Suitability of dust and bioaerosols from a pig stable as inoculum for biological air filters

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ABSTRACT

Biofiltration for removing ammonia and odour compounds from ventilation air of pig stables is a promising approach. In order to reduce the time for starting up a well-functioning biofilter a good inoculum suited for the environment is needed. In this study the microbial identity and quantity of dust and bioaerosols coming from a pig stable were analyzed for its suitability as inoculum. Inoculation of biofilters with dust had similar good ammonia removal capabilities as biofilters inoculated with activated sludge, although analysis of the microbial dust community revealed clear differences. The organic fraction of the dust particles seems to be important for mediating biofilm development on the filter material.

1 INTRODUCTION

Increasing productivity combined with a more centralized production of pigs has the last decades created odour nuisance problems and a high load on the environment through air emissions. Biofiltration is a promising and low cost technology for treatment of large volumes of air containing low concentrations of different compounds, such as ventilation air from a pig stable. The quantitatively most important components in ventilation air from pig stables are organic acids and ammonia, which can readily be oxidized by heterotrophic and nitrifying bacteria, respectively.

A well-functioning biological air filter requires microorganisms capable of utilizing air components and adapted to live under extreme conditions. Inoculations by addition of activated sludge or material from existing filters often speed up the process of establishing a new biofilm. However, activated sludge might not be optimal

as the microorganisms herein are not adapted to the conditions on the new filter material nor the substrate fed into the biofilter.

Microorganisms indigenous to the ventilation air, aerosols, and dust particles may already be pre-adapted to life in the environment and thus better suited as inoculum. However, only few investigations, most of which applying culture-dependent techniques, have dealt with the composition of airborne dust and aerosols from pig stables. By analyzing the microbial community herein its potential use as inoculum of nitrifying bacteria and heterotrophic microorganisms into biofilters can be evaluated.

In the present study we have applied cultivation-independent molecular techniques to characterize the microbial community composition in dust and bioaerosols from a pig stable, and compared this to established biofilters treating air from the same stable.

2 MATERIALS AND METHODS

2.1 Sampling

Dust and bioaerosols was obtained from a closed gestation unit with 450 sows near Aalborg, Denmark. Dust and bioaerosol samples were collected from the air by introducing a 2 cm thick (30 cm in diameter) water resistant cellulose filter with a pore size of <2 μm. Samples were collected in periods ranging from 2 to 4 days with an average air flow of 200 $m^3\ h^{-1}$ in order to obtain homogenous samples. The dust load of the air was determined from the mass accumulated over time in the filters and the air flow (Windmaster, Kaindi). Dust and aerosol particles were extracted from the filters by washing them thoroughly in sterile filtered tap water on which all following analysis has been made unless otherwise stated. Biofilm samples from the biofilters were obtained by thoroughly shaking an aqueous suspension of the biofilter material until no more material fell off.

2.2 Biofilter setup

Four identical biofilters were used to test the suitability of dust/bioaerosols as inuculum. The filters were cylindrical packed bed reactor (630 mm in diameter and 700 mm in height) packed with 300 mm of Lightweight Expanded Clay Aggregate beads, 10-20 mm in diameter (LECA®; Maxit A/S,). The filters had an initial porosity of 46% and a specific surface area of 300 $m^2\ m^{-3}$ and a density of 215 kg m^{-3}. The pig stable air was introduced through the bottom of the columns for counter-current flow operation and maintained with an airflow of 40-44 $m^3\ h^{-1}$. The empty bed residence time was 8.1-8.7 s. The filters were kept moist by a discontinuous water addition of 3700 mL day^{-1}. Homogenized activated sludge (400 mL), from nitrogen removing wastewater treatment plant (Aalborg East wastewater treatment plant) was added to two filters.

2.3 Cultivation and identification of the microbial community

Quantification of viable microorganisms in the dust was done by serial dilutions of the dust sample using sterile filtered tap water. 100 µL of each dilution was plated on triplicate trypticase soy agar plates for bacteria and incubated for 48 h at room temperature (22ºC) and on triplicate malt extract agar plates (Smid *et al*., 1989) supplemented with 0.1 g L^{-1} chloramphenicol for quantification of fungi for 48 h at room temperature (22ºC).

Different staining procedures were applied to the samples in order to gain information about the bacterial community. Total cell counts was obtained by DAPI (4',6-diamidino-2-phenyl indole) staining a sample concentrated on a polycarbonate filter (Osmonics Inc.) as described by Frølund *et al*. (1996). BacLight Live/Dead staining (Molecular Probes) and incubation with 1 mM Cyanoditolyl Tetrazolium Chloride (CTC) (Polysciences Inc.) was used to examine the viability of the cells in the sample.

Identification and quantification of the major bacterial phyla in the dust and biofilters was performed with fluorescence *in situ* hybridization (FISH) and a range of oligonucleotide probes (Table 1). Further details on these probes are available in probeBase (www.probeBase.net). Cells were fixed by paraformaldehyde and ethanol as previously described (Kong *et al*., 2005) Fixed samples were stored at -20°C in 50% ethanol and 50% phosphate buffered saline until analysis. FISH was performed on gelatine-coated glass slides according to the method of Manz *et al.*, (1992). Application of gelatine-coated slides and addition of 1% agarose on top of the sample reduced the loss of cells during FISH from 35-80% to only 7%. 52% of the total DAPI counts were detectable by hybridization of the general bacterial probemix (EUBmix).

An Axioscop II (Zeiss) and a 630X magnification was used for all visualizations. For counting, a minimum of 400 cells were counted per sample distributed between at least 10 randomly chosen counting grids.

DNA extraction was carried out using FastDNA SPIN kit for Soil (Vista). Undiluted extracted DNA was amplified by polymerase chain reaction (PCR) with the primers 8F and 1492R, purified, ligated, cloned and sequenced as previous described by Kong *et al*. (2005).

2.4 Nitrification activity

Nitrification activity in the dust was evaluated by measuring the production of NO_x under aerobic conditions (with and without the presence of 5mg L^{-1} *N*-allylthiourea) in dust suspended in 0.5 L 0.9% NaCl. No effect of NO_x and excess of organic material supporting growth of heterotrophic microorganisms, was observed by implementing various pre-incubations prior to addition of 5 mM NH_4^+.

2.5 Analytical methods

The water content and the content of organic matter in the dust was determined after drying at 105°C for 24 h followed by 550°C for 3 h. The fat content was quantified by mixing a diluted dust sample with Nile red to a concentration of 6.5 μg mL^{-1} and determining the fluorescence (excitation at 510 nm and emission at 580 nm) by fluorescence spectrophotometer, while protein was quantified with the CBQCA Protein Quantification Kit (Molecular Probes). The fluorescence was measured and quantified using oleic acid or gelatine as standard compounds. NH_4^+, NO_2^- and NO_3^- concentrations were determined after extraction with 1 M KCl for 2 h for biofilter samples and after sterile filtration for dust samples. They were analyzed by an autoanalyzer (Technicon TRAACS 800).

3 RESULTS AND DISCUSSION

Inorganic filter material has the advantage over organic materials that it is not or only very slowly degraded. However, it often requires inoculation in order to minimize startup time. Addition of a proper inoculum can help reach the required performance of the filter faster. The challenge is to find the right inoculum. Complex microbial consortia, such as activated sludge are often chosen for a biofilter treating air with a broad specter of organic substrates, while commercially available monocultures are favored for treatment of a particular contaminant. Activated sludge is rich in diversity, but the cells herein might not be well-adapted for treating the components and the actual concentrations of the contaminated air. Thus better inoculum candidates should be found.

An unknown fraction of the microorganisms indigenous to dust and aerosols in contaminated air systems can be speculated to be pre-adapted for living in the contaminated air.

3.1 Dust composition and microbial detection

In the present study we have investigated the microbial composition of dust and aerosols, compared it to the composition of biofilters treating the same air, and evaluated íts potential as inoculum for biofilters. Initial experiments aimed to analyze the quality of the air regarding load and composition of microorganisms. The dust load in the ventilation air was measured to 0.7 mg TS m^{-3} (total solids) of which 80% of the TS were carbohydrate, 15% were protein and 5% fatty acids. The culturable fraction of the dust particles averaged 1.31×10^5 colony forming units (CFU) m^{-3} for bacteria and 1.33×10^4 CFU m^{-3} for fungi. These numbers are largely in agreement with measurement from other pig stable environments (Chi and Li, 2005; Predicala *et al*., 2002) and show a high microbial input into the biofilters. The culturable part of

the total bacterial community, however, only constituted less than 1% as 3.45×10^7 cells m^{-3} was detected by DAPI. The equivalent number for activated sludge was 5×10^9 cells ml^{-1} with a culturable fraction of 10-15%. BacLight Live/Dead staining revealed 65% living cells in the dust, of which most were highly respiratory active, as determined by CTC staining.

3.2 Nitrification activity

One of the main advantages of biofiltration is the high removal of ammonia through microbial nitrification. The biofilter system analyzed, when running steadily, had ammonia removal efficiencies varying between 70-90% (Figure 1) which are in agreement with other surveys (Melse and Mol, 2004; Sheridan *et al*., 2002).

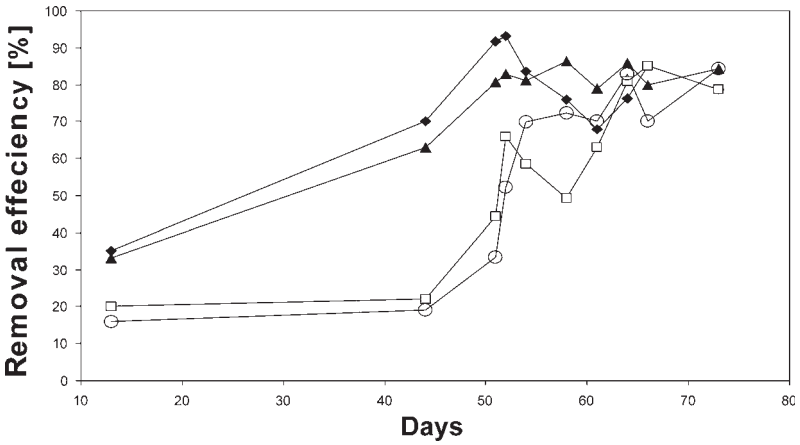

Figure 1. Removal efficiency of ammonia in four biofilters receiving approximately 44 $m^3 h^{-1}$ air from a pig stable. Solid symbols indicate biofilters inoculated with activated sludge, while open symbols show biofilters which have only received air with 0.7 mg dust particles per m^3.

More than 60% removal efficiency of ammonia was seen after approximately 44 days for the biofilters inoculated with activated sludge, while the two filters only supplied with dust, reached the same level after approximately 52 days (Figure 1). The corresponding control receiving filtered air without dust particles did not reach an ammonia removal efficiency above 60% within 120 days (results not shown). These results indicate the importance of inoculating the biofilters, and that a continuous supply of dust particles to the filters is almost as efficient as inoculation with activated sludge. The low dust load from the stable (most likely a consequence of the addition of fat to the pig feed to reduce dust), could further be speculated to be limiting for an even faster startup time of the filters. Other values of airborne dust concentration has

been reported to be between 1.66-21 mg m^{-3} (Crook *et al*., 1991). The relatively fast build up of ammonia removal obtained by inoculation with dust, compared to the control with dust free air, could be the result of providing a suitable microbial consortium to the filters with the dust, and/or a supply of organic coating of the filter for establishing a proper biomass. The nitrifying activity of the dust was investigated and it was found that ammonia was removed with a rate of approximately 0.05 µmol m^{-3} h^{-1} (74.8 µmol g TS^{-1} h^{-1}). However, no measurable accumulation of nitrite or nitrate was observed, indicating that the removal of ammonia was most likely due to heterotrophic activity by being incorporated into the biomass. Further analyses of the microbial consortia were therefore carried out to evaluate this.

3.3 Identification of the microbial community

The identity of the bacteria in the dust particles were analysed by FISH. Only 52% of all microorganisms were detected by this approach, with the majority (85%) being gram positive cells distributed with 10% *Actinobacteria* and 75% *Firmicutes* (Table 1). Higher taxonomic analysis revealed that 50% of the FISH-detected community derived from the genus *Veillonella* and 36% from the genus *Streptococcus*. *Alpha-, Beta-, Gamma-*, and *Deltaproteobacteria* made up only ca. 1% of the community each. In agreement with the rate measurements, use of specific probes for ammonia oxidizers showed, that they constituted less than 1% of all FISH detectable cells. No nitrite oxidizers (*Nitrobacter* or *Nitrospira*) were found.

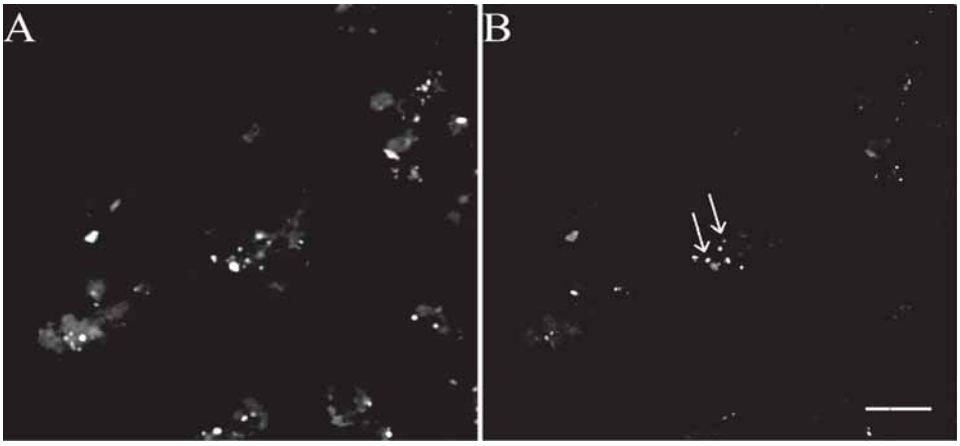

Figure 2. Fluorescence *in situ* hybridization of dust particles with the general probe mix EUBmix (A) and a specific probe targeting *Veillonella* (B). The color has been artificially changed to black and white nuances to increase the contrast. The arrows indicate the presence of *Veillonella* positive cells which is not targeted by the EUBmix. Bar equals 20µm.

Table 1.
List of microorganisms analysed with FISH showing phylogroup, probe used and its specificity and reference to each tested probe. The portion of the tested organism group compared to *Eubacteria* is presented in percent.

Probe name	**Specificity**	**Reference**	**Percentage of *Eubacteria* [%]***
ALF968	*Alphaproteobacteria*	Neef, (1997)	<1
BET42a	*Betaproteobacteria*	Manz et al., (1992)	1
Nso190	Ammonia oxidizing *Betaproteobacteria*	Mobarry et al., (1996)	<1
GAM42a	*Gammaproteobacteria*	Manz et al., (1992)	1
SRB385 + SRB385Db	Most *Deltaproteo-bacteria* and some other *Bacteria*	Amann et al., (1990) Rabus et al., (1996)	1
HGC69a	Most *Actinobacteria*	Roller et al., (1994)	10
HGC236	Many *Actinobacteria*	Erhart, (1997)	1
LGCmixLGC354b+A+C	*Firmicutes*	Meier et al., (1999)	77
Str	*Streptococcus*	Trebesius et al., (2000)	36
Veil223	*Veillonella dispar, V. parvula, V. atypical*	Harmsen et al., (2002)	52
Enc131	Most enterococci	Meier et al., (1997)	1

* Percentage of specific probe of EUBmix (Amann et al., 1990; and Daims et al., 1999).
** Hybridization of dust with the following probes did not result in positive FISH signals: Actino1011 (HGC1011) (Some *Actinobacteria*) (Liu et al., 2001), HGC1351 (Many *Actinobacteria*) (Erhart, 1997), Myb736a (*Mybobacterium* complex) (De los Reyes et al., 1997), Sau (Kempf et al., 2000), Lis637 (*Listeria*) Schmid et al., (2003), CF319a+b (*Bacteroidetes*) Manz et al., (1996), Gnsb941+CFX1223 (Phylum *Chloroflexi*) (Gich et al., 2001), GCB532 (Green sulphur bacteria) (Tusehak et al., 1999) Ntspa662 (*Nitrospira*) (Daims et al., 2000), NIT3 (*Nitrobacter*) (Wagner et al., 1996) Arch915 (*Archaea*) (Amann et al., 1990) Pla46 (*Planctomycetes*) (Neef et al., 1998), NonEUB338 (Nonsense) (Wallner et al., 1993).

Through FISH analysis it was observed that 60% of the Veil223 positive cells were not targeted by the EUBmix probeset (Figure 2) although its phylogenetic affiliation in the phylum of *Firmicutes* is generally considered to be targeted by this probe mixture. The low fraction of FISH-detectable cells, was thus significantly higher than the 52%, and could be calculated to be around 83% of all DAPI counts. The fraction of FISH-detectable cells of all DAPI counts is often, although not entirely correct, used to interpret the actual activity level of the sample (Bouvier and del Giorgio, 2003). In the case of our dust and aerosol sample we have further seen that most (65% of all cells in the sample were viable and most of these were metabolically active as determined by Live/Dead and CTC staining. Therefore, very likely they could be

transferred to and trapped in the biofilters and there be actively respiring from the contaminants in the ventilation air. Both *Veillonella* and *Streptococcus* are known to carry out fermentation and therefore if established in the biofilm may play a role in the removal of organic odours compounds present in the air.

Approximately 57% of all FISH-detectable cells in the biofilters inoculated with activated sludge were nitrifiers, while only 19% were nitrifiers in the biofilters receiving dust. The identity of these nitrifiers were tested with more specific oligonucleotide probes, but only minor fractions were identified. In the biofilters inoculated with activated sludge ca. 1% of the ammonia oxidizers were found to belong to the genus of *Nitrosomonas* and ca. 1% of cells belonging to the genus *Nitrosococus mobilis*. In the biofilters inoculated with dust, approximately 50% of all ammonia oxiders were affiliated within the *Nitrosomonas oligotropha*-lineage. No nitrite oxidizers (*Nitrobacter* or *Nitrospira*) were found in any of the biofilters.

A generally higher number of FISH-detectable cells of all DAPI counts were seen in the biofilters inoculated with dust (ca. 86%) compared to biofilters inoculated with activated sludge (ca. 51%). No significant difference in the total number of DAPI counts (ca. 5-8 × 10^5 cells g dry LECA®) were seen between the two types of biofilters.

To further investigate this difference in microbial consortia between the dust and biofilters we have screened the constructed clone library has revealed mainly two populations within the dust both belonging to the phylum of *Firmicutes*, namely sequences closely related to *Clostridium* and *Bacillus* species which originated from pig intestine samples. From cultured isolates *Bacillus* has been identified in dust and bioaerosols from swine barns (Predicala *et al*., 2002).

Further investigations will focus on expanding the molecular investigations of the microbiology of the dust in order to compare larger fractions of the entire communities.

In conclusion it appears that the microbial consortia in the dust is not directly reflected in the biofilters. Almost no nitrifiers were observed in the dust and aerosols indicating that the organic fraction of the dust particles is important for mediating biofilm development on the filter material. A clear difference in the diversity was observed between biofilters inoculated with activated sludge and with biofilters only receiving dust although no clear difference is seen between the performance of these biofilters. Thus it cannot be concluded from this study to which extent dust is preferable to activated sludge as an inoculum. However, dust particles and bioaerosols seems to hold sufficient capacity to be considered as a good candidate for inoculum for biofilters treating ventilation air from pig stables.

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