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Review of methods to measure bioaerosols from composting sites

Better regulation science programme
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Steve Killeen

Head of Science

Executive summary

A new report by the Environment Agency explores the best methods to measure bioaerosols released from composting sites that could pose a potential risk to human health.

Waste composting is an important part of the UK's recycling strategy. In recent years there has been a steady increase in waste composting, resulting in an increase in the number of composting sites as well as greater capacity in existing ones. Although this brings many benefits to the UK, there remain concerns over human exposure to bioaerosols from waste composting. Because micro-organisms are fundamental to the composting process, they will always be present in large quantities in the bulk material. Any handling process, such as turning the compost to give it air, is likely to create airborne dust that will contain micro-organisms.

Studies have shown that exposure to the pathogenic fungus *Aspergillus fumigatus* can trigger asthma, bronchitis and allergic responses. Those most at risk are likely to be workers on site. Workers and residents near to composting facilities will be less exposed because of the dilution and dispersion of bioaerosol emissions. However, there is still uncertainty over the concentration to which a compost bioaerosol must be reduced to be considered 'safe'.

Because of these concerns, the Environment Agency has adopted a policy position on composting and the potential human health effects of exposure to bioaerosols generated from composting. This places limitations on the location of composting facilities to prevent, where possible, the siting of composting activity within 250 metres of a workplace or boundary of a dwelling, unless justified by a site-specific risk assessment that shows the risks to be acceptable.

Thus, for many compost site operators some form of bioaerosol monitoring is required, for example before starting new operations or making big changes to existing ones, during normal operations to establish typical bioaerosol emissions, and after adopting control measures to reduce emissions.

In 2004, the Environment Agency commissioned WS Atkins to review methods for monitoring bioaerosols from compost sites, and to propose a standard sampling method for measuring bioaerosols associated with composting. The WS Atkins review forms Part 1 of this report. This report was subsequently sent for independent review to the Health and Safety Laboratory (HSL) and their review forms Part 2 of this report.

Part 1 reviews the following sampling/collection methods: impaction, impingement, filtration, cyclone scrubbing, electrostatic precipitation and sedimentation.

It discusses their advantages and drawbacks with respect to monitoring bioaerosols on composting sites in terms of collection efficiency, sampling time, ease of use, cost and robustness in the field, and other qualities such as the ability to distinguish between different micro-organisms and preferably, to primarily detect those that might pose a risk to human health.

The report also looks at counting methods to measure the pathogens collected, based on culturable and non-culturable approaches such as direct staining and fluorescence microscopy, flow cytometry and biomarkers measured with polymerase chain reaction (PCR).

To conclude (Part 1), the standard method proposed by the report to measure bioaerosols in the vicinity of a green waste composting site is the Andersen single-stage sampler fitted with a hemispherical baffle, with the micro-organisms collected onto either nutrient agar or tryptone soya agar (TSA) and malt extract agar (MEA).

The outputs from the sampling will be expressed as colony-forming units per cubic metre, with the results from the nutrient agar or TSA plates providing a figure for the total number of culturable mesophilic bacteria present, and enumeration of colonies of *Aspergillus fumigatus* present on the MEA plates providing a measure of the fungal content of the bioaerosol.

Part 2 of the report stresses the need for accurate sampling to better understand the health consequences of bioaerosols emitted from compost sites. Many types of samplers have been used over the years, including liquid impingers, solid impactors, filters and electrostatic precipitators. Although direct agar impaction methods, mainly the Andersen sampler, are still considered to be the benchmark for bioaerosol sampling, the practical limitations of these methods mean that future sampling strategies are likely to move away from these and towards filtration as the most likely alternative ahead of liquid impingers.

Impingers have the advantage of being compact, able to run for extended periods and to collect in liquid which allows for multiple analyses from the same sample. However, liquid collection is not compatible with size fractionation. Filtration methods, in addition to being simple, include the potential for size fractionation, ease of handling and transport of samples, multiple analyses from single samples and compatibility with collection methods already used for environmental pollution monitoring, such as particulate (PM10) monitoring. These advantages must be balanced against potential limitations due to dehydration stresses affecting the micro-organisms collected.

In terms of analytical methods, there is scope to further explore culture methods to target the main species found in compost. Thermophilic actinomycetes and *Aspergillus fumigatus* are the most representative of composting material and also the agents most likely to present a respiratory hazard. Although mesophilic bacteria may be an easily cultured general indicator of microbial contamination, there is little evidence of it presenting a health hazard in waste handling.

The greatest scope for progress, however, is in the development of molecular-based methods. Methods for detecting target organisms by their unique DNA sequences are well established, but characterising mixed populations would be the goal for monitoring compost bioaerosols. In the longer term, the development of microarray-based detection systems offers the greatest potential in this area. Although the set-up costs may be high, an established system could be a simple and cost-effective monitoring method.

PCR-based methods are very sensitive in complex, mixed DNA samples, and do not require the presence of a culturable or living organism.

Practical problems in monitoring bioaerosol emissions from composting are equally important. The approach proposed by the Department for Environment, Food and Rural Affairs for further investigation is of a multi-level monitoring system, in which simple detection methods, perhaps even electronic particle counters, are used for basic monitoring and are supported by more in-depth (culture-based or other) analysis when required.

Part 2 of the report concludes that the Association for Organics Recycling (2009) standardised protocol, '*A standardised protocol for the monitoring of bioaerosols at open compost facilities*' should be followed by those carrying out bioaerosol monitoring at compost sites. This protocol, first developed in 1999 by The Composting Association (now the Association for Organics Recycling) entitled '*Standardised protocol for the sampling and enumeration of airborne micro-organisms at composting facilities*' has been re-designed and updated through funding from the Environment Agency. The re-designed protocol, describes two sampling approaches for collecting culturable micro-organisms. It will be available from the Association for Organics Recycling website in 2009. In future, this protocol should be used in conjunction with the '*Guidance on the evaluation of bioaerosol risk assessments for composting facilities*' (to be published on the Environment Agency website in 2009), which will assist those completing risk assessments and will provide an understanding of what the Environment Agency expects to be included in bioaerosol risk assessments and monitoring.

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1 Overview

Waste composting is an important component of the UK strategy for recycling of materials. As a result, there has been a steady increase in waste composting from ca one million tonnes in 2000/2001 to 3.4 million tonnes by 2005/2006 (Association for Organics Recycling, 2008). This has been achieved by increasing the number of composting facilities as well as capacity at existing facilities. Although this is beneficial to the UK, there continues to be concern over exposure to microbial aerosols and their products (bioaerosols) from waste composting and potential effects on human health. Because micro-organisms are fundamental to the composting process, they will always be present in large quantities in the bulk material. Any handling process, such as turning to promote aeration or separation into size fractions (screening), is likely to create airborne dust that will contain micro-organisms.

Previous studies have demonstrated the potential for respiratory ill health, primarily asthma, bronchitis and allergic alveolitis, but also infection from exposure to the opportunist pathogenic fungus *Aspergillus fumigatus*, as reviewed by Swan *et al.* (2003). Those most at risk are likely to be workers on site, because of their potential for greater exposure. Workers and residents peripheral to compost facilities will be less exposed because of dilution and dispersion effects of bioaerosol emissions. However, there is still uncertainty in the concentration to which a compost bioaerosol must be reduced to be considered at a 'safe' level. Because of these concerns, the Environment Agency has adopted a policy position on composting and potential human health effects of exposure to bioaerosols generated from composting (Policy number: 405_07 available at <http://www.environment-agency.gov.uk>). This places limitations on the location of composting facilities to prevent, where possible, the siting of composting activity within 250 metres of a workplace or boundary of a dwelling, unless justified by a site-specific risk assessment that shows risk to be acceptable.

The implication for many compost site operators is that some form of bioaerosol monitoring is required in a site-specific risk assessment. This could be required:

- before starting new operations or making significant changes to existing operations, to provide a benchmark;
- during representative operations to establish typical bioaerosol emissions;
- after implementing any control measures aimed at reducing emissions, to determine their effectiveness.

The Environment Agency should therefore be able to recommend bioaerosol monitoring strategies that are:

- acceptable to the Environment Agency in terms of the data generated;
- transparent in providing understandable data to support risk assessments;
- consistent with or comparable to data from other sites, to develop a broader picture of potential exposure and effectiveness of controls;
- acceptable to the site operator in terms of ease of execution and cost.

In 2004, the Environment Agency commissioned WS Atkins to review methods for monitoring bioaerosols from compost sites. This review was intended to provide an update on the monitoring protocol '*Standardised protocol for the sampling and enumeration of airborne micro-organisms at composting facilities*', published by the Composting Association (now Association for Organics Recycling (AFOR)) in 1999. In addition, the review aimed to propose, with justification, a standard sampling method

for measuring bioaerosols associated with composting. The WS Atkins review forms Part 1 of this report. This report was subsequently sent for independent peer review to the Health and Safety Laboratory (HSL) and their review forms Part 2 of this report.

2 Part 1 - Introduction

A review by Swan *et al.*, (2003) for the Health and Safety Executive (HSE), described the potential health consequences of exposure to bioaerosols and the state of knowledge of waste compost bioaerosols at that time. The information presented in this report is intended to address one of the recommendations in the HSE report, namely the development of a standard approach to measure bioaerosols at waste composting sites. The development of such an approach will enable the Environment Agency to support and review its current policy position.

2.1 Purpose of the study

This report reviews methods available for the measurement of bioaerosols, and proposes a standard approach for measuring bioaerosols at green waste composting sites, although for in-vessel systems where there is an external processing element, the methods for measuring bioaerosols are equally applicable. For the purposes of this report, 'measurement of bioaerosols' includes both the collection of the micro-organisms and quantification of the numbers collected.

Bioaerosols are defined as 'airborne particles, large molecules, and volatile compounds that are living or were released from a living organism' (Griffiths *et al.*, 1997). The components of a bioaerosol range in size from 0.02 to 100 µm (diameter) (Pillai and Ricke, 2002), and are released from a wide range of activities and processes (Folmsbee and Strevett, 1999). The focus of this report is bioaerosols released from green waste compost processing facilities.

A wide range of techniques have been used for determining levels of micro-organisms in air at and around composting facilities (Henningson and Ahlberg, 1994; Crook, 1996 and Pillai and Ricke, 2002). However, because of the variety of techniques used, it is difficult to make comparisons between the results from published studies. Differences in both the sampling efficiencies of individual collection techniques and in the methods used to enumerate the micro-organisms sampled are known to make a significant difference in the concentrations of micro-organisms reported (Henningson and Ahlberg, 1994; Pillai and Ricke, 2002). The lack of a standard approach has been identified as one of the areas limiting further research into the health effects of bioaerosols (Reinthalder *et al.*, 1999 ; Pillai and Ricke, 2002). A standard measurement method would help to reduce variability and allow for a greater ease of comparison between monitoring studies.

Significant work to develop a standard approach has already been conducted by experts AFOR in their document '*Standardised protocol for the sampling and enumeration of airborne micro-organisms at composting facilities*' (Gilbert and Ward, 1999). However, the background literature review which was undertaken to support that document was never published. The aim of this report is to explore any further developments in this field since 1999, and to propose a standard method for measuring bioaerosols and the rationale supporting it.

2.2 Purpose of the monitoring protocol

Given the variety of techniques available for the sampling and enumeration of micro-organisms in bioaerosols, it is important to select techniques most relevant to measure the micro-organisms of interest and to operate in the intended environment. Sampling

strategies and methods must be relevant to the environment in which they are to be used to ensure the samples (and results) are representative (Crook and Sherwood-Higham, 1997).

The standard monitoring protocol required by the Environment Agency must be suitable to determine the numbers of micro-organisms present at a point within 250 metres of the composting facility.

Comparing levels of bioaerosols to background levels is necessary because there are currently no dose-response data on the level of exposure which causes an adverse effect to human health (Eduard and Heederik, 1998; Pillai and Ricke, 2002; Sanchez-Monedero and Stentiford, 2003). In the absence of such dose-response information, the Environment Agency ensures that bioaerosol emissions from composting sites do not exceed the 'acceptable levels' of 1000 cfu/m³ for total bacteria and fungi and 300 cfu/m³ Gram-negative bacteria (based on findings from Wheeler et al. (2001)). To do this bioaerosol 'background level' needs to be determined upwind of a compost facility. This will represent the level of bioaerosols that would be present irrespective of whether there was a compost site there or not. Bioaerosol levels are then determined downwind of the site and adjacent to the nearest sensitive receptor to ensure levels are maintained at an 'acceptable level' at the sensitive receptor. Should dose-response data become available for micro-organisms released from green waste compost, the monitoring strategy will require updating.

The standard protocol should therefore measure micro-organisms in green waste compost that may cause an adverse effect to human health, at a minimum detection level of around 1000 colony-forming units (cfu) per cubic metre (m³), and be suitable for use in an outdoor environment. The minimum detection level proposed (1000 cfu/m³) is based on the findings of Sanchez-Monedero and Stentiford in 2003 who reported background levels of 10² to 10³ cfu/m³ micro-organisms at composting sites in the absence of composting activities.

2.3 Selection criteria

Some of the criteria that must be considered when selecting a monitoring strategy, such as operation in an outdoor environment, and the sampling of micro-organisms known to cause adverse human health effects, have already been mentioned. The purpose of this section is to summarise the other factors relevant to the selection of an appropriate strategy. The criteria that may need to be addressed include:

- Collection efficiency – the ability of the sampler to collect the bioaerosol present. This is usually expressed as a percentage relative to reference samplers (Fannin, 1981).
- Sampling time – there is a risk of overloading agar collection plates with micro-organisms, as well as desiccating vegetative micro-organisms collected on solid surfaces, and losing liquid from impinger-based and cyclone-based samplers. Longer sampling times are, however, reported to provide a better estimate of mean microbial numbers than a shorter sampling period (Li and Lin, 1999).
- Sampler reliability – relates to the dependability and robustness of the instrumentation used. This is of particular importance for the monitoring of composting bioaerosols outdoors, often in locations that may only be accessible on foot (Fannin, 1981).
- Ease of sterilisation

- Maintenance of collected sample viability – field sampling means that enumeration of micro-organisms collected is unlikely to be possible at the point of sampling. Therefore, survival of the micro-organisms post-collection and pre-enumeration is an important consideration.
- Ease of sample assay – this has implications for cost and requirements for trained personnel.
- Ease of operation – also has cost and training implications.
- Remote operation capability – this includes a requirement for the technique to operate on battery power as mains power is unlikely to be available in the field. Samplers with greater remote operation capacity may also offer more versatility in the field than those that require constant attention (Fannin, 1981).
- Particle size discrimination – this is a key consideration for the identification of micro-organisms likely to cause respiratory infection. An ideal sampler for human aeroallergens and aeropathogens should be able to collect the same size fraction and concentrations as are inhaled and collected by the human respiratory system (Griffiths *et al.*, 1997).
- Micro-organisms to be monitored – should include those identified as causing potential adverse effects to human health, such as the fungus *Aspergillus fumigatus* (Crook and Lacey, 1988; Pillai and Ricke, 2002) and Gram-negative bacteria (Crook and Lacey, 1988). Thermophilic micro-organisms such as *Saccharopolyspora* sp. and *Thermoactinomyces vulgaris* that are characteristic of compost environments might also be monitored (Crook and Lacey, 1988; Pillai and Ricke, 2002). However, because of possible allergenic effects caused by a variety of components of bioaerosols (including micro-organisms and endotoxins) it may be more suitable to employ less-specific monitoring and measure total numbers of bacteria or fungi.
- Cost.

Irrespective of the method used to collect and quantify micro-organisms present in the bioaerosol, the approach employed for the collection of samples in the field, including the number of samplers used and their position relative to the compost windrows and relevant receptors, is a key component of the overall sampling protocol.

The Association for Organics Recycling conducted a series of studies to determine the most appropriate sampling strategy. The findings from these studies are presented in the *Standardised protocol for the sampling and enumeration of airborne micro-organisms at composting facilities* (Gilbert and Ward, 1999). The protocol provides details of the sampling procedure, diagrams illustrating the best positions for the samplers at various sites, and the frequency of sampling required. Key points that should be considered when setting out a sampling strategy are presented in the following section. These have been taken from the protocol, and it is recommended that the full text be obtained before any sampling takes place.

2.4 Sampling strategy – summary of main points

The following is a summary of the strategy developed by the Association for Organics Recycling for the collection of airborne micro-organisms at composting facilities:

Sampling procedure:

- minimum of two samples collected at each sampler location point;
- samples stored at 4°C after collection, and processed within 12 hours of sampling;
- control samples should be taken - if impaction samplers are used, control samples will be unopened Petri dishes containing the chosen culture medium.

Sampling locations:

- sampling conducted at a minimum of three locations – upwind of the site, downwind of the site, and adjacent to the nearest sensitive receptor (occupied building);
- sampling upwind of the site conducted concurrently with either downwind sampling or receptor sampling;
- terrain between the compost windrow(s) and sampler locations should be similar, thereby reducing variability in wind characteristics and dispersion of micro-organisms;
- maintain notes of wind direction and speed during sampling;
- sampling should not be conducted during periods of precipitation (rain, sleet, snow), or when the air temperature is below 5 °C (as this can cause unacceptable levels of condensation in the sampler).

Frequency of sampling:

- all samples to be collected on a single day;
- sampling at various points during the year may be required to identify any seasonal fluctuations in microbial numbers. The actual number of sampling days should be determined on a risk assessment basis.

3 Methods for collecting micro-organisms

Although Wolfe (1961) commented that the number of methods available for the collection of micro-organisms is roughly equal to the number of investigators, there are in fact a limited number of approaches suitable for collecting micro-organisms from compost bioaerosols. The purpose of this section is to review the methods available and to discuss the advantages and disadvantages of each strategy with respect to monitoring bioaerosols in the vicinity of composting sites.

The principle concepts for the collection of airborne micro-organisms are impaction, impingement, filtration, cyclone scrubbing, electrostatic precipitation and sedimentation; with the first three encompassing most of the methods reported to date:

- Impaction – uses inertial forces to collect particles or micro-organisms in the air. The air is drawn through the impaction sampler and forced to change direction. This causes the particles with too high an inertia to become impacted onto a solid surface (Henningson and Ahlberg, 1994).
- Impingement – uses the same approach as impaction except that the particles are collected in a liquid rather than onto a solid medium (Henningson and Ahlberg, 1994; Pillai and Ricke, 2002).
- Filtration – collects micro-organisms by drawing the air through a porous material, usually a membrane filter. The collection efficiency of this process depends on the physical properties of the particle and the filter, and the flow rate of the air (Pillai and Ricke, 2002).
- Cyclone scrubbing – the air is forced into a centrifugal motion and particles with a high enough inertia are forced onto the wall of the sampler (Henningson and Ahlberg, 1994).
- Electrostatic precipitation – with this process, airborne particles (including micro-organisms) are electrically charged on entering the sampler, causing them to drift and be deposited onto a suitable collection substrate. Electrostatic precipitation is reported to be a less severe collection strategy than impaction or impingement due to the significantly lower velocities encountered by the particles (Pillai and Ricke, 2002).
- Sedimentation – this method (also described as gravimetric sampling) is the simplest sampling strategy available, with micro-organisms collected on agar plates following their passive deposition from air. Although this approach is reported to be suitable for studies seeking to identify the presence of particular micro-organisms (McCartney *et al.* 1997), it cannot give a value for the number of micro-organisms present per unit volume of air as the volume of air sampled is not determined (it is a non-volumetric method) (Henningson and Ahlberg, 1994). Sedimentation methods are therefore not suitable for monitoring bioaerosols and are not discussed further in this report.

3.1 Impaction

Impaction-based methods are one of the more common approaches to collecting micro-organisms from bioaerosols (Pillai and Ricke, 2002), with a number of different samplers available commercially. Examples include the Andersen six-stage, two-stage and single-stage samplers (Jensen *et al.*, 1992), the Casella slit sampler, the Marple eight-stage impactor and the MAS-100 sampler (Li and Lin, 1999).

The Andersen six-stage sampler (along with the AGI-30 all glass impinger) has been suggested as the sampler of choice for the collection of viable micro-organisms by the International Aerobiology Symposium and the American Conference of Governmental Industrial Hygienists (Jensen *et al.*, 1992). The Anderson single-stage sampler has been adopted by the UK's Association for Organics Recycling in their standard protocol for the sampling and enumeration of airborne micro-organisms at composting facilities (Gilbert and Ward, 1999).

Impaction-based samplers differ in characteristics of the inlet point (size and shape of aperture), number of collection 'chambers' within the sampler and whether the micro-organisms are impacted onto a solid (glass slide) or semi-solid (agar plate) surface, or, in the case of the Marple eight-stage impactor, a filter or gelatine (Macher and Hansson, 1987).

3.1.1 Collection surface

Impaction onto a glass slide is often used to collect aerosolised fungal spores whilst the semi-solid agar surface is used to collect bacteria (Pillai and Ricke, 2002). However, agar plates have been used routinely for the collection of both fungi and bacteria (Li and Lin, 1999; Predicala *et al.*, 2002; Sanchez-Monedero and Stentiford, 2003). Impaction samplers using agar plates are described as culture-based impactors. Non-culture based impactors such as spore traps and rotating arm samplers are more suitable for the collection of larger fungal spores and pollen. The collection efficiencies of these samplers for smaller particles such as bacterial cells are therefore relatively low, making such samplers unsuitable for compost monitoring (Eduard and Heederik, 1998).

The primary advantage of using agar plates as the collection medium is that the collected micro-organisms are cultured and then counted on the plates; thus, samples require no further processing post-sampling to determine the numbers of micro-organisms present (except for incubation at the required temperature) (Crook and Lacey, 1988; Li and Lin, 1999 and Nesa *et al.*, 2001). This is a key reason for the widespread use of impaction samplers over impingement methods (Li and Lin, 1999), and it may also reduce the costs of the sampling programme. Using glass or liquid collection surfaces means that some post-collection processing is required if the numbers of micro-organisms are to be determined by counting colonies on agar growth media. Collection or re-suspension in a liquid medium is, of course, required if other enumeration systems such as flow cytometry or direct counting are to be used.

The use of agar plates also enables enumeration at low concentrations (a few micro-organisms per m³ air). This is one of the key advantages over and above the use of filters and impingers.

However, because micro-organisms are collected directly on to the culture medium, there is a danger that the agar plates can become overloaded with micro-organisms. This makes the enumeration of colonies that grow on the plates difficult, as they overlap and become indistinguishable from one another. Therefore, some information on the likely number of micro-organisms present is required before sampling. In

environments with potentially high levels of micro-organisms, for example adjacent to the compost windrows during turning, a shorter sampling time should be used to prevent overloading the agar plates (Eduard and Heederik, 1998)

Because of the potential for overloading, impaction systems are more suitable for monitoring less-contaminated air, whereas impingers are more suitable for use in more contaminated environments (Willeke *et al.*, 1998).

Impaction of the micro-organisms onto a solid or semi-solid surface causes significant stress to the micro-organisms collected (Stewart *et al.*, 1995), and may result in the more fragile organisms, such as vegetative bacterial cells, dying off (or becoming non-culturable) on the impaction surface. Gram-positive bacteria have been found to be more resistant than Gram-negative strains to the physical stresses incurred (Stewart *et al.*, 1995; Li and Lin, 1999). This 'selective pressure' may distort the findings of the monitoring study towards those micro-organisms capable of surviving the collection process. The issue of culturability and whether the results are truly representative of the micro-organisms present in the bioaerosol is discussed in Section 4.1.

For samplers using agar plates as the collection medium, the collection efficiency of the sampler has been found to be affected by:

- the sampling time;
- the concentration of the agar (most likely because this alters the 'springiness' of the agar surface); (Juozaitis *et al.* 1994) and
- the depth of agar in the collection plate (Cipriano, 1979; cited in Henningson and Ahlberg, 1994)

Altering the agarose content of the agar will change the potential for the agar to dry out. Desiccation of the surface layer of the agar results in more micro-organisms rebounding from the surface during impaction and becoming re-entrained in the air and lost from the system (and therefore not collected) (Willeke *et al.*, 1992). Increased sampling time, high temperatures and low relative humidity will also increase desiccation of the agar.

Changing the agar thickness in the plate (in collection stage two) from 4.00 to 4.75 mm increases the collection efficiency of 5-10 μm particles by a factor of two. Cipriano (1979) (cited in Li and Lin, 1999) also reported that the collection efficiency, when using plastic agar plates, was only 80 percent of the efficiency achieved when using the glass plates that the method was originally designed for. Loss of water from the agar (and subsequent desiccation of the plates) is an issue with long sampling periods (Li and Lin, 1999), and may be more significant for plates with a high concentration of agarose. Desiccation will adversely affect the growth of sensitive vegetative strains (Li and Lin, 1999).

The issue of plastic versus glass plates was also investigated by the Composting Association in the development of their standard protocol (Gilbert and Ward, 1999). Because the plastic plates were thinner, they were found to sit slightly lower in the collection chamber than the glass plates. The capacity (volume) of the plastic dishes was also greater than the glass ones (which had thicker walls). The lower position of the plastic plates in the sampler meant that the surface of the agar was at a different height than if glass plates had been used. This was overcome by raising the height at which the plastic plates were located in the collection chambers and by using plates containing 40 ml of agar rather than the 27 ml used in the glass plates by Andersen (Gilbert, 2003 *personal communication*).

3.1.2 Number of collection stages

An advantage of the impaction (and impingement) approach is that because impaction of the micro-organisms is determined (in part) by the air flow through the sampling device, by varying the velocity of the flow different-sized particles can be collected. By using an impaction sampler with multiple collection chambers or stages, different collection chambers can be designed to collect different-sized particles. Multi-stage (or cascade) impaction samplers are able to provide information on the particle size distribution of the aerosol (Henningson and Ahlberg, 1994; Pillai and Ricke, 2002).

3.1.3 Inlet characteristics

The inlet characteristics of the samplers will strongly influence the collection and recovery efficiencies of the samples. A theoretical study by Agarwal and Liu in 1980 (cited in Henningson and Ahlberg, 1994) showed that the Andersen six-stage sampler gave no sampling bias for 10 μm particles in still air, and therefore inlet (and collection) efficiency was strongly dependent on wind velocity at the time of sampling, and the orientation of the sampler to the wind. At a wind velocity of above 5 m s^{-1} with the inlet aperture facing the wind (with the long axis of the sampler parallel to the ground), the number of 10 μm particles was overestimated by 50 percent. However, when the experiment was repeated with the sampler in an upright position (and the wind blowing across the inlet aperture), less than five percent of the 10 μm particles were collected (Willeke *et al.*, 1992).

Similar results were reported by Grinshpun *et al.* (1994). They reported that collection efficiencies of the Andersen six-stage sampler were 90-150 percent when sampling in the same orientation to horizontal air flows (long axis of the sampler parallel to the ground), but 8-100 percent when orientated vertically.

To reduce the effects of wind speed (and therefore minimise one of the factors creating variability between tests), the Association for Organics Recycling proposed that a hemispherical baffle be placed around the inlet of the Andersen sampler to create still air conditions over the inlet (Gilbert and Ward, 1999). The details of the baffle are described in detail by May (1966) (cited in Gilbert and Ward, 1999) but it should extend in height at least 15 cm above the top of the inlet aperture (May, 1966).

3.2 Impingement

Impingement-based methods operate along the same principle as impaction-based approaches, except that the micro-organisms are collected into a liquid medium. A number of commercial impinger samplers are currently available including the All Glass Impinger 30 (AGI-30) (the successor to the AGI-4 or Porton impinger) (Henningson and Ahlberg, 1994), the SKC Biosampler (employs a centrifugal air motion to reduce losses through re-entrainment) (Willeke *et al.*, 1998), the Burkard multistage sampler, the modified personal impinger (MPI), the multi-orifice impinger (MOI), and the multi-stage liquid impinger (MLI) (constructed of aluminium and stainless steel (modified MLI)). Most impinger samplers are made of glass, making them cheaper than metal samplers such as the Andersen sampler, but affecting their robustness in the field.

The AGI-30 has been recommended (along with the Andersen six-stage impaction sampler) as a standard sampler for the collection of bioaerosols since 1964 (Brachman *et al.*, 1964, cited in Henningson and Ahlberg, 1994). Both impingement and cyclone-based methods have been reported as more suitable than impaction-based samplers for the study of *Legionella sp.* (Dennis, 1990; cited in Henningson and Ahlberg, 1994).

As with impaction samplers, both single and multi-stage impingement samplers have been developed (Henningson and Ahlberg, 1994). Impingement samplers also differ in the collection medium and size and shape of the inlet aperture. The use of multi-stage impingement samplers such as the AGI-30 will provide information on the particle size distribution within the aerosol.

As impingers would need to be sterilised before re-use, replicate samples would require a 'fresh' impinger, which increases costs. Andersen samplers could be wiped with 70 percent (v/v) ethanol or industrial methylated spirits (IMS) and dried before re-use with new agar plates, meaning that the same sampler could be re-used many times in one day, especially at 'background' concentrations.

3.2.1 Collection medium

Although impingement-based samplers are characterised by the use of a liquid collection medium, there is some variation in the type of liquid used. In all cases the liquid needs to be an isotonic or buffered solution to avoid imposing osmotic stresses on micro-organisms following their collection. Betaine or peptone solution has been recommended to protect bacteria from osmotic shock (Eduard and Heederik, 1998).

A disadvantage of using a buffered water medium such as Ringers solution is that if the sampler is used for long periods of time, buffer will be lost from the sampler through evaporation. This affects the collection efficiency of the device (Willeke *et al.*, 1998). Losses of up to 10 percent of the collection liquid have been reported from an AGI-30 impinger during a 30-minute sampling period (Henningson and Ahlberg, 1994). In such situations the use of a mineral oil may be more suitable, due to its minimal evaporation potential (Pillai and Ricke, 2002), although Willeke *et al.* (1998) reported that the AGI-30 impinger cannot be used with glycerol or any other liquid whose viscosity is substantially higher than that of water. Therefore, where losses of collection liquid through evaporation are likely (if the sampler is to be used for long sampling periods, or in high temperature environments), a sampler such as the SKC sampler (which can use glycerol) (Willeke *et al.*, 1998) may be more suitable than the AGI-30.

Whilst impingement systems may impose less physical stress on the micro-organisms than impaction-based systems, losses have still been reported with some impinger samplers (Pillai and Ricke, 2002). Juozaitis *et al.* (1994) reported that only 10 percent of the bacterium *Pseudomonas fluorescens* collected by the AGI-30 sampler could be cultured, and that this low recovery efficiency was due to the physical stresses encountered by the pseudomonads when collected with that sampler. The SKC Biosampler (described by its manufacturer as an improved design over the AGI-30) (Pillai and Ricke, 2002), however, has an expected recovery efficiency of 90 percent for particles of under one μm in diameter (Pillai and Ricke, 2002). This improved recovery efficiency is in part due to the swirling motion of air through the sampler which significantly reduces particle 'bounce' from the collection surface (even when the surface is dry) (Willeke *et al.*, 1998).

A reported limitation with liquid collection mediums is that they may not be as suitable for the collection of fungal spores as for bacteria. Spores from many fungi are hydrophobic and when collected in liquid, may float to the surface and be re-entrained into the exit airflow and lost. Spores are also more 'bouncy' than vegetative cells (Willeke *et al.*, 1998) and this may reduce their collection relative to vegetative cells in samplers where re-entrainment levels are high. Subsequent serial dilutions of suspensions before plating out may also break up aggregates, thereby increasing apparent concentrations.

3.2.2 Inlet characteristics

The inlet characteristics of the sampler influence the collection and recovery efficiencies achieved. For example, the inlet nozzle of the AGI-30 is designed so that 100 percent of particles under one μm in diameter are collected at wind speeds of under 5 m s^{-1} (Willeke *et al.*, 1992).

Increased flow rate is reported to be correlated with reduced recovery efficiency. One of the reasons for the development of the AGI-30 from the AGI-4 was to improve recovery efficiency by raising the jet nozzle above the surface of the collection liquid (from four mm to 30 mm) in order to get a more gentle impaction surface (Henningson and Ahlberg, 1994; Eduard and Heederik, 1998). The MOI sampler has four inlet jets compared to the single inlet of the AGI samplers, in order to achieve a lower jet velocity (Henningson and Ahlberg, 1994).

As with the impaction-based samplers, collection and recovery efficiencies are affected by wind speed at the inlet. Studies with the MLI sampler showed that even at a wind speed of 4.5 m s^{-1} across the inlet, the collection efficiency was just 9.6 percent, whereas when a baffle was used to create still air conditions, the collection efficiency increased to 99 percent (May, 1966; cited in Henningson and Ahlberg, 1994).

3.3 Filtration

Filtration-based samplers provide a relatively simple, inexpensive and effective means of collecting micro-organisms from bioaerosols. However, because only one filter is usually employed, information on the particle size distribution of the aerosol is not obtained. An exception is the filter sampler reported by Kenny *et al.* (1999) which is capable of collecting the inhalable dust fraction and further subdividing that into thoracic and respirable particle fractions. This sampler was reported to perform as well as the standard IOM (Institute of Occupational Medicine) filter but with the added advantage of size fractionation. The applicability of using this sampler for the collection of micro-organisms was not investigated (Kenny *et al.*, 1999).

The filter method is commonly used in personal samplers worn by workers at relevant facilities. Due to their small size, such samplers are not suitable for the more general sampling required for the Environment Agency's standard protocol. Predicala *et al.* (2002) concluded that filter-based samplers (when used as non-personal samplers) were more suitable for qualitative assessments of micro-organisms in bioaerosols, whereas impaction samplers such as the Andersen six-stage sampler were suitable for both qualitative and quantitative sampling. Microbial counts recorded by the Andersen sampler were significantly higher than those recorded by the membrane filter system (Predicala *et al.*, 2002).

As with impaction samplers, filters may become overloaded with micro-organisms when employed in highly contaminated environments, rendering the enumeration of the micro-organisms present (by direct counting or culture) impossible (Eduard and Heederik, 1998).

The key problem with filter systems is desiccation of the micro-organisms on the filter post-collection (Pillai and Ricke, 2002). Whilst fungi and spore-forming bacteria are likely to survive on the filter, vegetative bacterial cells, particularly Gram-negative bacteria, are reported not to survive the desiccation stresses incurred (Wang *et al.*, 2001). Poor survival due to desiccation was the reason the Association for Organics Recycling dismissed filters as the collection system for their Standard Protocol (Gilbert, 2003, *personal communication*).

Another problem with the filter method is sampling efficiency. Studies by the Association for Organics Recycling using IOM head filtration units (designed for occupational use) reported significant effects due to wind movements. The addition of a baffle (to mimic a human torso) was unsuccessful as this made the whole sampling apparatus unstable in windy conditions (Gilbert, 2003, *personal communication*).

The level of loss due to desiccation varies according to sampling time and relative humidity. For sampling times over 30 minutes, Wang *et al.* (2001) reported that only fungal propagules retained their viability, whilst vegetative bacterial cells became non-viable. However, the reduction in viability of the bacterial cells decreased when the relative humidity increased from 30 to 85 percent, highlighting the importance of recording the meteorological conditions at the time of sampling. Recovery of bacterial cells could also be improved if the filters were vortexed and agitated in an ultrasonic water bath prior to processing (Wang *et al.*, 2001). Again, vortexing will break up aggregates of spores, and dislodge cells/spores that are attached to vegetative particles, thereby increasing the counts.

3.4 Cyclone sampling

With cyclone systems, the micro-organisms are collected in a liquid medium and are reported to offer similar recovery efficiencies to impingement samplers such as the AGI-30. A study with the Gram-negative bacterium *Escherichia coli* reported a recovery efficiency of 100 ± 10 percent relative to the AGI-30 (Henningson *et al.*, 1988). Liquid losses due to evaporation are, however, slightly higher than for liquid impingers with under 15 percent of liquid lost from the cyclone sampler over a 30-minute sampling period (compared to 10 percent for the impinger) (Henningson and Ahlberg, 1994).

The advantages of cyclone systems over impaction or impingement methods is that cyclones are less susceptible to particle 'bounce' and loss through re-entrainment (Willeke *et al.*, 1998). They are also reported to be relatively easy to sterilise (Henningson and Ahlberg, 1994). This is an important consideration where multiple samples need to be taken.

3.5 Electrostatic precipitation

Electrostatic precipitation offers a less physically rigorous approach to the collection of micro-organisms from bioaerosols, compared to impaction or impingement-based methods (Stewart *et al.*, 1995; Pillai and Ricke, 2002). A reduction in the level of physical damage incurred by the cells should result in better recovery efficiencies.

The collection substrate used in electrostatic precipitation samplers can be solid or liquid. However, the choice of collection substrate has been found to affect the recovery efficiency (Mainelis *et al.*, 1999). For example, in a study using spores of *Bacillus subtilis var. niger*, the lowest recovery efficiency (15-22 percent) was recorded when water was used as the collection medium. When agar was used the recovery efficiency was between 50 and 60 percent, and when the spores were collected on filters, the recovery efficiency was much higher, at 90 percent (Mainelis *et al.*, 1999).

The findings reported by Mainelis *et al.* (1999) suggest that further studies to determine the performance characteristics of electrostatic precipitation might be required before it could be proposed as a standard method for the collection of bioaerosols.

3.6 Hybrid samplers

Hybrid samplers designed to combine the advantages of several of the approaches discussed above have also been developed. The virtual impactor developed by Marple and Liu (1989) (cited in Henningson and Ahlberg, 1994) is based on a combination of electrostatic precipitation and impaction, with the sample collected on a metal foil. However, the system was not designed to collect samples for subsequent viable analysis, with particles analysed instead by gas chromatography (GC). Such samplers are probably not relevant to monitoring green waste compost bioaerosols, as information on the types of micro-organisms present would not be obtained.

3.7 Direct counting

Direct counting differs from the other techniques discussed in that particles are counted as they are sampled, usually using optical techniques (Eversole *et al.*, 2001). There are no separate collection and enumeration stages of the process. Particle-counting systems such as the Coulter counter (Henningson and Ahlberg, 1994) have been used widely in the direct measurement of dust levels. However, these systems are limited for the monitoring of compost aerosols as they cannot distinguish between biological and non-biological particles, and are therefore likely to overestimate numbers of micro-organisms present.

The prototype single particle fluorescence analyser (SPFA) described by Eversole *et al.* (2001) is an exception, as it is able to distinguish between dust particles and micro-organisms in aerosols, by measuring the particle's intensity of UV fluorescence relative to a calibrated reference level.

The advantages of direct-counting techniques are that they can provide the results immediately, and may be used for continuous or long-term monitoring with none of the problems of overloaded culture medium, evaporation of collection liquid or loss of viability due to desiccation encountered with the other systems described. However, detection efficiencies of existing direct-counting techniques are still relatively low compared to the other methods discussed (Eversole *et al.*, 2001). Therefore, direct-counting techniques are probably not suitable at present, although with further development they may provide a realistic alternative to collection and enumeration-based methods.

4 Enumeration of micro-organisms

With the exception of direct-counting techniques, enumeration of the micro-organisms present forms the second stage of all monitoring strategies. Enumeration methods are divided into culturable and non-culturable approaches. As discussed in the previous chapter, impaction-based methods are limited to culturable approaches, whilst other collection methods can use either culturable or non-culturable approaches. Numbers of micro-organisms can be determined by:

- Counting individual cells (non-culturable) – direct staining and observation by fluorescence microscopy or by flow cytometry. Some cells that have a characteristic morphology may be identified and enumerated by light microscopy. This latter method is, however, limited to a few species of micro-organisms (mainly fungi) (Eduard and Heederik, 1998). Observation by light microscopy cannot differentiate between *Aspergillus* and *Penicillium* species of fungi, for example (Eduard and Heederik, 1998). Enumeration by light microscopy is therefore considered to be of limited use in monitoring compost bioaerosols.
- Counting individual colonies (culturable) – culturing on selective growth media.
- Statistical evaluation of numbers of cells present (culturable) – most probable number (MPN) counting.
- Measuring levels of specific microbial biomarkers (non-culturable) – particular fatty acids, endotoxins, antigens or nucleic acid sequences.
- Measuring levels of metabolic activity in the sample (non-culturable) – such as levels of adenosine triphosphate (ATP).

The selection of culturable versus non-culturable methods for enumeration of micro-organisms from environmental samples is dominated by the fact that culturable methods can only measure numbers of micro-organisms that are both viable and culturable. Viable but non-culturable cells (VNBCs) are not measured, and therefore culturable methods are not representative of all the micro-organisms present.

4.1 Culturable versus non-culturable methods and the relevance to monitoring composting sites

Culturable methods are defined as those in which the micro-organisms are collected from the environment and grown or cultured under particular conditions (usually on solid agar growth media). After a period of incubation (the conditions of which, such as time, temperature and oxygen availability, will vary according to the micro-organism(s) being cultured), the number of distinct colonies of micro-organisms present on the growth media are counted, with the count expressed as colony-forming units (cfu). As each colony is assumed to have grown from a single micro-organism, the number of cfus gives a figure for the number of micro-organisms present in the sample.

Culture-based methods are therefore relatively simple, easy to use and of low cost. However, they are only useful for the enumeration of micro-organisms that are both

viable and culturable under the culture conditions imposed (type of growth media and incubation conditions).

This is the major limitation with culture-based methods, as only a small proportion of micro-organisms present in a bioaerosol (probably under 10 percent) are culturable. This figure is based on reports that under 10 percent of all micro-organisms in the environment are culturable (Torsvik *et al.*, 1994). A similar value has been reported for the proportion of culturable micro-organisms in indoor aerosols (Heidelberg *et al.*, 1997).

Even those micro-organisms that are viable and culturable will not grow under the same culture conditions. For example, thermophilic micro-organisms such as *Saccharopolyspora sp.* and *Thermoactinomyces sp.* that are characteristic of compost prefer culture temperatures over 50 °C (Droffner *et al.*, 1995; Pillai and Ricke, 2002), whereas mesophilic micro-organisms such as *Bacillus subtilis* and *Escherichia coli* grow best at temperatures of 5 to 55 °C (Neidhart *et al.*, 1990).

Thus, any culture-based method will be selective towards a fraction of the micro-organisms present and will not be representative of all micro-organisms in the bioaerosol.

The counter-argument is that in addition to being relatively simple and easy to use, much of our understanding of microbiology, in terms of classification of micro-organisms, and the identification of pathogenic strains, comes from studies using culture-based methods. Whilst molecular and phenotypic-based techniques are now widely used in the identification of specific micro-organisms, they are less simple and more costly to use. They also provide a level of detail that may not be needed for monitoring micro-organisms in bioaerosols at compost sites.

As discussed at the start of this report, it is important to select a monitoring strategy appropriate for the environment and objectives of the strategy. The monitoring strategy required is, by design, a comparative study that must be able to distinguish between 'background' levels of micro-organisms and those levels present during composting operations (windrow turning for example). As long as the same techniques are used to determine 'background' and 'operational' levels, and representative groups of micro-organisms are studied (for example, total culturable mesophilic bacteria), culture-based techniques are suitable, and should not be dismissed purely because they are limited to measuring viable and culturable micro-organisms.

4.2 Culture-based methods

Culture-based methods involve growing (culturing) the micro-organisms on some form of semisolid growth medium, with results expressed as colony-forming units. Micro-organisms may be collected onto the growth medium directly from air (as for impaction-based methods), or collected in liquid (impingement systems) and then spread onto the growth medium. Micro-organisms collected onto filters may be enumerated using culture-based methods by laying the filter directly onto the growth medium and counting the colonies that grow on the filter, or by washing the filter in liquid buffer and subsequently spreading the buffer onto the growth medium.

The advantages of culture-based methods are that they are simple and relatively easy and cheap to perform. They are applicable to the enumeration of bacteria, fungi and viruses. The growth medium is usually produced in plastic Petri dishes, although for some samplers strips of agar or gelatine are used (agar strips are approximately four to ten times more expensive than Petri dishes).

There are a number of limitations with culture-based methods (in addition to the issue of non-culturability already discussed). Nevertheless, the impact of these can be limited by careful design and conduct of the sampling experiments. For instance:

- Prior information is required on the micro-organisms chosen for study. As discussed, growth requirements for different groups of micro-organisms vary widely and no one medium can be used for the culture of all species. Therefore, growth media must be selected appropriate for culturing the micro-organisms of interest. For example, nutrient agar or Tryptone Soya agar (TSA) are used widely for the enumeration of total culturable mesophilic bacteria (Terzieva *et al.*, 1996; Heidelberg *et al.*, 1997), whilst malt extract agar is commonly used for culturing fungi (Eduard and Heederik, 1998; Wu *et al.*, 2000).
- If large numbers of colonies (above 300) (Eduard and Heederik, 1998) are present on the plate, the colonies are likely to merge and overlap as they grow. This makes accurate counting difficult. The optimum number of colonies per Petri dish is 30 to 300 (Chang *et al.*, 1995). If micro-organisms are collected directly (impaction sampling), information is required on the numbers of colonies likely to be collected per Petri dish. Where numbers are likely to be high and cause overloading of the growth medium, sampling time will need to be reduced. If micro-organisms are collected in a liquid medium (impingement sampling), the issue of overloading is less significant as different serial dilutions of the collection liquid can be cultured and the most suitable one counted.
- For impaction samplers such as the Andersen sampler, micro-organisms are impacted onto the agar plates at specific points that correspond to the inlet holes in the collection chamber. Several studies have recommended that only those colonies that grow within the areas corresponding to the inlet holes are counted (Andersen, 1958). Colonies present elsewhere on the agar plate may be due to contamination or 'particle bounce'.
- The growth of some micro-organisms on the culture media may inhibit or enhance the growth of other taxa (Eduard and Heederik, 1998). For example, the growth of fungi on nutrient agar plates is likely to mask many bacterial colonies present, unless specific growth inhibitors such as cyclohexamide for fungi are added to the growth media. This may need to be addressed in the selection of the culture medium used.

4.2.1 Most probable number counting

Most probable number (MPN) assays are methods used to quantify the number of micro-organisms in aqueous samples without actual counting (Makkar and Casida, 1987). Serial dilution of the sample estimates the density of micro-organisms present on the basis that one micro-organism will produce a positive result after incubation.

Although MPN methods can only count culturable micro-organisms, growth conditions (liquid culture) are much less stressful to the micro-organisms than growth on semi-solid agar, and therefore more micro-organisms are likely to be detected. MPN assays are relatively easy to perform but rely on statistical calculations to determine the number of micro-organisms present. If cells are present in aggregates in the inoculation medium, rather than as single cells, then an incorrect result is likely. There is a good probability that aggregates of cells will be present.

4.2.2 Other culture-based techniques

Other culture-based techniques such as the Biolog™ or Pheneplate™ systems have been developed to identify individual or groups of bacteria according to their ability to use specific substrates (Bochner, 1989; Fulthorpe and Allen, 1994; Katouli *et al.*, 1996). Such systems are not suitable for the quantitative analysis of microbial populations and are therefore not relevant for the enumeration of compost bioaerosols.

4.3 Non-culture based methods

4.3.1 Direct staining and fluorescence microscopy

Micro-organisms collected in liquid buffer or on a filter can be stained using fluorescent stain (a fluorochrome) and observed (and counted) by epifluorescence microscopy (Eduard and Heederik, 1998). Both fungi and bacteria can be counted in this way (Palmgren *et al.*, 1986).

Different fluorochromes are available for different types of cells, and cells in different metabolic states. Epifluorescence microscopy can be used, for example, to differentiate between viable and non-viable cells (irrespective of their culturability) and may therefore be more representative of the total number of micro-organisms present. Fluorochromes are available that indicate cell viability by differences in cytoplasmic redox potential, electron transport chain activity, enzymatic activity, cell membrane potential and membrane integrity (Kepner and Pratt, 1994).

The two fluorochromes most frequently used to enumerate micro-organisms in environmental samples are acridine orange (3,6-bis[*N,N*-dimethylamino]acridinium chloride) and DAPI (4',6-diamidino-2-phenylindole). Both acridine orange and DAPI have been applied to bioaerosol monitoring, with DAPI reported as less susceptible to non-specific binding. Both fluorochromes are nucleic acid stains and allow micro-organisms to be distinguished on the basis of colour.

Acridine orange binds to both DNA and RNA. Single stranded nucleic acid emits an orange-red fluorescence and double stranded nucleic acid fluoresces green. DAPI fluoresces blue or bluish-white when bound to DNA and yellow when bound to non-DNA material (Kepner and Pratt, 1994). However, because DNA retains its staining properties even in non-viable cells, acridine orange and DAPI cannot be used to differentiate between dead, metabolically inactive but living, and living cells (Kepner and Pratt, 1994).

A limitation of epifluorescence microscopy is that with environmental samples such as soil and sediment, the fluorochrome may also bind to organic matter present, leading to overestimation of the number of micro-organisms present (Kämpfer *et al.*, 1991). If the sample contains too much organic matter, or too many microbial cells, the sample is likely to appear as a mass of colour and the differentiation and enumeration of individual cells will not be possible. Because aerosols from composting sites are characterised by high levels of abiotic particulate matter, non-specific binding of the fluorochrome to this material may limit the use of this technique for bioaerosol monitoring. The use of the Baclight fluorescent stain may minimise 'false-positive' results generated from binding to abiotic particles as this stain has been found to be less susceptible to binding to such material (and non-viable cells) relative to acridine orange which will bind to humic material (Kidesco and Nielsen, 1997). Both Baclight and acridine orange have been used as fluorochromes for the enumeration of airborne micro-organisms (Terzieva *et al.*, 1996).

Eduard and Heederik (1998), however, concluded that microscopic methods were the most suitable for the assessment of exposure to micro-organisms in highly contaminated environments. This may have been due to their dismissal of culture-based methods (on the basis that culturability is not a requirement for health impacts), thereby leaving microscopic techniques as the most appropriate of the non-culturable methods available.

The advantage of this technique is that as a non-culturable approach it enables all viable cells (culturable and non-culturable) to be counted. Kämpfer *et al.* (1991) found that epifluorescence microscopy identified ten times more micro-organisms from water and soil samples than culturing on selective media. Also, if the counting is automated with the use of a computer-based image analysis system, a high throughput of samples can be achieved (Kildeso and Nielsen, 1997).

The limitations of enumeration by fluorescence microscopy are binding of the fluorochrome to abiotic material, and human error in counting the number of micro-organisms present and differentiating between microbial cells and abiotic material (dust particles for example) (Pillai and Ricke, 2002). Counting cells by eye is also a rather time-consuming process; this may reduce the number of samples that can be processed and increase costs. As discussed, sample throughput can be improved using an image analysis automated system (Crook and Sherwood-Higham, 1997), although this also has limitations in that it will only count particles that fall within the size parameters set by the programmer. Overlapping cells are unlikely to be counted.

In a comparative study of samples taken from a waste-sorting yard, numbers of micro-organisms (as determined by epifluorescence microscopy with acridine orange as the fluorochrome) were found to vary significantly between the three laboratories processing the samples (Eduard *et al.*, 2001). Differences were due primarily to the enumeration of small bacteria of under 0.2 μm in diameter which were counted by one laboratory but not the others. The study concluded that whilst epifluorescence microscopy was suitable for the enumeration of fungal propagules and large bacteria, the recognition of small bacteria may represent a major source of error in the technique, especially where their size approaches the optical resolution of the microscope and their size cannot be observed satisfactorily. Better fluorochromes are also required (Eduard *et al.*, 2001). Similar observations have also been reported by the Composting Association (Gilbert, 2003 *personal communication*).

Micro-organisms can also be detected and enumerated by immunofluorescence microscopy in which the fluorochrome, such as fluorescein isothiocyanate (FITC) (or other marker) is attached to the cell by an antibody-antigen interaction. The technique is limited by the non-specific binding of the fluorescent-labelled antibodies to abiotic particles (Lindahl and Bakken, 1995). Therefore, whilst this technique has been used to measure fungal spore levels in environments of patients with anticipated mould allergy (Popp *et al.* 1988; cited in Eduard and Heederik, 1998), it is likely to be less suited to monitoring bioaerosols from compost facilities where levels of abiotic matter are high.

4.3.2 Flow cytometry

Flow cytometry is a technique for quantifying components or structural features of cells primarily by optical means (Muirhead *et al.*, 1985), and can be used on both fungi and bacteria (Porter *et al.*, 1997). The advantage of the technique is that it can analyse many thousands of cells within seconds (Porter *et al.*, 1997). Under optimum conditions, the automated process can count $1\text{-}2 \times 10^3$ cells s^{-1} (Davey and Kell, 1996).

The limitations of the technique are that it may also count abiotic particles of the same size as microbial cells, and that it can only count micro-organisms present as single

cells. The presence of many micro-organisms in bioaerosols as aggregates of cells means that collected cells may require agitating or vortexing to break the cell clusters prior to analysis (although vortexing creates a sample that is not representative of the aggregates that would be inhaled/respired). Vortexing may affect the viability of some of the cells present. De-agglomeration may also increase cell numbers as it will break the clumps into single cells (Jensen *et al.*, 1992; Terzieva *et al.*, 1996). The application of flow cytometry is limited for the analysis of highly particulate matter such as soil (Porter *et al.*, 1997), and therefore also for aerosols from compost windrows.

As flow cytometry requires the cells to be in an aqueous suspension, it should be used with impingement, filtration or cyclone sampling techniques. Once in suspension, the cells are passed single-file through a laser beam by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light, the amount of light scattered being dependent on the size of the particle and the presence or absence of specific cell surface features. For the quantification of micro-organisms in environmental samples, some type of fluorochrome is required as the light scatter characteristics are not sufficient to differentiate cells in environmental samples.

The combination of flow cytometry with fluorescent in situ hybridisation (FISH) has the potential to quantify and/or sort cells on the basis of differences in nucleic acid sequences. FISH methods involve the labelling of specific nucleic acid sequences inside intact cells using so-called phylogenetic stains (Porter *et al.*, 1997). This technique has been used for the detection of aerosolised bacteria (Lange *et al.*, 1997)

4.3.3 Biomarkers

Measuring levels of particular biomarkers has been proposed as a realistic alternative to direct counting or culture-based techniques (Crook and Sherwood-Higham, 1997; Pillai and Ricke, 2002). The biomarkers measured may include specific cell components such as fatty acids, ergosterol (marker of fungal biomass) or muramic acid (marker of peptidoglycan, and therefore of bacterial biomass) (Pillai and Ricke, 2002), or cell products such as endotoxins (markers of Gram-negative bacteria, and also the causative agents of some allergenic effects) (Crook, 1996; Eduard and Heederik, 1998).

Measurement of the endotoxin content in aerosols using the *Limulus* amoebocyte lysate (LAL) assay can be conducted using commercially available kits (Crook and Sherwood-Higham, 1997), with the results reported to correlate well with acute respiratory health effects. Although the assay has been used for monitoring bioaerosols, Eduard and Heederik (1998) commented that a generally accepted protocol was still not available for conducting the LAL assay, and that this would be required if any comparisons with findings from other tests were to be made. Further possible limitations with the method are that it may not be valid under all conditions as different constituents of dust can interfere with the assay (Hollander *et al.*, 1993). Other microbial agents such as glucans may also give positive results with some of the commercial LAL assays (Tanaka *et al.*, 1991; cited in Eduard and Heederik, 1998).

The advantages of measuring particular biomarkers are that limitations with the 'whole cell' techniques discussed above (such as culturability and non-specific binding of fluorochromes) are avoided. Also, because some of the adverse health effects caused by exposure to bioaerosols are a consequence of the presence of particular cell products such as endotoxins, and not the viable micro-organisms themselves, monitoring levels of these compounds may be more relevant to assessing exposure than measuring levels of viable micro-organisms.

However, with the exception of endotoxins, it is unclear which biomarkers present in bioaerosols should be monitored to measure exposure to non-infectious micro-

organisms (Pillai and Ricke, 2002) (this limitation has also been levelled at culture-based methods where the growth media are selective to some degree in the micro-organisms that grow). The biomarker selected should be appropriate and representative of the group of micro-organisms chosen for study, and should be used in both the measurement of 'background' and 'operational' levels of bioaerosols.

4.3.4 Nucleic acid markers

Molecular-based analytical methods offer great potential in terms of specificity, sensitivity and speed of analysis (Crook and Sherwood-Higham, 1997). The use of gene-specific probes, in conjunction with amplification of the sample using PCR (polymerase chain reaction) can be used to detect the presence of microbiological material in any sample, and has been applied to the analysis of bioaerosols under simulated field conditions. As with other non-culture based methods, the detection of particular DNA or RNA sequences is applicable for the detection of both non-culturable and culturable microorganisms (Nugent *et al.*, 1997; Speight *et al.*, 1997).

Whilst nucleic acid-based techniques can be used to quantify micro-organisms in bioaerosols, the method is of most relevance to studies where information on numbers of specific micro-organisms is required, especially if those micro-organisms are present at low numbers. However, the absence of dose-response data for bioaerosols means that information on specific micro-organisms is of no greater benefit than total culturable counts data for the risk assessment. Therefore, whilst nucleic acid-based markers could be used to quantify bioaerosol emissions from green waste composting sites, the method offers no advantages over other approaches reviewed in this report.

4.3.5 Metabolic activity

Analysis of adenosine triphosphate (ATP) levels has also been used as a method for measuring microbial numbers in samples (Crook and Sherwood-Higham, 1997). The method is described as quantitative but non-specific (Speight *et al.*, 1997) in that it cannot distinguish between different microbial taxa. Measurement of ATP indicates the presence of metabolically active microbial cells, but as ATP levels vary with cell size and metabolic state, a direct relationship between levels of ATP and numbers of micro-organisms does not always exist (Crook and Sherwood-Higham, 1997).

The measurement of ATP has been used as an alternative to culture-based techniques to detect airborne micro-organisms (Stewart *et al.*, 1997). However, whether the method is suitable to assessments of green waste composting sites is questionable. Micro-organisms released from compost windrows may be expected to be more metabolically active than the background microflora. If this is the case, the ATP-bioluminescence method will not be suitable.

5 Selection of sampling technique

The selection of an appropriate sampling technique must consider the advantages and disadvantages of both the collection and quantification/enumeration systems used. For example, the use of some collection systems restricts the user to just one enumeration system. Micro-organisms collected by impaction-based samplers can only really be enumerated using culture-based techniques, whereas impingement or filtration methods are not restricted to the choice of enumeration method (Fannin, 1981).

Selection must also consider the purpose of the intended monitoring programme, the environment in which it is to be conducted and the information which may need to be derived from the results. If data are required on levels of a specific micro-organism for example, methods unable to identify that fungus or bacterium would not be suitable.

The purpose of this section is to outline the rationale for selecting a collection and enumeration system for measuring micro-organisms in bioaerosols from green waste composting sites.

5.1 Choice of collection technique

Of the collection strategies discussed, impaction or impingement systems are probably the most appropriate methods for sampling micro-organisms in bioaerosols released from green waste composting sites. Filtration systems are too susceptible to desiccation and are probably more suited to personal samplers for workers operating in more highly contaminated environments. Cyclone and electrostatic precipitation-based systems have the potential to be suitable samplers, especially given their good collection and recovery efficiencies. However, as noted by Mainelis *et al.* (1999), electrostatic precipitation requires further study before it can be proposed as a standard method. Losses of collection medium due to evaporation in cyclone systems may also need to be reduced before these systems can be recommended as a standard method.

A summary of the advantages and disadvantages of each of the collection systems is presented in Table 5.1.

Table 5.1: Advantages and disadvantages of collection techniques

Collection technique	Advantage	Disadvantage (limitation)
Impaction	<p>Direct collection of micro-organisms onto culture medium.</p> <p>Technique already widely used. Therefore considerable amount of data on collection and recovery efficiencies.</p> <p>Can process multiple samples in a day without having to sterilise the samplers in between samples.</p> <p>Single stage Andersen samplers will only collect the inhalable fraction.</p>	<p>Restricted to just culture-based enumeration methods.</p> <p>Risk of overloading culture plate when sampling in highly contaminated environments.</p> <p>Wind speeds during sampling may also affect sampling efficiencies, although the effects are less significant than those reported for filtration methods.</p>
Impingement	<p>No problem of overloading collection medium.</p> <p>Better survival of collected micro-organisms in liquid rather than solid collection medium.</p> <p>No restriction on subsequent enumeration methods used.</p> <p>Technique already widely used. Therefore considerable amount of data on collection and recovery efficiencies.</p>	<p>Samples require further processing post-collection before numbers can be quantified. This must be done without further growth or die-off of the micro-organisms collected.</p> <p>Question-mark over the robustness of impinger samplers (except the metal MLI).</p> <p>Problem of loss of collection liquid through evaporation.</p> <p>Will require sterilisation between samples.</p> <p>Wind speeds during sampling may also affect sampling efficiencies, although the effects are less significant than those reported for filtration methods.</p>
Filtration	<p>Easy to use.</p> <p>No restriction on subsequent enumeration methods used.</p>	<p>Relatively low recovery efficiencies due to desiccation of micro-organisms on the filter.</p> <p>Samples require further processing post-collection before numbers can be quantified. Must be done without further growth or die-off of the micro-organisms.</p> <p>Risk of overloading the filter when sampling in highly contaminated environments.</p> <p>Wind effects on sampling efficiencies.</p>
Cyclone	<p>Good collection efficiency due to reduced particle 'bounce' and loss through re-entrainment</p> <p>Easy to sterilise.</p>	<p>Problem of loss of collection liquid through evaporation.</p> <p>Relatively limited use of this system for collection of compost bioaerosols to date.</p>
Electrostatic precipitation	<p>Good recovery efficiency due to reduced stress on the micro-organisms during collection.</p>	<p>Limited study on this technique to date.</p>

Impingement and impaction-based samplers reported in the literature as suitable for monitoring bioaerosols in a field environment are the AGI-30 impinger and multi-stage liquid impinger; and the Andersen, SAS (Surface Air Systems), Burkard and Casella slit impactors. A number of studies have conducted comparative tests on some of these samplers, and have reported the following results (unless comparisons are made with different samplers used at the same time, comparisons of results in a quantitative

fashion may not be appropriate due to differences between experiments; comparisons presented below are thus purely indicative of the relative performance of methods):

- A comparison of the Andersen two-stage, Burkard portable and SAS Super 90 samplers found that the Burkard sampler has the highest collection efficiency for total bacteria, followed by the SAS Super 90 and the Andersen two-stage. However, there was no statistical difference between the three, indicating comparable collection efficiencies of the three techniques. The findings are consistent with a previous study investigating the collection of fungal spores (Mehta *et al.*, 1996).
- A comparison of various samplers found that the Andersen six-stage and AGI-30 samplers generally showed the highest yields of culturable micro-organisms among samplers using direct cultured plates and dilution plating respectively (Eduard and Heederik, 1998).
- Numbers of micro-organisms recorded by impingement methods are usually higher than those recorded using direct culture plates (impaction samplers). This is due to aggregates of micro-organisms collected in liquid impingers being broken up before culturing and is not a function of any greater sampling efficiency (Eduard and Heederik, 1998).
- The SAS (Surface Air Systems) high flow portable sampler recovered consistently lower levels of fungal spores compared to the Andersen single stage sampler (Bellin and Schillinger, 2001).
- Both the SAS and RCS samplers are not efficient collectors of small particles (Jensen *et al.*, 1992) and are therefore more suitable for the collection of fungal spores or pollen.
- A comparison of the Andersen six-stage sampler with the Reuter centrifugal sampler (RCS) found that the Andersen sampler collected a broader range of fungi (Tavora *et al.*, 2003).
- A comparison of the Andersen six-stage, the Andersen two-stage and the AGI-30 liquid impinger using aerosols of the bacteria (*Escherichia coli* and *Bacillus subtilis*) gave comparable results in their collection efficiencies (Jensen *et al.*, 1992). Recovery efficiency was not addressed.
- Liquid impingement samplers perform better than filtration methods for sampling airborne bacterial bioaerosols (Li *et al.*, 1999). This is a consequence of the poor survival of vegetative cells on the filter.
- Compared with the AGI-30, the SKC Biosampler was found to have a better collection efficiency (Willeke *et al.*, 1998). This was due in part to a much lower degree of re-aerosolisation of particles following their entrainment into the collection liquid.
- A comparison of the Andersen two-stage and Andersen six-stage samplers using aerosols of micro-organisms under one μm in diameter and at a concentration below 1,000 particles m^{-3} found that the two-stage sampler gave lower results (cfu/m^3) than the six-stage one (Gillespie *et al.*, 1991).
- An assessment of the Andersen single-stage and six-stage samplers found the two instruments comparable for estimating total airborne fungal levels (Jones *et al.*, 1995).

5.2 Choice of enumeration technique

All of the enumeration techniques reviewed here are suitable for the measurement of bioaerosols. The choice of method is therefore determined by the purpose of the monitoring strategy, and the probable funds and equipment available. Culture-based methods using selective isolation plates are probably the cheapest of the methods described and require very little in the way of technical equipment. Direct counting by epifluorescence microscopy is also relatively cheap to perform, but requires access to a suitable microscope, as well as to an image analysis system if a sufficient number of samples are to be processed.

For the purpose of the monitoring strategy, if information on the presence or absence of specific micro-organisms is required then the selective isolation plate method is probably the most suitable. However, if information on the total numbers of micro-organisms present in the bioaerosol is required, direct counting by epifluorescence microscopy is more appropriate. This method will provide higher counts than culture-based approaches and is more representative of total numbers present.

The advantages and limitations of the various methods are presented in Table 5.2.

Table 5.2: Advantages and disadvantages of enumeration techniques

Enumeration technique	Advantage	Disadvantage (limitation)
Culturable methods		
Selective isolation plates	Low cost and easy to use. Requires little technical equipment. Can be used to identify specific taxa of micro-organisms.	Will only identify viable and culturable micro-organisms. Non-viable cells (the majority of those likely to be present in the bioaerosol) will not be detected. Therefore not representative of the micro-organisms in the bioaerosol. Poor precision of measurement.
Most probable number	Relatively quick and easy to perform. Less susceptible to the culturability issues that affect selective isolation plate methods as the micro-organisms are grown in liquid media.	A statistical test and does not measure actual numbers of micro-organisms. Result may be affected by aggregates of cells. This is likely to limit the suitability of this method to analysis of bioaerosols.
Non-culturable methods		
Epifluorescence microscopy	Able to count both culturable and non-culturable cells. Therefore results are more representative of total numbers of micro-organisms in the bioaerosol. Relatively cheap operating costs. High throughput of samples possible if image analysis system used.	Limited ability to identify specific taxa of micro-organisms. Possibility of false-positive results with some fluorochromes binding to abiotic particles. Image analysis system may count abiotic particles within the same size parameters as microbial cells. Not suitable for counting aggregates of cells. Overestimation due to binding to abiotic material.
Flow cytometry	Same as for epifluorescence microscopy	Same as for epifluorescence microscopy
Biomarkers	Can be used to identify certain taxa of micro-organisms. Not prone to many of the limitations of culturable or non-culturable methods as whole cells are not measured.	No standard approach to which biomarkers should be monitored to provide certain information. Some biomarker tests such as the LAL assay are affected by dust or other microbial cell components. This is likely to be a significant problem with bioaerosols.

5.3 Choice of micro-organisms for study

As discussed, the micro-organisms measured will be determined in part by the choice of sampling programme, and in particular the type of enumeration method selected. Non-culturable methods will by design just measure numbers of broad groups of micro-

organisms, such as cells smaller than certain size parameters. Immunofluorescence methods may provide more specific monitoring, although this technique is not really suited to monitoring bioaerosols due to the non-specific binding of the fluorescent labelled antibodies to abiotic particles (Lindahl and Bakken, 1995).

The selection of specific micro-organisms for study, such as *Aspergillus fumigatus*, is therefore only possible if samples are cultured on selective isolation plates (although the continuing development of biomarker techniques may mean that these will soon be suitable for the identification of a wide range of individual microbial taxa).

The range of selective isolation media available means that monitoring programmes may be designed to identify a wide range of micro-organisms. However, the standard protocol requires monitoring of just one or two groups of micro-organisms (the absence of dose-response information means that information on numbers of many different micro-organisms is of no greater benefit than data on levels of more general groups, such as total fungi).

The purpose of the standard protocol is to assess relative exposure of people to micro-organisms released from the compost that may cause adverse effects to their health. It is therefore best to monitor for micro-organisms that are released from green waste compost and may cause adverse effects to human health.

Compost microflora includes fungi and both mesophilic and thermophilic bacteria. Whilst thermophilic bacteria such as *Saccharopolyspora sp.* and *Thermoactinomyces sp.* have been used as key indicators of compost microflora (Pillai and Ricke, 2002), they have not been identified as causing significant adverse effects to human health, compared to the fungi and mesophilic bacteria which cause both direct effects (as pathogenic micro-organisms) and indirect effects (through the production of toxins and allergenic materials) (Eduard and Heederik, 1998; Pillai and Ricke, 2002).

Micro-organisms proposed as indicator organisms are therefore fungi and mesophilic bacteria. Information on numbers of these two groups is sufficient to provide the Environment Agency with an understanding of the relative level of exposure of micro-organisms in compost which may cause health effects to people in the vicinity of the compost site. Both groups of organisms can also be enumerated relatively easily using selective isolation plates.

The culture medium adopted most frequently for the isolation and enumeration of fungi from environmental samples is malt extract agar (MEA) (Lacey and Dutkiewicz, 1976; Wu *et al.*, 2000). MEA media is also recommended by the ACGIH (Wu *et al.*, 2000), and has been used for the enumeration of fungi in bioaerosols (Bellin and Schillinger, 2001; Huang *et al.*, 2002; Sanchez-Monedero and Stentiford, 2003). Streptomycin (50 mg/l) and novobiocin (10 mg/l) can be added to the MEA media to suppress the development of bacterial colonies (Sanchez-Monedero and Stentiford, 2003).

On culturing the MEA plates, it is more relevant to count numbers of *Aspergillus fumigatus* colonies rather than total mesophilic fungal colonies. Work by Gladding *et al.* at various waste sites (Gilbert, 2004 *personal communication*) has indicated that *A. fumigatus* is a good indicator of fungi at composting sites. Other fungi are present in high numbers in bioaerosols produced from other sources, such as crop harvesting, but *A. fumigatus* is reported to be specific to composting.

The culture media commonly used for the isolation and enumeration of mesophilic bacteria are nutrient agar and TSA (tryptone soya or tryptic soy agar) (Lacey and Dutkiewicz, 1976). Both types of agar have been used for the enumeration of bacteria in bioaerosols (Crook and Lacey, 1988; Terzieva *et al.*, 1996; Heidelberg *et al.*, 1997; Mehta *et al.*, 1996; Sanchez-Monedero and Stentiford, 2003). Reducing the strength of the agar (to half- or quarter-strength) is reported to reduce the diameters of the colonies (Chang *et al.*, 1995), thereby reducing the potential for overlapping of colonies

and overcrowding on the plates. Such an approach might be useful when sampling in relatively contaminated environments.

5.4 Selection of sampling technique

Based on the information presented in this report, the sampling technique proposed as the standard method required by the Environment Agency for the monitoring of levels of micro-organisms in bioaerosols from green waste compost facilities is the Andersen single-stage sampler with selective isolation plates to measure total mesophilic bacteria (using nutrient agar or TSA incubated at 37°C) and total fungi (using MEA incubated at 40°C). The media used to culture the bacteria should contain 50 mg/l cyclohexamide (anti-fungal agent). The sampler should be fitted with a baffle to ensure still air above the inlet aperture.

The main reasons for this choice are:

Ease and simplicity of use

Both sampling and enumeration of micro-organisms are relatively easy to perform and do not require highly trained personnel. Micro-organisms are collected directly onto the selective isolation plates which require little further processing to count numbers.

The lack of further processing (except incubation) is viewed as a key advantage of impaction samplers over liquid impingers, even if the method restricts the user to the measurement of culturable micro-organisms.

The Andersen single-stage sampler has been recommended over the two-stage version because of ease of use. With both the single-stage and six-stage versions the top of the cone is held down by springs, and is therefore relatively easy to remove to change the plates during sampling. However, with the two-stage version the cone is screwed down, making it that much harder to change the plates (and could potentially lead to warping of the cone).

Information provided

Outputs from the technique (cfu of culturable micro-organisms per m³ of air) provide the level of information required by the Environment Agency to assess composting facilities.

As discussed, the information required is comparative, comparing 'operational' levels of micro-organisms with 'background' levels. In the absence of dose-response data, quantitative information on actual numbers of micro-organisms present is not required. The fact that non-culturable micro-organisms will not be measured is not considered important.

When (if) dose-response levels are developed, an enumeration method capable of measuring a greater proportion of the micro-organisms present may be required, as details on actual exposure levels will be needed (rather than just exposure to culturable micro-organisms as is measured with the proposed method). Under these circumstances, the metal MLI impinger with micro-organisms counted by epifluorescence microscopy would probably be a more suitable system.

With respect to the choice of the Andersen single-stage sampler over the other versions (two- and six-stage), one report has suggested a relatively poor collection efficiency for the two-stage sampler (Gillespie *et al.*, 1981), although Jensen *et al.* (1992) reported comparable collection efficiencies for the six-stage and two-stage samplers. The single-stage and six-stage samplers have been found to have

comparable collection efficiencies as well (Jones *et al.*, 1985; Gilbert, 2003 *personal communication*).

The single-stage sampler has been found to be more suitable for use at low concentrations of micro-organisms (Gilbert, 2003 *personal communication*). At low concentrations (such as those encountered when measuring 'background' levels) many of the culture plates in the six-stage sampler are blank. This leads to significant errors when cfu m⁻³ values are calculated (Gilbert, 2003 *personal communication*).

Cost

Whilst the technique will require a significant start-up cost to purchase the Andersen sampler, operation of the equipment and the making of selective isolation plates are relatively cheap. The Andersen single-stage sampler is the cheapest of the three (six-, two- and single-stage) to operate, as only one agar plate is required per sampling run.

Field suitability

The robustness and portability of the Andersen sampler means that it is suitable for use in the field. The all-glass construction of many of the liquid impingers means that they are more fragile and less suitable (Fannin, 1981). The exception is the metal MLI.

5.5 Guidance on QA procedures

Because the approach applied by the Environment Agency is a comparative system, measures should be taken to ensure that the approach used to measure 'background' levels of micro-organisms is the same as that to measure 'operational' levels.

Whilst the use of the baffle around the inlet aperture to the sampler should minimise the effects of wind speed, it would be beneficial to conduct the two sampling activities ('background' and 'operational' analysis) under similar meteorological conditions (temperature, relative humidity, wind direction). There should ideally be no rain at the time of sampling, as this is likely to reduce dispersion and size of the bioaerosol. Key meteorological parameters should be recorded at each sampling period.

Measures should be taken to minimise the level of microbial contamination of the culture plates, and ensure that the micro-organisms on the culture plates were collected from the bioaerosol during the sampling period. Measures proposed by the Association for Organics Recycling (Gilbert and Ward, 1999) are the type of approach that should be adopted.

If plastic Petri dishes are used, measures should also be taken to ensure that the plate(s) are located in the sampler so that the surface of the medium is at the height (within the collection chamber) specified by the manufacturer. Where plastic dishes are used, they will need to contain media such that the height of the agar is equivalent to that in the glass plates. For 90 mm plastic Petri dishes, 40 ml of agar is required.

Where possible, sampling of the micro-organisms should be performed in accordance with the principles set out in the Department of Health's Good Laboratory Practice compliance programme (Advisory Leaflet no.2: the Application of GLP Principles to Field Studies) (DoH, 1990; cited in Gilbert and Ward, 1999).

5.6 Summary of proposed method for measurement of bioaerosols

The standard method proposed for the measurement of bioaerosols in the vicinity of a green waste composting site uses the Andersen single-stage sampler fitted with a hemispherical baffle, with the micro-organisms collected onto either nutrient agar or TSA and MEA.

The outputs from the sampling will be expressed as cfu/m³, with the results from the nutrient agar or TSA plates providing a figure for the total number of culturable mesophilic bacteria present, and enumeration of colonies of *Aspergillus fumigatus* present on the MEA plates providing a measure of the fungal content of the bioaerosol.

Part 2 - Peer review and update

6 Overview

The Environment Agency commissioned the Health and Safety Laboratory (HSL) to peer review the WS Atkins report (Part 1) and specifically answer the following questions:

- Were the culturing methods described adequate?
- Were the sampling methods appropriate?
- Had the appropriate micro-organisms and microbially associated human health hazards been reviewed, with the necessary sampling, culturing and analytical techniques described?
- Had all the suitable methods for these aspects been adequately assessed, described and reviewed?

6.1 Conclusions of the peer review

The peer review of Part 1 came to the following conclusions:

- The review provided a useful overview, including a review of bioaerosol sampling devices in terms of the mechanisms by which they work (that is, how they collect airborne particles) with reference to some of the devices available, and the advantages and limitations of each mechanism.
- The review of samplers, however, mainly focused on two sampling mechanisms - impaction (mainly onto agar media) and impingement into liquid - with less focus on filtration as a potential method for monitoring bioaerosols on composting sites. The filtration methods discussed mainly focused on the use of low flow rate samplers such as IOM samplers. These are primarily designed for personal (breathing zone) air sampling but can with some limitations be used as fixed point samplers outdoors. However, little consideration was given to higher flow rate fixed point filtration samplers such as Partisol samplers used in air pollution monitoring. There is potential value in considering these methods in the context of Environment Agency requirements for the following reasons:
 - The equipment may be integral, with built-in sampler, programmable timer and pump. This would make its operation simpler.
 - Some equipment is battery-operated for use remote from power supply.
 - Sampling devices such as PM₁₀ heads could be used to collect health-relevant aerosols. Such methods would be compatible with other environmental dust and particulate pollution monitoring.
 - Although cost of equipment may be little different from Andersen samplers, it would be less labour intensive to set up and run. This would overcome other problems such as sampling affected by adverse weather and the availability of site personnel.

- Samplers could be run continuously for long periods to provide a more representative temporal picture of bioaerosol release at site boundaries. Some devices have automated sample changers for timed sample collection during the monitoring period.
 - Post-sample handling could be simplified. If necessary, samplers could be stationed at a site for long periods and samples despatched to the analyst. This is less applicable to Andersen sampling.
 - Although a potential problem with filtration sampling is dehydration causing loss of bioaerosol culturability, this may be less of an issue with fungal spores and actinomycete bacterial cells – the agents of greatest health consequence on compost sites.
 - Other analyses, such as total cell counts, biomarkers, molecular markers, could be analysed from the same sample.
- In the review of analytical methodology, culture-based methods referred only to a limited number of agar media. Cranfield University have developed a novel method for the isolation of actinomycetes using a soil compost media at an incubation temperature of 44 °C and seven days' incubation (Taha *et al.* 2007). This method has overcome masking of conventional agar plates, as well as reducing analysis time and costs. For fluorescence microscopy, the description of fluorochromes available was limited. However, sources of error exist with fluorescent microscopy counting (not fully detailed) which need to be addressed if it is to be used as more than a research tool. For instance, Prigione and Filipello Marchisio (2004a) found that the very thick and resistant cell walls of spores and conidia of most fungi could severely impede staining with fluorescent dyes, which could limit epifluorescence microscopy as a means of direct detection and quantification of spores.
 - In reviewing the measurement of biomarkers, endotoxin measurements were described but there was no reference to workplace endotoxin measurement protocols outlined in British Standard/European Standard guidelines (BS EN 13098:2001, *Workplace atmospheres. Guidelines for measurement of airborne micro-organisms and endotoxins*, and BS EN 14031:2003, *Workplace atmospheres. Determination of airborne endotoxins*).
 - The review made only limited reference to molecular-based analytical methods, which undersells the potential for this area of analysis in characterising compost bioaerosols and comparing with background data.
 - Appropriate sampling techniques for monitoring compost bioaerosols were summarised, including their advantages and limitations. However, for collection of bioaerosols into liquid (impingers and cyclones), the practical considerations of storage and transport of samples, such as refrigeration to maintain sample stability, was not mentioned.
 - Although the introduction to the review summarised the practical requirements for sampling, those practicalities were not fully considered in the final recommendations. For example, although the Andersen impaction sampling method is a well recognised and valuable technique, the following needs to be considered in the process of monitoring:
 - Andersen sampling requires the use of expensive equipment (in multiples for simultaneous sampling).

- The method is labour-intensive, therefore costly to undertake, partly because of the short sampling times which require frequent reloading of samplers.
- The equipment is bulky, requiring separate vacuum pumps, and either mains power supply or generators.
- There is a question as to whether the short sampling times are pertinent to the information required by the Environment Agency. Short sampling times may be used as part of a sampling strategy to tie in with specific work tasks. This could be valuable for measuring peak exposure events, including occupational exposure, but for site boundary and sensitive receptor exposure issues it may be more relevant to undertake long-term monitoring.

Practicalities and strategies when sampling bioaerosols on composting sites are considered further in Section 6.7.

In summary, Part 1 recommended the same choice of sampling and analysis as had the ‘*Standardised protocol for the sampling and enumeration of airborne micro-organisms at composting facilities*’ (1999). The following sections aim to provide some updated information to supplement Part 1.

6.2 Overview of molecular techniques to monitor bioaerosols from compost

Microbiology has seen a huge transformation over the past two decades as a result of the way in which microorganisms can be studied. It has long been realised that culture-dependent methods are selective, as only a fraction (0.001 to 15 per cent) of bacteria from environmental samples can be cultured (Amann *et al.*, 1995). To avoid the bias inherent in culturing, molecular methods are increasingly used to assess microbial diversity in a wide range of habitats.

Molecular techniques are increasingly used to investigate the microbial content and diversity of composting materials. A logical extension of this is to apply these techniques, where possible, to isolate and characterise bioaerosols emitted from composts. Current work at the National Physical Laboratory (funded by Department for Environment, Food and Rural Affairs (Defra) WR0605) in the UK aims to develop methods in this area and is testing a new real-time monitoring network to quantify emissions of potentially harmful biological species in the immediate vicinity of waste processing sites. The following section summarises a few of the more common molecular techniques that could be applied to study microorganisms in compost or in aerosols. Such techniques include PCR amplification followed by cloning and/or sequencing (Fierer *et al.* 2008), DNA fingerprinting (Halet *et al.* 2006), real-time PCR (Novinscak *et al.* 2007) and microarrays (Franke-Whittle *et al.* 2005).

6.2.1 Polymerase chain reaction (PCR)

Since the development of DNA sequencing (determining the order of nucleotides in a DNA fragment) in the late 1970s and polymerase chain reaction (PCR) in early 1980s, advances in molecular techniques now make possible the detection, identification and quantification of airborne fungi, bacteria and viruses. The power of PCR lies in its ability to amplify and copy small amounts of target DNA from environmental samples. It is probably the most versatile yet precise of all biological techniques. Short pieces of DNA can be amplified about a million-fold in a short space of time (minutes to a couple of

hours). A series of heating and cooling cycles are used to drive the splitting, annealing and extension phases. At the end of many cycles, large amounts of target DNA are available for further analysis. For more detailed information see http://en.wikipedia.org/wiki/Polymerase_chain_reaction.

6.2.2 DNA fingerprinting

One approach that would elucidate the microbial composition of compost or bioaerosols involves the analysis of part of the bacterial genome, that is, a targeted piece of DNA such as 16S rRNA gene. This approach usually involves a PCR-mediated step. Essentially genomic DNA is extracted from the sample and subjected to several rounds of PCR. Either the whole gene or a fragment of the gene can be amplified. The genes can then be cloned directly and sequenced for identification or they can be analysed using a fingerprinting technique such as amplified ribosomal DNA restriction analysis (ARDRA) or denaturing gradient gel electrophoresis (DGGE) analysis.

ARDRA is a technique used to compare bacterial communities. It is based on comparison of the analysis of polymorphisms within a DNA fragment which are obtained as a result of DNA cleavage using a restriction enzyme. Briefly, a previously amplified fragment of DNA is digested with a restriction enzyme (cuts DNA at a specific DNA sequence). The DNA fragment is then split into a variable number of fragments of different size. These DNA fragments are separated on a high-resolution agarose gel and a restriction pattern is visualised. This technique is often combined with cloning, which is a means of separating individual DNA fragments out of a complex mix to make interpretation simpler. Individual fragments can be sequenced for identification.

DGGE is a techniques used to separate amplicons that are the same length, but vary in their nucleotide sequence. It is based on the principle that double stranded DNAs migrate at a faster rate compared to partially single stranded DNAs in a polyacrylamide gel. Briefly DNA is extracted from your sample and your target gene amplified using universal primers. Denaturing (urea gradient) polyacrylamide gels are used to separate the double stranded fragments according to their melting properties. Dissociation of the double stranded DNA fragments causes branching and the branched structure becomes entangled in the gel matrix and mobility in the gel is retarded. The melted stands migrate at different rates through the gel thus separating the fragments of different DNA sequence and a multiple banding pattern or community profile should appear on the gel. These bands are indicative of the different target sequences in the original sample. The resultant bands can be sequenced for identification.

Further information available from:

http://en.wikipedia.org/wiki/Amplified_Ribosomal_DNA_Restriction_Analysis

http://en.wikipedia.org/wiki/Temperature_gradient_gel_electrophoresis

6.2.3 Real-time PCR (or quantitative PCR)

Real-time PCR or quantitative PCR (qPCR) has established itself as a sensitive qualitative and quantitative technique that has become important in all areas of microbiology. It follows the general pattern of PCR, but the DNA is quantified after each round of amplification; this is the "real-time" aspect of it. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-strand DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA (cDNA).

The basic idea behind real-time PCR is that the more abundant a particular cDNA is in a sample, the earlier it will be detected during repeated cycles of amplification. Various systems allow the amplification of DNA to be followed and often involve the use of a fluorescent dye which is incorporated into newly synthesised DNA molecules during real-time amplification. Real-time PCR machines, which control the thermocycling process, can then detect the abundance of fluorescent DNA and thus the amplification progress of a given sample. Typically, amplification of a given cDNA over time follows a curve, with an initial flat phase followed by an exponential phase. Finally, as reagents are used up, DNA synthesis slows and the exponential curve flattens into a plateau. This technique is an enormously powerful tool and theoretically amplification can be achieved from as little as a single starting template. Valasek and Rep (2005) provide a comprehensive overview of real-time PCR. They discuss the basic concepts, chemistries, and instrumentation of real-time PCR and its advantages and limitations.

6.2.4 Microarrays

DNA microarrays go by many other names, including DNA chip, PhyloChip, genome chip, expression chip and gene array. In addition, a Genechip® (registered trademark of Affymetrix Inc.) is a type of microarray manufactured using a process analogous to that used for making computer chips.

Microarrays offer a fast, high-throughput alternative for the parallel detection of microbes from virtually any sample. Microarrays are composed of many discretely located probes on a solid substrate such as glass. Each probe is composed of a sequence that is complementary to an organism-specific gene sequence. PCR is used to amplify one or more genes and the products are then hybridised to the array to identify species-specific polymorphism within one or more genes. They can also be used to 'fingerprint' bacterial isolates and to identify diagnostic markers suitable for developing new PCR-based detection assays. In recent reviews (Bodrossy and Sessitsch, 2004; Sergeev *et al.*, 2004; Loy and Bodrossy, 2006), the application potential is described as it spreads across most sectors of life sciences, including environmental microbiology and microbial ecology; human, veterinary, food and plant diagnostics; water quality control and industrial microbiology.

6.3 Use of molecular techniques to study bacteria

Harvey *et al.* (2001) used ARDRA to develop a rapid method of identifying thermophilic organisms. The impetus for this was that pulmonary diseases such as extrinsic allergic alveolitis (hypersensitivity pneumonitis) can be caused by thermophilic mycelial bacteria such as *Saccharopolyspora rectivirgula*, *Saccharomonospora viridis*, *Thermoactinomyces sacchari*, and *Thermoactinomyces vulgaris*. However, air sampling analyses in highly contaminated environments to evaluate exposure to these species are limited by their fastidious growth requirements and the difficulty of identifying them by conventional techniques. ARDRA was successfully applied to 49 thermophilic actinomycete-like strains from sawmill samples and the authors recognised its potential application to other environments such as compost plants.

Ishii *et al.* (2000) studied microbial succession during a laboratory-scale composting process of garbage using DGGE. The DGGE band pattern was found to change during the composting process, with fermenting bacteria dominating the mesophilic phase and thermophilic bacillus the thermophilic phase; after the cooling phase, bacterial populations were more complex than in previous phases. Thus, the DGGE method

was found to be useful in revealing microbial succession during a composting process. In further related work, Ishii and Takii (2003) compared microbial communities during four full-scale composting processes, which were again analysed by DGGE combined with measurement of physicochemical parameters. Comparison of the four processes indicated that the concentration of dissolved organic carbon was higher in food waste composting than in sewage sludge composting, and microbial communities varied with composting substrate. The tendency for different microbes to appear in the composting process with different concentrations of dissolved organic carbon agreed with previous studies, and suggested that the main factor affecting microbial communities was the concentration of dissolved organic materials. The authors claimed that this research was the first to study composting mechanisms using molecular methods.

Recent developments in DNA microarray chip technology have also made it possible to characterise microbial populations and this has been used for compost population studies (Kim *et al.*, 2004; Franke-Whittle *et al.*, 2005).

Kim *et al.* (2004) developed a tool, based upon a DNA microarray chip, to identify specific bacteria from activated sludge, using the hybridisation of genomic DNA with random probes. In this chip, the sets of target probes were immobilised on amine-coated glass and constructed with PCR products derived from randomly fragmented genomic DNAs extracted from pure cultures of the three target strains. Initial hybridisation results, when pure cultures were employed, showed the specificity of the probes as well as the resolution of the system, demonstrating the capabilities of this system to identify specific bacterial strains. The microarray was also tested for its ability to distinguish specific bacteria from among mixed bacterial communities, such as in sludge, soil or spiked genomic DNA samples. The results showed that the probes were specific, with only mild cross-hybridisation in a small number of cases. Furthermore, the chip clearly discriminated between all three strains when they were alone or together within mixed samples. Moreover, using the spot intensity and DNA hybridisation kinetics, the starting genomic DNA concentrations could be estimated relatively well, which would make it possible to predict the number of specific bacteria present within test samples. In conclusion, the random genomic hybridisation approach, without any sequence information available for the probes, is a practical protocol for identifying and screening for specific bacteria within any complex bacterial community from environmental samples such as activated sludge.

Franke-Whittle *et al.* (2005) developed a molecular tool to allow screening for the presence or absence of different micro-organisms within compost samples. A microarray consisting of oligonucleotide probes spotted on aldehyde-group coated glass slides, targeting variable regions of the 16S rRNA gene, was designed and tested for the investigation of microbial communities in compost. Probes were designed for micro-organisms previously reported in the composting process and for plant, animal and human pathogens. Details of the target species are available at http://www.microbial-ecology.net/probebase/array.asp?array_id=8. The microarray was found to have a detection limit of 10^3 cells, although when using microlitre sample volumes from gram quantity extracts of compost material, in compost spiking experiments, the detection limit was reduced to 10^5 cells because of the presence of humic acids extracted and co-purified with the nucleic acid extraction.

Brodie *et al.* (2007) developed a custom high-density DNA microarray (PhyloChip) to detect and monitor bacterial populations in urban air. They looked at how the composition of aerosols changed over time and with location. The tool they developed allows bacteria to be identified and monitored in any type of sample without the need for cultivation. Developed at Berkeley Lab in the USA, this microarray quickly and accurately identifies known and unknown bacteria in any sample. Unknown bacteria are classified based on their similarities to known micro-organisms. The high-density microarray format which combines multiple probes with a paired mismatch-control

probe for each perfect match probe significantly reduces the chances of misidentifying a specific micro-organism. When the microarray was tested in urban air in two Texas cities, over 1,800 types of bacteria were found. Before this study, there was no sense of the actual diversity of micro-organisms in air.

6.4 Use of molecular techniques to study fungi

Zeng *et al.* (2006) developed two real-time PCR systems to accurately quantify levels of *Cladosporium*, one of the most common airborne moulds found indoors and outdoors. *Cladosporium* spores are important aeroallergens, and prolonged exposure to elevated spore concentrations can provoke chronic allergy and asthma. The real-time PCR systems were found to be highly specific and sensitive for *Cladosporium* detection even in a high background of other fungal DNAs, and were used to quantify the fungus in aerosols of five different workplace and domestic indoor environments.

Cruz-Perez *et al.* (2001) used laboratory cultures to establish a protocol for the rapid detection and quantification of *A. fumigatus* using genetic amplification. Oligonucleotide primers and a fluorescent-labelled probe were designed for use with qPCR. Primers and probe were tested for selectivity, specificity and sensitivity of detection of the target organism using a fluorogenic nuclease assay and a sequence detector. The primer set developed was specific for *A. fumigatus* and had a sensitivity of below 20 template copies. These primers amplified all *A. fumigatus* isolates tested and did not amplify DNA extracted from other *Aspergillus* species or 15 other fungal genera. This research resulted in a qPCR method for the detection and quantification of *A. fumigatus*.

McDevitt *et al.* (2004) investigated the potential advantage of molecular methods such as qPCR over culture and optical methods for estimating human exposure to fungi. Using qPCR, the analysis of airborne *A. fumigatus* samples collected over extended time periods provided a more representative assessment of chronic exposure. The assay detected fewer than ten *A. fumigatus* conidia per qPCR reaction and quantified conidia over a four-log₁₀ range with high linearity and low variability among replicate standards in less than four hours. The sensitivity and linearity of qPCR for conidia deposited on filters was equivalent to conidia calibration standards. *A. fumigatus* DNA from eight isolates was consistently quantified using this method, while non-specific DNA from 14 common fungi, including six other *Aspergillus* species, was not detected. This method therefore provided a means of analysing long-term air samples collected on filters, and a potential alternative to culture-based methods for assessing compost bioaerosols, as well as a method for correlating airborne *A. fumigatus* conidia concentrations with adverse health effects.

Haugland *et al.* (2004) developed a total of 65 quantitative qPCR assays and tested them for the detection of selected *Aspergillus*, *Penicillium* and *Paecilomyces* species. The assays varied in specificity from species or subspecies to closely related species groups. A generic assay for all target species of *Aspergillus*, *Penicillium* and *Paecilomyces* was also developed and tested. Estimated conidia detection limits for target species ranged from less than one to several hundred per sample for the different assays. After spiking samples with target organisms, qPCR analyses indicated that enumeration was within approximately a one-half log range of the expected values 95 per cent of the time.

6.5 Summary of molecular techniques

The studies above provide a brief summary of where molecular tools have been applied in environmental monitoring, and demonstrate their potential to uncover the composition and dynamics within different environmental compartments. They may be capable of characterising a diversity of micro-organisms that might not be possible using traditional culture techniques. In terms of using such techniques to assess bioaerosols emissions from compost, the choice of technique depends on whether qualitative or quantitative information is needed.

DNA fingerprinting and microarray techniques are more qualitative and provide an insight into the types of micro-organisms present in a sample and how these can change over time. Such techniques would be useful to identify prevalent species. These prevalent species could then be used as biomarker species and probes developed for their detection and quantification using qPCR.

All of the above techniques rely on PCR amplification of a mixed product from a complex DNA sample. These methods, like most, are not without limitations and PCR itself is subjected to amplification bias. However, limitations are likely to be outweighed by the speed and accuracy in determining microbial composition compared to traditional culture techniques, and bias such as template saturation during PCR can be avoided by effective optimisation of protocols.

6.6 Bioaerosol sampling and detection methods - updated

There is no single method of bioaerosol measurement, and the sampling mechanisms used may influence the results obtained.

To understand the advantages and limitations of the range of bioaerosol samplers used, a number of papers have compared methods and devices currently available. Many of these have been described in Part 1, but more recent developments are discussed in the following section.

An *et al.* (2004) investigated the physical and biological performances of a portable centrifugal sampler for viable bioaerosols, RCS High Flow. In laboratory tests, the RCS High Flow sampler was found to enumerate approximately 40 per cent of *Bacillus globigii* (BG; a bacterium commonly used in evaluation studies) spores and cells relative to another sampler, the BioSampler, used as a reference. A similar ratio was found during testing in an indoor environment. However, this ratio decreased to below 10 per cent when testing was performed outdoors. Therefore, it was concluded that while the RCS High Flow sampler offered certain advantages over other samplers for viable bioaerosols, being lightweight, battery-operated, and collecting viable micro-organisms at a high flow rate directly on agar media, the results obtained would have to be adjusted to avoid potential underestimation of micro-organism concentration in the air, especially if sampling was performed outdoors.

Many different bioaerosol sampling devices are commonly used for indoor air quality studies. Four of these, the RCS, Andersen N6 Single-Stage and Surface Air System Super 90 culturable sampling devices, and the Air-O-Cell particulate sampling device, were used to take about 300 side-by-side measurements in 75 public buildings (Lee *et al.*, 2004b). The results obtained from these instruments showed high linear correlations, but were not directly comparable. To compare data from research studies it would be necessary to know the relationships between the equipment performances and would require inter-instrument calibration. The researchers developed regression

models to provide the basis for inter-instrument comparisons, by converting concentrations measured using one instrument to estimates of concentration using another, for example a 'reference' instrument such as the Andersen N6. The range of measurements, detection limits and environmental factors may affect comparisons, and while direct comparisons between sampling data were not possible, the regression models they reported explained 60 to 85 per cent of the variance in fungal concentrations, and emphasised the effect of environment on measurement.

More recently, Yao and Mainelis (2007) analysed the collection efficiencies of the MAS-100, Microflow, SMA MicroPortable, Millipore Air Tester, SAS Super 180, BioCulture, and RCS High Flow portable microbial samplers when sampling six bacterial and fungal species ranging from 0.61 to 3.14 micron in aerodynamic diameter. The efficiencies with which airborne micro-organisms were deposited on samplers' collection medium were compared to the particle inhalation and lung deposition convention curves, that is, the international standard models for entry of particles into the human lung. When sampling fungi, RCS High Flow and SAS Super 180 deposited 80 to 90 per cent of airborne spores on agar, which was the highest of the samplers tested, with other samplers showing collection efficiencies of only 10 to 60 per cent. When collecting bacteria, RCS High Flow and MAS-100 collected 20 to 30 per cent, whereas other samplers collected less than 10 per cent. Comparing the samplers' collection efficiencies with particle inhalation convention curves showed that RCS High Flow and SAS Super 180 could be used to assess inhalation exposure to particles larger than 2.5 micron, such as fungal spores. The RCS High Flow sampler was also considered to reflect the particle lung deposition pattern when sampling both bacteria and fungi. MAS-100 and SAS Super 180 matched the total deposition curve fairly well when collecting bacterial and fungi species, respectively. For other tested samplers, the authors observed substantial discrepancies between their performances and particle deposition efficiencies in the lung. Their results, therefore, differed from previous comparisons in terms of rating the performance of some samplers. The paper also showed that the feasibility of using portable microbial samplers for exposure assessment depends on a particular sampler model and microbial species.

Aizenberg *et al.* (2000a) assessed the performance of three devices for the total enumeration of airborne bacterial and fungal spores. The devices – the Air-O-Cell sampling cassette, the Burkard personal volumetric air sampler and the Button Aerosol Sampler – were evaluated under controlled laboratory conditions. The first two are glass-slide impactors, while the third collects spores on a filter. The samplers were tested with polystyrene latex particles, two bacterial species (*Streptomyces albus* and *Bacillus subtilis*) and three fungi (*Cladosporium cladosporioides*, *Penicillium brevicompactum*, and *Penicillium melinii*). Collection efficiency of the Button Aerosol Sampler was close to 100 per cent for the entire particle size range studied. Acridine orange (with epifluorescent microscopy) and lactophenol cotton blue (with bright light microscopy) staining techniques were used to enumerate spores microscopically. Counts were not significantly different between the two techniques with the Button Aerosol Sampler filters, but when lactophenol cotton blue staining was used to compare total microbial counts yielded by all three samplers, the Button Sampler showed significantly higher counts for the smaller size micro-organisms (*B. subtilis* and *C. cladosporioides*). For the larger micro-organisms (*Penicillium* species), all three samplers yielded similar results. These results suggest that filter collection using the Button Aerosol Sampler can be advantageous for the enumeration of total airborne spores. This performance was confirmed by the authors (Aizenberg *et al.*, 2000b) in further studies in the field.

Witschger *et al.* (2004) also evaluated existing personal aerosol samplers. Most of these had been characterised primarily with wind tunnel tests conducted at relatively high wind speeds, whereas modern indoor occupational environments usually have very slow moving air. Thus, the first objective of the study was to design and test an

experimental protocol for measuring the sampling efficiency of personal inhalable aerosol samplers in the vicinity of the aerosol source, when the samplers operate in very slow moving air. The second objective was to evaluate three widely used aerosol samplers: the IOM Personal Inhalable Sampler, the Button Personal Inhalable Aerosol Sampler and the 25 mm Millipore filter holder (closed-face C25 cassette). The Button Sampler efficiency data showed good agreement with the standard inhalable convention and especially with the low air movement inhalability curve. The 25 mm filter holder was found to considerably under-sample particles larger than 10 microns and its efficiency did not exceed seven per cent for particles of 40-100 micron size. The IOM Sampler facing the source was found to over-sample compared with data obtained previously with a slow-rotating, freely suspended sampler in a low air movement environment. Particle wall deposition in the IOM metallic cartridge was also found to be rather significant and particle size dependent. For each sampler (IOM, Button and C25), the precision was characterised through the relative standard deviation (RSD) of the aerosol concentration obtained with identical samplers in a specific experiment. The average RSD was 14 per cent for the IOM Sampler, 11 per cent for the Button Sampler and 35 per cent for the 25 mm filter cassette.

Button Personal Inhalable Samplers were used to compare personal and stationary sampling to assess farmers' exposure to airborne fungi on pig, dairy and grain farms (Adhikari *et al.*, 2004). The total concentration of airborne fungi ranged from 1.4×10^4 to 1.2×10^5 spores per m^3 in the three locations. Grain unloading and handling activity generated the highest concentrations of airborne fungi compared to the other two activities. Lower coefficients of variation were observed for the fungal concentrations measured by personal samplers (7-12%) compared to the concentrations measured by stationary samplers (27-37%). No statistically significant difference was observed between the stationary and personal measurement data for total concentrations of airborne fungi. The results indicated that personal exposure of agricultural workers in confinements may be adequately assessed by placing several Button Samplers simultaneously operating in a static stationary mode throughout the work site.

The personal bioaerosol sampler CIP 10 is a well-characterised and efficient sampler for airborne particles for gravimetric analysis. An adaptation of this, the CIP 10-M (M-microbiologic), has been developed to measure worker exposure to airborne biological agents (Gorner *et al.*, 2006). This sampler is battery-operated, is light and easy to wear and offers full work shift autonomy. It can sample much higher concentrations than biological impactors and limits the mechanical stress on micro-organisms. Biological particles are collected in 2 ml of liquid medium inside a rotating cup at an air flow rate of 10 litres per minute. Three particle size selectors allow health-related aerosol fractions to be sampled according to international conventions. The sampled microbiological particles can be easily recovered for counting, incubation or further biochemical analysis, for example for airborne endotoxins. The device's physical sampling efficiency has been laboratory tested and field trials have been carried out under industrial waste management conditions. The results indicate satisfactory collection efficiency, whilst experimental application has demonstrated the usefulness of the CIP 10-M personal sampler for individual bioaerosol exposure monitoring.

A number of papers have described new bioaerosol sampling and detection methods which may prove useful in the future, but are currently only applicable as research tools. Many of these are aimed at biodefence-based bioaerosol sampling. For example, Fergenson *et al.* (2004) described a mass spectrometry-based analytical technique for the real-time and reagentless characterisation of individual airborne cells without sample preparation. Rapid chemical analysis of individual cells was considered to have potential application to bioaerosol detection for biodefence and cellular diagnostics for clinical medicine. Clark Burton *et al.* (2005) evaluated filter materials and extraction methods for the sampling of *Bacillus anthracis* (anthrax). A mixed cellulose ester and polytetrafluoroethylene (PTFE) filters in combination with vortexing

and shaker extraction demonstrated the best performance for the filter collection and extraction of BG spores. Although not directly applicable to compost bioaerosol sampling, the extraction methods may be useful for optimising filtration-based sampling methods.

Lee *et al.* (2004a) described a new design of an electrostatic precipitator (ESP) for bioaerosol sampling primarily aimed at the biodefence market. An important parameter affecting the behaviour of airborne micro-organisms is their surface electrical charge, and the aim was to use this in a novel bioaerosol sampler. Sivasubramani *et al.* (2004) developed and evaluated a fungal spore source strength tester (FSSST), which aerosolised spores from growth surfaces and sampled the airborne fungi into a bioaerosol sampler. The method was developed to assess indoor fungal sources with respect to their spore aerosolisation potential. It was hypothesised that airborne fungal spore concentrations measured with air samplers during specific time intervals do not adequately represent maximum spore concentration levels, because of the sporadic nature of spore release. The FSSST was able to provide optimum conditions for spore aerosolisation and could potentially be used in the field to assess the maximum spore release from a fungal source. This method could be useful for assessing bioaerosol sources from industrial materials such as compost.

6.7 Bioaerosol sampling on composting sites: practicalities and strategies

Under ideal circumstances, bioaerosol sampling on and around compost facilities would be carried out to a consistent standard under well-controlled conditions. In practice, conditions on a composting site make it difficult to measure bioaerosols consistently and reproducibly. However, measures can be introduced to minimise site influences on the outcome of bioaerosol monitoring, and these may stimulate ideas for changing the manner of monitoring to eliminate variables. In the following table (Table 6.1), the criteria listed in Section 2.3 for bioaerosol monitoring, and the sampling strategy requirements in the AFOR protocol ‘*Standardised protocol for the sampling and enumeration of airborne micro-organisms at composting facilities*’ (1999), are set against practical problems or questions arising from monitoring exercises undertaken by HSL.

Table 6.1: A comparison of ideal requirements and the practical problems involved in bioaerosol monitoring

Ideal requirements for compost site bioaerosol monitoring	Practical problems to achieving requirements; relative importance to success of bioaerosol monitoring
Criteria for efficient bioaerosol monitoring (Section 2.3)	
Collection efficiency – the ability of the sampler to collect the bioaerosol present. This is usually expressed as a percentage relative to reference samplers. These are defined in terms of their ability to collect according to the international standard convention for human particle inhalation.	Of fundamental importance to all bioaerosol sampling, although small differences in collection efficiency between sampling methods are less critical compared to other variables affecting bioaerosol concentrations. Some bioaerosol components will be collected with greater efficiency than others for some methods (see Section 5). Of greater importance is establishing a common method which, even if not the

Ideal requirements for compost site bioaerosol monitoring	Practical problems to achieving requirements; relative importance to success of bioaerosol monitoring
	most efficient, allows for comparability of results.
<p>Sampling time. Longer sampling times may overload bioaerosol samplers collecting directly onto agar plates, dehydrate vegetative micro-organisms collected on solid surfaces, or lose liquid from impinger-based and cyclone-based samplers. Conversely, longer sampling times are likely to provide a better estimate of mean microbial numbers.</p>	<p>This has major implications for the method of choice of Andersen sampler. It can be run for no more than a few minutes when sampling close to a major bioaerosol source. Extrapolating to the broader picture of bioaerosol emissions must be questioned, along with whether short sampling times provide the information required by Environment Agency. Short sampling times may be used as part of a sampling strategy to tie in with specific work tasks. This may be valuable for measuring peak exposure events, including occupational exposure, but for site boundary and sensitive receptor exposure, it may be more relevant to undertake long-term monitoring.</p>
<p>Sampler reliability, that is, dependability and robustness of the instrumentation used. This is of particular importance for the monitoring of composting bioaerosols outdoors, often in locations that may only be accessible on foot.</p>	<p>This is a critical factor. Samplers need to be able to withstand exposure to a harsh external environment. Although sampling would not be done in high winds or rain, equipment may be placed on site for extended periods while waiting for appropriate sampling conditions or a specific event to be monitored.</p>
<p>Ease of sterilisation.</p>	<p>Critical to ensure that the sample collected is bioaerosol and not cross contamination, especially from soil or dust</p>
<p>Maintenance of collected sample viability. Field sampling means that enumeration of micro-organisms collected is unlikely to be possible at the point of sampling. Therefore, survival of the micro-organisms post-collection and pre-enumeration is an important consideration.</p>	<p>A critical factor for culture-based analysis. Impaction onto agar favours survival of less robust cells in this instance, although more robust cells may be able to withstand sampling stresses and post-sampling storage prior to processing for analysis. Less critical for non-culturable methods. Development of, and confidence in, microscopic or molecular analysis or health biomarkers may reduce the criticality of this parameter.</p>
<p>Ease of operation, with implications for cost and requirements for trained personnel.</p>	<p>If a simple method can be used, sampling could be done by technical support staff or by site representatives, with samples sent to analytical support laboratories.</p>
<p>Ease of sample assay, which also has cost and training implications.</p>	<p>Culture-based analytical methods are relatively simple but are often labour intensive. Microbiological characterisation</p>

Ideal requirements for compost site bioaerosol monitoring	Practical problems to achieving requirements; relative importance to success of bioaerosol monitoring
	requires skill. Alternative methods such as molecular-based ones may be more costly in reagents and still require skill, but there may be savings in staff time and less subjective results. Some may lend themselves to greater automation. If specific targets are identified, they may even be developed as field assays.
Remote operation capability, including a requirement for the technique to operate on battery power, as mains power is unlikely to be available in the field. Samplers with greater remote operation capacity may also offer more versatility in the field than those that require constant attention.	Battery-operated samplers or operation from portable generators is feasible, but may increase the size and weight of sampling equipment. Of fundamental importance to locating samplers where required, or being able to change locations according to site activities or wind direction.
Particle size discrimination. This is a key consideration for the identification of micro-organisms likely to cause respiratory infection. An ideal sampler for human aeroallergens and aeropathogens should be able to collect the same size fraction and concentrations as are inhaled by the human respiratory system.	The Andersen agar impactor is capable of collecting up to six size fractions depending on the type used, but its design pre-dates the ISO/CEN convention for health-based particle size fractions (inhalable, thoracic, respirable particles) and the size discrimination approximates to this convention. More recently designed bioaerosol samplers do not necessarily follow the ISO/CEN convention. Other particulate samplers are better characterised to the above, or to criteria such as PM ₁₀ or PM _{2.5} .
Micro-organisms to be monitored should include those identified as causing potential adverse effects to human health, such as the fungus <i>Aspergillus fumigatus</i> and Gram-negative bacteria. Thermophilic micro-organisms such as <i>Saccharopolyspora</i> sp. and <i>Thermoactinomyces vulgaris</i> that are characteristic of compost environments might also be monitored. However, because of possible allergenic effects caused by a variety of components in bioaerosols (including micro-organisms and endotoxins), it may be more suitable to employ less specific monitoring and measure total numbers of bacteria or fungi.	Critical. However, data is still needed on the importance of key microbial components in relation to health burden. It may be best to monitor for specific predominant agents, especially for 'fingerprinting' a bioaerosol emission source and attributing it to a compost site or specific activity. However, in terms of allergenic burden and health-based monitoring, it may be better to measure markers such as total protein content of an aerosol.
Cost of bioaerosol sampling equipment.	Critical to the wider ability for monitoring. The cost of individual items of sampling equipment, multiplied by requirements for sampling at numerous locations, places

Ideal requirements for compost site bioaerosol monitoring	Practical problems to achieving requirements; relative importance to success of bioaerosol monitoring
	routine sampling outside the budget of many. Alternatives, which may be explored by Defra in their current research programme, would be simple top level monitoring supported by in-depth sampling and analysis if required.
Sampling strategy requirements from AFOR Standardised Protocol. This is based on the use of Andersen agar impaction samplers.	
Minimum of two samples collected at each sampler location.	Needed to collect samples for different microbial groups, such as bacteria and fungi. Would be less critical for a sampling device where a single sample could be subdivided, such as with collection into liquid or onto filters.
Samples stored at 4°C after collection, and processed within 12 hours of sampling.	This is necessary to maintain viability and culturability within a sample, or to prevent outgrowth if sampling into liquid. Processing within 12 hours poses a logistical challenge for a longer monitoring exercise, or where variable conditions on site cause delays in sample collection. Most critical for samples not collected directly onto agar. Even with these, it may be ideal to start incubation as soon as possible, requiring the use of portable incubators. An alternative would be initial sample processing shortly after collection, for example by placing samples into a preservation medium.
Control samples should be taken. If impaction samplers are used, then the control samples should be unopened Petri dishes containing the chosen culture medium.	Fundamental requirement for quality control purposes.
Sampling conducted at a minimum of three locations: upwind of the site, downwind of the site, and adjacent to the nearest sensitive receptor (occupied building).	The greater the number of sample locations, the more representative the monitoring. This has cost implications in terms of the equipment needed, and with labour intensive sampling methods such as Andersen samplers it means further staff costs. With more automated methods, the costs would be equipment only. In this instance, staff costs would be mostly in site attendance, in which case better value would be obtained from more sampling locations.
Sampling upwind of the site conducted concurrently with either downwind	Important in establishing background comparisons. See above for practical and

Ideal requirements for compost site bioaerosol monitoring	Practical problems to achieving requirements; relative importance to success of bioaerosol monitoring
sampling or receptor sampling.	cost implications.
Terrain between the compost windrow(s) and the sampler locations should be similar, thereby reducing variability in wind characteristics and dispersion of micro-organisms.	May be difficult to achieve on many sites where there may be various activities, or sites may be sloping. Liable to variation according to which windrows are operable.
Maintain notes of wind direction and speed during sampling.	Critical, and achievable through the use of portable weather stations. Some automated sampling devices may be operable according to wind speed or direction.
Sampling should not be conducted during periods of precipitation (rain, sleet, snow), or when the air temperature is below 5°C (as this can cause unacceptable levels of condensation in the sampler).	Sampling in adverse conditions would be irrelevant because of the effects of removing bioaerosol from the atmosphere. Low air temperature effects are more critical to Andersen impactor sampling. Filtration-based or impinger sampling could be done at lower temperatures. This has a potentially large impact on the logistics of monitoring. In practical terms, while the above is entirely justified it represents a considerable burden on organising a monitoring exercise to match representative on-site activity with near-optimal environmental conditions.
All samples to be collected on a single day.	See logistical considerations above.
Sampling at various points during the year may be required to identify any seasonal fluctuations in microbial numbers. The actual number of sampling days should be determined on a risk assessment basis.	See logistical considerations above.

7 Summary

To better understand the health consequences of bioaerosols emitted from compost sites, accurate sampling is fundamental. Many types of samplers have been used over the years, including liquid impingers, solid impactors, filters, electrostatic precipitators, and many others. The efficiencies of these samplers depend on a variety of environmental and methodological factors.

Although direct agar impaction methods, mainly the Andersen sampler, are still considered to be the benchmark for bioaerosol sampling, the practical limitations of these methods mean that future sampling strategies are likely to move away from these and towards filtration as the most likely alternative ahead of liquid impingers.

Impingers have the advantage of being compact devices, able to be run for extended periods and to collect in liquid which allows for multiple analyses from the same sample. The method is compatible with culture and molecular-based analysis and biomarkers including endotoxins. There would be a need to control for limitations caused by collection in liquid, which could increase the possibility of changes in constituents during the period after collection and during storage and transport to the analytical laboratory. Also, collection in liquid would not be compatible with size fractionation.

The advantages of filtration methods, in addition to being simple, include the potential for size fractionation, ease of handling and transport of samples, multiple analyses from single samples, comparable collection by personal samplers or high volume fixed point samplers and compatibility with collection methods already used for environmental pollution monitoring, such as PM₁₀ monitoring. These advantages must be balanced by potential limitations due to dehydration stresses affecting culturable collection.

In terms of future development of analytical methods, there is scope for further examination of culture methods, to target prevalent compost species. As already mentioned, work has been carried out to develop compost-based nutrient agar media to improve recovery of actinomycete species (Taha *et al.* 2007). Agar media such as DG18 are beneficial in collecting fungal bioaerosols, because of their ability to restrict fast-growing species. With regard to target species, thermophilic actinomycetes and *Aspergillus fumigatus* are the most representative of composting material and also the agents most likely to present a respiratory hazard. However, mesophilic bacteria are an easily cultured general indicator of microbial contamination.

The greatest scope for progress, however, is in the development of molecular-based methods. Methods for detecting specific target organisms by their unique DNA sequences are well established, but characterising mixed populations would be the goal for monitoring compost bioaerosols. In the longer term, the development of microarray-based detection systems offers potential in this area. Although the set-up costs may be high, an established system could be a simple and cost-effective monitoring method. PCR-based methods are very sensitive in complex, mixed DNA samples, and do not require the presence of a culturable or living organism (Kuske, 2006).

Practical problems in monitoring bioaerosol emissions from composting are equally important. As described in this report, many factors may affect the ability to take representative samples. Simple methodology may be most appropriate, even if it may reduce collection efficiency to some extent. The approach proposed by Defra for further investigation in their *Waste and resources R&D programme* is of a multi-level monitoring system, in which simple detection methods, perhaps even electronic particle counters, are used for basic monitoring and are supported by more in-depth (culture-based or other) analysis when required.

The conceptual challenge is posed by how to interpret the results. As described in previous reviews, including Swan *et al.* (2003) and in this report, the absence of definitive health-based data on dose-response relationships between bioaerosols and respiratory allergy or infection makes it impossible to state that a certain level of exposure poses no risk. Therefore, the approach that can be taken is to benchmark exposure, as influenced by compost bioaerosols, against at one end of the spectrum 'typical' background bioaerosols in the absence of a significant emission source and at the other end against bioaerosols encountered in workplaces where there is a known and significant emission likely to cause respiratory ill health. Where the bioaerosol does not greatly exceed the background level, it must be assumed that there is no excess risk. This is complicated by the variable nature of 'typical background' bioaerosols used as a benchmark, which may be affected by orders of magnitude by climatic conditions and the presence of vegetation. Even when a compost bioaerosol emission exceeds background, it is not easy to establish at what point this starts to represent an excess risk, or the tolerability of any excess risk, when for example other bioaerosol emissions such as those downwind of agricultural activities are generally considered tolerable. Some of the more recently published papers may assist in providing points of comparison both for typical background levels and for work activities where there is a concern over bioaerosol exposure and respiratory health. For example, see Cooper *et al.* 2003; Dacarro *et al.* 2005; Dutkiewicz *et al.* 2001; Dutkiewicz *et al.* 2002; Gora *et al.* 2004; Jo and Seo 2005; Jones and Harrison 2004; Krysinska-Traczyk *et al.* 2004; Lee *et al.* 2006; Leenders *et al.* 1999; Liao and Luo 2005; Perdelli *et al.* 2006; Portnoy and Barnes 2003; Prazmo *et al.* 2003a and b; Shelton *et al.* 2002; Skorska *et al.* 2005; Smit *et al.* 2006; Spaan *et al.* 2006; Thorne *et al.* 2004; Toivola *et al.* 2002; Toivola *et al.* 2004; Tsai and Macher 2005.

Future work in the *Waste and resources R&D programme* funded by Defra will address the above and may assist progress towards a definitive answer.

8 Conclusion

A recent review by Kuske (2006) summarised bioaerosol monitoring as follows: *'There is a need for baseline information about the normal abundance, distribution and composition of bacteria in the atmosphere. Studies face significant challenges, including the broad diversity of bacteria that can be carried into the air from soil and plant sources, and the tremendous variability (both locally and regionally) in microbial load and composition owing to seasonal effects, local climate, weather patterns, local human activities, and local wind currents'*.

At present, it is recommended that the new AFOR protocol 'A standardised protocol for the monitoring of bioaerosols at open compost facilities' (in preparation and available from the AFOR website in 2009) be followed by those undertaking bioaerosol monitoring surveys at compost sites. In future this should be used in conjunction with the *Guidance on the evaluation of bioaerosol risk assessments for composting facilities* (in preparation and available from the EA website in 2009). The new AFOR protocol is currently being updated in a project funded by the Environment Agency. It is intended to aid the assessment of the risk and impact of a composting facility on the airborne concentration of micro-organisms. This new protocol will reflect developments in technology over the past ten years and has been re-designed so that it may be revised and updated more regularly in the future as new information and new technology develops. For example, current projects include work on endotoxin emissions at Cranfield University, and work to develop molecular-based rapid monitoring methods for bioaerosols at National Physical Laboratory.

Whilst molecular-based methods, such as real-time quantification, can provide rapid and semi-quantitative detection, more research is needed to enable their use in the field. Potential issues to be addressed before wider adoption of these methods are:

- Do the information needs justify the reagent and equipment costs and technical specialism needed to conduct such tests?
- Is the information too detailed for what is required for simple compost site bioaerosol monitoring?
- Is the information obtained likely to be in a familiar enough format? Measurement of bioaerosols in colony forming units (cfu) per m³ may not provide the entire picture, but cfu is a widely understood unit of measurement in occupational health, food hygiene and so on. With molecular detection, a benchmark to cfu equivalents may be needed.

However, the above issues should not be considered a barrier to future development and adoption of bioaerosol measurement methods. In conclusion, there is a need for greater understanding of the potential effect of compost bioaerosol exposure on human health. Key to that understanding is the ability to measure typical and representative bioaerosol levels accurately, to compare them to typical background levels and to correlate exposures with health endpoints. Only then can questions about the effects of bioaerosols on health be addressed.

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List of abbreviations

AFOR	Association for Organics Recycling (was the UK Composting Association)
ARDRA	Amplified Ribosomal DNA Restriction Analysis
cfu/m ³	Colony forming unit per cubic metre
cDNA	Complimentary DNA
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
HSL	Health and Safety Laboratory
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
µm	Micrometre (one millionth of a metre)

Glossary

Actinomycetes: a specific group of bacteria that are capable of forming very small spores.

Aerosol: a suspension in a gaseous medium of solid particles, liquid particles or solid and liquid particles having a negligible falling velocity.

Agar: a gelatinous material derived from marine algae, used as a base for bacterial culture media and as a stabiliser and thickener in food.

Amplification: an increase in the number of copies of a specific DNA fragment.

***Aspergillus fumigatus*:** species of fungus with spores that can cause allergic reactions in some people.

Bacteria: a group of micro-organisms with a primitive cellular structure, in which the genetic material is not retained within an internal membrane (nucleus).

Bioaerosol: generic term used to describe micro-organisms and their products made airborne.

Biomarker: a cellular or molecular indicator of a biological state of an organism.

Buffer: buffer solutions are used to keep pH at a constant value and maintain a constant environment for micro-organisms.

Colony forming units (cfu): unit of measure for micro-organism numbers that relies on bacteria to grow to form colonies on nutrient plates that can be subsequently counted.

Desiccation: is a state of extreme stress caused by drying or dehydrating.

Dissociation: is a general process in which molecules separate into smaller molecules e.g double stranded DNA separates and become single stranded.

Dose response: this is the relationship between exposure level and an adverse effect in an organisms.

DNA (Deoxyribonucleic Acid): the molecule that encodes genetic information: DNA is a double-stranded helix held together by bonds between pairs of nucleotides.

DNA fingerprinting: these are techniques used to identify and organisms based on its unique DNA sequence.

DNA sequences: the succession of letters (nucleotides – adenine, cytosine, guanine, thymine A, C, G, T) in a strand of DNA.

Endotoxin: certain (toxic) substances found within bacterial cells and which are released only on cell lysis.

Fatty acid: a chemical molecule consisting of carbon and hydrogen atoms bonded in a chainlike structure.

Fungi: a group of micro-organisms with a more complicated cellular structure than bacteria, in which the hereditary genetic material is retained within an internal membrane, forming a nucleus.

Gene: a length of DNA which codes for a particular protein, or in certain cases a functional or structural RNA molecule.

Genomic DNA: a whole organisms DNA.

Glucan: polysaccharides with immune stimulating abilities that is found on the cell walls of yeast.

Gram-negative bacteria: a group of bacteria that can be identified by their inability to hold the crystal violet stain due to fundamental differences in cell biology.

Gram-positive bacteria: a group of bacteria that can be identified by their ability to hold the crystal violet stain due to fundamental differences in cell biology.

Mesophilic: the temperature range most conducive to the maintenance of optimum digestion by mesophilic bacteria 20 – 45 °C.

Microarray: a component of a device for screening genomic or cDNA for mutations, polymorphisms or gene expression. The array is a small glass slide or other solid surface on which thousands of immobilized oligodeoxynucleotide probes have been synthesized or robotically deposited in a predetermined array, so that automated recording of fluorescence from each of the spots may score successful hybridizations. An array may be designed for the detection of all known genes of a species or selected specific sequences. The array may also consist of different antibodies or proteins

Micro-organism: an organism too small to see with the naked eye that is capable of living on its own.

Mycotoxins: toxic substances produced by fungi.

Nucleic acid: a large molecule made up of a sequence of phosphorylated nitrogen-containing bases Adenine, Cytosine, Guanine, Thymine (ACGT). DNA and RNA are both nucleic acids.

Oligonucleotide probe: a sequence of nucleotides that have been synthesized and are used as a molecular probe to detect a target piece of DNA.

Phenotypic: these are the observable characteristics of an organism such as morphology.

Polyacrylamide gel: matrix used to separate proteins or DNA fragments.

Polymerase chain reaction (PCR): an enzyme-based method to generate copies of a target DNA sequence for subsequent analysis.

PM10: airborne particles with a diameter less than 10 micron.

Real-time PCR: also called quantitative PCR (qPCR) is based on the polymerase chain reaction where it amplifies a target DNA sequence. It is used to detect the presence and quantify a specific sequence in a piece of DNA.

Serial dilution: is a stepwise dilution of a substance in solution. The dilution is achieved by removing one part of the solution, placing in a new tube and adding water to a known volume in a new tube.

Thermophilic: microbial species with a preference for growth at high temperature, typically 50°C+, such as thermophilic actinomycete species.

Thermotolerant: microbial species able to tolerate and grow at high temperature, typically 40°C+, for example *Aspergillus fumigatus*.

Vegetative bacterial cell: bacterial cell that is capable of growing under favourable conditions.

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