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DETECTION OF AIRBORNE MICROBES IN A COMPOSTING FACILITY BY CULTIVATION BASED AND CULTIVATION-INDEPENDENT METHODS

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Abstract: Standard methods for quantification of airborne bacteria are based on cultivation and counting of grown colonies. From complex natural environments it is known that only a small fraction of the total number of cells can be cultivated on routinely used agar-media. Direct microscopic cell counting after DNA-staining usually generates higher concentrations of one to two magnitudes. The objective of the presented study was to compare the concentrations of airborne bacteria sampled in a composting facility by using for any sample the cultivation on trytic soy agar (TSA) – agar, as well as direct counting after DAPI-staining. The concentrations after counting grown colonies were within a range of 10^5-10^7 cfu m^{-3}. Concentrations of direct counted cells ranged between 10^6-10^9 microbes m^{-3}. In these comparative measurements only 1.5-15.3% of the airborne bacterial cells enumerated by direct counting formed countable colonies after incubation on TSA-agar. Obviously, cultivation based methods underestimate the real amount of airborne microbes. In addition, from literature it is known that inactive or even dead cells can also have the potential to cause health effects. Consequently, a risk assessment based only on measuring colony forming units may, in some cases, not be sufficient.

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INTRODUCTION

The composting process as part of waste management is based on the degradation and humification of organic material by highly diverse microbial communities, including members of *Bacteria, Fungi and Archaea* [6]. It has been known for a long time that composting material contains microorganisms as vegetative cells or spores in concentrations up to 10^{10}-10^{11} cells per gram dry weight [5]. During processing, the compost-material is moved and pathogenic and non-pathogenic microbes will be aerosolised (e.g. shown in Fig. 1). These bioaerosols contain up to 8×10^6 cfu of bacteria and 2×10^7 cfu of fungi per m^3 [23] which can be inhaled by the employees at these workplaces. The inhalation or ingestion of viable pathogenic microbes may cause infection. However, for the initiation of allergic responses the viability of airborne microbes is not a prerequisite because dead cells as well as cell debris may also provoke allergies. Therefore, quantification of airborne microorganisms is essential for the risk assessment especially at workingplaces handling those biological agents. Concentrations can be measured in different ways, including as colony forming units after cultivation, microscopically cell counting after DNA-staining (e.g. DAPI, acridine-orange) respectively, fluorescence *in situ* hybridisation (FISH), or more recently by quantitative PCR [e.g. 9, 10, 13, 14, 15, 16, 17, 19]. Currently, the quantification of airborne microorganisms (fungi and bacteria) is most often based on cultivation on agar-based-media after sampling [21]. By these methods only microbes which survived the sampling

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as well as transport stress are potentially detectable. The ability of viable microorganisms of reproduction under used cultivation and incubation conditions is a second factor limiting the detectability. Microorganisms which can be cultured by standard techniques may therefore account for only a minor proportion of the total amount. It is estimated that only 1-10% of microorganisms observable in environments such as soil or freshwater are typically cultivated by using standard cultivation techniques [1, 3, 4, 7, 9, 17, 20]. Information is rare about the proportion of cultivable microorganisms from the total number of microbial cells present in bioaerosols of composting plants by using standard cultivation techniques and current types of standard media [22]. Therefore, the purpose of this work was to determine the portion of cultivable microorganisms using TSA-Agar, compared to total counts obtained after DNA-staining by using DAPI or acridinorange.

Figure 1. The Picture shows the dust aerolisation a few seconds after facility workers starts to turnover the compost heaps. Samples were taken direct in the dusty cloud.

MATERIALS AND METHODS

Examined facility. Air sampling was performed in a municipal composting plant located in Germany which processed 8,000 t biowaste (50-70% domestic waste and 30-50% plant residues) per year. The main or intensive composting was carried out on force-aerated static piles covered with semi-permeable membranes for 4 weeks without turning. Afterwards, the process continued with the curing stage for 12 weeks. In this phase of processing, the piles were mechanically turned weekly by using a self-powered turning machine. The whole process was performed outside a closed building covered only by a roof.

Microbiological examination of the air. Air samples were taken in the composting facility while piles at the curing stage were turned. For each measurement 2 different sampling devices (AGI 30-Impinger and MD8 filtration-sampler) were mounted on a mobile arrangement (a specially prepared tool trolley) so that samples could be taken at a constant distance from the turning process for the whole time, although the turning machine was mobile while working. The procedure is shown in Figure 1.

Airborne microorganisms were sampled using filtration samplers MD8 (Sartorius, Göttingen, Germany) or all glass liquid impinger-30 (AGI-30, Zinsser Analytic, Frankfurt, Germany). An air volume of 1.0 m^3 was collected by filtration within 10 min (using a flow rate of 100 l min^{-1}) through gelatine membrane filters (pore size 3.0 µm; Sartorius, Göttingen, Germany). The AGI-30 contained 50 ml of sterile saline solution (0.9% NaCl). Sampling was carried out for 10 minutes at an air flow of 12.7 l min^{-1} (sampled air volume 0.127 m^3).

The filters were processed in the laboratory after a maximum storage of 24 h. For indirect processing, each of the filters was dissolved in 10 ml of 0.9% NaCl solution (containing 0.01% Tween-80 at pH 7), shaken in a temperature-controlled water bath for 15 min at 35°C until they became clear. Samples from Impingement were transported in a cooling device at 4-8°C and the solutions could be used directly.

Cultivation based method. After a serial dilution up to 10^{-9}, Caso-agar plates (synonym Tryptic Soy Agar – TSA – with Cycloheximide [g l^{-1}]: Peptone from casein 15.0, peptone from soymeal 5.0, sodium chloride 5.0, cycloheximide (actidione) 0.3, agar 16.0) were inoculated with 0.1 ml per dilution each in triplicate and incubated at 36°C for 7 days. Grown visible colonies were counted after 4 and 7 days. The data were reported as cfu and calculated per cubic meter of air (cfu m^{-3}).

Cultivation independent method. For total cell counting the sampled microorganisms in the impingement fluid were fixed with 1% (v/v) formaldehyde as final concentration and could be stored at -20°C afterwards. Suspended gelatine from the used filters were precipitated by contact with formaldehyde so that these samples could not be fixed and had to be dyed directly.

DNA-Staining of fixed samples. Fixed cells were mixed with the DNA-binding dye 4‘,6-Diamidino-2-phenylindol-dihydrochlorid (DAPI, Boehringer, Mannheim, Germany) at a ratio of 5:1. The blue fluorescent dye DAPI was dissolved in a phosphate buffer system (PBS, 130 mM NaCl/ 10 mM Na_2HPO_4, pH 7.2) to a concentration of 1 µg ml^{-1}. The mixtures were incubated for 10 min in the dark at room temperature. DAPI stained cells were filtrated by using sterile polycarbonate filter (25 mm, diameter, 0.2 µm pore size) fixed in a glass vacuum filtration unit (Schleicher and Schuell, Dassel). The air-dried filters were immersed in Citifluor (Citifluor Ltd., London, UK) on a glass slide. Analysis of the filters were carried out microscopically (magnification ×1000) with an Epifluorescencemicroscope (Axioplan 2; Zeiss, Göttingen, Germany) by using a counting ocular. For statistical purposes, 20 counted microscopic

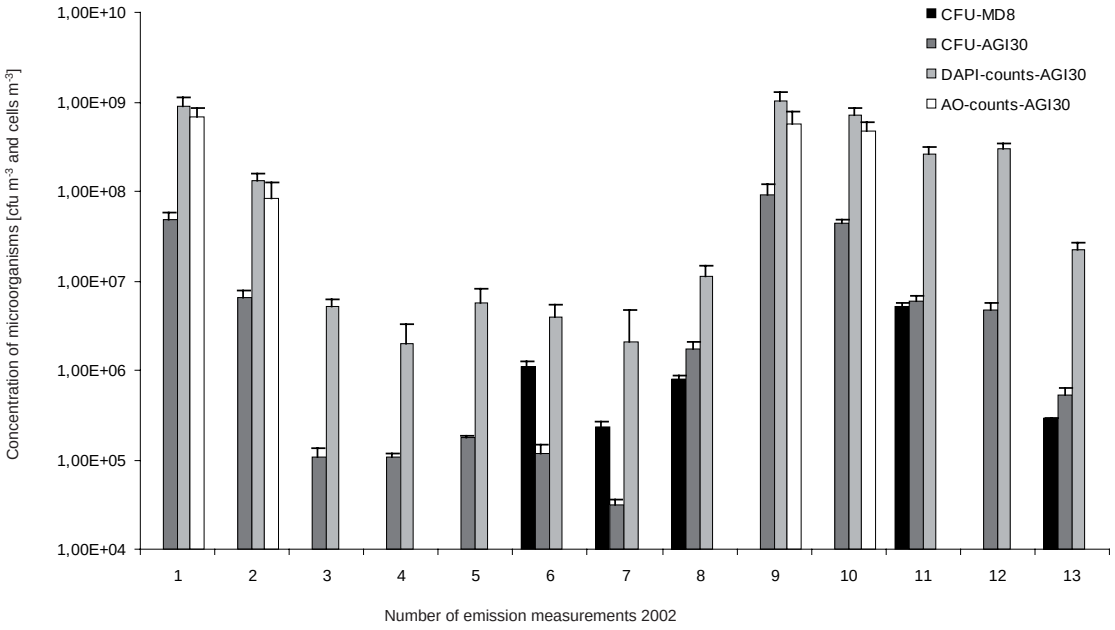

Figure 2. Comparison of the total cell number (after DAPI and Acridinorange staining) and colony forming units (on TSA-Agar) in bioaerosol samples collected at a composting plant. Sampling was performed either with the filtration methode (MD8) or with the impingment method (AGI30). Cfu-values are means of n=3 ±SD; DAPI values are means of n=20 counted microscopical fields ± SD.

fields of view corresponding to 600-1,000 stained cells were counted per filter. The counted area and the amount of cells allowed the calculation of cells per m^{-3} sampled air.

Some of the samples were stained with acridine-orange according to Palmgren *et al.* [15]. The detection limit of the direct counting methods after DNA-staining depends on sampling volume. For the method used and statistical precission this was down to about 20,000 microbial-cells per sample.

RESULTS

The results of the examination are presented in Figure 2 and Table 1. Concentrations of airborne microorganisms at the same sampling-point varied depending on the analysing method. Figure 2 clearly shows that cultivation independent methods (DNA-staining with DAPI or acridine-orange) generate considerably higher concentrations compared to the cultivation dependent method which is

Table 1. Parameters of measurements compared with results for cultivability.

Sampling	Facility depending activity	Distance to emission source [m]	Relative humidity [%]	Filters	Cultivability+ (% of DAPI-Counts)	Evaluation by AO-staining
1	turnover	1	80-90	not performed	5.37	yes
2	turnover	1	95; decreasing	not performed	5.14	yes
3	turnover	1-2	86	stuck to holder*	2.04	not enough material
4	turnover	1-2	88	stuck to holder*	5.38	not enough material
5	turnover	1-2	90	stuck to holder*	3.17	background fluorescence
6	turnover	30-40	70	evaluated	3.06	not enough material
7	turnover	40-50	68	evaluated	1.48	not enough material
8	simulated turnover**	10-20	66	evaluated	15.27	not enough material
9	simulated turnover**	2	66	not performed	8.89	yes
10	simulated turnover**	2	66	not performed	5.98	yes
11	riddling	2	82	evaluated	2.22	no
12	riddling	2	85	stuck to holder*	1.56	no
13	riddling	2	81	evaluated	2.34	no

AO = acridine-orange; *no work up, evaluation not possible; **facility depending break in activity: simulated by shovelling some compost material; + based on AGI samples

currently used as the standard method. Measured concentrations of simultaneously taken samples differ by about 2 orders of magnitude. The concentrations of cultiviable bacteria were between 10^5-10^7 cfu m^{-3} compared to 10^6-10^9 microbes m^{-3} generated by direct counting methods. The percentage of cultivation based microorganisms concentrations ranged from 1.5-15.3% in comparison with the total cell number counted after DAPI-staining (Tab. 1).

Results on the basis of cell-counting after staining by DAPI is more reliable than those evaluated with acridine-orange. This result is based on the fact that the background fluorescence in total was weaker and the discrimination between compost particles and microbial cells was better after DAPI-staining. Due to these described visual effects, fewer microbial-cells were counted after staining with acridine-orange, but the differences varied at the same magnitude.

Measuring during turnover of compost piles by using a filtration system with gelatine filters cannot be recommended. Under the wet and warm conditions of the resulting vapour loaded air the gelatine often stuck to the holders and therefore the filters cannot be analysed (Tab. 1).

DISCUSSION

The recorded concentration detected via the cultivation approach is in the similar range found earlier, e.g. by [24, 25, 26]. Concerning to normal concentrations of airborne bacteria of about 10^1-10^2 cfu m^{-3} in areas free from biological contamination the concentrations of 10^5-10^7 cfu bacteria m^{-3} (Fig. 1) shows that the exposition at a composting plant is very high. In contrast to cultivation based quantification, the direct microscopic counts of airborne microorganisms mainly originating from compost material were between 25-66-fold greater. The total numbers of microorganisms measured for these investigations reached up to 10^9 cells per m^3 compared to 10^7 cfu per m^{-3}. Very little is known about the microbial community at different stages of composting [6]. However, focussing on microbial diversity, the biological degradation processes in composting windrows are comparable to that in other environments, especially in soils. Heterogeneous environments provide many different ecological niches that vary in either space and time. Not only abiotic environmental factors determine the microbial diversity but also the interaction of different microbes that takes form from the antagonistic to the beneficial. The result of this complex environmental heterogeneity is a highly diverse microbial community of which only a small fraction of microbes is typically culturable [8]. Compared to other investigations with different soils [19] or lake water [2], our results show a similar tendency by comparing cultivation with total counts after DAPI-staining. Similar results were also observed in experimentally generated bioaerosols from composting household waste [22]. Depending on the medium used for cultivation, a maximum of about 1% of total cell number were detected [22].

The culturability, and therefore the enumeration, of microbes will be determined by the nutritional and growth requirements of the individual species. Consequently, the efficiency of recovering microorganisms from environmental samples increases by using different cultivation media in principle. Many of "cultural-resistant" microbes will in fact be readily culturable but their cultural and growth requirements might be unknown. Previously reported results of the recovery of sampled airborne bacteria cultivated on a variety of media (including TSA) after aerosolization in an aerosol chamber show that no single medium recovered all species of bacteria. Furthermore, only a small fraction of the airborne bacterial concentrations could be recovered on any of the tested media compared to enumeration of the total number of cells by direct microscopy after acridine orange staining [18]. Of course, using a number of different agar-media is not possible for standard measurements of airborne microbes because it is too time-consuming. On the other hand it has been shown by [27] that the selectivity of a "selective" media allows not only the growth of the target microorganisms. A detailed study of species composition even on one selective medium, therefore, is possible only with a high analytical investment and only after isolation of grown species. Therefore, this strategy cannot be used for quantitative measurements of all or selected microbes because each sampled microbe which is potentially culturable must have been grown on the medium on which it was plated. Additionally, an agglomerate of 2 or even more cells or species may give rise only to one colony produced from the fastest growing cell, so that the presence of the other cells remains unrecognized. For all of that, it is generally accepted that enumeration of microorganisms counted as colony forming units significantly underestimates the total number. The direct staining of DNA for quantitative investigations of samples from a complex matrix such as compost or soil, is currently favoured compared with techniques based on DNA-cloning, because matrix compounds that bind DNA or inhibit the enzymatic reactions required for cloning [17] can be neglected.

During the composting of organic material, rapid substrate changes in pile conditions, such as temperatures and pH seem to favour shifts in microbial community composition, as observed, e.g. by Michel *et al.* [12]. The species composition emitted from heaps can vary with material ages. Therefore, the analysis of different bioaerosol sources with only one medium limits also the quality of culture based results.

Nonetheless, the so-called „viable but non-culturable" (VBNC) microbes, a stress-induced non-physiological survival-state of microbes, will not be detected [19] by cultivation using standardised methods. Aerosolisation can induce bacteria entering the VBNC state [18]. It is well known that VBNC may be resuscitated but the factors which are required therefore are usually unclear. Environmental factors or signals from other actively growing microbes are suspected. It is possible that VBNC retains the

capacity of virulence and could cause diseases if they were resuscitated. While these inactive cells persist for long periods of time, dead cells disappear very rapidly in the environment [11].

Although the cultivation based method results in a clear lower detection compared to the culture independent method, techniques based on direct counting do not differentiate between living and dead cells. The detection of microbes with the potential of cause an infection is one of the main focuses of work hygiene. Only living microbes may cause an infection so that cultivation up to now is the standard technique to enumerate these microorganisms. But it must be taken into account that VBNC cells can be activated upon their passage through the host [11]. High concentrations of airborne dead cells can result in toxigenic of allergenic inhalation effects. For all of this, an important factor for assessing health risks is not solely the concentration of bacteria which form colonies on routinely used agar-media but also the total number of bacteria cells [18].

For the reasons discussed above, it is obligatory to use cultivation methods for accessing the hygiene situation of a workplace atmosphere although they have substantive limitations. However, for the initiation of allergic responses, the viability of airborne microbes is not a prerequisite. The counts of total cell numbers are better compared and can provide additional information about the basic exposure.

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