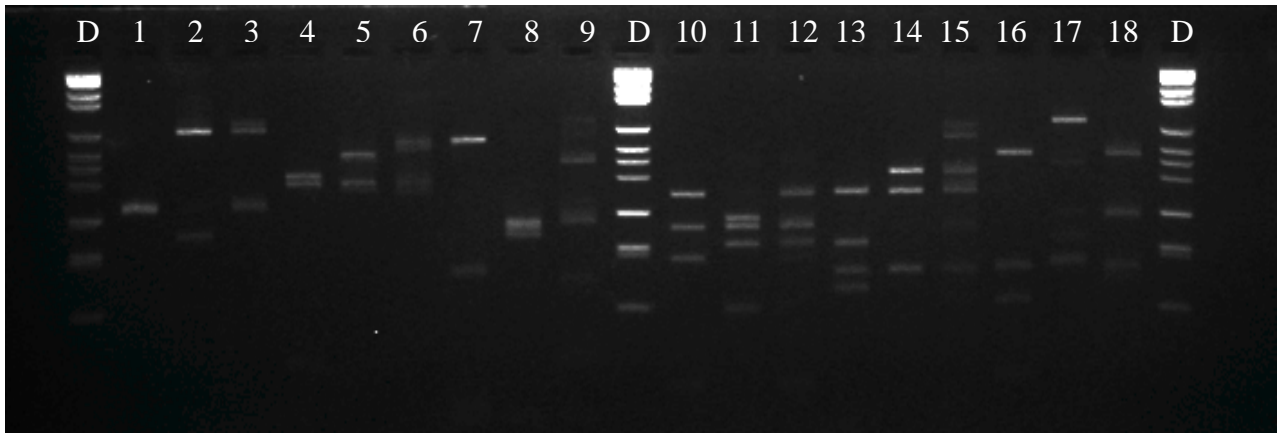


Figure 2. Distinction of biosolids from animal faecal material by restriction enzyme digestion of F1/F2 PCR amplified DNA. *Ban II*: Lanes 1-3; *Dde I*: Lanes 4-6; *Hae III*: 7-9; *Hpa II*: 10-12; *Rsa I*: 13-15; *Taq I*: Lanes 16-18. Cow faecal material: Lanes 1, 4, 7, 10, 13 and 16; biosolids: Lanes 2, 5, 8, 11, 14 and 17; sheep faecal material: Lanes 3, 6, 9, 12, 15 and 18; DNA molecular weight ladder VI: Lane D.

Despite significant advances towards developing a system of indicators for faecal source tracking, conflicting opinions remain regarding their effectiveness (Fogarty and Voytek 2005; Gilpin 2003; Scott 2002). The mostly commonly used faecal indicators micro-organisms (coliforms, faecal coliforms, *Esherichia coli*, and enterococci) are found in the faeces of both humans and animals and, thus, can give no indication from which source the contamination originates (Dorai- Raj *et al.* 2005). This is because of the adventitious levels of such *enterobacteriaceae*, which persist in the environment (Menaia 1998; Bitton 2005; Layton 2006).

Recently, the attention has focussed on two new and promising markers in *Bacteroides spp* and *Bifidobacteria spp* (Bernhard & Field 2000a, 2000 b). These anaerobes predominate the intestinal microflora of both human and animal digestive systems and together outnumber coliforms by several hundred-fold (Allsop & Stickler 1985). The development of human-specific *Bacteroides* markers have increased the value of these potential indicators (Bernhard & Field, 2000a, 2000b) which previously were not recognised because of difficulties in culturing anaerobes in the laboratory (Nebra 2003). Significantly, some species are specific to humans whereas, others are found exclusively in animals (Bonjoch, 2004; Dorai-Raj, 2005).

Several priming sequences on the 16S rRNA gene have been reported to be useful for the identification by PCR amplification (and variations from this technique) of both *Bacteroides spp* (Bernhard & Field, 2000a,b) and *Bifidobacteria spp* (Kaufmann, 1997; Matsuki, 1999).

Work planned or in progress: There are a number of projects within this project yet to be completed. These include:

- increase the sample size and species range,
- identify the lowest concentration of known faecal sample before detectability is compromised,
- collect and monitor water samples for nitrate concentration and faecal contamination at various intervals prior to the biosolids application and following harvest,
- determine probable nitrate concentrations in water samples contamination with known rates of biosolids.

The movement of biosolids particles would be dependent of a number of environmental conditions including land slope and rainfall. Direct particle movement and nutrient losses are primary considerations (Smith 1996). The research described herein uses a before and after approach. It tests the assumption that if constituents in biosolids move from the known site of application, movement would occur during runoff events induced by increased rainfall & high soil moisture conditions. Under heavy rainfall, the discharge by rivers & streams increase and the therefore the source area for runoff water expands. This situation could be encountered anytime between late April to November in watersheds of all sizes throughout the agricultural region of Western Australia.

Conclusion

The aim of this research is to assess the effectiveness of recombinant DNA techniques in distinguishing biosolids from animal faecal material, i.e. cow, sheep, pig, chicken, horses and kangaroo. The use of *Bacteroides spp* and *Bifidobacterium spp* as biosolids indicators was found to be promising for the differentiation process. This was conditional however, on: the use of a combination of primer pairs to minimise the risk of misidentification; that the PCR amplification was being performed at the highest permissible annealing temperature and; the use of restriction enzymes that confirm the identity of the host.

Given that land application of biosolids is restricted in close proximity to sensitive areas including waterways it is uncertain at this stage if PCR amplification will identify the presence of biosolids material or be able to differentiate between biosolids and animal faecal material. Overall, our evaluations show recombinant DNA techniques have the potential to distinguish samples of biosolids from other sources of faecal material.

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