HORIZONTAL- Desk study

DRAFT For Information not to be cited

Sample handling protocols for sludges and treated biowaste for microbiological analysis

Authors: D C Lambkin[1], S Nortcliff[1], and T C White[2]

[1]Department of Soil Science, The University of Reading
Whiteknights, Reading, UK
[2]Marquis and Lord, Stratford upon Avon, UK

Literature review of sample handling protocols for sludges and treated biowaste for microbiological analysis

Contents

List of Tables

Executive Summary

This report evaluates the existing standards for sample handling protocols for sludges and treated biowaste for microbiological analysis. The objective is to identify those areas where the advice either does not reflect current wisdom or insufficient information is included.

It was concluded that the current standards do include enough information for container selection and transporting samples, although the level of detail contained in each standard varies. It is recommended that any new standard should bring together this information.

The sample storage conditions in the current standards do not reflect differences between the organisms of concern to hygienic parameters. The current holding times are based on recommendations for water samples and are considered to be inadequate for sludge and treated biowaste samples.

Information on current practice was collated from published commercial laboratory recommendations and research papers. Suitability of current practice was reviewed in light of published research investigations. Recommendations are made for individual sample holding conditions for separate groups of organisms.

Current standards recommend that samples should be kept in the dark to prevent degradation of the sample. Some standards recommend brown or amber glass for this purpose. However, plastic containers are also recommended, especially where there is a risk of gas pressure build-up. The literature supports the need for light exclusion, particularly some UV light wavelengths. There is still a degree of transparency through some apparently opaque materials, which could be important in Southern Europe. The advice in standards should be extended to include checking that the plastic absorbs the relevant wavelengths.

Current advice is that sample containers should be sterilised before use. Sterilisation may not be necessary for all samples, for example sludges with high bacterial populations, but no evidence was found in the literature to support either sterilising or not sterilising containers. Relaxing the requirements for sterilisation could produce a saving on sampling costs and it is recommended that the necessity to sterilise containers should be investigated. If investigated further, it may be possible to advise that sterile containers are necessary only where the analytical sensitivity requires a zero background; for example, the examination of advanced treated sludges.

Maximum holding time is the time between sample collection and analysis, which is the sum of the time to transport the sample from the field and storage time at the laboratory. This is not made clear in the current standards, but it is important because it is more difficult to control temperature outside the laboratory and, unless refrigerated vans are used, transportation time becomes important.

Any new standard needs to differentiate between the temperature at which samples are stored and temperatures that are practicable during transportation. This advice is currently provided in one standard.

All the advice provided by standards is aimed at reducing the temperature of samples as quickly as possible to minimise changes, including deterioration. Whilst cooling samples is a problem during summer months, particularly at low latitudes, it is possible that sample freezing could be a problem in winter months, especially at high latitudes. None of the standards consider this possibility and no reports could be found in the literature. It is recommended that further investigation of the possibility of freezing is undertaken and the results are reflected in any new standard.

There is some evidence that localised freezing of water samples can be caused by certain coolant packs. As a minimum, standards should point out that this is a possibility for samples with high water content and suggest preventative action such as making sure that the sample and coolant are not in direct contact.

When samples are to be analysed for more than microbiological parameters, due regard must be taken of the variation in suitable storage conditions. Sample analysis should be prioritised such that the organisms most susceptible to change are analysed first.

In the U.S.A a project is commencing in 2005 to develop scientifically defensible methods for collecting and handling representative samples for microbiological examination from biosolids matrices (liquid, cake, compost) for various treatment processes and applications (e.g. land application and reuse) that are expected to result in more consistent, accurate results. The project is expected to report in the winter of 2007 and it is strongly recommended that the results be critically analysed with a view to incorporating them in Horizontal/CEN standards.

1 Introduction

1.1 General

Sewage sludge and treated biowaste are applied to agricultural land to provide nutrients and to improve the soil condition. The use of these materials for land application is regulated by EU Directives that require the collection and analysis of samples of soil and the materials to be landspread.

Monitoring takes place either to check the quality of the final product (treated sludge or biowaste) before land-spreading, or to demonstrate pathogen reduction during treatment.

The aim of this document is to review the procedures for handling and transporting samples of sludge and treated biowaste that are collected and analysed for hygienic characteristics for regulatory purposes. Matters relating to experimental design, sampling patters and statistical aspects are specifically not covered and will be the subject of an additional report.

1.2 Scope

This review covers sampling and sample handling of sludge and treated biowaste in the context of the European legislation. It does not cover soil, soil improvers and growing media. However, reference may be made to documents that report soil studies where it is considered that the results contribute to this review.

The parameters considered are those within the HORIZONTAL-HYG remit:

- *Escherichia coli*
- *Salmonella* spp.
- *Clostridium perfringens*
- Intestinal enterococci
- Viable helminths
- Viruses
- Bacteriophages
- Plant pathogens

This review of sample handling covers that part of sampling that ensures sample integrity. It does not include a detailed review of processes that need to be taken into account in sampling design: sampling pattern, depth, seasonal influences, logistics or safety in the field. Nor does it include laboratory sample preparation procedures such as sub-sampling.

1.3 Literature searched

- Current and proposed European legislation
- CEN and ISO Standards that are current or in preparation
- Information from CEN Technical Committees
- Published journal articles
- Books
- Reports
- Laboratory web sites

2 Existing Legislation and Standards

2.1 Legislation

To the best of our knowledge there is no European legislative mandate to monitor soils for hygienic parameters. The Sewage Sludge Directive 86/278/EEC, 1986 [#84] permits the use of untreated sludge in soils, provided it is injected and the time delay required before specific uses is adhered to. It specifies sampling and analysis for metals, dry matter, organic matter, pH and nutritional parameters, but there is no requirement for hygienic analysis.

There are four other European regulations (two in draft form) (Table 1) that specify hygienic parameters for treated sludge and wastes. These specify the minimum requirements, but several Member States have their own legislation that requires additional hygienic analysis.

Table 1 European legislation relevant to the control of sludges and biowastes

EU regulation	**Specified hygienic parameters**
Sewage Sludge Directive 86/278/EEC, 1986 [#35]	Hygienic parameters not specified
Working Document on Sludge, 3rd Draft, 2000 [#36]	Specifies (for sludge only): *Salmonella seftenberg W775*, *Salmonella* spp., and *Escherichia coli*
Biological Treatment of Biowaste, 2nd draft, 2001 [#37]	Specifies for compost/digestate end-product: *Salmonella* spp. and *Clostridium perfringens*
Animal By-Products Regulation 2003 [#50]	Specifies for composted wastes: *Clostridium perfringens*, *Salmonella* and *Enterobacteriaceae*. Test on receipt or first working day after (equates to 72 hours). Refrigerate 2-8°C until required.
Regulation (EC) No 1774/2002 (Animal By-Products) [#38]	Specifies for composted wastes: *Clostridium perfringens*, *Salmonella* and *Enterobacteriaceae*

Sludge

European Directive 86/278/EEC [#84] regulates the use of sewage sludge for application to land. This Directive is implemented by Members States in national regulations. Direct application of these regulations in other Member State jurisdictions have not been studied as part of this review. However, the UK has been used to provide examples of how these regulations have been applied. Primarily this has taken the form of The Sludge (Use in Agriculture) Regulations 1989 [Statutory Instrument No. 1263] and amendments [#49], which implement the EU Directive [#84]. A code of practice [#28] complements the regulations and describes advice on "best practice". The code also supplements the information contained in the "Safe Sludge Matrix" [#1] with its cropping and grazing guidance.

The Working Document on Sludge [#36] requires that the results of *Salmonella* spp. and *Escherichia coli* analyses are to be supplied as indicators that the sludge has received the required level of treatment (advanced or conventional) appropriate to its end-use. The data required depends on the type of sludge treatment. Advanced Treatment is validated through removal or reduction of test organisms such as *Salmonella Senftenberg W775* and *E. coli*. "The treated sludge shall not contain *Salmonella* spp. in 50g (wet weight) and the treatment shall achieve at least a $6Log_{10}$ reduction in *Escherichia coli* to less than $5x10^2$ CFU/g". Conventional Treatment is validated through a $2Log_{10}$ reduction in *Escherichia coli.* "The sludge treatment shall at least achieve a $2Log_{10}$ reduction in *Escherichia coli"*.

The UK regulations [#49], for example, introduce definitions for "conventionally treated sludge" and "enhanced treated sludge" that specifically relate to microbiological standards and describe requirements for monitoring and control; these definitions are essentially identical to those laid out in the Working Document on Sludge [#36] for Advanced and Conventional treatments. Specifically, a conventional treatment process is one that is designed so as to reduce the amount of *E. coli* present in sludge by not less than 99% (2 log_{10} reduction), is monitored in accordance with the regulations and satisfies end-product tests for *E. coli*. An enhanced treatment process is designed to reduce the amount of *E. coli* in the present in sludge by not less than 99.9999% (6 log_{10} reduction) is monitored in accordance with the regulations and satisfies end-product tests for *E. coli* and *Salmonella* spp. [#99].

The Working Document on Sludge [#36] proposes that sampling and analysis shall be carried out as given by CEN standards. If CEN standards are not available, and until they are developed, ISO international or national standards shall apply. Annex VII of this document lists standards relevant to the examination of sludge. The list includes EN ISO 5667-13:1998, Water Quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works [#5] and methods for characterising sludges for characteristics laid out in the regulations. Although they are listed, there are no standard methods given for *Salmonella senftenberg W775*, *Salmonella* spp. or *Escherichia coli*.

The UK regulations identify units for expressing *E. coli* test results (as colony forming units per gram dry weight of sludge) and state that the presence or absence of *Salmonella* species shall be determined by reference to 2g of dry weight of sludge. These regulations prescribe a monthly sampling frequency with a set of five samples (100 ml liquid sludge or 100g dried sludge) being taken at random from a batch of sludge. Each of the five samples is to be

analysed. The sampling frequency for enhanced treated sludge can be reduced where the sludge consistently meets the standards.

It is required that end-product samples, each consisting of five random samples of not less than 100ml of liquid sludge or 100g dried sludge, should be analysed at monthly intervals to ensure satisfactory operation. Samples should be analysed for *E. coli* in conventionally treated sludge and for *E. coli* and *Salmonella* spp. in enhanced treated sludge. Other Member States may have their own, and additional, hygienic requirements, for example those listed in Table 2.

Table 2 Some European limit values for pathogens concentrations in biosolids

Country	**Salmonella**	**Other pathogens**
France [#19, #42]	8 MPN/10g DM	Enterovirus: 3 MPN/10g DM Helminth eggs: 3MPN/10g DM
Italy [#52]	1000 MPN/g DM	
Luxembourg [#19]		Enterobacteria: 100/g No egg or worm likely to be contagious
Poland [#19]	Biosolids cannot be used in agriculture if it contains Salmonella	'Parasites' 10/kg DM
Norway [#8, #76]	None	No viable helminth ova

Biowaste

Biological Treatment of Biowaste 2nd Draft [#37], Annex II specifies that an indicator organism shall be used in order to determine the effectiveness of the treatment in sanitising biowaste. The proposed indicator is *Salmonella senftenberg W775* (H_2S negative) [under review]. Annex IV specifies that, for biological treatment plants producing more than 100 tonnes of treated green and wood waste per year or 50 tonnes of treated biowaste per year, *Salmonella* spp. and *Clostridium perfringens* should be absent in test samples in order for the materials to be regarded as sanitised. Sample collection, preservation and analysis must assure valid and representative results. For sampling, the draft regulation makes reference to EN 12579:2000 Soil improvers and growing media – sampling [#9].

Pathogen testing usually involves testing for the presence of specific micro-organisms, such as *Salmonella* and faecal coliforms, to support the process-oriented 'temperature-time' regimes in seeking to ensure a hygienic product [#113]. Additional hygienic requirements are aimed at prevention of harm to humans, animal and the environment. For example, BSI PAS (Publicly Available Specification) 100:2005 [#25] requires that *Salmonella* spp. shall be

absent in a sample of 25 g fresh mass (tested in accordance with BS EN ISO 6579) and the concentration of *Escherichia coli* shall be less than 1,000 CFU g^{-1} fresh mass (tested in accordance with BS ISO 11866-3). In addition, if the compost passes the pathogen tests required by the Animal By-Products Regulations or EU Regulation 1774, no additional pathogen tests are necessary for demonstrating PAS 100 compliance. The European Composting Association also requires that *E. coli* O157:H7 should be absent in finished compost. German compost quality regulations (LAGA M10) require testing for Tabak-Mosaik-Virus (Tobacco Mosaic Virus, TMV). In Sweden composted biowaste is monitored using faecal *Streptococcus* and *Enterobacteriae* as indicator organisms. PD CR 13455:1999 lists the requirements for soil improvers and growing media in some nations (Table 3).

Table 3 Some European provisions for pathogens in soil improvers and growing media (Reproduced from PD CR 13455: 1999 [#8]

	Application area	**Pathogens**	**Approval level**
EC "eco-label" 488/98 EEC	Gardening	*Salmonella* spp. *E. coli*	none <1000 MPN/g
Austria *Draft compost ordinance 10/98*	Land reclam. Agriculture sacked Sport/playground Technical use	*Salmonella* spp. *Salmonella* spp. *E. coli* *Salmonella* spp. *E. coli* *Campylobacter* *Yersinia* spp. *Listeria* -	none none If found, recomm. for the safe use none none none none none no requirements
Belgium *VLACO*		*general* Eelworms	none none
Germany *Biowaste Ordinance*		*Salmonella senftenberg* *Plasmodoph. brass.* *Nicotiana virus 1*	none infection index:<0.5 guide value biotest:<8/plant none in 50g sample
Italy		*Salmonella sp.* *Enterobacteriaceae* *Fecal Streptococcus* *Nematodes* *Trematodes* *Cestodes*	none in 25g sample <$1x10^3$ CFU/g <$1x10^3$ MPN/g none in 50g sample none in 50g sample none in 50g sample
Norway		*Salmonella* *Viable eggs of parasites* *Thermotolerant coliform bacteria*	none none <2500/g dry solids
The Netherlands		*Eelworms* *Rhizomania virus* *Plasmodoph. brass.*	none none none

2.2 Standards

In an overview of Project HORIZONTAL [#44], a small group of International and European standards were identified as relevant to sampling of sludge, treated biowaste, waste and fertilisers: EN ISO 5667-13:1997 (Sludge); EN 12579:2000 (Treated biowaste, Fertilisers); TC 292 Working Instructions (Waste); EN 1482:1996, 13040:2000 (Fertilisers).

These standards, and any they refer to for additional information, are listed in Table 4. In general, where conditions are specified, the standards require sterile containers to be used. Two of the standards require the containers to be made of glass. Where sample preservation is recommended, all the samples advise cooling the sample and most specify that the samples should not be frozen. Storage duration is short, between 6 and 8 hours.

The earlier version of EN ISO 5667-3 (1995) [#4] provides advice for both waste water and sludge samples. This standard has been superseded by a later version (2003) [#11], which does not refer to sludge samples or to microbiological examination. This topic is now covered in EN ISO 5667-15 [#7].

None of the standards provide published evidence to support the recommended sample handling guidance and the holding times can appear arbitrary when a single set of instructions is applied to a large group of organisms. The terms *microbial testing* or *bacteriological examination* can include a wide range of organisms, some of which may be more or less sensitive to storage times or temperature. A criticism that has been levelled at standards is that sample holding times were originally established for aqueous media and then blindly applied to other media [#90]. This problem has been recognised in the United States and two projects have been initiated to investigate holding time and temperatures.

The first project, *Characterization and monitoring: Sample holding time re-evaluation*, funded by the United States Environmental Protection Agency (USEPA) is being carried out by their National Exposure Research Laboratory, Environmental Sciences Division [#90] and is due to report in 2005. The work is investigating the effect of time and temperature on analysis of chemical constituents. Specifically these are: semi-volatile compounds, PCBs, pesticides and TOC, metals and Cr^{6+}. The study will cover preservation techniques, including cooling to 4°C, freezing at -20° and the addition of chemical preservatives.

The second project, *An investigation into biosolids sampling and handling methods for USEPA-Approved Microbial Detection Techniques*, is being funded by the Water Environment Research Foundation (Project number 04-HHE-7, http://werf.org). The two year project will develop scientifically defensible methods for collecting and handling representative samples for microbiological examination from biosolids matrices (liquid, cake, compost) for various treatment processes and applications (e.g. land application and reuse) that are expected to result in more consistent, accurate results. The project was put to tender in February 2005 and is expected to report in the winter of 2007.

It is recommended that, once these projects have reported their findings, the results are critically analysed with a view to incorporating them in CEN standards.

Table 4 Standards relevant to handling procedures for hygienic parameters

Standard	Matrix	Parameter	Containers	Preservation	Storage conditions	Storage duration	Notes
EN ISO 5667-13:1997 Water quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works [#5]	Sludge	Refers user to ISO 5667-3 and ISO 5667-16					
EN ISO 5667-3:1995 Water quality – Sampling – Guidance on the preservation and handling of samples [#4]	Waste water & Sludge	Total bacteria,Total coliforms, Thermotolerant coliforms, Faecal streptococci, Salmonella, Shigella, etc.	Sterile container	Cooling to between 2°C and 5°C	2 to 5°C, in the dark. Do not freeze	8 hours (drinking water, surface water, ground water and sludges)	Superseded by EN ISO 5667-3:2003
EN ISO 5667-3:2003 Water quality – Sampling – Part 3: Guidance on the preservation and handling of water samples [#11]	Waste water, not sludge	Biological analysis, but not microbiological analysis					
EN ISO 5667-15:1999 Water quality – Sampling – Part 15: Guidance on the preservation and handling of sludge and sediment samples [#7]	Sludge	Bacteriological examination	Sterile glass	Refrigerate	2 to 5°C, dark, airtight. Never frozen or dried.	6 hours	
EN 12579:2000 Soil improvers and growing media. Sampling [#9]	Treated biowaste, Fertiliser	Microbial testing	Sterile containers	Do not freeze or subject to temperature extremes.			
Pr CEN/TR 15310-4:2004 Guidance on sampling of waste (draft) [#14]	Waste	Bacteriological Examination	Sterile glass	Refrigerate	2 to 5°C, dark, airtight	6 hours	Method from ISO 5667-15
EN 13040:2000 Soil improvers and growing media – Sample preparation for chemical and physical tests, determination of dry matter content, moisture content and laboratory compacted bulk density [#10]	Fertiliser	Chemical & physical			1 to 5°C, not frozen, airtight.		

3 Previous HORIZONTAL work

The topic of sampling and sample handling has been discussed to varying degrees in four Project HORIZONTAL desk studies [#57, #59, #63, #102].]. Three of these reports deal with microbiological issues while the fourth discusses a chemical parameter.

The desk studies recognise that one of the most frequently encountered problems with evaluation of pathogens is ensuring representative sampling and appropriate sample preservation. However, it is acknowledged that the only international standards are guidance documents only [#59] and little has been published on protocols for microbiological sampling of sludges, soil, and treated biowaste. In addition, two of these reports acknowledge that this is a key area that needs to be addressed [#102, #111].

Samples may be collected for chemical (inorganic, organic), physical or biological (including microbiological) examination, or some combination of these, and the methods of sampling and preservation of samples for each examination will differ. Therefore storage of the samples, including methods and speed of transport to the investigation laboratory, should be implemented in accordance with the requirements of the analysis to be carried out [#57].

The report of Horizontal WP2 [#57] lists the qualities required of a sample container. A suitable container should:

- Preserve the components of the sample that are to be examined;
- Prevent cross-contamination either between samples or from the environment;
- Prevent loss of sample, for example by leakage from bottles or tearing of bags;
- Be appropriate for the size of sample to be collected;
- Preserve the structure of the sample in the case of undisturbed samples.

This is reiterated in the report of Horizontal WP3 [#102], which states that storage and transport of the sample should be undertaken in a manner designed to minimise change, including deterioration, and avoid contamination. Also, all samples should be taken in an appropriate container (e.g. polyethylene or polypropylene) in a correct manner and that glass containers should be avoided owing to potential fermentation and gas pressure build-up.

The importance of correct preservation and transportation has been stressed in a number of reports. Horizontal WP6 [#59] points out that the conservation of the sample after sampling depends strongly on the conditions in which it transported and conserved at the laboratory

until the analysis is performed. However, they caution against adopting an across-the-board requirement to keep samples at 4°C during transport to the laboratory.

The authors of Horizontal WP3 [#102] support the need to cool samples as soon as possible, stating that it is very important that once samples have been taken, the conditions for each sample should remain under the same specified conditions (e.g. 3-5°C) until arrival at the analysing laboratory within a specified time period from the sampling. However, they also point out that the temperature variance in Europe can be as much as 60°C. Northern Finland can routinely experience ambient temperatures of -20°C whilst Greece can routinely reach 40°C.

The authors of Horizontal WP2/4 [#63] suggest that sample preservation methods similar to those for other microbial parameters would be suitable, for example the methods in ISO 5667-15:1999 (2-5°C, dark, 6 hours to analysis) [#7]. But they also note that bacteriophage survival at 4°C has been reported for extended periods of time up to 72 or 96 hours.

4 Current Practice

4.1 Introduction

This section of the report reviews current practice in commercial laboratories and scientific research. This is followed by a review of the evidence for retaining or amending current sample handling instructions. Separate consideration is given to the varying matrices and microbiology that are covered within the Scope of the report.

4.2 Commercial laboratories

Commercial laboratory websites were identified by using a standard web search engine. Many commercial laboratories either do not publish sample handling procedures on their websites, or were excluded because they do not specify procedures for sludge and treated biowaste for the parameters of interest. The results of the search are given in Table 5.

Matrices: A variety of terms are used for matrices: compost, solid waste, environmental solids, sludge, sewage sludge and biosolids.

Containers*:* A wide range of container types is recommended: plastic, glass, high density polyethylene and jar (assumed to be glass, but no information provided). Some laboratories specify that these should be sterile. There is no pattern relating the container specification to the matrix type or parameter.

Bacteria: Almost all the laboratories recommend refrigeration or cooling to 4°C and one laboratory [#18] cautions against freezing sludge samples. Two laboratories [#32, #69] do not specify temperature. The maximum holding time is generally 24 hours, with three exceptions. One laboratory [#104] recommends 48 hours for *coliform* and other bacteria in compost. For environmental solids [#81] a maximum holding time of 30 hours is recommended for total coliform and 6 hours for faecal *coliform* or faecal *streptococci*. Two laboratories [#32, #69] do not specify maximum holding time.

Viruses: A range of storage conditions are recommended: freezing at -70°C, cool 4°C, cool 2°C-10°C or up to 25°C. One laboratory [#69] does not specify a temperature. The maximum holding times recommended depend on the temperature conditions but, in general, the lower the temperature the longer the holding time. Some companies recommend that frozen samples can be held as long as 2 weeks whereas the holding time for cooled samples is cited as being measured in hours.

Helminth ova: Almost all recommendations are for refrigeration or cooling to 4°C. One laboratory [#18] cautions against freezing sludge samples and one laboratory doesn't specify conditions. Three different holding times are recommended: 48 hours, 5 days, 1 month.

Giardia and Cryptosporidium: No data were found for oocysts and cysts.

Table 5 Holding times and temperature recommended by commercial laboratories

Parameter	Matrix	Container	Thermal Storage Conditions	Maximum Holding Time	Ref.
Coliform & other bacteria	Compost	P, G S	4°C	48 hours	[#104]
Faecal coliform	Compost	P, G, HDPE	Cool, 4°C (Ice Pack)	24 hours	[#112]
Bacteria	Solid waste	P		24 hours	[#32]
Total coliform	Environmental solids	S P	Cool, 4°C	30 hours	[#81]
Bacti-faecal coliform	Sludge	G			[#69]
Faecal coliform	Biosolids	P, G	Cool to 4°C	24 hours	[#20]
Faecal coliform	Environmental solids	S P	Cool, 4°C	6 hours	[#81]
Faecal coliform	Sewage sludge	P, G	Cool to 4°C	24 hours	[#27]
Faecal coliform	Sewage sludge		Refrigerate (do not freeze)	24 hours	[#18]
Faecal coliform	Sludge	Jar		24 hours	[#69]
Coliform (total, *E. coli*, Enterococci)	Surface water	S P	Cool 4°C	6 hours	[#71]
Heterotrophic plate count	Environmental solids	S P	Cool, 4°C	24 hours	[#81]
Faecal streptococci	Environmental solids	S P	Cool, 4°C	6 hours	[#81]
Faecal streptococci	Sludge	P			[#69]
Salmonella	Biosolids	P, G	Cool to 4°C	24 hours	[#20]
Salmonella	Compost	P, G, HDPE	Cool, 4°C	24 hours	[#112]
Salmonella	Sewage sludge	P, G	Cool to 4°C	24 hours	[#27]
Salmonella	Sewage sludge		Refrigerate (do not freeze)	24 hours	[#18]
Salmonella	Sludge	Jar		24 hours	[#69]
Enteric virus	Compost	P, G, HDPE	Cool, 4°C/ Freeze	24 hours/ 2 weeks	[#112]
Enteric virus	Compost	S G	-70°C	>8 hours	[#104]
Enteric virus	Compost	S P, G	4°C	8 hours	[#104]
Enteric virus	Sewage sludge	P, G	Up to 25°C/ 2-10°C	2 hours/ 48 hours	[#27]

Parameter	Matrix	Container	Thermal Storage Conditions	Maximum Holding Time	Ref.
Enteric virus	Sewage sludge		Cool/ Frozen	24 hours/ 2 weeks	[#18]
Enteric virus	Biosolids	P, G	Up to 25°C/ 2-10°C	2 hours/ 48 hours	[#20]
Virus assay	Sludge	Jar		72 hours	[#69]
Helminth ova	Compost	P, G, HDPE	Cool, 4°C	1 month	[#112]
Helminth ova	Compost	S P, G	Cool, 4°C	1 month	[#104]
Helminth ova	Biosolids	P, G	Cool to 4°C	5 days	[#20]
Helminth ova	Sewage sludge	P. G	Cool to 4°C	5 days	[#27]
Helminth ova	Sewage sludge		Refrigerate (do not freeze)	1 month	[#18]
Helminth ova	Sludge	Jar		48 hours	[#69]

S = Sterile, P = Plastic, G = Glass, HDPE = High Density Polyethylene

4.3 Scientific research papers

Not all scientific research papers describe exactly the sample containers and transport and holding conditions; some give no details at all. A list of conditions reported in the literature is given in Table 6.

Two papers specify sampling according to standard protocols [#29, #109]. Of those papers that report containers used, all except one [#114] report that the containers were sterile. In general, all sample transport and holding conditions entail keeping the samples cool and keeping the transport time to the laboratory short.

Bacteria and phage analysis was initiated within 48 hours (30min, 6h, 8h, 12h, 24h, 48h). Where only presence/absence was evaluated, samples were analysed within 72 hours. Sample storage at 4°C for 2-3 weeks was recorded for *Giardia* and *Cryptosporidium* in two papers [#47, #85] and for bacteriophages in one paper [#56].

Table 6 Holding times and temperature reported in published papers

Matrix	Container	Parameter	Transport and Holding Conditions	Ref.
STW, Input liquid & Treated sludge cake		Enterococci, Coliforms, E. coli, Clostridiae, Salmonella, Listeria, Cryptosporidium, Campylobacters, VTEC O157	Cold, <12°C during transport. Analysis began within 24h after sampling	[#87]
Biosolids compost	LDPE bags	Respiration	Packed in ice in a cooler. Shipped to lab the same day	[#114]
WWTP, untreated and treated sewage sludge	sterile containers	E. coli, faecal streptococci, bacteriophages, culturable enteroviruses	Transferred to lab within 2 hours of collection. Stored at 4°C (max overnight)	[#67]
Environmental waters, combined sewer overflow treatment, WWTP		Cultivatable viruses Coliphages Faecal coliform, E. coli, Enterococcus	48h (MPN); 72h (presence/absence) 48h (PFU); 72h (presence/absence) 6-12h or 72h, depending on method	[#65]
Environmental waters	sterile containers	E. coli, faecal streptococci, bacteriophages, culturable enteroviruses	Transport on wet ice, analyse immediately	[#80]
Untreated & treated sewage sludge	sterile containers	Bacteria, bacteriophages	Kept at 4°C for <8h, until analysis	[#62]
STW Untreated and treated waters		Faecal coliforms, enterococci	Collected and stored in accordance with standard protocols: ISO 5667-3, USEPA protocol for water and wastewater	[#109]
Treated sewage sludge		Giardia, Cryptosporidium, Clostridium perfringens, E. coli	Samples stored at 4°C. Indicator bacteria analysis within 2 days; Microscopy within 2 months	[#85]
WWTP feed sludge		E. coli, faecal streptococci, bacteriophages, culturable enteroviruses	Collected and transported to lab with 1h. Chilled to 4°C	[#52]
WWTP influent and effluent liquids	sterilised glass bottles	Total coliform, faecal coliform, Clostridium perfringens	Immediate transport to lab in ice boxes under sterile conditions	[#88]
STW untreated, MAD and final product		E. coli, faecal streptococci, bacteriophages, culturable enteroviruses	Samples were taken in accordance with EN ISO 5667-13:1998	[#29]
WWTP at several stages in the treatment process	Sterilised tubes	Coliphages	Samples kept at 4°C and transported to lab within 24h.	[#100]
WWTP		Faecal coliforms	Samples composited over 24h, kept at 6°C. Returned to lab refrigerated. Analysed within 6h	[#45]

Matrix	Container	Parameter	Transport and Holding Conditions	Ref.
Pulp & Paper mill wastewater		Bacteria	Composited samples collected over 24h. Shipped on ice. Arrived at lab within 48h	[#46]
Sewage influent		Giardia, Cryptosporidium	10 point composite. Added 1 capful (10-15ml) Tween 20 prior to mixing and sub-sampling. Samples shipped immediately on ice to lab, stored at 4°C. Analysed within <2weeks	[#47]
Activated sewage sludge		Bacteria, bacteriophages	Bacteria isolated immediately. Samples stored at 4°C for 2-3 weeks until bacteriophage isolation	[#56]
STW influent & treated sludge	sterile containers	Bacteriophages, enteric viruses	Transported to lab within 2h of collection. Stored (max overnight) at 4°C then processed	[#58]
STW activated sludge	stoppered sterile flasks	Bacteria, bacteriophages	Kept on ice before processing	[#39]
Soils		Enteric viruses (inocculated into soil then transported to test labs)	Samples transported on ice using overnight delivery service. Tested within 24h of arrival at labs	[#53]
STW untreated sewage	sterile glass bottles (liquid) sterile plastic bags (sludge)	Bacteriophages, coliphages, enteroviruses	Samples kept at 4°C. Waters analysed within 6h of collection, sludge within 12h	[#101]
Soil treated with sewage sludge	sterile cuvette/sterile glass container	Bacteria	Soil transferred to sterile cuvette (enamelled metal). Mixed in situ then transferred to lab in sterile glass container	[#71]
Compost		Bacteria	Unless analysed immediately, stored at 4°C	[#83]
Seawater/sewage sludge mix	collected in foil-wrapped, sterile glass bottles	Phages, faecal coliforms, enterococci	Kept in dark for transport to lab. Holding time between collection and assay typically 30 minutes or less	[#93]

5 Review of the literature

5.1 Introduction

The ultimate aim of sampling is analysis of a sample that is representative of the whole. This requires taking a portion of material, transporting it to the laboratory, analysing it and producing a report of the results. An important stage in this process is sample handling; ensuring that the sample integrity is not compromised.

When a sample is collected it goes through a number of handling stages, any of which could affect sample integrity. First the sample is transferred to a container, sealed and labelled with unique identification. The container is packed with others into a mode of transport and transported to the laboratory. Once at the laboratory, the sample is checked in and either analysed immediately or placed in temporary storage. Finally the sample is analysed and the results recorded.

The initial steps taken in the field frequently are critical to laboratory analysis performed hours, days or even weeks after a sample is obtained. The time and conditions of sample storage between sampling and analysis must be described [#30]. The description should include details of sample containers, cleaning and sterilisation procedures and transport and storage conditions appropriate for the sample material and parameters to be measured.

The goal of sample preservation is to maintain sample integrity between collection and analysis and should limit biological, chemical and physical changes to the sample. Sample preservation is governed by the sample matrix, the desired quality objectives of the analysis, the nature of the parameter of interest and the analytical method. Requirements for sample preservation in the field and transport to the laboratory should be determined during the planning phase when analytical protocols are selected [#107].

Two papers have identified that the storage and transport of the sample should be undertaken in a manner designed to minimise change, including deterioration, and avoid contamination [#99, #102]. Containers and packing should be chosen to protect the samples from cross-contamination, breakage or leakage. Storage time should be considered as a whole, both during transport and in the laboratory, so that the analysis is carried out before the sample is beyond its shelf life. The following sections discuss these stages in relation to the different microbiological properties of bacteria, viruses, helminth ova, and (oo)cysts. Very little research has studied the effects of cold storage on compost samples [#21] or biosolids [#99].

Several researchers have investigated the effects of cold storage on soil samples [#21], but most of the available literature concerns water samples.

Only one standard, EN ISO 5667-3:1995 [#4] provides separate advice on sample preservation and storage for different microbiological parameters. However, the advice is identical for both water and sludge samples, and the standard has been superseded. EN ISO 5667-3:1995 has been replaced by EN ISO 5667-3:2003 [#11] for preservation of water samples and EN ISO 5667-15:1999 [#7] for preservation of sludges. EN ISO 5667-15:1999 recommends a single set of storage conditions for samples for bacteriological examination. The guidance given in Pr CEN/TR 15310-4:2004 [#14] is identical to that in EN ISO 5667-15:1999.

Standards for Soil Improvers and Growing Media, EN 12579:2000 [#9] and EN 13040:2000 [#10] do not provide detailed advice on sample storage and preservation for microbiological analysis other than to avoid extremes of temperature or freezing.

A draft standard, ISO/CD 19458:2003 *Water quality – Sampling for microbiological analysis*, does include separate holding times and temperatures for a wide variety of micro-organisms, which reflect current understanding of inactivation rates in water samples. These conditions may not be suitable for sludge samples.

5.2 Factors affecting sample microbiological properties

The principal biological, chemical and physical phenomena that may cause changes in the samples are changes in water content, biological activity, chemical changes (reactions with the atmosphere or container) and volatile losses [#13]. In general, refrigeration will reduce biological activity and moisture will induce microbial activity unless the temperature is very low. Refrigeration will also slow down; reduce, or even arrest, losses of volatile materials and generally slow down some chemical reactions depending on the system being considered. These factors may also influence the survival of microbiological activity at these temperatures.

It should be remembered that microbiological activity in the sample can affect other properties that may be of interest, for example by degrading organic compounds [#60]. In this case samples may need to be kept under conditions that minimise microbial degradation.

Pathogen survival depends primarily on:

- The type of organism (some are more resistant)
- Moisture (longevity is longer in moist conditions)
- pH (neutral pH favours longevity)
- Organic matter (a food/energy source)
- Other micro-organisms present (competition, predation)
- Temperature (pathogens generally survive longer at lower temperature)
- Sunlight (has been known to reduce survival time).

Epstein [#33] summarised the physical, chemical and biological factors that can inactivate pathogens, see Table 7. The importance of each of these factors varies within and between groups of micro-organisms.

Table 7 Physical, chemical and biological factors affecting pathogen activity

(Reproduced from Epstein (2003) [#33].

Physical	**Chemical**	**Biological**
Temperature Desiccation Applied fields: Microwave radiation IR radiation Ultrasonication Magnetic fields Pulsing electrostatics/ electrolytics	pH (acids/alkali) Ozone Ammonia Nitrous acids Phosphoric acid Nitric acid Alkaline agents Sulphuric acid	Antagonistic organisms Digestion (aerobic/anaerobic) Composting Alkaline composting

5.2.1 Bacteria

5.2.1.1 Factors affecting bacteria survival

Faecal coliform bacteria, *Escherichia coli* and *Salmonella* spp. are used for routine evaluation of sewage treatment plant performance and sludge quality [#67]. *E. coli* has been selected as a surrogate to determine pathogen reduction through the treatment process because it has been shown to have similar survival characteristics to pathogens [#29].

Data for evaluations of microbial indicator density that support current holding time recommendations are limited, particularly for *E. coli* [#80]. Most of the available literature concerns effects on human and animal health and genetics research. For example, control in food production and storage, survival in soil after sludge application, sources and

concentrations in water bodies and water supply, reduction during sewage treatment (e.g. mesophilic-anaerobic-digestion, MAD), and use as indicator organisms.

Tolerance to factors such as temperature, acidity and moisture varies between bacteria species, with consequences for their survival in environmental samples. A range of factors, including sunlight, temperature, ciliate predation and chlorination [a chemical disinfectant treatment] affect persistence in the environment of faecal coliform, enterococci and sulphite-reducing bacteria [#109]. Concentrations of enteric micro-organisms in seawater are also affected by dilution, salinity and sunlight [#94].

Bacteria have been demonstrated to be light sensitive, moving away from light sources [#15], but no study of this phenomenon in biosolids or treated biowastes was found in the literature. Fujioka and Yoneyama [#41] assessed the inactivating effect of sunlight on faecal bacteria (*Escherichia coli and E. faecalis* [previously classified as *Streptococcus faecalis*]) using purified and washed cultures suspended in clean buffer or seawater. They found that in the absence of sunlight the bacteria were stable for at least 6 hours. Under summer sunlight conditions *E. coli* suffered a 6 log10 inactivation after 1 hour of exposure and *E. faecalis* was inactivated by 3 log10 over 4 hours. Inactivation of *E. coli* was similar under both summer and winter sunlight conditions, but inactivation for *E. faecalis* was clearly slower during winter sunlight exposure. The authors concluded that *E. coli* is so sensitive to sunlight inactivation that it should not be used to monitor recreational waters for the presence of human enteric viruses.

Sinton *et al*. [#93] assessed the inactivating effect of sunlight on faecal coliforms in sewage-polluted seawater, comparing the effect of four optical filters (polyester, acrylic, polycarbonate and orange acrylic) to inactivation in the dark or under full summer sunlight. Faecal coliforms were inactivated by a wide range of solar wavelengths, but inactivation rates were low in the dark and highest under full sun. Inactivation was least, and not much greater than in the dark, under the orange acrylic filter (wavelengths below 556 nm excluded).

The results of these experiments indicate that *E. coli* in water is very sensitive to sunlight inactivation, but the effects can be reduced by using containers with suitable light filtration properties. Although no evidence was found in the literature, *E. coli* in sludges may be protected to some extent because light doesn't deeply penetrate the sample.

Bacteria have been shown to persist longer in soil under wetter and cooler conditions [#68]. It has been observed that pathogens stay viable in soil for two months or more, especially in

damp and shady areas [#34]. The survival of bacteria in the soil depends on many parameters such as temperature, moisture pH, soil composition and the presence of other micro-organisms.

It has been reported that microbial population density increases with temperature and that the growth rates of micro-organisms double with every 10ºC increase in temperature until a species specific optimum temperature is reached (Hawkes, 1963; Rao *et al*. 1974; Gaudy and Gaudy, 1980 all quoted in #88). Temperatures below 10ºC are generally considered acceptable for *E. coli* preservation [#80].

In 1952, the U.S. Public Health Laboratory carried out extensive studies on coliform bacteria in water samples stored overnight at room temperature (16-23ºC) or in a refrigerator (2-5ºC). In his review of the research, Heulelekian [#48] reported that [statistically] significant increases were found in 15% of the samples stored at room temperature and 7% of the refrigerated samples. However, [statistically] significant decreases were found in 19% of the room temperature samples and 17% of the refrigerated samples.

In 1988, Milligan [#66] re-evaluated the recommended sample holding times for *Escherichia coli* and enterococci in water samples. Water samples were collected and kept on ice for either 6 or 24 hours before analysis. Ten replicates each of three water samples were analysed. No statistically significant difference was found for *E. coli* counts, but enterococci counts were significantly different. Based on their results, the authors recommended that water samples could be kept for up to 24 hours before analysis for *E. coli*, but for enterococci the holding time should not be extended beyond the currently recommended 6 hours.

In 2002, Sonzogni *et al*. [#95] investigated survival of *E. coli* in well water samples to evaluate the holding times recommended by USEPA. Triplicate samples were prepared and tested across a matrix of four temperatures (4, 10, 20 and 30ºC) and four time periods (8, 30, 48, 72 hours). All samples, including those containing very low levels of bacteria can be preserved for at least 48 hours if held at 4ºC, the temperature usually achieved by shipping samples packed in wet ice.

The data showed that the cooler the samples, the longer the holding time. The authors concluded that the current practice of allowing up to 48 hours without cooling is too lax. Preservation at 10ºC for 30 hours was deemed suitable for all samples. Most samples could be held for 30 hours at 20ºC. Samples held at 30ºC were deemed unsuitable for *E. coli* testing and samples submitted during summer months should be preserved by cooling. A maximum

holding time of 30 hours and a requirement to cool the samples to 10ºC or less was recommended.

Pope *et al*. [#80] investigated *E. coli* in water samples stored at 5, 10, 20 and 35ºC and analysed after 8, 24, 30 and 48 hours. Based on the results it was recommended that samples could be held at less than 10ºC, provided that they were not allowed to freeze, with no significant effect on the results. For five of the seven samples tested, no significant decrease in *E. coli* densities were found, but for the other two samples there was a significant decrease after 8 hours.

Both time and temperature have an impact on total coliform densities [#80]. Researchers evaluating total coliform in drinking water found that when samples were kept at 5ºC densities were 34% lower at 24h compared to 0h (McDaniels and Bordner, 1983 quoted in #80). In the same study, there was an 87% decrease over 24 hours for samples stored at 22ºC. Similar results have been reported by other researchers. Totanzos and McFeters (1997 quoted in #80) observed that total coliform density after 6 hours was 23% lower in samples kept at 5ºC and 47% lower at 22ºC. After 30 hours the densities had decreased by 33% (5ºC) and 62% (22ºC).

Recommended maximum holding times for *E. coli* vary from 8 to 24 hours [#80]. The results from a study of water samples kept at 2-4ºC indicated that the difference in holding time produced a difference of less than 20% in 85% of the samples (Standridge and Lesar, 1977 quoted in #80). This difference was considered acceptable when compared to analytical variability.

The pathogens in wastewater are usually associated with insoluble solids [#89] and many micro-organisms survive better when they are associated with solids than when they are suspended in water [#67]. Therefore holding conditions recommended for water samples may be over-conservative if applied to samples of biosolids or treated biowaste.

Low temperatures help the survival of bacteria in soil with survival levels tending to decrease as the temperature increases. Moisture is fundamental in controlling temperature as water has a high calorific value. The alternating cycles of freezing and thawing in winter also have an influence on the survival of pathogens in soil. A low incubation temperature and high soil moisture aid the survival of *E. coli* and enterococcus sp.

Parker and Martell [#75] reviewed survival of micro-organisms under freezing conditions. Bacteria can be injured or die as a result of rapid chilling without freezing (cold shock), freezing, storage at low or sub-zero temperatures, and subsequent warming. For bacteria that have been frozen and thawed, both the rates of cooling and warming affect survival. The extent of damage depends on the micro-organism; faecal coliforms and *E. coli* being more susceptible to freezing than many other bacteria species.

Vieira and Nahas [#108] examined the changes in different groups of bacteria and fungi in soil samples stored at 5ºC and -12ºC over a 32-week period. After one week there was a decrease in total bacteria and *Bacillus* spp. at both temperatures, but there was no difference in total bacteria at -12ºC. Sanin *et al*. [#89] investigated freeze/thaw as a method for pathogen reduction in sludge that might be feasible in regions where natural freezing is available. They tested the effect of temperature, freezing rate and time on reduction of faecal coliforms and *Salmonella* spp. At a freezing temperature of -7ºC faecal coliforms was not reduced at all in 1 day and *Salmonella* were reduced by 0.26 log10. Reduction in numbers of both organisms was greater at lower temperatures, longer freezing times and faster freezing rates. This indicates that short-term temporary freezing, for example by contact with ice packs during transportation, may not affect bacterial concentrations.

Survival of *E. coli* O157:H7 in soil was compared with survival of non-pathogenic forms by Mubiru *et al*. [#68]. Inoculated soil was subdivided among polythene bags and stored at 25ºC for the duration of the experiment (8 weeks). *Escherichia coli* death was modelled using a first-order decay equation from which half-life was calculated. *E. coli* half-life (the time required for half the *E. coli* population to disappear) measured in the two soils tested was 2.2±0.89 and 3.3±0.90 days. The difference in death rates between the soils could be due to differences in exchangeable bases, soil organic matter and total nitrogen, which have been associated with increased faecal bacteria survival in soil. Higher clay content in one soil could have caused greater water stress.

Selvakumar *et al*. [#91] investigated the effect of extended holding times on concentrations of micro-organisms in sanitary water collected from the influent at a wastewater treatment facility. A wide range of micro-organisms was tested; 5 indicator organisms: total coliform, faecal coliform, faecal streptococcus, enterococcus, *Escherichia coli*, and two pathogens: *Pseudomonas aeruginosa* and *Streptococcus aureus*. One set of sub-samples were analysed immediately; the others were stored at 4ºC until the designated days of analysis.

Concentrations of total coliform, faecal coliform and faecal streptococcus were lower on day 2 than day 1. Measured concentrations of *E. coli*, *P. aeruginosa* and *S. aureus* were higher on day 2 than day 1. However, the differences were only significant for *E. coli*. It was concluded that, for sanitary wastewater, 4ºC was regarded as being a suitable temperature for preserving the samples and the holding time could be increased beyond 24 hours for all organisms tested, except *E. coli*. It was recommended that, where samples are to be analysed for a number of bacteria, analysis for faecal streptococcus, enterococcus, *S. aureus* and *E. coli* should be analysed first.

In another study soil was mixed with sludge and incubated in the laboratory at 25ºC and controlled moisture of 80%; samples were analysed after 5, 10, 20, 40 and 80 days [#34]. All three micro-organisms (faecal coliform, *E. coli* and enterobacteriaceae) were observed to behave in a similar manner and there was no difference in behaviour between sludge types. Over the first 5 days there was a rapid decrease in population, followed by an increase between day 5 and 10. Subsequently the number of bacteria decreased significantly and faecal coliforms could be considered absent after 40 days. The decrease in colony forming unit count was attributed to the temperature and moisture content of the mixtures.

The USEPA Part 503 Rule [#105, #106] recommends that biosolids samples for bacterial analysis are cooled promptly to <4°C, but not frozen, and analysed within 24 hours.

5.2.1.2 Conclusions

The current standards recommend that samples for bacterial analysis are refrigerated (2-5°C), not frozen, kept airtight and in the dark; analysis should commence within 6-8 hours. Current practice in commercial laboratories and published research is to keep samples under similar conditions to those recommended by the standards, but the time to analysis is generally longer: 24-48 hours. The evidence from the literature tends to support a longer maximum holding time than 6-8 hours.

It is recognised that cooling samples to refrigerator temperatures is not easily achieved in the field. However, this problem can be overcome if samples are transported quickly to the laboratory.

It is recommended that the new standards should take the following points into consideration:

- When ambient temperatures are above 10ºC, samples should be cooled as soon as possible after collection. This is particularly important for samples collected during warmer periods such as the summer months.

- Samples should be kept at 10ºC or less, preferably at 0-5ºC.
- Samples should be shipped to the laboratory without delay so that analysis can be completed within 24-48 hours after collection.
- Freezing should be avoided, especially samples with high water content. Temporary freezing at temperatures above -12ºC might not have a significant affect on bacteria numbers.
- Samples should be kept in the dark and measures should be taken to avoid changes in sample moisture content.
- Sample analysis should be prioritised such that bacteria, which are susceptible to change, are analysed first.

5.2.2 Viruses

5.2.2.1 Factors affecting virus survival

Most of the literature on viruses is aimed at laboratory methods for virus extraction and enumeration, animal and human epidemiology, persistence in the environment, investigation of indicator organisms and evaluating the hygienic function of treatment processes in wastewater treatment works. Information on bacteriophages in sludges is scarce [#58]. Most studies involving bacteriophages in sewage treatment plants have been concerned with evaluating the hygienic function of the treatment process [#39]. A problem with much of the research reported is that few experiments are conducted on sewage sludge or treated biowastes and most work is carried out on spiked samples.

Viruses can survive for extended periods in faeces, untreated and treated wastewater and sludges. Studies have demonstrated that persistence is dependent on several factors including the virus type, waste type, temperature and other environmental conditions and processes [#97].

Sobsey and Meschke [#97] produced a wide-ranging review of virus survival in the environment. They listed the main factors affecting survival in faecal material (Table 8).

Of the chemical constituents in liquid or semi-solid (faeces, human night soil, biosolids, animal manure, etc.) environmental matrices, the amount and types of organic matter and specific antiviral chemicals, such as ammonia at elevated pH levels (pH > 8.5), play a role in virus survival. Of the physical factors influencing virus survival in liquid media, temperature,

sunlight and virus associations with solids are among the most important factors influencing survival [#97].

Table 8 Important factors influencing virus survival in faeces and faeces-contaminated environmental media.

Reproduced from Sobsey and Meschke (2003) [#97].

Factor	**Effects**
Physical	
Heat or thermal effects	Increasing activation at higher temperature; pasteurise
Desiccation or drying	Increased inactivation at lower moisture content or relative humidity (RH); effects of RH differ between enveloped and non-enveloped viruses
Aggregation	Clumping protects viruses from inactivating agents
Adsorption to particles or surfaces	Adsorption protects viruses from inactivating agents; some specific chemical surfaces (heavy metals) are virucidal
Encapsulation or embedding	Viruses within membranes or larger particles are protected from inactivation
Chemical	
Hydrogen ions; pH	Viruses survive best at near neutral pH and worst at pH extremes
Organic matter	Many viruses are stabilised and protected by dissolved, colloidal and solid organic matter, including faecal organics and natural organic matter (humic materials)
Ammonia	NH_3 has virucidal activity; manifest at higher pH (>pH 8)
Salts and ionic strength	Increased concentrations of salts (e.g. sodium chloride) are antiviral for many viruses; some viruses are destabilised and inactivated by water lacking stabilising salts (such as NaCl) ions such as Mg^{++}
Enzymes	Proteases and nucleases contribute to virus inactivation
Biological	
Microbial activity	Biological treatment and microbial activity/metabolism in soils, sediments, water; several contributing mechanisms
Proteolytic activity	Proeolytic enzymes inactivate/denature virion proteins
Microbial predation	Engulfment, ingestion, etc. by protozoa, helminths, etc.
Biofilms	Virus adsorption too biofilms can be protective or microbial activity in biofilms can cause virus inactivation and degradation

Enteroviruses survive well in the environment. Eisenberg *et al*. [#31] reviewed survival times. Sorber & Moore (1986) reported that the half-life in soil ranges from 5 or 6 days to 30 days depending on various environmental factors such as temperature. Ahmed & Sorensen (1995, 1997) observed reduction rates in biosolids from 0.08 to 0.02 log10 reduction per day, again depending on a variety of environmental factors. Sludge treatment processes such as mesophilic-anaerobic-digestion have been shown to reduce enteroviruses by 1 to 4 log10 (Ahmed and Sorensen, 1997; Feachem, 1983; Sorber and Moore, 1986; Tata *et al*. 2000, all quoted in #31).

Viruses in human and animal faecal wastes and faeces-contaminated water generally are inactivated more rapidly at higher temperatures. At moderate and low temperatures and

intermediate pH (5-9) viruses can persist for considerable periods of time that may range from hours to days to weeks or even months in the case of the most persistent viruses. The rate of inactivation and the extent of survival vary depending on the virus type, temperature, sample matrix and other environmental conditions [#97].

Parker and Martel [#75] reviewed virus survival at low and freezing temperatures. Laboratory and field studies have shown that many viruses can persist for months in water and soil during cold weather, and that viral survival is greater at low temperatures (4-5°C compared to 22°C). Even at sub-zero temperatures, survival is greater than at warmer ambient temperatures. Most viruses can withstand freezing. Hurst *et al*. (1989 quoted in #75) found that, over a period 12 weeks, there was pronounced inactivation of three human enteroviruses (Coxsackievirus, echovirus and poliovirus) in surface freshwater at 22°C, but losses were reduced at 1°C and greatly reduced at -20°C.

Pesaro *et al*. (1995 quoted in #97) showed that, depending on ambient temperature, pH, and type of animal waste, the time required for a 90% (1 log 10) reduction of virus varied from less than 1 week for herpes virus to more than 6 months for rotavirus. Under conditions of higher temperature or drying conditions persistence was not as great. Temperature is one of the most important factors determining virus survival, survival decreasing as temperature increases, but the magnitude of the effect varies [#97]. In their review Sobsey and Meschke [#97] reported the results of several experimental investigations.

An investigation of the effects of temperature on the persistence of Aujesky's disease virus in pig slurry during anaerobic storage at 5, 20, 35, 40, 45, 50 and 55ºC showed that virus inactivation rates increased with increasing temperature. At 5ºC and 20ºC the virus was inactivated in 15 weeks and 2 weeks respectively. At higher temperatures the virus was inactivated in 5 hours or less (Botner, 1991 quoted in #97). The effect of temperature varies between viruses. Another investigation comparing Aujesky's disease virus and bovine enterovirus in liquid cattle manure showed that the enterovirus was more persistent (Biermann *et al*., 1999 quoted in #97).

Lasobras *et al*. [#58] carried out an investigation to obtain information on the persistence of three groups of bactoeriophages (somatic coliphages, F-specific bacteriophages, phages infecting *Bacterioides fragilis*) in de-watered, mesophilic-anaerobically-digested sludges at different storage temperatures. Containers were wrapped in foil to avoid the effect of light and placed at 4, 20 and 37ºC. Aliquots were analysed at 0, 10, 20 and 45 days. The results showed

that bacteriophages persistence depends on temperature with longer persistence at low temperatures. Only minor differences in numbers were found in sludge stored at 4ºC over a period of 45 days.

Ajariyakhajorn *et al*. [#3] investigated the effects of temperature on virus survival in pig slurry at three temperatures (4, 25, 37ºC). Samples were analysed at times 0, 1, 2, 4, 6 and 12 hours, and at 1, 2, 4, 6, 8, 10, 12, 14 days. The pH of the slurry was between 7.0 and 8.5 during the experimental period. The results are shown in Table 9.

Table 9 Effect of temperature on the survival of viruses in swine slurry.
Reproduced from Ajariyakhajorn *et al*. 1997 [#3]

		Survival at:		
Organism	**Fraction**	**4ºC**	**25ºC**	**37ºC**
Pseudorabies virus	Supernatant	8d	6h	2h
Pseudorabies virus	Sediment	8d	6h	2h
Porcine reproductive and respiratory syndrome virus	Supernatant	8d	12h	2h
Porcine reproductive and respiratory syndrome virus	Sediment	14d	1d	6h

Pesaro *et al*. (1995 quoted in #97) concluded that at ambient temperatures (<20ºC) thermal effects were only indirectly related to viral inactivation and that other factors were more directly contributing to virus inactivation.

In sewage sludge and sewage sludge eluates, indigenous viruses have been shown to remain stable (less than 1 log10 reduction) for up to 27 days at room temperature. Virus survival in a variety of media has been shown to be much longer at lower temperatures. Poliovirus survived for up to 66 days at 4ºC in digested sludge (Clark, 1961 quoted in #97). Canin coronavirus survives well frozen at -20ºC, but does not survive well above 4ºC (Tennant, 1994 quoted in #97). At temperatures of 2ºC or -70ºC viruses were shown to be stable for greater than 160 days (Hurst and Goyke, 1986 quoted in #97).

Ajariyakhajorn *et al*. [#3] demonstrated that virus survival (at 4°C) is affected by pH. They ran a set of experiments to test the effect on virus survival in pig slurry of three pHs (4.0, 7.0, 10.0). The results are shown in Table 10.

Table 10 Effect of pH on the survival of viruses in swine slurry.
Reproduced from Ajariyakhajorn *et al*. 1997 [#3]

		Survival at pH:		
Organism	**Fraction**	**4.0**	**7.0**	**10.0**
Pseudorabies virus	Supernatant	12h	4d	12h
Pseudorabies virus	Sediment	6h	8h	12h
Porcine reproductive and respiratory syndrome virus	Supernatant	2h	8d	<2h
Porcine reproductive and respiratory syndrome virus	Sediment	6h	14d	<2h

Light has both direct and indirect mechanisms of virucidal activity. The direct activity is likely due to radiation at wavelengths below 370 nm (ultraviolet radiation) being absorbed by proteins and nucleic acids. The most active UV wavelengths against viruses are in the ranges of UVB (280-320 nm) and UVC (185-280 nm) because wavelengths in the range 200-280 nm are highly absorbed by nucleic acids. UVA (320-400 nm) is the most abundant UV in sunlight, but it is less virucidal than the lower wavelengths [#97].

Fujioka and Yoneyama [#41] assessed the inactivating effect of sunlight on three human enteric viruses (poliovirus, Coxsackievirus, echovirus) using purified and washed cultures suspended in clean buffer or seawater. They found that in the absence of sunlight the viruses were stable for at least 6 hours, but under summer sunlight conditions the viruses suffered a 3 log10 activation reduction over the same time period. The viruses were inactivated by winter sunlight to a lesser extent, a 1 log10 reduction.

The effects of natural biological activity on the survival of viruses are well-documented in surface waters, faecal wastes and soils. Viruses consistently demonstrate less persistence in natural waters and other environmental media compared to the same media that have been sterilised or pasteurised. Bacteria and other microbial predators play a role in viral inactivation either through production of metabolites that adversely affect the virus particles or by direct use of the virus as a nutrient source [#97].

Many bacteria produce proteolytic enzymes that inactivate enteric viruses. Viruses may serve as a nutrient source for bacteria. It was concluded from a study of virus survival in mixed-liquor suspended solids of activated sludge that micro-organisms are responsible for virucidal activity because inactivation or removal of the micro-organisms caused a loss of virucidal activity. The most probable process is chemically mediated microbial antagonism due to the action of enzymes, the release of metabolic products and the use of viruses and their

components as a nutrient source [#97]. Therefore, samples should be kept cool to reduce bacterial activity.

The effect of relative humidity on the survival of viruses varies greatly with virus type. Tjotta (1991) found no difference in survival time for HIV in phosphate buffer with 2% bovine serum in solution or dried. Similar results were found for hepatitis B virus (Avero, 1974). However, results have been different for other viruses. Mahl and Sadler (1975) observed that viruses tended to survive longer at low relative humidity rather than mid or high humidity. Mahl (1975) tested Coxsackievirus, vaccinia, and adenovirus and found that they were minimally affected by humidity. Yet other viruses have been shown to be very sensitive to desiccation, for example rhinovirus (Buckland, 1962) and Herpes Simplex Virus type 1 (Bardell, 1994) [#97].

The USEPA Part 503 Rule [#105, #106] recommends that biosolids samples for virus analysis are either cooled promptly to <4°C and analysed within 24 hours or within 2 weeks if stored frozen. USEPA recommend that samples should be frozen immediately and stored at -70°C; freeze-thaw should be kept to a minimum [#17]. The draft standard, ISO/CD 19458 [#12] also recommends that samples for virus analysis can be kept at -70°C for up to one month.

5.2.2.2 Conclusions

The current standards make no specific recommendations for samples for virus analysis, only bacterial analysis. In this case they recommend that samples are refrigerated (2-5°C), not frozen, kept airtight and in the dark; analysis should commence within 6-8 hours.

Current practice in commercial laboratories is to analyse samples within a timeframe appropriate to the sample holding temperature. Samples kept at up to 25°C are analysed immediately (with 2 hours) and cooled samples are analysed within 24-72 hours. However, if samples are frozen the holding time can be extended to 2 weeks. There are few examples in published research for virus samples, but they generally cool samples and test within 24 hours of arrival at the laboratory.

The evidence from the literature tends to support a longer maximum holding time than 6-8 hours. It is recognised that cooling samples to refrigerator temperatures is not easily achieved in the field. However, this problem can be overcome if samples are transported quickly to the laboratory.

Extending sample holding time by freezing the samples has been demonstrated to be a viable option. Current practice is to analyse frozen samples within 2 weeks. This time period is also recommended by the USEPA for biosolids samples.

It is recommended that the new standards should take the following points into consideration:

- When ambient temperatures are above 10ºC, samples should be cooled as soon as possible after collection. This is particularly important for samples collected during warmer periods such as the summer months.
- Samples should be kept at 10ºC or less, preferably at 0-5ºC.
- Samples should be shipped to the laboratory without delay so that analysis can be completed within 24-48 hours after collection.
- Samples should be kept in the dark and measures should be taken to avoid changes in sample moisture content.
- Sample holding time can be extended to 2 weeks by freezing. Samples that are also to be analysed for bacteria should not be frozen.
- Sample analysis should be prioritised such that viruses, which are susceptible to change unless frozen, are analysed first.
- Provided sub-samples for viral analysis are stored frozen, viral analysis can be assigned a lower priority than bacterial analysis.

5.2.3 Helminth ova

5.2.3.1 Factors affecting helminth survival

The most common forms of sludge stabilisation are digestion, composting, pasteurisation, or the use of chemicals such as lime [#89]. Sludge treatment aims to kill pathogens by raising the pH, drying or the temperature-time effect of digestion or composting. One criterion for safe sludge disposal is the number of viable helminth ova present. These ova are ubiquitous and relatively resistant to most forms of treatment [#22]. Helminth ova are very resistant in the environment and their survival in soils for periods up to 6 months and one year have been reported [#43].

Ascaris suum, a pig pathogen, is very similar to *Ascaris lumbricoides*, a human pathogen [#51]. Experiments to assess ova destruction typically are done with *Ascaris suum* because they are one of the hardiest *Ascaris sp*. It is assumed that if *A. suum* ova are destroyed, then so are the ova of less hardy species. *Ascaris suum* ova are therefore used as ‘indicators’ for

inactivation studies of helminth ova in wastewater treatment systems [#22, #55].

Most investigative work on helminth ova concerns their destruction during sludge treatment or their loss of viability in the environment. However the aim of sample preservation and storage is to maintain viability until they are analysed.

The eggs of *Toxocara canis* are resistant to several chemical agents but are quite sensitive to desiccation and extremes of temperature [#24]. Plachy *et al*. (1995, #77) demonstrated that survival of *Ascaris suum* eggs in sludge depends on temperature, pH and dry matter content and chemical oxygen demand (COD).

Gaspard *et al*. [#43] studied the effect of different conditions that could influence the survival of eggs in various soil types. Storage temperature was found to be the most important factor. Having tested storage at 4ºC, 19ºC and 30ºC, survival was best at the lowest temperature with little difference in survival rate between 20ºC and 30ºC. The second most important factor was humidity. Eggs survived better deep in the soil where they were protected from drying.

Ascaris suum survival was studied in sludge drying beds at sewage treatment plants [#78]. Egg survival decreased with increasing exposure time and dry matter as the sludge dried. Drying bed temperature and air temperature significantly affected the viability of eggs.

It can be concluded that, in order to maintain ova survival samples should be kept cool and prevented from drying out.

The purpose of a study by O'Donnell *et al*. [#72] was to investigate the destruction rate of parasite eggs stored in sludge under controlled conditions to gain insight on their destruction rates in lagoons. Survival rates under long-term storage conditions were examined using samples of sludge and topsoil. Samples were stored at 4ºC, 25ºC and 'in ground', under ambient outdoor conditions and subject to normal temperature fluctuations. Samples were analysed at three-month intervals over a period of 33 months. Four species were studied: *Ascaris* spp., *Toxocara* spp., *Trichuris* spp. and *Hymenolepis* spp.

Destruction of eggs occurred, especially within the first 3 months, and the recovery rate decreased inversely with the storage temperature. Storage temperature had the greatest effect on egg viability and the effect varied between species. The greatest number eggs were recovered from the soil samples stored at 4ºC. Egg viability decreased very slowly when

stored at 4ºC. These results show that samples should be stored at 4ºC and that the storage period must be less than 3 months.

Holmqvist and Stenström [#51] investigated viability of *A. suum* during the composting process, but during the experiment two controls were kept at 4ºC; one was compost and the other was a suitable storage solution (no details given). At the end of the experiment (31 days) ova viability in the compost control had reduced to 60% of the initial level but the ova kept in solution showed no reduction in viability. Therefore compost samples should be stored at 4ºC for a period of less than 31 days.

Ascaris suum ova survival was assessed during storage of dewatered biosolids incubated for up to 62 days at various temperatures (5, 22, 38, 49.5ºC) under anaerobic and aerobic conditions [#2]. Destruction of pathogens occurred at all temperatures, but rates increased with increasing temperature. There was no statistically significant difference between aerobic and anaerobic sludges. At 50ºC the decay rate of *A. suum* eggs was estimated to be 0.21 log10 reductions per day.

Papini and Cardini [#74] kept unembyonated and embryonated *Baylisascaris transfuga* eggs at -20ºC or at +37ºC to study the effect of temperature on egg viability. Unembryonated eggs were viable when kept at -20ºC for 33 days, but those kept at 37ºC for 1 day underwent degeneration. Embryonated eggs survived better at the lower temperature and proved to be infective to mice for up to 33 days compared to 13 days when kept at 37ºC.

In view of the rapid degeneration of eggs at 37ºC and above, it would be prudent to cool samples as quickly as possible when ambient temperatures are high.

Sanin *et al*. [#89] explored the effect of freeze/thawing of sludges as a method of sludge conditioning and pathogen reduction. The effects of freezing temperature, freezing rate and time frozen on several pathogens were investigated. It was found that freezing produced a significant reduction of most of the pathogenic micro-organisms tested. However, freezing at -25ºC for 7 days had no effect on the viability of *Ascaris* spp. *Ascaris* spp. is known to be the hardiest of all helminths, so the results may not be transferable to other species. O'Lorcain [#73] found that embryonated *Toxocara cati* eggs exhibited a greater resistance to freezing than embryonated *Toxocara canis*, but only 22% of the eggs remained viable after freezing in the freezer compartment of a domestic refrigerator for 1 month. It appears that some helminth eggs may be able to survive short-term sub-zero temperatures, but freezing cannot be considered a feasible method of sample preservation.

No literature was found that discussed the effect of sunlight on helminth ova. However, Capizzi-Banas and Schartzbrod [#22] did investigate the suitability of irradiation as a method for disinfecting sludge. Two suspensions of *Ascaris* sp. ova were prepared fresh or stored in deionised water for two months at 4ºC. The freshly prepared ova were more proof against irradiation. Significant differences in D_{10} value (dose for 90% inactivation) were obtained for the two suspensions: 1125±145 Gy and 661±45 Gy for the fresh and stored suspension respectively. The effects of irradiation on the ova of living parasites mostly involve chemical changes but are also dependent on physical and physiological factors. Irradiation quality, dose rate and dose distribution are key physical parameters for ova survival. However, although storage increases the vulnerability of ova to radiation, temperature and moisture content are the most important physiological and environmental parameters [#22].

The USEPA Part 503 Rule [#105, #106] recommends that biosolids samples for helminth ova analysis are cooled promptly to <4°C, but not frozen, and analysed within one month.

5.2.3.2 Conclusions

The current standards make no specific recommendations for samples for helminth analysis, only bacterial analysis. In this case they recommend that samples are refrigerated (2-5°C), not frozen, kept airtight and in the dark; analysis should commence within 6-8 hours.

Current practice in commercial laboratories is to keep samples refrigerated at 4°C and analyse samples within either 5 days or 1 month. None of the published research provided detailed information on sample holding conditions.

The evidence from the literature supports a longer maximum holding time than 6-8 hours. Samples should be kept cool, but it is recognised that cooling samples to refrigerator temperatures is not easily achieved in the field. However, this problem can be overcome if samples are transported quickly to the laboratory.

If samples are cooled to <4°C, holding time can be extended to at least 2 weeks and probably 1 month. A holding time of 1 month is also recommended by the USEPA for biosolids samples.

It is recommended that the new standards should take the following points into consideration:

- When ambient temperatures are above 10ºC, samples should be cooled as soon as possible after collection. This is particularly important for samples collected during warmer periods such as the summer months.
- Samples should be kept at 10ºC or less, preferably at 0-5ºC.
- Samples should not be frozen.
- Samples should be kept in the dark and measures should be taken to avoid changes in sample moisture content.
- Sample holding time can be extended to 1 month if samples are kept cool.
- Sample analysis should be prioritised such that the organisms most susceptible to change are analysed first.
- If samples are kept cool 0-5°C, helminth enumeration is less urgent than bacterial or viral analyses.

5.2.4 Cysts and oocysts

5.2.4.1 Factors affecting cyst and oocyst survival

In the following paragraphs the term (oo)cyst is used when referring to both cysts and oocysts.

There is a large body of literature on oocysts and cysts but it is concerned mostly with water treatment methods (e.g. ozone and chlorine treatment), environmental surveys, infectivity and pathways. Investigations of *Cryptosporidium* oocysts and *Giardia* cysts in sewage sludge have been mostly confined to removal efficiency during sludge treatment. This not altogether surprising since the *Cryptosporidium* parasite was not recognised as an agent causing waterborne disease in humans until 1987 and the public health significance of *C. parvum* and *C. hominis* only became apparent in 1993 [#23].

Non-viable (oo)cysts that pose no threat to public health may be present in the environment, therefore it is imperative that (oo)cyst viability be determined [#23] and this is reflected in regulatory requirements. Consequently, every effort should be made to control those factors that could cause a loss of viability between sample collection and analysis. Reports of controlled experiments on sample holding time and temperature are limited, and most experimentation is done using *Cryptosporidium parvum*, but it is still possible to extract some information relevant to this review.

In general, *Cryptosporidium* oocysts and *Giardia* cysts are environmentally resistant. Important stresses include temperature extremes, freeze-thaw cycling and extreme water potential, especially desiccation [#110]. *Giardia* cysts and *Cryptosporidium* oocysts are rapidly inactivated on pastures and fields by desiccation, UV light and bacterial degradation (Sischo *et al*. 2000, quoted in #47).

Sunlight has been reported as being the single most important factor affecting the inactivation of bacteria in the environment (Chamberlain and Mitchell, 1978 quoted in #26). Nasser *et al*. (2003, quoted in #26) also reported that oocyst infectivity decreased significantly more rapidly in seawater and tap water in the presence of sunlight than in the dark. It has been demonstrated that both medium and low UV radiation is able to inactivate *C. parvum* oocysts in drinking water samples, whereby the oocyst remains intact, but non-infective (Bukhari *et al*., 1999; Craik *et al*., 2001 quoted in #23).

Temperature has a large effect on (oo)cyst survival [#23] and has been identified as the most influential factor (in the absence of sunlight) on oocyst inactivation in soil [#26].
Cool temperatures preserve (oo)cysts and it has been demonstrated that cysts can remain viable for almost 2 months at 0-2°C and oocysts for almost 6 months at 4°C. Higher temperatures reduce viability and (oo)cysts are effectively inactivated at 54-55°C [#85].
Rapid freezing inactivates oocysts in comparison to the slow freezing that is typically found in the natural environment [#23].

Robertson and Gjerde [#86], while acknowledging that it is preferable to complete analysis as soon as possible after sample collection, found that in many instances it was practically impossible to comply with the sample holding times specified in the U.S. Environmental Protection Agency methods (Method 1622 and Method 1623). Samples frequently failed to reach the laboratory within 24 hours and samples often could not be shipped on the day of collection. In many of these instances, the temperature at which the samples were held could not be regulated.

To test the viability of (oo)cysts in their samples, Robertson and Gjerde [#86] conducted an investigation of sample holding time for water samples comparing storage at 0-4°C in the dark with room temperature (18-22°C) in the light and sampling 24h, 48h, 72h, 1 week and 2 weeks after spiking. They found no significant difference in the numbers of *Cryptosporidium* oocysts or *Giardia* cysts over the two-week period or between treatments and concluded that the holding method for *Cryptosporidium* or *Giardia* is less critical than the standard (U.S.A.)

methods suggest. They proposed that the holding time could be extended to 2 weeks if necessary and that keeping the sample cool might not be as critical as suggested.

Robertson *et al*. (1992, quoted in #23) showed that storage time was an important factor in survival. After 178 days storage at 4ºC, 78% of the oocysts in samples of human faeces had died. Jenkins *et al*. (2003, quoted in #23) found that *C. parvum* oocysts remained infective for seven months when incubated in a circulating water bath at 15ºC, a temperature commonly encountered in the environment.

Increases in temperature have been shown to accelerate oocyst degradation in fresh water and seawater. Pokorny *et al*. [#79] found that as temperature increased from 4 to 23ºC the duration of *C. parvum* oocyst infectivity decreased. However, although warmer temperatures can accelerate degradation, oocysts are known to remain infective for up to 12 weeks when stored in water at 25ºC (Fayer *et al*., 1996 quoted in #23).

Sherwood *et al*. [#92] stored *Cryptosporidium* sp. in three different media (distilled water, phosphate-buffered saline, 5% bovine serum albumin) at 4ºC and in phosphate-buffered saline at 15-20ºC and 37ºC. There was a progressive loss of infectivity in all media at 4ºC with no detectable infectivity in distilled water after 2 months. Complete loss of infectivity occurred at 15-20ºC within two weeks and at 37ºC within 5 days.

Freezing has been investigated as a method of sample preservation. *Cryptosporidium parvum* oocysts have been shown to be resistant to freezing, depending on the temperature and duration of freezing. In general, cysts can endure very low temperatures, especially if they are partially desiccated, but in aqueous environments cysts are less resistant to very low temperatures because the water that is contained in them may crystallise [#75]. Survival rate is low under conditions of rapid freezing and thawing, but greater when frozen and thawed slowly [#75].

Fayed and Nerad [#40] evaluated *Cryptosporidium parvum* oocyst survival at various temperatures. Oocysts were suspended in deionized water and stored at 5ºC or frozen at -10ºC, -15ºC, -20ºC and -70ºC for up to 168 hours then thawed at room temperature (21ºC). Oocyst viability decreased as the freezing temperature decreased and as the storage time increased. No viable oocysts were found in samples frozen at -70º.

Thawed oocysts appeared similar regardless of the temperature at which they were frozen, but more detailed analysis showed that freezing at -70ºC produced the most oocysts with cracked

walls. The authors concluded that freezing at any of the temperatures tested for even the shortest period rendered a portion of the oocysts non-infectious. From these studies it appeared that freezing, rather than prolonging infectivity, had destroyed infectivity.

These results are supported by Robertson *et al*. (1992, quoted in #23) who found that 67% of *Cryptosporidium parvum* oocysts were dead after 21h and 100% after 152h freezing at -22ºC. Other studies have also reported that oocysts were not infectious after thawing (e.g. Ernest, 1986; Fayer *et al*., 1991, quoted in #40).

Walker *et al*. [#110] examined the effect of freeze-thaw (-14 to 10ºC) cycles on *C. parvum* survival. The effect of freeze-thaw cycling was extreme and about four times the effect of freezing alone. The damage increased as the oocysts were subjected to more freeze-thaw cycling events. The damage was possibly caused by mechanical damage to the oocyst wall.

Although sunlight and temperature have been identified as the most important influential factor on oocyst inactivation, other effects are also important.

Oocysts are susceptible to inactivation by desiccation. Robertson *et al*. (1992, quoted in #23) found that following 2 hours of air-drying at room temperature (12-20ºC) on glass slides only 3% of *C. parvum* oocysts remained viable and after 4 hours more than 99% of the oocysts were dead. Udeh *et al*. [#103] observed that no oocysts survived after 24 hours air-drying on glass slides at room temperature (20°C).

Biological interactions also affect oocyst survival. Olsen *et al*. (1999, quoted in #23) noted that oocysts were inactivated more rapidly in natural soils as compared to autoclaved soil.

Walker *et al*. [#110] tested the hypothesis that the interaction between temperature and water potential stresses enhances *C. parvum* oocyst degradation, leading to rates of population decay that are higher than those previously reported and used for risk assessment. They investigated three exposure times (1, 15, 29 days), three temperatures (-14, 4 and 30ºC) and three levels of water potential (-4, -12 and –33 bars). The results indicated that, with increasing water potential stress, estimates of the degradation rate increase. Degradation rate also increased with increased temperature. Water potential stress enhanced the degradation rate even under minor temperature stress.

Jenkins *et al*. [#54] studied the effects of soil type, temperature and moisture on the inactivation rates of *C. parvum*. Oocysts were incubated in three soil types (silty clay loam,

silt loam, loamy sand) at three temperatures (4ºC, 20ºC and 35ºC) and three soil water potentials (-0.033, -0.5 and –1.5 MPa). At the higher temperatures non-irradiated and gamma-irradiated soils were compared. The soil water potentials investigated did not affect oocyst inactivation at any temperature. Rates of oocyst inactivation increased significantly between 4 and 20ºC, but not between 20 and 30ºC.

Davies *et al*. [#26] considered inactivation of *Cryptosporidium* oocysts in soil in the absence of sunlight under a range of temperature, moisture and biotic status regimes (± gamma-irradiation). The moisture characteristics of the soil and the biotic status appeared to have little effect on the inactivation rate. In the absence of sunlight, temperature was the most influential factor affecting oocyst inactivation. The inactivation rate was significantly different at the three different temperatures with greatest inactivation at 35ºC and the least inactivation at 4ºC. The difference between inactivation rates was greatest between 35ºC and 20ºC than between 20ºC and 4ºC.

5.2.4.2 Conclusions

The current standards make no specific recommendations for samples for (oo)cyst analysis, only bacterial analysis. In this case they recommend that samples are refrigerated (2-5°C), not frozen, kept airtight and in the dark; analysis should commence within 6-8 hours.

No details were found for current commercial laboratory practice. Details in published papers were sparse, but when samples were kept cool (4°C) they were analysed in either 2 weeks or 2 months. One paper quoted analysis within 24 hours for samples kept at <12°C.

The evidence from the literature supports a longer maximum holding time than 6-8 hours. Samples should be kept cool, but it is recognised that cooling samples to refrigerator temperatures is not easily achieved in the field. However, this problem can be overcome if samples are transported quickly to the laboratory.

If samples are cooled to <4°C, holding time can be extended to at least 2 weeks and probably 1 or 2 months. A holding time of 1 month is also recommended by the USEPA for biosolids samples.

It is recommended that the new standards should take the following points into consideration:

- When ambient temperatures are above 10ºC, samples should be cooled as soon as possible after collection. This is particularly important for samples collected during warmer periods such as the summer months.
- Samples should be kept at 10ºC or less, preferably at 0-5ºC.
- Samples should not be frozen.
- Samples should be kept in the dark and measures should be taken to avoid changes in sample moisture content.
- Sample holding time can be extended to 1 month if samples are kept cool.
- Sample analysis should be prioritised such that the organisms most susceptible to change are analysed first.
- If samples are kept cool, 0-5°C, (oo)cyst enumeration is less urgent than bacterial or viral analysis.

5.3 Containers

5.3.1 General considerations

All samples should be taken in an appropriate container that is fit for purpose. They must be non-toxic, protect the sample from spillage or contamination and be large enough to contain enough sample material for all the planned analyses, taking into account the volume required for each of the analytical tests to be carried out on the sample and the number of replicate analyses. The size and shape of the container may need to be taken into account if the samples are to be preserved and stored prior to further processing [#6].

Guidance for container selection provided in standards varies greatly in detail. EN 12579:2000 [#9] simply states that containers for microbiological testing should be sterilised. Pr CEN/TR 15310-4:2004 [#14] provides detailed guidance on selecting suitable containers, listing the factors to be considered when choosing a suitable container, including the shape and size of the container. No details are provided on how to sterilise, although the standard recommends that containers are sterilised.

Guidance on container selection in the standards for water sampling is spread across the suite of standards, each standard providing bits of advice. EN ISO 5667-13:1997 [#5] directs the reader to EN ISO 5667-3 for specific guidance, but gives some general requirements for a suitable container. EN ISO 5667-13:1997 cautions against the use of glass containers where gas build-up might be an issue and states that double polyethylene bags are acceptable, except for trace organics.

EN ISO 5667-16:1998 [#6] points out that it is a standard for biotesting of samples, not bacteriological testing. However, it does provide detailed guidance on the factors to be considered when choosing a container: container volume, shape and materials, the number of analyses, replicates and repeat analyses that might be required, how much sample is required for each test and how much storage space is required to hold the samples.

EN ISO 5667-3:1995 [#4] does not provide detailed information on container selection, other than containers have to withstand the sterilisation process, they should be free of compounds that could affect microbial activity and that glass is not suitable for freezing samples. This standard has been replaced by EN ISO 5667-3:2003 [#11] and EN ISO 5667-15:1999 [#7].

EN ISO 5667-15:1999 [#7] recommends the use of sterile glass containers, but states that disposable commercial plastic containers could be used, provided they were verified not to interfere with analyses. EN ISO 5667-3:2003 [#11] provides detailed guidance on container selection and cleaning using solvent washing or acid washing, but provides no information about sterilisation.

There are several physical and chemical characteristics to consider when selecting a suitable container for shipping and storing samples. These include the container materials and it size, shape and method for ensuring a proper seal. Sample containers must provide reasonable assurance of maintaining physical integrity against breakage, rupture or leakage during handling, transport and storage [#6, #107]. Sample containers should:

- Be appropriately clean, e.g. sterile for pathogens
- Be made from appropriate materials, e.g. glass, plastic, metal
- Be of appropriate colour, e.g. typically brown or amber glass is used to preclude light
- Be sufficiently large to contain enough material for all the required analyses
- Have a large enough opening to get the sample in and out with ease
- Have a secure closure made of appropriate material. The closure may need to withstand internal pressure increases.

5.3.2 Sterility

Containers should be clean and dry. Existing standards (Table 4) and most laboratory websites (Table 5) and research papers (Table 6) report or recommend the use of sterile containers. However it could be argued that for most sludge sampling purposes it is usually sufficient to use suitably sized clean containers and proof of sterility may not be essential owing to the nature of the material being sampled [#99]. Where non-sterile containers are

used it may be appropriate to include a portion of blank samples to demonstrate that the containers do not make a significant contribution to the result [#99]. The USEPA [#106] recommends conservative microbiological practice such that containers are sterilised for all treated sludges. However, in the absence of evidence for or against the practice, it may be prudent to err on the side of caution and recommend that containers for biosolids and treated biowaste should be sterile and subject to the same quality control procedures as for water sample containers. Certainly, if sludges have received enhanced treatment, it would be advisable to use sterile containers.

At least two standards [#4, #14] caution against re-use of containers if it can be avoided. New, disposable plastic containers are sufficient for sludge sampling requirements [#99]. Food quality plastic containers are used by at least one water company in the UK (pers. comm.). If sample containers are to be reused they should be both easy to clean and sterilise. The sample material should be suitable for the chosen sterilisation method if required.

There are three alternative methods for obtaining clean and sterile containers:

- Purchase pre-cleaned or sterilised containers [#96]. If pre-sterilised containers are used they should be supplied with adequate records of their sterility and the manufacturer's expiry or use-by date [#99].
- Obtain the containers from a sub-contracted laboratory with approved container cleaning and handling protocols [#96]. This would probably be the laboratory carrying out the sample analysis.
- The containers are cleaned and maintained by the sampling organisation [#96].

If sample containers are to be reused they should be both easy to clean and sterilise. The sample material should be suitable for the chosen sterilisation method if required. Any cleaning and sterilisation process should not induce, or accelerate, corrosion or degradation of the container material.

There are a number of methods of sterilising containers e.g. autoclaving, oven sterilisation, ethylene oxide, gamma rays. Methods for cleaning containers are covered in the Standard Operating Procedures of the Florida Department of Environmental Protection [#96]. Container sterilisation is also covered in the draft standard ISO/CD 19458, *Water quality-Sampling for microbiological analysis* [#12], which specifies methods for sterilising by autoclaving or heating in a dry oven. Three alternative methods (immersion in boiling water,

ethylene gas or gamma irradiation) are provided as notes [#12]. This draft standard also includes advice on inactivation of disinfectants and quality control of sample bottle sterility.

5.3.3 Container materials

There is a large number of container material types on the market to choose from including low-alkali borosilicate glass (e.g. Pyrex®, Corex®), other non-corrosive glass, plastics (e.g. high density polyethylene, low density polyethylene), polystyrene, polycarbonate, polyvinyl chloride (PVC) polymethylpentene, polypropylene or polytetrafluoroethylene (PTFE, Teflon®) [#107]. Some of these are not suitable for sludges and treated biowastes because they cannot withstand pressure build-up and some are not suitable when samples are to be subjected to microbiological analyses because they may leach chemicals that affect microbial survival. The materials should be free of substances that are inhibitory or toxic to micro-organisms [#99]. It should be noted that container materials that are suitable for microbiological analysis might not be appropriate for other analyses. For example, plastic containers are unlikely to be suitable for samples that are to be analysed for organic compounds. In this case multiple containers should be used to preserve sample integrity as necessary.

Glass containers (jars or bottles) are not usually recommended for sludges because they are easily broken, particularly if there is a pressure build up from gases emitted from fermenting samples [#98, #99, #102]. This may also apply to certain polystyrene containers [#99]. If the container becomes pressurised there is a risk of explosion causing harm through flying glass and infectious material and/or pathogenic aerosols. Detailed guidance on dealing with the risk of gas build up is given in EN ISO 5667-13:1998 [#5] and EN ISO 5667-15:1998 [#7].

The US Composting Council [#104] recommends that for most feedstock or compost samples, containers can be made of stainless steel, plastic, glass or Teflon® because they will not change compost chemical quality. However, there are no specific recommendations for containers for microbiological quality.

Micro-organisms have been shown to be inactivated by sunlight; therefore containers should be made of materials that exclude as much sunlight as possible. For example, if glass containers are used, they should be amber glass, not clear. Alternatively, the containers should be wrapped in materials that exclude light, such as aluminium foil or kraft paper or kept in the dark (e.g. a cool-box during transportation).

ISO/CD 19458 [#12] provides a list of container types and the advantages and disadvantages of each type.

5.3.4 Opening and closure

A suitable container should be shaped appropriately for the purpose [#107]. Wide mouthed containers are preferred because they are easier to fill and to extract sub-samples in the laboratory with less risk of cross-contamination. Lid options include plastic or metal screw caps or plastic press-on lids. The lid should provide a good seal to prevent any leakage or drying out of the sample and must also be non-toxic to the sample. Metal caps can cause toxicity problems; especially those made from aluminium.

Press-on plastic lids attached to the bottle or jar are often recommended because they facilitate filling and pipetting, so reducing the risk of cross-contamination. However, press-on lids may not be suitable for liquid samples because they do not ensure a proper seal [#107].

5.3.5 Conclusions

It is recommended that containers for biological analysis:

- Preferably should not be re-used. When re-use is necessary the containers should be chosen such that they are easy to clean and sterilise.
- Should be sterilised with traceable quality control procedures.
- Preferably should be made of plastic, but other materials can be used if they do not affect the microbiological quality of the sample. Glass containers should not be used unless care is taken to avoid gas pressure build-up.
- Should be large enough to contain enough material for all the analyses.
- Should have a large opening to enable easy filling and extraction of sub-samples.
- Should have a secure closure that can be cleaned and sterilised to the same quality as the container.
- Should be made of materials that exclude sunlight, wrapped to exclude sunlight or kept in the dark.

The current standards cover all of these recommendations, but no single standard covers all of the points relative to hygienic parameters. A new standard could be produced by extracting the necessary information from existing standards.

Current advice is that sample containers should be sterilised before use. Sterilisation may not be necessary for all samples, for example sludges with high bacterial populations, but no evidence was found in the literature to support either sterilising or not sterilising containers.

Relaxing the requirements for sterilisation could produce a saving on sampling costs and it is recommended that it should be investigated. Depending on the results of any such investigation, it may be possible to advise that sterile containers are necessary only where the analytical sensitivity requires a zero background; for example, the examination of advanced treated sludges.

5.4 Packing and transporting samples

5.4.1 General introduction

Once the sample is collected it should be transported hygienically under strict conditions of time and temperature to preserve its microbiological status [#61]. At all times correct handling and documentation procedures should be followed. A well-documented procedure should be used to control transport and prevent damage to the samples.

Samples should arrive at the laboratory at the correct temperature and unharmed. Packaging and transport conditions should therefore be carefully specified to assure that the microbiological condition of the sample is not significantly changed. The maximum temperature during transport should be specified.

Of the water sampling suite of standards, EN ISO 5667-3:2003 [#11] has the most detail on sample preservation and transportation. There is a section that discusses the general considerations for sample preservation. This part of EN ISO 5667 describes the most commonly used preservation techniques, in particular cooling and freezing of samples. The section on cooling or freezing of samples is the most detailed provided in the standards reviewed, although much of the guidance is specific to water samples.

Guidance on sample preservation and transportation appears in other parts of EN ISO 5667, but it is much less detailed (Part 13 [#5], Part 15 [#7] and part 16 [#6]). In each of these standards the guidance is essentially the same, with some variation in wording. The sample should be kept at a temperature lower than that during filling. Cooling of samples is only truly effective if applied immediately after sample collection. Use cool boxes with melting ice or refrigerators (2-5°C) in vehicles at the sampling site. Store the samples in the dark. Store samples in a cool place, preferably at a temperature lower than that prevailing when the sample was taken, giving due regard to local climatic conditions. Samples should be protected from breakage, external contamination and temperature increases. Melting ice can spoil

identification labels that are not waterproof. If the travel time exceeds the recommended time to analysis, this should be noted.

EN 12579:1999 [#9] simply states that samples should be despatched as soon as possible, no more than two days after sample collection. EN 13040:2000 [#10] provides further guidance, for example keeping the sample at 1-5C, but not frozen. However, neither of these standards is concerned with taking samples for biological analysis.

Pr CEN/TR 15310-4:2004 [#14] guides the reader through a selection process, starting with a discussion of sample preservation in general, and giving consideration to the factors that cause change in samples and common methods of preservation. Specific instructions are given for sample preservation for different components (e.g. volatile components), but not for biological determinations. However, this document does provide guidance on preserving different types of samples, such as liquids, sludges, like substances and solids. Further detail is given for each of the preservation methods, for example, how to keep a sample airtight, in the dark or cool. No guidance is given on how to pack and transport samples.

5.4.2 Sample protection

Samples should be tightly sealed, packed upright to prevent leakage and cushion from shock [#64]. Packing material should be used to prevent the containers shifting during transport, which may cause them to leak or break.

5.4.3 Sample temperature

It is important that samples for microbiological analysis are kept cool so the sample does not deteriorate and the analytical results are representative of the material that was sampled. The US Composting Council [#104] recommends that, although a wide range of sample holding times and conditions may be required for different analyses, all compost samples should be chilled immediately upon collection. This is a sensible precaution to keep microbial activity to a minimum. However, care should also be taken to prevent the samples from freezing. Temporary freezing may eliminate freeze-sensitive cells of *Clostridium perfringens* or reduce the contamination of other enteric pathogens [#61].

The purpose of chilling the samples is to slow down biological activity and minimise changes in the sample after sample collection and before laboratory analysis. When a sample arrives at an inappropriate temperature, for example, when parts of the sample are frozen or chilled samples arrive at unacceptably high temperatures, the interpretation of any analysis is difficult. Lightfoot and Maier pointed out that, in some cases, it is possible that samples that

are not appropriately temperature-controlled should not be analysed; although this was in reference to food and water samples that could be the subject of litigation [#61].

Similarly, it is possible that sludge and treated biowaste samples collected for regulatory purposes should not be analysed if they are not appropriately temperature-controlled. However, such a decision is a matter for regulatory direction and not within the scope of a standard, which aims to recommend prescribed time and temperature conditions based on the best available scientific evidence. However, regulations could require compliance with such a standard.

The temperature of the sample has to be controlled in two different environments: during transportation to the laboratory and in laboratory storage awaiting analysis. It is easier to maintain sample temperature in the laboratory since usually there are storage refrigerators specifically for this purpose. It is more difficult to attain and maintain controlled temperature conditions during transportation. Therefore this is the period when samples are most at risk from temperature-induced changes.

It is recognised within the EN ISO 5667 suite of standards that cooling samples to 0-5°C in the field is not practicable without refrigeration. In this case it is recommended that samples are stored in a cool place, preferably at a temperature lower than that prevailing when the sample was taken, giving due regard to local climatic conditions.

It has been pointed out [#102] that ambient temperature in Europe can range from 40ºC in the summer in Greece to -20ºC in winter in Finland. All the literature looked at was concerned with keeping samples cool. No literature was found that addressed the alternative scenario: what to do when the sampling conditions are below freezing.

Samples typically are transported in small refrigerators or refrigerated vans or in insulated cool boxes with coolant materials such as wet ice or ice packs. Regardless of the method used, samples cannot instantaneously be cooled to 4ºC, the most frequent recommended temperature. The rate of cooling will be determined by many factors including: the thermal properties of the sample matrix, sample volume, container shape and material, cooling method, ratio of coolant to sample, properties of the packaging materials, initial sample temperature, ambient temperature.

Any change in the temperature of the samples depends on the cooling rate and time. If time is kept short then, unless the cooling rate is fast, the temperature change will be minimal. Therefore, if samples are quickly transported to the laboratory temperature control may be less of an issue. It is not possible to define how short this time period should be. There is a paucity of reports of sample temperature investigations and no report has been found that explores this period of sample holding. When details are provided, method sections of research papers report that samples are transported to the laboratory within 1-2 hours [e.g. #52, #58, #67, #93]. The transportation time will be more critical when the ambient temperature is high, for example during summer months in southern European countries.

Samples that cannot be delivered to the laboratory within a short time, say 1-2 hour, will need to be cooled. This can be achieved by putting the sample into refrigerated conditions or by adding cooling materials to the packing boxes with the samples. When long delays in shipment to the laboratory are anticipated, it is recommended that individual samples should be stored in a refrigerator until they are transported [#104]. Samples are likely to cool more rapidly under refrigerated conditions. Once samples arrive at the laboratory they should be transferred to a refrigerator for storage until processing.

Dry ice should never be used to cool samples for microbiological analysis. Commonly used cooling materials include wet ice, ‘blue ice’, ‘picnic packs’, ‘cool packs’, ‘ice packs’ and refrigerant gel packs. Each has advantages and disadvantages. (NOTE: the following text discusses these)

When loose ice melts, the contents of the cooler are free to shift, potentially allowing contamination of the samples. If ice is used it should be bagged, preferably double- or triple-bagged to guard against spillage should the inner bag split or leak. Any leakage could contaminate the samples.

Artificial ice packs (picnic packs, cool packs, etc.) exert a greater cooling effect than true ice and do not melt [#12]. This means there is less risk of labels becoming detached, ink marks being obliterated or sample contamination. However, some types of artificial ice pack could cause localised freezing if they are in direct contact with samples.

No literature was found that looked at keeping sludge or treated biowaste samples cool during transport. Those few papers that did discuss or investigate the topic investigated water samples.

In 1998, an inter-laboratory study was carried out in Hawaii to investigate analytical proficiency for microbiological examination of water samples [#16]. Sample temperature was monitored during transport to the laboratories. Four of the nine laboratories taking part in the trial reported transport temperatures at or above 10ºC. Although the results were not statistically different for *Clostridium perfringens* or faecal coliform, it was found that *enterococci* counts were consistently biased lower for laboratories with the longest transport time.

Pope *et al*. [#80] monitored the temperature of water samples kept cool with different methods. The aim was to use 'real world' storage practices so they tested wet ice and UTEK® refrigerant gel packs. The samples were collected from seven sites within a two-hour drive of the laboratory. An iButton® temperature-logger, set to record at 15-minute intervals, was placed in an identical filled container with each batch of samples. In general, samples temperature was maintained at <10ºC and above freezing. However, they observed that sometimes the samples froze during storage regardless of whether wet ice or gel packs were used. Some of the frozen samples showed a significant decrease in *E. coli* concentration, which supports the argument for taking precautions to prevent samples from freezing.

At least one organisation [#82] recommends that blue ice or other types of commercial freezing containers that have freezing points below 0ºC are not used because the low temperatures can cause bottles to freeze resulting in ruined samples or broken bottles. Other organisations specify that wet ice shall be used in cooling samples to 4ºC and that dry ice, blue ice and chemical cooling packs are not acceptable [#70, #96].

Ice and ice packs work by absorbing heat from the sample and the air around the sample. Heat is transferred also from the air outside the insulating packaging e.g. the cool box. This heat transfer will cause the coolant materials to defrost and warm up. Once the cooling materials have reached an equilibrium temperature with the samples they are no longer effective. The time to reach this point will depend on a number of factors, as listed above in relation to the rate of cooling.

When transporting samples to the laboratory, the main points to consider with respect to temperature control are how long it will take to transport the samples to the laboratory (a function of distance and travel conditions) and ambient temperature conditions. Using this information the necessary arrangements to control sample temperature can be made:

- If samples cannot be transported immediately, keep them stored under controlled conditions, such as a refrigerator.

- When transporting the samples keep them as cool as possible by packing with coolant materials.
- Consider the risk to the samples from the cooling materials that may be caused by cross-contamination or by freezing and take appropriate preventative action.
- Consider how long the cooling method will be effective, taking into account the ambient temperature, the thermal properties of the cooling system and the coolant-to-sample ratio.

5.4.4 Cross contamination

All packaging and coolant materials are a potential source of cross-contamination. Cool boxes should be kept exclusively for the purpose, kept clean and dry, particularly on the inside, and should be regularly disinfected [#99]. Van storage racks, boxes or coolant packs should be similarly treated.

5.4.5 Chain of custody

All samples should be uniquely labelled and accompanied by the correct documentation. The labelling and documentation that is required is independent of the type of sample or analysis and is extensively covered in existing standards [#4, #5, #7, #9, #14].

5.4.6 Sample transportation time

The sample should spend as little time as practically possible in transport and laboratory reception [#61]. This is the period when samples are at risk of breakage and when temperature is difficult to control unless refrigerated transport is used.

The draft ISO standard, ISO/CD 19458, *Water quality – Sampling for microbiological analysis* [#12], prepared by ISO TC 147/SC 4, provides extensive advice on transporting samples to the laboratory. Although this is a standard for water sampling, the same principles apply to transportation of biosolid and treated biowaste samples.

However, if samples are packed in insulated boxes with ice or ice packs the temperature of the samples will probably not be any higher than ambient.

5.4.7 Conclusions

The critical factors in packaging and transporting samples for microbiological analysis are sample safety, hygiene, temperature and time.

- All recommendations are that samples are cooled to 0-5°C as soon as possible after collection.
- It is not always possible to cool samples to 0-5°C in the field. In this case the temperature should be reduced as much as possible and the delay in delivery to the laboratory should be minimised.
- If there are unavoidable delays in transport to the laboratory the samples should be stored under refrigerated conditions.
- Other than for virus analysis, the samples should not be frozen.
- The method of cooling should take into account the sample-coolant ratio and the effect on the sample with respect to localised freezing, damage to sample identification labels and cross-contamination.
- Samples should be uniquely labelled and accompanied by chain of custody documentation.

All of these points are covered in existing standards, although the standards vary in how much detail is provided.

Standards provide advice on what points to consider for keeping samples cool during transport to the laboratory: ambient and sample temperature, sample container size and shape, ice box volume and characteristics, mass of ice (or other coolant). However, the evidence for and against different coolants is conflicting and there is no method of deciding how much coolant to use.

All the advice provided by standards is aimed at reducing the temperature of samples as quickly as possible. Whilst cooling samples is a problem during summer months, particularly at low latitudes, it is possible that sample freezing could be a problem in winter months, especially at high latitudes and altitudes. None of the standards consider this possibility and no reports could be found in the literature.

6 Critical Points and Recommendations

6.1 Current standards

6.1.1 Only one standard, EN ISO 5667-3:1995 [#4], lists separately the microbiological parameters to which it applies. The other standards refer variously to 'biological, not microbiological analysis', 'bacteriological examination' or 'microbial testing'. However, EN ISO 5667-3:1995 has been superseded by EN ISO 5667-3:2003 so, in effect, no standard for sludges or treated biowaste refers to the full list of microbiological parameters that current and proposed EU Directives set out to control.

6.1.2 A draft standard, ISO/CD 19458:2003 does include individual holding times for a wide variety of organisms, but for water samples not sludge or treated biowaste.

6.1.3 In the current standards the preservation and storage techniques are essentially the same for all hygienic parameters, primarily because they are grouped together under one heading. These are keep the sample cool (1-5ºC or 2-5ºC), in the dark, not frozen or dried, and do not subject to extreme temperatures. These instructions do not take into account any variability between parameters; for example, it is acceptable to freeze samples for viral analysis.

6.1.4 Recommended storage times in current standards for sludge and treated biowaste samples are based on times for water samples, a criticism that has frequently been levelled at the standards. Standards for sludge and treated biowaste need to take into consideration that the matrices have different physical, chemical and biological properties to water.

6.2 Containers

6.2.1 It is recommended that containers for biological analysis:

- Preferably should not be re-used. When re-use is necessary the containers should be chosen such that they are easy to clean and disinfect.
- Should be sterilised with traceable quality control procedures.
- Preferably should be made of plastic, but other materials can be used if they do not affect the microbiological quality of the sample. Glass containers should not be used unless care is taken to avoid gas pressure build-up.
- Should be large enough to contain enough material for all the analyses.
- Should have a large opening to enable easy filling and extraction of sub-samples.

- Should have a secure closure that can be cleaned to the same quality as the container.
- Should be made of materials that exclude sunlight, wrapped to exclude sunlight or kept in the dark.

Although the current standards cover all of these recommendations, no single standard covers all the points. It is recommended that the necessary information is extracted from existing standards to produce a new document.

6.2.2 Light can inactivate viruses and the effect varies with the wavelength of the light. The current standards provide advice on choosing containers that prevent light penetration, for example brown or amber glass. As an alternative to glass, there are many plastic containers that could be used and current standards point out that certain chemicals could leach from plastics that affect micro-organism viability. There is still a degree of transparency through some apparently opaque materials, which could be important in Southern Europe. The advice should be extended to include checking that any plastic used absorbs the relevant wavelengths. Of most concern are wavelengths below 370 nm (ultraviolet radiation), paricularly UVB (280-320 nm) and UVC (185-280 nm).

6.2.3 Current advice is that sample containers should be sterilised before use. Sterilisation may not be necessary for all samples, for example sludges with high bacterial populations, but no evidence was found in the literature to support either sterilising or not sterilising containers. Relaxing the requirements for sterilisation could produce a saving on sampling costs and it is recommended that the necessity to sterilise containers should be investigated.

6.3 Sample transportation

6.3.1 The critical factors in packaging and transporting samples for microbial analysis are sample safety, hygiene, temperature, time and chain of custody (including labelling) . All of these points are covered in existing standards, although the standards vary in how much detail is provided.

6.3.2 Maximum holding time is the time between sample collection and analysis, which is the sum of the time to deliver the sample to the laboratory and storage time at the laboratory. This is not made clear in the current standards, but it is important because it is more difficult to control temperature outside the laboratory and, unless refrigerated vans are used, transportation time becomes important.

6.3.3 All recommendations in current standards are to cool samples to less than 5ºC (without freezing) as soon as possible after collection. However, it is not always possible to achieve such a low temperature without refrigeration, particularly when ambient temperatures are high. EN ISO 5667-3:2003 [#11] points out that the purpose of coolants during transport is to reduce the temperature of the air surrounding the sample and prevent the sample temperature from increasing.

6.3.4 All the advice provided by standards is aimed at reducing the temperature of samples as quickly as possible to minimise changes, including deterioration. Whilst cooling samples is a problem during summer months, particularly at low latitudes, it is possible that sample freezing could be a problem in winter months, especially at high latitudes. None of the standards consider this possibility and no reports could be found in the literature. It is recommended that further investigation of the possibility of freezing is undertaken and the results are reflected in any new standard.

6.3.5 Standards provide advice on what points to consider for keeping samples cool during transport to the laboratory: ambient and sample temperature, sample container size and shape, ice box volume and characteristics, mass of ice (or other coolant). However, the evidence for and against different coolants is conflicting and there is no method of deciding how much coolant to use. There is some evidence that localised freezing of water samples can be caused by certain coolant packs. As a minimum, standards should point out that this is a possibility for samples with high water content and suggest preventative action such as making sure that the sample and coolant are not in direct contact.

6.4 Sample storage conditions

6.4.1 The current standards do not differentiate between groups of micro-organisms and provide one set of storage conditions for all micro-organisms. The advice given is based on recommendations for water samples and does not reflect current practice in commercial laboratories or the results of research.

6.4.2 There is some general advice for sample storage conditions that is already contained in the standards.

- When ambient temperatures are above 10ºC, samples should be cooled as soon as possible after collection. This is particularly important for samples collected during warmer periods such as the summer months.
- Samples should be kept at less than 10ºC, preferably 0-5ºC.
- Samples should be shipped to the laboratory without delay.
- Samples should be kept in the dark and measures should be taken to avoid changes in sample moisture content.

6.4.3 Sample storage temperatures and times depend on the micro-organism being enumerated. Based on the results of the literature search, separate holding conditions can be recommended for the different groups of micro-organism, Table 11.

Table 11 Recommended holding conditions for samples for microbiological analysis

Parameter	Temperature	Time	Notes
Bacteria	0-5ºC	Complete analysis within 24-48 hours after collection	Do not freeze
Viruses	0-5ºC OR Freeze	Complete analysis within 24-48 hours after collection Analyse within 2 weeks	
Helminth ova	0-5ºC	Analyse within 1 month	Do not freeze
Oocysts	0-5ºC	Analyse within 1 month	Do not freeze

6.4.4 When samples are to be analysed for more than one microbiological parameter, due regard must be taken of the variation in suitable storage conditions. Sample analysis should be prioritised such that the organisms most susceptible to change are analysed first. If samples are kept cool, oocyst and helminth ova enumeration is less urgent than bacterial or viral analysis. When samples are to be stored frozen to delay viral analysis, this can only be done if sub-samples have previously been extracted for bacterial, helminth and oocyst enumeration since viability of these organisms deteriorates when frozen.

6.4.5 In the U.S.A a project, *An investigation into biosolids sampling and handling methods for USEPA-Approved Microbial Detection Techniques*, is being funded by the Water Environment Research Foundation. The project will develop scientifically defensible methods for collecting and handling representative samples for microbiological examination from biosolids matrices (liquid, cake, compost) for various treatment processes and applications (e.g. land application and reuse) that are expected to result in more consistent, accurate results. The project is expected to report in the winter of 2007

and it is strongly recommended that the results are critically analysed with a view to incorporating them in Horizontal/CEN standards.

7 References

1. Agricultural Development and Advisory Service, Water UK and the British Retail Consortium. (2001) *The Safe Sludge Matrix. Guidelines for the Application of Sewage Sludge to Agricultural Land*. 3rd ed. April 2001. http://www.assuredproduce.co.uk/_code/common/item.asp?id=4033093

2. Ahmed, A.U. & D.L. Sorensen (1995) Kinetics of pathogen destruction during storage of dewatered solids. *Water Environment Research*, **67**:143-150.

3. Ajariyakhajorn, C., S.M. Goyal, R.A. Robinson, L.J. Johnston & C.A. Clanton (1997) The survival of Salmonella anatum, pseudorabies virus and porcine reproductive and respiratory syndrome virus in swine slurry. *Microbiologica*, **20**:365-369.

4. Anon (1995) *EN ISO 5667-3:1995 Water quality – sampling – Guidance on the preservation and handling of samples*. CEN, Brussels.

5. Anon (1998) *EN ISO 5667-13:1998, Water Quality – Sampling – Part 13: Guidance on sampling of sludges from sewage and water treatment works*. CEN, Brussels.

6. Anon (1998) *EN ISO 5667-16:1998, Water Quality – Sampling – Part 16: Guidance on biotesting of samples*. CEN, Brussels.

7. Anon (1998) *EN ISO 5667-15:1998, Water Quality – Sampling – Part 15: Guidance on preservation and handling of sludge and sediment samples*. CEN, Brussels.

8. Anon (1999) *PD CR 13455:1999, Soil improvers and growing media – Guidelines for the safety of users, the environment and plants*. BSI, London.

9. Anon (2000) *EN 12579:2000, Soil improvers and growing media – Sampling*. CEN, Brussels.

10. Anon (2000) *EN 13040:2000, Soil improvers and growing media – Sample preparation for chemical and physical tests, determination of dry matter content and laboratory compacted bulk density*. CEN, Brussels.

11. Anon (2003) EN ISO 5667-3:2003 *Water quality – sampling – Guidance on the preservation and handling of samples*. CEN, Brussels.

12. Anon (2003) *ISO/CD 19458, ISO TC 147/SC 4 N 0228. Water quality – Sampling for microbiological analysis*. Draft International Standard. ISO, Geneva.

13. Anon (2004) *ISO/CD18512: Soil Quality – Guidance on long and short-term storage of soil samples*. ISO, Geneva.

14. Anon (2004) pr CEN/TR 15310-4, *Characterization of waste – Sampling of waste materials – Part 4: Guidance on procedures for sample packaging, storage, preservation, transport and delivery*. CEN/TC 292 WI292019

15. Armitage, J.P. (1997) Behavioural responses of bacteria to light and oxygen. *Archives of Microbiology*, **168**: 249-261.

16. Asato, B. (2005) Voluntary split-sample program for environmental laboratories improves bacteriological data. Hawai'i Water Environment Association (HWEA) Laboratory Committee, Hawai'i. http://www.hwea.org/committees/lab01.htm

17. Berg, G., R.S. Safferman, D.R. Dahling, D. Berman, & C.J. Hurst (1984) *USEPA manual of methods for virology*. Chapter 4, Quality Assurance. U.S. Environmental Protection Agency. Washington, DC.

18. BioVir Laboratories (2002) Biosolids pathogens storage and handling times. BioVir Laboratories, Inc, Benicia, CA. http://www.biovir.com/Biosolids.holding.time.html

19. Board on Environmental Studies and Toxicology (BEST) (2002) *Biosolids applied to land: Advancing standards and practices*. The National Academic Press, Washington, DC. http://www.nap.edu/books/0309084865/html

20. Brobst, R. (2005) Biosolids Reference Sheet: Part 2C. EPA Region VIII, Denver, CO. http://www.epa.gov/region08/water/wastewater/biohome/biosolidsdown/handbook/handbook2.pdf

21. Butler, T.A., L.J. Sikora, P.M. Steinhilber & L.W. Douglass (2001) Compost age and sample storage effects on maturity indicators of biosolids compost. *Journal of Environmental Quality*, **30**:2141-2148.

22. Capizzi-Banas, S. & J. Schwartzbrod (2001) Irradiation of *Ascaris ova* in sludge using an electron beam accelerator. *Water Research*, **35**:2256-2260.

23. Carey, C.M., H. Lee & J.T. Trevors (2004) Biology, persistence and detection of *Crptosporidium parvum* and *Cryptosporidium hominis* oocyst. *Water Research*, **38**:818-862.

24. Chung, L.-Y., B.-H. Fang, J.-H. Chang, S.-M. Chye & C.-M. Yen (2004) The infectivity and antigenicity of *Toxocara canis* eggs can be retained after long-term preservation. *Annals of Tropical Medicine & Parasitology*, **98**:251-260.

25. Composting Association, WRAP & BSI (2005) *Publicly Available Specification 100 (PAS 100:2005)*. BSI, London.

26. Davies, C.M., N. Altavilla, M. Krogh, C.M. Ferguson, D.A. Deere & N.J. Ashbolt (2005) Environmental inaction of *Cryptosporidium* oocysts in catchment soils. *Journal of Applied Microbiology*, **98**:308-317.

27. DEP (2000) Sampling manual for pollutant limits, pathogen and vector attraction reductions in sewage sludge. 3620-Bk-Dep2214 Rev. 12/2000. Department of Environmental Protection, Bureau of Water Quality Protection, Division of Wastewater Management, Pennsylvania, USA.

28. DoE (1996) *Code of Practice for the Agricultural Use of Sewage Sludge*. Department of the Environment. HMSO, London.

29. Eccles, J.P., R. Searle, D. Holt & P.J. Dennis (2004) A comparison of methods used to enumerate Escherichia coli in conventionally treated sewage sludge. *Journal of Applied Microbiology*, **96**:375-383.

30. Egli, H., M. Dassenakis, H. Garelick, R. van Grieken, W.J.G.M. Peijnenburg, L. Klasinc, W. Kördel, N. Priest & T. Tavares (2003) Minimum requirements for reporting

analytical data for environmental samples. IUPAC Technical Report. *Pure & Applied Chemistry*, **75**:1097-1106.

31. Eisenberg, J.N.S., J.A. Soller, J. Scott, D.M. Eisenberg & J.M. Colford, Jr. (2004) A dynamic model to assess microbial risks associated with beneficial uses of biosolids. *Risk Analysis*, **24**: 221-235.

32. Environmental Laboratory Services (2005) *Environmental Laboratory Services Guide*. http://www.els-lab.com/pdf/Guide.pdf

33. Epstein, E. (2003) *Land application of sewage sludge and biosolids*. Lewis Publishers, CRC Press LLC, Boca Raton, Florida.

34. Estrada, I.B., A. Aller, F. Aller, X. Gómez & A. Morán (2004) The survival of *Escherichia coli*, faecal coliforms and enterobacteriaceae in general in soil treated with sludge form wastewater treatment plants. *Biosource Technology*, **93**: 191-198.

35. European Commission (1986) Council Directive on the protection of the environment, and in particular the soil, when sewage sludge in used in agriculture. 86/278/EEC. *Official Journal of the European Communities*. 4.7.86 No. L 181/6-12.

36. European Commission (2000) *Working Document on Sludge*. 3rd draft. ENV.E.3/LM, Brussels, 27 April 2000. http://europa.eu.int/comm/environment/waste/sludge/sludge_en.pdf

37. European Commission (2001) *Biological treatment of biowaste*. Working Document, 2nd draft. European Commission, Directorate-General Environment. Brussels, 12 February 2001.

38. European Commission (2002) Regulation (EC) No 1774/2002 of the European Parliament and of The Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. *Official Journal of the European Communities*. 10.10.2002 No. L 273/1-95.

39. Ewert, D.L. & M.J.B. Paynter (1980) Enumeration of bacteriophages and host bacteria in sewage and the activated-sludge treatment process. *Applied and Environmental Microbiology*, **39**: 576-583.

40. Fayer, R. & T. Nerad (1996) Effects of low temperature on viability of *Cryptosporidium parvum* oocysts. *Applied and Environmental Microbiology*, **62**: 1431-1433.

41. Fujioka, R.S & B.S. Yoneyama (2002) Sunlight inactivation of human enteric viruses and fecal bacteria. *Water Science and Technology*, **46**:291-295.

42. Gantzer, C., P. Gaspard, L. Galvez, A. Huyard, N. Dumouthier & J. Shwartzbrod (2001) Monitoring of bacterial and parasitological contamination during various treatment of sludge. *Water Research*, **35**:3763-3770.

43. Gaspard, P., Y. Ambolet & J. Schwartsbrod (1997) Urban sludge reused for agricultural purposes: Soils contamination and model development for the parasitological risk assessment. *Bulletin de L'Academie de Medecine*, **181**:43-57.

44. Gawlick, B.M., H.A. van der Sloot, F. Ulberth, S. Nortcliff, T. Simonart, B. Cooper, R. Leschber, K. Andersen & K. Wichman (2004) On the development of horizontal CEN

standards supporting the implementation of EU Directives on sludge, soil and biowaste – Project HORIZONTAL. *Trends in Analytical Chemistry*, **23**:686-703.

45. George, I., P. Crop & P. Servais (2002) Fecal coliform removal in wastewater treatment plants studied by plate counts and enzymatic methods. *Water Research*, **36**:2607-2617.

46. Gilbride, K.A. & R.R. Fulthorpe (2004) A survey of the composition and diversity of bacterial populations in bleached kraft pulp-mill wastewater secondary treatment systems. *Canadian Journal of Microbiology*, **50**:633-644.

47. Heitman, T.L., L.M. Frederick, J.R. Viste, N.J. Guselle, U.M. Morgan. R.C.A. Thompson & M.E. Olson (2002) Prevalence of *Giardia* and *Cryptosporidium* and characterization of *Cryptosporidium* spp. isolated from wildlife, human and agricultural sources in the North Saskatchewan River Basin in Alberta, Canada. *Canadian Journal of Microbiology*, **48**:530-541.

48. Heulelekian, H. (1953) Microbiology of water and sewage. *Annual Reviews in Microbiology*, **1953**:461-472.

49. H.M. Government (1990) *The Sludge (Use in Agriculture) Regulations 1989, Statutory Instrument No. 1263, as amended by The Sludge (Use in Agriculture) (Amendment) Regulations 1990, Statutory Instrument No. 880*. HMSO, London.

50. H.M. Government (2003) *The Animal By-Products Regulations 2003*. Implementing Regulation (EC) No. 1774/2002 of the European Parliament and the Council, laying down health rules concerning animal by-products not intended for human consumption. HMSO, London.

51. Holmqvist, A. & T.A. Stenström (2001) Survival of *Ascaris summ* ova, indicator bacteria and *Salmonella typhimurium phage 28B* in mesophilic composting of household waste. *Internet Dialogue on Ecological Sanitation* (15 Nov.-20 Dec. 2001). http://www.ias.unu.edu/proceedings/icibs/ecosan/holmqvist.html

52. Horan, N.J., L. Fletcher, S.M. Betmal, S.A. Wilks & C.W. Keevil (2004) Die-off enteric bacterial pathogens during mesophilic anaerobic digestion. *Water Research*, **38**:1113-1120.

53. Hurst, C.J., S.A. Schaub, M.D. Sobsey, S.R. Farrah, C.P. Gerba, J.B. Rose, S.M. Goyal, E.P.Larkin, R. Sullivan, J.T. Tierney, R.T. O'Brien, R.S. Safferman, M.E. Morris, F.M. Wellings, A.L. Lewis, G. Berg, P.W. Britton & J.A. Winter (1991) Multilaboratory evaluation of methods for detecting enteric viruses in soil. *Applied and Environmental Microbiology*, **57**: 395-401.

54. Jenkin, M.B., D.D. Bowman, E.A. Fogarty & W.C. Ghiorse (2002) *Cryptosporidium parvum* oocyst inactivation in three soil types at various temperatures and water potentials. *Soil Biology and Biochemistry*, **34**:1101-1109.

55. Kato, S., E. Fogarty & D.D. Bowman (2003) Effect of aerobic and anaerobic digestion on the viability of *Cryptosporidium parvum* oocysts and *Ascaris suum* eggs. *International Journal of Environmental Health Research*, **13**:169-179.

56. Khan, M.A., H. Satoh, H. Katayama, F. Kurisu & T. Mino (2002) Bacteriophages isolated from activated sludge processes and their polyvalency. *Water Research*, **36**: 3364-3370.

57. Lambkin, D.C., T.D. Evans, S. Nortcliff & T.C. White (2004) *HORIZONTAL WP2: Towards producing harmonised methods, with quantified precision, for sampling sludges, treated biowastes and soils in the landscape*. Dept Soil Science, University of Reading, UK.

58. Lasobras, J., J. Dellunde, J. Jofre & F. Lucena (1999) Occurrence and levels of phages proposed as surrogate indicators of enteric viruses in different types of sludges. *Journal of Applied Microbiology*, **86**: 723-729.

59. Lepeuple, A.S., G. Gaval, M. Jovic & M.R. de Roubin (2004) *Literature review on levels of pathogens and their abatement in sludges, soil and treated biowaste.* HORIZONTAL – 6, WP3. Hygienic parameters. Anjou Recherche, France.

60. Leschber, R. (2004) *Evaluation of the relevance of organic micro-pollutants in sewage sludge. Results of a JRC-Coordinated survey on background values.* Provisional Report for Commenting. EUR XXXXX EN. B.M. Gawlick and G. Bidoglio (eds). Office for Official Publications of the European Communities, Luxembourg.

61. Lightfoot, N.F. & E.A. Maier (eds) (1998) *Microbiological analysis of food and water: Guidelines for quality assurance*. Elsevier Science B.V., Amsterdam, The Netherlands, 266pp.

62. Lucena, F., A.E. Duran, A. Morón, E. Calderón, C. Campos, C. Gantzer & S. Straber (2004) Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. *Journal of Applied Microbiology*, **97**:1069-1076.

63. Lucena, F., A.R. Blanch & J. Jofre (2005) *HORIZONTAL Desk study: Bacteriophages (and viruses) to be monitored in EU in sludges, soils and treated biowastes*. Dept. Microbiology, University of Barcelona, Spain.

64. Maine Department of Environmental Protection (2000) *Sampling/ analysis work plan guidance*. Draft May 2000. Maine Department of Environmental Protection, Augusta, ME. http://mainegov-images.informe.org/dep/rwm/publications/pdf/guidance.pdf

65. Massachusetts Water Resources Authority (2004) *Study of anthropogenic virus in Boston Harbor, Charles River, Cottage Farm CSO Treatment Facility and deer island Treatment Plant 1995-2003*. Report ENQUAD 2004-15. Environmental Quality Department, Massachusetts water Resources Authority, Massachusetts, NE.

66. Milligan, J.D. (1988) Evaluation of sample holding time extension for *Escherichia coli* and *Enterocci* measurements. *Chemosphere*, **17**:1241-1245.

67. Mocé-Llivina, L. M. Muniesa, H. Pimenta-Vale, F. Lucena & J. Jofre (2003) Survival of bacteria indicator species and bacteriophages after thermal treatment of sludge and sewage. *Applied and Environmental Microbiology*, **69**:1452-1456.

68. Mubiru, D.N., M.S. Coyne & J.H. Grove (2000) *Escherichia coli* pathogen O157:H7 does not survive longer in soil than a nonpathogenic fecal coliform. *Agronomy Notes*, **32(3)**, 7pp.

69. MWH Laboratories (2005) General laboratory Analyses 2005 Test list as of 5/10/05. http://www.mwhlabs.com/files/MWHLabsTestlist2005.pdf

70. N.C. Division of Water Quality (2005) *Guidance for Sample Submission.* Revision Mar. 11 2005, North Carolina Division of Water Quality (DWQ), Department of Environment and Natural Resources. http://h2o.enr.state.nc.us/lab/qa/sampsubguide.htm

71. Niewolak, S., S. Tucholski, S. & E. Radziejewska (2002) Sanitary-bacteriological evaluation of meadow soil irrigated with biologically treated sewage. *Polish Journal of Environmental Studies*, **11**: 141-149.

72. O'Donnell, C.J., K.B. Meyer, J.V. Jones, T. Benton, E.S. Kaneshiro, J.S. Nichols & F.W. Schaefer III. (1984) Survival of parasite eggs upon storage in sludge. *Applied and Environmental Microbiology*, **48**:618-625.

73. O'Lorcain, P. (1995) The effects of freezing on the viability of *Toxocara canis* and *T. cati* embryonated eggs. *Journal of Helminthology*, **69**:169-171.

74. Papini, R. & G. Cardini (1997) Effects of temperature on viability and infectivity of *Baylisascaris transfuga* eggs. *Revue de Medecine Veterinaire*, 148:903-906.

75. Parker, L.V. & C.J. Martel (2002) *Long-term survival of enteric microorganisms in frozen wastewater*. Cold Regions Research and Engineering Laboratory Technical Report ERDC/CRREL TR-02-16. US Army Corps of Engineers Engineer Research and Development Center, Hanover, NH, USA.

76. Paulsrud, B., B. Gjerde & A. Lundar (2004) Full scale validation of helminth ova (*Ascaris suum*) inactivation by different sludge treatment processes. *Water Science and Technology*, **49**:139-146.

77. Plachy, P., I. Placha & M. Vargova (1995) Effect of physico-chemical parameters of sludge aerobic exothermic stabilization on the viability of *Ascaris suum* eggs. *Helminthologia*, **32**:233-237.

78. Plachy, P. & P. Juris (1995) Survival of the model helminth *Ascaris suum* eggs in the sludge drying beds of sewage treatment plants. *Veterinarni Medicina*, **40**:23-27.

79. Pokorny, N.J., S.C. weir, R.A. Carreno, J.T. Trevors & H. Lee (2002) Influence of temperature on *Cryptosporidium parvum* oocyst infectivity in river water samples as detected by tissue culture assay. *Journal of Parasitology*, **88**:641-643.

80. Pope, M.L., M. Bussen, M.A. Feige, L. Shadkix, S. Gonder C. Rodgers, Y. Chambers, J. Pulz, K. Miller, K. Coneell & J. Standridge (2003) Assessment of the effects of holding time and temperature on *Escherichia coli* densities in surface water samples. *Applied and Environmental Microbiology*, **69**:6201-6207.

81. Premier Laboratory (2003) *Environmental sampling guide*. 2003 Revision. Premier Laboratory, Dayville CT. http://www.premierlaboratory.com/SupportFiles/SamplingGuide2003.pdf

82. Radtke, D.B (ed.) (2004) Handling and shipping of samples. Chapter A5.5 (version 2.2, 9/2004) in *National Field Manual for the Collection of Water-Quality Data: Techniques of Water-Resources Investigations*. Book 9 Handbooks for Water-Resources Investigations. U.S. Geological Survey. http://water.usgs.gov/owq/FieldManual/chapter5/html/Ch5_contents.html

83. Ranalli, G., G. Bottura, P. Taddei, M. Garavani, R. Marchetti & C. Sorlini (2001) Composting of solid and sludge residues from agricultural and food industries. Bioindicators of monitoring and compost maturity. *Journal of Environmental Science and Health*, **A36**: 415-436.

84. Rila, J.-P., W. Dott & A. Eisentraeger (2003) Long-time respiration of contaminated soil samples during laboratory storage under different conditions. *Soil and Sediment Contamination*, **12**:481-496.

85. Rimhanen-Finne, R., A. Vuorinen, S. Marmo, S. Malmberg & M.-L. Hänninen (2004) Comparative analysis of *Cryptosporidium*, *Giardia* and indicator bacteria during sewage sludge hygienization in various composting processes. *Letters in Applied Microbiology*, **38**:301-305.

86. Robertson, L.J. & B. Gjerde (2000) Effect of sample holding time on recovery of *Cryptosporidium* oocysts and *Giardia* cysts from water samples. *Applied and Environmental Microbiology*, **66**:1724-1725.

87. Sahlström, L., A. Aspan, E. Bagge, M.-L. Danielsson-Tham & A. Albihn (2004) Bacterial pathogen incidences in sludge from Swedish sewage treatment plants. *Water Research*, **38**:1989-1994.

88. Saleem, M., A.A. Bukhari & M.H. Al-Malack (2003) Seasonal variations in the bacterial population in an activated sludge system. *Journal of Engineering Science*, **2**:155-162.

89. Sanin, F.D., P.A. Vesilind & C.J. Martel (1994) Pathogen reduction capabilities of freeze/thaw sludge conditioning. *Water Research*, **28**:2393-2398.

90. Schumacher, B.P. (2001). *Characterization and monitoring: Sample holding time re-evaluation*. Project outline. National Exposure Research Laboratory Environmental Sciences. http://www.epa.gov/nerlesd1/cmd/tasks/holding.htm

91. Selvakumar, A., M. Borst, M. Boner & P. Mallon (2004) *Effects of samples holding time on concentrations of microorganisms in water samples*. Water Environment Research, **76**:67-72.

92. Sherwood, D., K.W. Angus, D.R. Snodgrass & S. Tzipori (1982) Experimental *Cryptosporidiosis* in laboratory mice. *Infection and Immunity*, **38**:471-475.

93. Sinton, L.W., R. K. Finlay & P, A. Lynch (1999) Sunlight inactivation of fecal bacteriophages and bacteria in sewage-polluted seawater. *Applied and Environmental Microbiology*, **65**: 3605-3613.

94. Skanavis, C. & W.A. Yanko (2001) *Clostridium perfringens* as a potential indicator for the presence of sewage solids in marine sediments. *Marine Pollution Bulletin*, **42**: 31-35.

95. Sonzogni, W., J. Standridge & M. Bussen (2002) Preservation and survival of E. coli in well water samples submitted for routine analysis. Final report DNR Project #166. Wisconsin State Laboratory of Hygiene, Environmental Health Division, Madison, Wisconsin. 48pp. http://www.dnr.state.wi.us/org/water/dwg/gw/research/reports/166.pdf

96. Southern DataStream Inc. (2005) *Standard Operating Procedures for laboratory operations and sample collection activities*. Florida Department of Environmental Protection - QA Section. http://www.residuals.ws/Methods/sop/chapt4_4.htm

97. Sobsey, M.D. & J.S. Meschke (2003) *Virus survival in the environment with special attention to survival in sewage sludge droplets and other environmental media of fecal or respiratory origin.* World Health Organization meeting 23-25 September 2003, Rome Italy.

98. Standing Committee of Analysts (SCA) (2003) *The microbiology of sewage sludge. Part I – An overview of the treatment and use in agriculture of sewage sludge in relation to its impact on the environment and public health*. Draft (20 August 2003). Booklet within the series: Methods for the examination of waters and associated materials. Standing Committee of Analysts, Environment Agency (National Laboratory Service), Nottingham, UK.

99. Standing Committee of Analysts (SCA) (2003) *The microbiology of sludge. Part 2 – Practices and procedures for sampling and sample preparation*. Draft (20 August 2003). Booklet within the series: Methods for the examination of waters and associated materials. Standing Committee of Analysts, Environment Agency (National Laboratory Service), Nottingham, UK.

100. Tanji, Y., K. Mizoguchi, M. Yoichi, M. Morita, N. Kijima, H. Kator & H. Unno (2003) Seasonal change and fate of coliphages infected to Escherichia coli O157:H7 in a wastewater treatment plant. *Water Research*, **37**:1136-1142.

101. Tartera, C., F. Lucena & J. Jofre (1989) Human origin of *Bacteroides fragilis* bacteriophages present in the environment. *Applied and Environmental Microbiology*, **55**: 2696-2701.

102. Thompson, K.C., R. Shepherd, & A. Hockin (2004) *Hygienic parameters feasibility of horizontal standards for Escherichia coli and Salmonella spp. in sludges, soil, soil improvers, growing media and biowastes.* Project Horizontal Final Report February 2004. http://www.ecn.nl/docs/society/horizontal/hor3a_ecoli_salm.pdf

103. Udeh, P.J., G. John & J.N. Veenstra (2003) Field inactivation of oocysts exposed to agricultural land. *Water, Air and Soil Pollution*, **142**:211-228.

104. US Composting Council (2001) *Test Methods for the Examination of Composting and Compost.* Chapter 2: Sample collection and laboratory preparation. The US Composting Council Research and Education Foundation. http://tmecc.org/tmecc/Table_of_Contents.html

105. USEPA (1994) Chapter 6: Sampling and Analysis. *A plain English Guide to the EPA Part 503 biosolids rule*. EPA/832/R-93/003. USEPA Washington, DC. Pp129-151.

106. USEPA (1999) *Environmental Regulation and Technology: Control of pathogens and vector attraction in sewage sludge (including domestic septage) under 40 CFR Part 503*. U.S. Environmental Protection Agency. Cincinnati, OH.

107. USEPA (2004) *Multi-Agency Radiological Laboratory Analytical Protocols Manual (MARLAP)*: Chapter 10. Field and sampling issues that affect laboratory measurements. July 2004. NUREG-1576; EPA 402-B-04-001C; NTIS PB2004-105421 www.epa.gov/radiation/marlap

108. Vieira F.C.S. & E. Nahas (1998) Microbial counts of dark red latosol samples stored at different temperatures. *Revista de Microbiologia*, **29**:129-136.

109. Vilanova, X., A. Manero, M. Cerdà-Cuéllar & A.R. Blanch (2004) The composition and persistence of faecal coliforms and enterococci populations in sewage plants. *Journal of Applied Microbiology*, **96**: 279-288.

110. Walker, M., K. Leddy & E. Hagar (2001) Effects of combined water potential and temperature stresses on *Cryptosporidium parvum* oocysts. *Applied and Environmental Microbiology*, **67**:5526-5529.

111. Warnes, S.L. & C.W. Keevil (2004) *Desk studies on feasibility of horizontal standard rapid methods for determination of Clostridium perfringens and enterococci in sludges, soil, soil improvers, growing media and biowastes.* Report by Project HORIZONTAL Work Package 3. Task 4. University of Southampton, UK.

112. Woods End Research Laboratory (2005) *Principles and practice: Compost sampling for lab analysis***.** Woods End Research Laboratory Inc., Mt Vernon, ME. http://www.woodsend.org/pdf-files/sampli%7E1.pdf

113. WRAP (2002) *Comparison of compost standards within the EU, North America and Australasia*. The Waste and Resources Action Programme (WRAP), Banbury, Oxon, UK.

114. Wu, L. & L.Q. Ma (2001) Effects of sample storage on biosolids compost stability and maturity evaluation. *Journal of Environmental Quality*, **30**:222-228.

8 Annexes

8.1 Terms and definitions

Autoclaving: Sometimes called steam sterilization, is the use of pressurized steam to kill infectious agents and denature proteins. This kind of 'wet heat' is considered the most dependable method of sterilizing laboratory equipment and decontaminating biohazardous waste. Autoclaves do not remove chemical contamination.

Advanced treatment: (hygienisation) Annex I: Sludge Treatment Process) Treatment is validated through a $6Log_{10}$ reduction of a test organism such as *Salmonella Senftenberg W775*. The treated sludge shall not contain Salmonella spp. in 50g (wet weight) and the treatment shall achieve at least a $6Log_{10}$ reduction in *Escherichia coli* to less than $5x10^2$ CFU/g.

Conventional Treatment: The treatment shall achieve at least a $2Log_{10}$ reduction in *Escherichia coli.*

Sterilisation

Sterilisation is the destruction of ALL infectious agents from an environment. This includes algae, bacteria, fungi, protozoa, viruses dormant endospores and poorly characterised agents such as viroids and the agents that are associated with spongiform encephalopathies.

Disinfection

Whereas sterilisation is an absolute phenomenon, the definition of disinfection is more nebulous. It refers to the removal from an environment of microbes that may cause disease.

Maximum Holding Time: The length of time a sample can be stored after collection and prior to analysis without significantly affecting the analytical results.

First order decay equation: An equation used to calculate the half-life (t½), the time required for half the microbial population to disappear.

log10A = -kt + constant

where A = the microbial population, k = the death rate, and t = time.