# DNA FOR BIOSOLIDS MANAGEMENT: A NEW ERA?

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## 1. ABSTRACT

Contact with contaminated soils has been identified as a possible mechanism of giardiasis infection. The inadequacy of current methods to reliably detect pathogens in waste highlights the need for development of new tools. Some molecular-based methods have been developed for the detection of pathogens in soils, compost products and biosolids. The new achievements will be reviewed and their application in the context of waste management discussed.

Keywords: Biosolids; *Giardia*; viability assay; Polymerase Chain Reaction; FISH.

## 2. INTRODUCTION

Composted organic waste can improve the physical, chemical and biological properties of a soil. Compost-based soil amendments are therefore used for gardening and landscaping as well as applied on agricultural land (Alderslade, 1981). Current assessment methods for determining the level of health risk from exposure to biosolids have relied on indicator organisms chosen as representative of the main pathogenic waste source. The thermotolerant coliforms *E. coli* and *Salmonella* are the most widely-monitored indicators, with standard methods available based on the Most Probable Number (MPN) method.

The Federal Guidelines for unrestricted use (ARMCANZ and ANZECC, 1997) applies to wastes with a microbial loading of less than 1 *Salmonella* in 50 g and less than 100 thermotolerant coliforms in 1 gram of biosolids. The NSW-EPA guidelines (NSW-EPA, 1997) show even greater stringency, with no provision for *Salmonella*, less than 100 *E. coli* in 1 gram, less than 1 enteric virus in 4 grams and less than 1 helminth ovum in 4 grams of biosolids. Both guidelines approve composting, pH and heating, heating and drying or long term storage as processes to achieve pathogen reduction to the recommended guideline values. *Legionella*, faecal Streptococci, coliphages, *Clostridium perfringens*, *Cryptosporidium* and *Giardia* are now being considered as indicators for biosolids management.

Parasitic protozoa commonly present in the faeces of animals such as human, cats and swine include *Giardia lamblia, Entamoebia histolytica, Dientamoeba fragilis, Naegleria fowleri, Acanthamoeba, Cryptosporidium spp., Sarcocytis spp., Toxoplasma gondii* and *Balantidium coli*. This paper will focus on *Giardia* as it has been identified as the main agent for protozoan infection in humans (Anon., 1995). It will discuss the risks associated with *Giardia* and the need for new technologies for the detection of *Giardia* cysts.

## 3. HEALTH RISKS ASSOCIATED WITH *GIARDIA SPP.*

The concentration of a pathogen in a treated waste material will be the result of initial pathogen numbers in sewage, its survival in the waste material, how the waste was processed (treatment) and the potential for pathogen regrowth. The pathogen numbers in sewage sludge depends on the nature of incoming wastewater and on pathogen removal during sewage treatment. Another factor is the excreting load of infected individuals. Feachem *et al.* (1983)

reported an excretion rate of more than $10^5$ *Giardia* cysts per gram. Pathogens become concentrated when solids are separated from liquid waste (Fradkin *et al.,* 1989).

A major aim of waste treatment is the reduction of volatile solids. Conventional sludge stabilisation processes such as dewatering, aerobic digestion, anaerobic digestion, air-drying and lime stabilisation indirectly reduce pathogens by reducing the volatile solids in sludge (De Bertoldi *et al.,,* 1991). One of the main contributing factors towards pathogen reduction in all these processes (heat drying, composting-pasteurisation and □-irradiation) is temperature. Inactivation of helminth ova requires temperatures higher than 55°C for 2 weeks while enteric bacteria are inactivated when treated at 70°C for 30 minutes (Pereira-Neto *et al.,* 1987).

The persistence of *Giardia* cysts in composted and heat-treated wastes is well documented (Chauret *et al.,* 1999; Soares *et al.,* 1994; Sykora *et al.,* 1991; Lewis-Jones and Winkler, 1991; Hu *et al.,* 1996). A study conducted in Perth (Australia) by McInnes *et al.* (1997) found *Giardia* cyst numbers ranging from 200 to 770 cysts per gram in samples of commercially composted biosolids produced in that area. *Giardia* cyst survival times of up to 6 months in biosolids depending on the temperature and bacterial activity were also reported. However, as *Giardia* exist as non-reproductive cysts outside an appropriate host, cyst numbers can only decrease over time. An appropriate storage time is thus needed for biosolids prior to its use, to minimise the risk from *Giardia* . To this end, it has been recommended that a storage period of 30 weeks be implemented for biosolids destined for use as a soil amendment (Sidhu *et al.,* 2001).

Questions have also been raised in recent years over the possible contribution of microorganisms in drinking water to endemic levels of gastroenteritis in the community, and the same questions could also be raised for the use of composted biosolids-based products. Giardiasis infection requires as little as 10 cysts depending on the immune status of the subject exposed to the agent (Rentdorff, 1954). Considering a concentration of 200 *Giardia* cysts per gram of compost, an adult with an average ingestion rate of 20 mg/day would ingest 40 cysts per day (Lepesteur, submitted). Using the model developed by Rentdorff (1954), this individual would have more than 53 % risk of being infected by *Giardia* each day of exposure.

No outbreak due to exposure to compost products has been reported to date. Lopez *et al.* (1980) however, demonstrated that 76 % of *Giardia* infections were asymptomatic. Furthermore, only about 30% of specimens from affected people will eventually have a pathogen identified (Hamlyn-Harris, 2001). Reporting of identified pathogens to surveillance systems is also variable, so that the number of infections identified and attributed to a specific pathogen represents only a fraction of the actual cases. An epidemiological approach would therefore be unable to detect a 50 % rate increase in gastrointestinal illnesses predicted in a quantitative risk assessment (Simmonds, 1999).

### 4. THE NEED FOR NEW TECHNOLOGIES

An essential part of calculating exposure to pathogens in any risk assessment is an accurate understanding of the type, concentration and distribution of infectious pathogens in waste. This information is critical to estimating the human health risk at different concentrations. The measurement of pathogen levels in composted biosolids based products is currently based on the detection of "indicator organisms" rather than on the detection of pathogens. The presence of faecal/thermotolerant coliforms or *E.coli* in compost-based products correlates

reasonably well with the possible presence of bacterial pathogens (NSW-EPA, 1997). Correlation with the possible presence of other classes of pathogen such as viruses and protozoa, however, is fairly poor. Despite considerable research efforts, no practical and broadly- applicable indicator of viral and protozoal risks has yet been identified.

*Giardia* present in environmental samples cannot be directly cultured on artificial media and therefore other detection methods need to be employed to reveal their presence. *Giardia* cysts are currently detected in water and biosolids samples by concentrating the cysts and staining the concentrate with fluorescently-labelled antibodies specific for *Giardia*. The stained sample is then examined under a high powered microscope for the presence of fluorescent, intact cysts (APHA 1995). The viability of *Giardia* cysts detected using this immunofluorescent method is difficult to assess due to the low numbers of cysts commonly detected (<1000 cysts per gram biosolids) and limitations in the technology used, which have caused erratic efficiency, low precision, lengthy analysis and lack of specificity in analysis of samples (APHA 1998). These factors, coupled with the need for a high degree of technical experience on the behalf of the operator, have resulted in the removal of this detection method from the Standard Methods for examination of Water and Wastewater. It has instead been recommended that a decision on a standard method be withheld until a reliable methodology is developed.

Such limitations as described above make the implementation of guidelines difficult when managing compost-based products. Technical problems encountered during the routine detection of pathogens in such a heterogeneous medium mean that pathogen concentrations are most likely well underestimated. The detection limits of currently-used routine methods are usually too high for reliable pathogen detection, and too high if an infectious dose of 10 cysts is considered. Current methods are also not able to distinguish between nonviable cysts and viable, infective cysts. As a result, overestimation of the risks associated with *Giardia* cysts may occur. There is therefore a real need for an accurate viability assay for emerging pathogens such as *Giardia*.

## 5. THE POTENTIAL OFFERED BY MOLECULAR TECHNOLOGY IN DETECTION AND VIABILITY ASSESSMENT OF *GIARDIA* IN BIOSOLIDS.

Recent advances in molecular technology have allowed the detection and study of microorganisms at a level of sensitivity that was previously unattainable. A number of these methods have been assessed for their ability to detect protozoan cysts in environmental samples (Betts *et al.,* 1995). One area of focus in biosolids management research is in determining the potential of these techniques to routinely detect target pathogens, with a particular emphasis on the assessment of the viability of detected micro-organisms.

The polymerase chain reaction (PCR) has been described by various authors as a useful diagnostic tool for determining the presence of *Giardia* cysts in water and sewage samples (Mayer and Palmer 1996; Rochelle *et al.,* 1997, Mahbubani *et al.* 1992). The PCR method involves the production of very high copy numbers (commonly $>10^{35}$) of a target section of nucleic acid from an organism. The presence of a large number of copies of the target nucleic acid is an indication of the presence of the sought organism(s). The target nucleic acid can be either DNA or RNA and can be highly specific for an individual species, or general for the detection of a wide group of loosely-related organisms.

PCR may not only be used in qualitative and quantitative detection, but also has potential in determining the viability of an organism. Several studies have used reverse transcription (RT) PCR to detect messenger RNA (mRNA) to determine the viability of *Giardia* cysts in water (Abbaszadegan *et al.,* 1997; Kaucner and Stinear, 1998; Mahbubani *et al.,* 1991). Abbaszadegan *et al.* (1997) amplified sequences of mRNA heat shock protein HSP70 from direct extraction of heat shocked cysts using freeze thaw fracture. Nonviable cysts did not show an increase in HSP70 mRNA when heat shocked. The primers used however, were specific for only one strain of *G. duodenalis* and did not amplify sequences from the other three known strains (S. Toze, CSIRO laboratories, personal communication).

Mahbubani *et al.* (1991) amplified a 171bp DNA fragment from a region of the giardin gene by PCR using freeze-thaw fracture to directly extract mRNA. The PCR product from this amplification did not distinguish directly between live and dead cysts. It was found however, that heat killing cysts increased mRNA production so that cysts inactivated by this method yielded a positive signal by RT-PCR. This was not observed in cysts killed by monochloramine or freezing. It was suggested that measuring mRNA levels before and after excystation induction by A260 or RT-PCR would indicate the viability status. Transcription occurred at significant levels in cysts during the induction process whereas no increase in mRNA production was noted in dead *Giardia* cysts. Conversely, Kaucner and Stinear (1998) used magnetic beads to capture mRNA from cells and found that heat killing cysts and oocysts did not produce an increase in mRNA. Gobet and Toze (2001) suggested that the differing results may have been due to mRNA decay which was initiated by the shortening of the poly A tail. Less mRNA would hence be captured by the magnetic beads than by the direct extraction method.

While the use of mRNA sources such as the heat shock protein can be useful for detecting the presence of viable cysts within a cluster of isolated cysts, it is not yet a technique that can be used to quantitatively distinguish between the number of viable and non-viable cysts present in a sample. This limitation is due to the fact that it is still unclear how much mRNA is produced by viable cysts, or if there is any change in the amount of mRNA produced as the cysts age or become damaged.

In addition, the DNA polymerase enzyme used in the PCR method is sensitive to a range of contaminants commonly found in the environment (eg humic compounds, divalent cations, heavy metals) (Wilson, 1997). Biosolids can contain many of these inhibiting compounds, and this can have a serious impact on the success of any attempted PCR reaction. Also, while the PCR method is very sensitive and capable of detecting very small numbers of targets per reaction, the methods also tend to be used on small reaction volumes (usually $\leq$ 50 $\mu$L). Thus, if there are few cysts dispersed in a large amount of biosolids, their detection would be difficult. These characteristics and limitations of the PCR method mean that there is a need for the development of methods capable of extracting and concentrating *Giardia* cysts from large amounts of biosolids, while removing the biosolids and soluble inhibitory compounds. The inability of current methods to simultaneously concentrate cysts while removing contaminating debris (without subsequent cyst loss) remains the major limiting factor for the use of PCR in biosolids.

Fluorescent *in-situ* hybridization (FISH) has also been suggested for detection of target organisms in environmental samples, and for viability assays. FISH has been used in a variety of cell metabolism or growth studies, many in conjunction with flow cytometry. FISH using oligonucleotide probes labelled with fluorescent dyes or biotin and digoxygenin have detected

microorganisms from a diverse range of environments such as soil and sewage sludge (Sandaa *et al.* 1999), activated sludge (Davenport *et al.* 1998), water and marine waters (Lindquist 1997, Vesey *et al.* 1998, Lee *et al.,* 1993 Ouverney and Fuhrman 1997), insect (Fukatsu *et al.* 1998) and animal tissue ( Boye *et al.* 1998). A common target for these labelled probes is regions within the small subunit ribosomal RNA. This is due to the comparatively larger numbers of ribosomes in a cell than DNA targets. One problem encountered in targeting rRNA for hybridisation signals was the lack of signal received from many cells. Even with optimised probes, starved or dormant cells in environmental samples may not contain sufficient rRNA to be detected by FISH (Kerkhof and Ward, 1993).

Reports on the use of FISH for *Giardia* detection are limited. A group at the University of Minnesota in Minneapolis reported the use of FISH for identification of *Giardia* trophozoites in human intestinal sections (Macechko *et al.*, 1998; Erlandsen *et al.,* 1994). Probes specific to the 16S rRNA of *Giardia duodenalis* (human pathogen) and *Giardia muris* (animal parasite) were developed. When human intestinal tissue infected with *G. duodenalis* trophozoites was hybridised with both of these probes, only samples with probes specific *to G. duodenalis* gave a fluorescent signal. This indicated the specificity of the probe was good. It was noted however, that both probes produced nonspecific staining of cells which the authors presumed to be leukocytes. Dorsch and Veal (2001) developed several *Giardia duodenalis* specific probes for hybridisation of cysts in water samples and reported sufficient fluorescent signal for adequate detection from two out of the six tested. In both these studies, the probes were designed for species identification and were not tested as a viability measure for the organism. It was assumed that the presence of fluorescence (and hence ribosomal RNA) indicated viable cysts or trophozoites.

A limitation to the use of FISH in biosolids is the production of background autofluorescence by the organic matter present. Biosolids have been noted to strongly fluoresce at the wavelengths used to detect the commonly-used fluorophores such as fluorescein and rhodamine (McInnes *et al.*, 1997). This background fluorescence can mask the fluorescent signal of the labelled cysts, many of which already have a weak signal. In addition to the problem of background fluorescence, the FISH method is only able to cope with small volumes of biosolids, making it necessary to examine many subsamples in an attempt to detect cysts. This is expensive, time consuming, tedious and requires a high level of expertise to get any results. Attempts to increase the amount of sample on one slide or filter would also increase the amount of biosolids material present which can obscure the field of view and prevent the detection of cysts by either physically covering the cysts or by imitating the size and shape of the cysts, resulting in false positive results. Sorting of samples mechanically by flow cytometry has similar difficulties, with samples requiring filtration (with possible cyst loss) prior to sorting and with autofluorescing objects of similar size and shape to cysts being selected during sorting. Thus, as with PCR, a suitable method to extract and concentrate cysts from large volumes of biosolids is needed for FISH to potentially be a viable detection method.

## 6. CONCLUSION
Standardized methods for the detection of enteric protozoan cysts from water and wastewater are currently in a state of flux as the more traditional methods have been found to be unsuitable, especially for sources where cysts are present in low numbers in large volumes of sample. This particularly pertains to sources such as soil and biosolids, where the removal of the background solid material is also a problem. Molecular-based methods are being employed in increasing numbers for the detection of target microorganisms in environmental

samples. Molecular methods have the advantage over other methods in being more sensitive, with a quicker throughput, and have the potential to assess the biological and physiological status of detected target microorganisms. Despite this, both the traditional and molecular methods are hampered by the current inability to handle small numbers of cysts in large sample volumes. The development of methods for the efficient concentration of cysts from background material and inhibitory compounds would have a significant impact on the usefulness of molecular methods in the detection of enteric pathogens such as *Giardia* in biosolids.

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