Title

Bait and scrape: An approach for assessing biofilm microbial communities on organic media used for gas-phase biofiltration

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1. Introduction

1.1. Focused microbial ecology can guide and improve biofilter performance

Bioreactors are an efficient alternative to traditional physical and chemical pollution control technologies (Delhoménie and Heitz 2005). Unlike other technologies, bioreactors harness bacterial and fungal microbial communities to capture and degrade pollutants. The use of a diverse and specialized microbial consortium enables the simultaneous treatment of pollutant mixtures of variable concentrations, giving bioreactors great promise for aqueous (Schipper et al. 2010), and gaseous agricultural

effluents (Nicolai et al. 2008). Despite their efficacy, low cost, and compatibility to most U.S. (Liu et al. 2014) and European farming systems (Hamon et al. 2012; Ubeda et al. 2013), bioreactor deployment has been hampered by unpredictable performance. Developing more reliable bioreactors for agricultural pollutant treatment will require an improved understanding of the microbial dynamics that underpin stable performance (Briones and Raskin 2003; Cabrol and Malhautier 2011).

For mitigating livestock emissions, biofilters are typically used. Biofilter are bioreactors which use microbial growths and their extracellular products attached to stationary reactor media surfaces - biofilms - to capture and degrade pollutants from a passing effluent (Mudliar et al. 2010). These systems can simultaneously treat volatile organic compounds (VOCs), hazardous odors (e.g. H_2S , NH_3), and greenhouse gas emissions (e.g. CH_4), but mitigation is highly variable with efforts to improve control hampered by inadequately understood, complex biofilm processes (Chen and Hoff 2009). As an example of this complexity, degradation of odors VOCs by biofilm heterotrophic bacteria is facilitated by their nourishment with nitrite and nitrate, byproducts of ammonia-oxidizing bacteria (Kristiansen et al. 2011). But, it has also been shown that ammonia-oxidizing bacteria are spatially restricted in biofilters due to inhibition by their own byproducts and by competition with VOC-degrading heterotrophs for oxygen (Juhler et al. 2009). Thus, biofilter function is dictated by the same nutritional cross-feeding considered essential to the stability of microbial communities (Seth and Taga 2014),

suggesting the whole biofilm community may be more important to biofilter performance than the study of individual functional groups.

1.2. Fungal biomass in biofilms may significantly influence biofiltration

The role of fungi in supporting biofilter performance and resilience have been particularly overlooked (Ralebitso-Senior 2012), despite their abundance in organic (Cardenas-Gonzalez et al. 1999) and synthetic media biofilters (Kristiansen et al. 2011; Xue et al. 2013). Several fungal attributes give these organisms unique potential to affect biofilter performance. These include: 1) their tolerance to desiccation (Kennes and Veiga, 2004) and acidification (Devinny and Ramesh 2005), both common stresses to biofilter stability; 2) their specialized oxidative capacities, in many cases driving cometabolism of aromatic pollutants and lignin (Estevez et al., 2005a; Jorio et al., 2009; Spigno et al., 2003), 3) their filamentous (hyphal) growth, which can extend into media pore space to increase the effective surface area interfacing with the effluent (Arriaga and Revah, 2005; van Groenestijn, 2001); and 4) their unique hyphal surface properties, which enable improved capture of emissions like hydrophobic volatiles (Kennes and Veiga 2004; Rene et al. 2013).

In the presence of hydrophobic VOCs, fungal biomass has been shown to increase in hydrophobicity and surface area (Vergara-Fernandez et al., 2006). The fungal: bacterial ratio has also been shown to correlate with the capture of emissions with low dissolution (Prenafeta-Boldú et al. 2012). These characteristics of fungi could be harnessed to filter livestock emissions in confined housings, where high exhaust rates reduce residence time and increase the role for capture, not oxidation. To date, the importance of fungal

biomass has not been explored in full-scale livestock emission biofilters and an adaptable biofilm monitoring approach tunable to various full-scale designs is required to enable such investigations.

1.3. Adapting monitoring general microbial biomarker approach for full-scale biofilters

Comprehensive monitoring of biofilter microbial communities demands high-throughput, to detect small spatial/temporal biofilm changes in large, heterogeneous bioreactors; this heterogeneity the result of bioreactor size, fluctuating emissions loading, and non-uniform organic media. Wood mulch is one of the most widely used media in US livestock production emission biofilters (Chen and Hoff 2009). It is preferred for its low-cost, wide availability, moisture holding capacity, bulk porosity, and ability to support a diverse microbial community (Schmidt et al. 2004). Although wood mulch promotes robust biofilm formation, its heterogeneity complicating microbial sampling (Cabrol et al. 2010), so that large sample numbers are required to ensure abiotic and biofilm representativeness.

Furthermore, monitoring strategies must target the surface biofilm communities where effluents and biocatalysis are at an interface. Unlike biofilms on impervious media, biofilms on wood mulch are accompanied by active microbial populations within the medium. Thus, while some members of these communities are significant to biofiltration (*i.e.* facilitating pollutant sorption, biodegrading pollutants, supporting/antagonizing growth of biocatalytic groups), the significance of others inside the wood may be less direct (*i.e.* wood degrading saprobes). Separation of biofilm and wood microbiota would

enable independent assessments of the impacts of their growths on pollutant biocatalysis and the interactions of these distinct populations.

One rapid, low-cost microbial monitoring approach suitable for high-sample numbers, biofilms, and wood microbiota is the detection of microbial biomarkers. Like any measure of microbial structure, biomarkers are not free of caveat and can only minimally resolve complex microbial consortia. However, microbial biomass measures can provide relevant functional information, as has been shown in many natural systems (Joergensen and Wichern 2008) and in engineered biofilters, to isolate the role of fungi in capturing hydrophobic emissions (Prenafeta-Boldú et al. 2012). Moreover, rapid biomarker sampling to complement or target other deeper microbial community assessments has been demonstrated in lab-scale biofilter research (Cabrol et al. 2009; Prenafeta-Boldú et al. 2012).

1.4. Aims

There were three main intentions of this research. Our first objective was to design a bait system that would provide a relevant substrate for monitoring bacterial and fungal biofilms colonizing biofilter packing media. Our second objective was to optimize the harvest and sampling strategies for these baits to ensure repeatable, low variability extraction of biofilm microbial biomarkers. Our third objective was to evaluate the approach in a full-scale biofilter to verify our ability to track key biofilter microbes in the field. By developing a bait and retrieve method, a more reproducible monitoring of biofilms should be achievable than what has been accomplished using the typical 'grab'

sample approach. By sampling in various areas of a biofilter, our study was geared to capture biofilm variability across biofilter packing. Biomarkers (microbial carbon (C), ergosterol) were chosen to monitor bacterial and fungal dynamics in biofilters for their functional significance in other systems, low-cost, high-throughput, and their potential to target more detailed molecular assessments.

2. Materials and Methods

2.1. Design of wood biofilm baits

Wood baits were developed to enable uniform biofilm sampling in the heterogeneous biofilter media, similar to the “coupon” baits used to monitor biofilms in aqueous systems (e.g. Deines et al. 2010). Baits consisted of 3 birch (*Betula papyrifera*) wafers (6.5 × 2.0 × 0.5 cm) cut from dried lumber with the largest face tangential, strung to a numbered aluminum tag with fishing line (Figure S1 in the Supplementary Material). The wafer dimensions resembled the average biofilter media size distribution, and replicate wafers enabled replicate biomass measures.

2.2. Optimizing the harvest and sampling of biofilms

To identify a harvest strategy that targeted biofilms and to identify required biofilm sample sizes for biomarker assessment, a microcosm was used to generate sample material. The microcosm consisted of a gas-tight plastic tub packed with sieved wood mulch media (1 cm mesh) from a biofilter treating swine odor. This was plumbed to a 55 gal swine manure storage which generated a polluted effluent for a lab-scale biofiltration system (Figure S2 in the Supplementary Material). Average inlet emissions were $23.5 \pm$

13.5 ppm NH₃, 565 ± 660 ppb H₂S, 518 ± 53 ppm CO₂, 38 ± 26 ppm CH₄, and 404 ± 80 ppb N₂O (n > 500). Baits were incorporated into the tub and the contents were mixed and watered weekly to promote homogenous biofilm growth and to maintain wood moisture content between 40-60% dry wt. (100°C, 48 h).

To test biofilm targeting, baits were harvested from the microcosm at 8 mo. and subsampled aseptically into 5 pieces using a sterile chisel. One subsample (0.5 × 2.0 × 0.5 cm) was used for measuring moisture content, and the remaining 4 subsamples (1.5 × 2.0 × 0.5 cm) were subsequently fractioned in different ways prior to microbial testing. Fractioning strategies to target biofilms included comparing ground and whole samples with biofilms either attached or detached from the media. To do so, with the remaining subsamples, the 1st with the biofilm attached was left whole (designated as *attached whole*), the 2nd with the biofilm attached was aseptically ground in a Midas Rex® Electric Bone Mill (Medtronic Inc., Minneapolis, MN, USA) with modified blades sharpened to an angle (*attached ground*), the biofilm of the 3rd was scraped free using a sterile single-edge razor blade (*detached biofilm*), leaving a biofilm-free bait (*wood whole*), and the 4th was similarly scraped but the bait was ground (*wood ground*). Microbial biomarker were measured on each sample fractions (n=10) and un-colonized bait controls (n=5). See Figure S1 in the Supplementary Material for a schematic of the sample fractioning.

To test biofilm sample size requirements, a large pool of biomass had to be generated and homogenized to ensure uniformity. To do this, >30 g of biofilm was scraped from the microcosm baits into a sterile blender containing 100 mL of sterilized Type I

water. The slurry was mixed for 30 s, transferred to a sterile glass petri dish, and the added water was evaporated by the sterile stream of air in a laminar hood. Intensive sample homogenization is required for the detection of cells in aggregates, but also liberates microbial C and introduces error in microbial biomass measurement (Jenkinson and Powlson 1976). To minimize this error, active cells were allowed to reabsorb the microbial C liberated by sample mixing by incubating the homogenized biofilm 2 d at 22°C. Microbial biomarkers were measured on biofilm subsamples of 10, 30, 50, 100, 150, and 200 g (n=10).

2.3. Field evaluation of the biofilm sampling technique

For the field component of this work, we used an up-flow, flat-bed biofilter constructed in 2011 on a swine nursery with deep-pit manure storage at the University of Minnesota West Central Research and Outreach Center in Morris, MN (Figure S1 in the Supplementary Material). Non-inoculated, fresh birch (*Betula papyrifera*) chips which passed a 5 cm mesh were used as biofilter media. We focused on a portion of the biofilter (L: 3.5 m, W: 1.25 m, H: 0.35 m) treating air from a continuously running pit fan (See Janni et al. 2014 for details). For the first year of operation, inlet and outlet air were semi-continuously sampled using an automated instrument trailer (Janni et al. 2014). Subsequently, inlet and outlet emissions were collected in duplicate into 50 L FlexFoil® bags similarly to Akdeniz et al. (2011) (Table S1 in the Supplementary Material).

For the field test, baits were placed at 4 locations in the media at the time of biofilter construction: 50 and 300 cm from the fan inlet (*near* and *far*, respectively), and 5

and 30 cm from the biofilter surface (*shallow* and *deep*, respectively) (Figure S1 in the Supplementary Material). After 20 mo., 10 baits were collected from each location, stored in sterile plastic baggies and returned to the laboratory on ice. Baits were subsampled for moisture content, biofilm material was harvested aseptically using a sterile razor blade, then pooled for each location into a sterile 15 mL centrifuge tube, and gently mixed. Baits were then sectioned aseptically using a sterilized chisel. Similar to the sampling method by (Jasalavich et al. 2000), a drill fitted with a sterile 2 mm dia. titanium bit was used to collect wood shavings from inside the bait, through the sectioned face (Figure S1 in the Supplementary Materials, Figure 4b). Wood shavings were also pooled in a sterile 15 mL centrifuge tube and gently mixed. Drilling was used over milling to improve targeting of internal wood communities and to speed aseptic sampling. Pooled biofilms and drillings were subsampled ($n = 6$) for microbial biomass determinations.

2.3. Microbial biomarkers

Total microbial biomass was determined by chloroform fumigation extraction (CFE) similar to the methods of Needelman et al. (2001), with sample moisture content raised above 50% for dry samples to enable maximal C flush (Ross 1989). Samples were extracted in 0.1 M K_2SO_4 (12.5 mL dry g^{-1}) with orbital shaking (100 rpm) for 1 h, then filtered and stored at -20°C. Extracts were later thawed, diluted 1:20 with Type I H_2O , and analyzed for non-purgeable organic C on a Shimadzu (Kyoto, Japan) TOC-VCPH analyzer. Extractant salinity was lowered to 0.1 M K_2SO_4 as no statistical difference between this concentration and the recommended 0.5 M solution was observed (Figure S3 in the Supplementary

Material) and the less saline extractant, in addition to sample dilution, helped minimize analyzer error and maintenance.

Fungal biomass was measured chromatographically as total ergosterol similar to the methods of Schilling and Jellison (2005). Free and esterified ergosterol were extracted from stored samples (-20°C in 5 mL methanol) by reflux, saponification with KOH and an additional reflux. Solids were removed by centrifugation and total ergosterol was separated from alkaline methanol by 3 pentane phase separations. Extracts were combined, evaporated, re-dissolved in methanol, filtered and measured by high performance liquid chromatography (HPLC) on an Agilent Technologies (Minnetonka, MN, USA) 1200 series HPLC.

2.4. Confocal microscopy

Confocal microscopy was used to visualize fungal hyphae in the biofilm and wood bait, similar to the methods of Schilling et al. (2013). Baits were tangentially sectioned (15 µm) with a Damon-IEC Minotome microtome cryostat (International Equipment Company, Chattanooga, TN, USA) after tissue freezing medium (TFM™) (Triangle Biomedical Sciences, Durham, NC, USA) infiltration and storage at -20°C. Sections were mounted onto a slide, stained in the dark with 10 µg mL⁻¹ wheat germ agglutinin-tetramethylrhodamine in 1× PBS (pH 7.4) (WGA-TMR, excitation 555 nm, emission 580 nm, Invitrogen, Calsbad, CA, USA) for 30 min, then rinsed with 1× PBS. Stained sections were then imaged using a Nikon A1si confocal system (Nikon Instruments Inc., Tokyo, Japan), equipped with a 32-channel PMT spectral detector, mounted on a Nikon Ti2000E inverted fluorescence scope.

The 561 and 405 laser bands were used to excite and separate fungal and wood tissues. Emissions were collected and NIS Elements imaging software (Nikon Instruments Inc., Tokyo, Japan) was used to unmix selected spectra. ImageJ 1.47 (National Institute of Health, Bethesda, Maryland, USA) was used to prepare images for publication.

2.5. Statistical analyses

For the sample fractioning experiment, the data could not be normalized through transformation due to the highly variable ranges in measurement when different fractions were being compared. As such, the Kruskal-Wallis test was used to test for differences in the biomarker measures of the different sample fractions. Tukey HSD was used post-hoc to test for differences in biomarker means of the different sample fractions. A separate Kruskal-Wallis test and Tukey HSD test was run for each biomarker response.

For the sample size experiment, normal distribution of data was verified by Shapiro-Wilk normality testing. One-way ANOVA was used to test for differences in the biomarker measures of the different sample sizes. Tukey HSD was used post-hoc to test for differences in biomarker means of the different sample sizes. A separate ANOVA and Tukey HSD test was run for each biomarker response. Additionally, coefficient of variation (CV) was calculated for each biomarker for each sample size to look at the change in measurement variability as sample size was increased.

For the field evaluation, normal distribution of data was verified by Shapiro-Wilk normality testing. One-way ANOVA was used to test for differences in the biomarker measures of the different sample locations. Tukey HSD was used post-hoc to test for

differences in biomarker means of the different sample locations. A separate ANOVA and Tukey HSD test was run for each biomarker response and the ergosterol: total microbial C ratios. All statistical analyses were conducted with R 3.0.1 (GNU Project) using the alpha level of 0.05.

3. Results

3.1. Targeting biofilm communities

The microbial biomass inside wood was partially extracted along with attached biofilms; therefore, we isolated biofilms from wood substrate (*detached*) by scraping prior to analyses (Figure 1, Table S2 in the Supplementary Material). Significant differences between sample fractions were found for both total microbial biomass, measured as microbial C ($p < 0.001$), and total fungal biomass, measured as ergosterol ($p < 0.001$) based on Kruskal-Wallis tests. Mean values for *attached whole* fractions statistically exceeded extractable biomarker values for *detached biofilm* fractions for both biomarkers based on post-hoc tests. The range and standard deviation of *attached whole* biomarker values for total microbial and fungal biomass were also larger than *detached biofilm* fractions. *Detached biofilm* fractions routinely had the smallest biomarker values of all the fractions tested. Similar patterns (data not shown) were seen for total DNA extracted by a modified CTAB extraction (Song et al. 2014).

Extraction of *attached whole* fractions overestimated *detached biofilm* microbial biomass, and in some cases better represented maximum extractable microbial biomass of the sample measured as *attached ground*, or approximated by the *sum* of surface and

wood biomarkers. For all mean biomarker values, there was no statistical difference between *attached ground*, the *sum of detached biofilm + wood whole*, and the *sum of detached biofilm + wood ground*. Exhaustiveness of the biofilm scrape was verified by the lack of difference between these values, and confirmed by confocal observations (data not shown). For total microbial and fungal biomass mean values, *attached whole* was also not statistically different from the *attached ground* baits. Variability was generally higher in samples that were ground. Similar patterns were seen with total DNA (data not shown).

3.2. Biofilm sample size requirements

Variability was typically higher for small sample sizes, and generally declined for all biomarkers when larger samples were used. ANOVA tests indicated statistical differences between sample sizes for both biomarkers ($p < 0.001$). Post-hoc analyses indicated microbial C and ergosterol mean values generally plateaued as sample size increased with accurate biomarker detection requiring ≥ 100 mg of biofilm per extraction (Table S3 in the Supplementary Material). Variability also stabilized at threshold sample weights between 50 and 150 mg for the biomarkers, with increasing biomass sample weight correlating well with the reduction in CV for both microbial biomarkers (Figure 2). Patterns with total DNA were more variable but similar (data not shown).

3.3. Biofilm fungi in full-scale biofilters

In the pooled biofilm samples, a significantly higher total fungal: total microbial biomass ratio was measured on baits in the far-shallow location than in the other locations (Figure 3, Table S4 in the Supplementary Material). ANOVA tests indicated

locations had significantly different mean ratio values ($p < 0.001$), but only the far-shallow location was different than other locations based on post-hoc tests ($p \leq 0.002$). The shift in the fungal biomass ratio in the far-shallow location was not driven by an increase in fungal biomass. Though statistically significant differences in ergosterol mean values across locations were found based on ANOVA testing ($p < 0.001$), the ergosterol levels of the far-shallow location did not change significantly from the other locations based on post-hoc tests ($p \geq 0.054$). Instead, a significant loss in total microbial biomass drove the change in the biomass ratio, as post-hoc tests indicated biofilms from the far-shallow location had significantly lower microbial C measures than all other locations ($p \leq 0.008$). The greatest microbial growths were measured in the far-deep biofilm samples where mean values of chloroform labile-C and total ergosterol were both elevated. Homogenized samples of wood drillings from the full-scale, flat-bed biofilter showed no difference in the ratio of total fungal: total microbial biomass using our protocol ($p = 0.527$) (Figure 3, Table S4 in the Supplementary Material). Microbial biomass was more concentrated in biofilms than in wood drillings. An inverse relationship between the mean biofilm fungal: total microbial ratio and the associated moisture content of each sample area was also apparent (Figure 3, Table S4 in the Supplementary Material). A negative correlation ($R^2 = -0.545$) was also found when biofilm ratios were compared to sample moisture content, regardless of the sample location.

In the far-shallow location, biofilms were typically variable with reduced thickness, compared to the thick, more uniform biofilms from locations like far-deep. On the more

desiccated samples, typical of the far-shallow location, biofilms were quite variable and were often observed with scattered growths of fungal aerial hyphae (Figure 4a, b). Confocal micrographs show connectivity between these aerial tufts of hyphae and the filamentous growth of fungal inside the wood matrix (Figure 4c).

4. Discussion

4.1. Wood baits are effective, and isolating biofilms improved measurement precision.

Wood baits were effective for sampling biofilter microbiota, and the biofilms on these baits were successfully targeted by their removal prior to biomarker measurement. Biofilm baits for aqueous systems, known as “coupons”, have been successfully used since the 1990s to monitor microbial biofilm communities in aqueous bioreactor systems (Deines et al. 2010). These field studies have significantly improved aqueous bioreactor stability (Simpa et al. 2010). While these coupons are typically made of piping materials, wood baits have been used similarly for monitoring biofilms in natural stream systems (Tank and Webster 1998). To our knowledge, this is the first use of a wood bait to sample microbial biofilms in gas-phase bioreactors. Like the use of coupons in aqueous systems, the use of biofilm baits in gas-phase biofilters facilitated precision sampling of natural biofilms growing in a complex, heterogeneous environment.

Scraping the biofilms free from bait surfaces prior to analysis was necessary for low variability, targeted microbial assessment. Small discrepancies between the mean values for summed fractions (*biofilm + wood*) and the entire sample (*attached ground*) were the likely result of milling artifacts and their impact on measurement accuracy. With the

exception of the *attached ground* ergosterol measurement, milling increased biomarker variability for both attached biofilm and scraped samples. Effective sampling of wood microbiota and complete biomarker extraction depends on effective pulverization of the wood matrix. However, this intensive processing can also degrade microbial cellular components and has been shown to increase measurement error for all biomarkers tested (Cabrol et al. 2010; Jenkinson and Powlson 1976; Newell et al. 1988). Although sampling microbial biomass in wood will always require some sample processing, scraping biofilms from the bait circumvented issues with milling and resulted here in small biomarker measurement variability. While not thoroughly explored, similar trends were also observed for extraction of DNA from the different sample fractions (data not shown).

Extraction of attached samples unsuccessfully targeted biofilms, and differences in biomarker extraction capacities complicate comparative analyses. When *attached whole* samples were extracted, biomarker measures were more variable and typically did not approximate *detached biofilm* fractions. Differences in the ability of extraction protocols to dissociate biofilms and penetrate the wood matrix may explain why biomarker measures of unfractionated samples overestimated the biofilm. This would be an important consideration for the extraction of DNA from intact biofilms for deeper probes of the microbial communities. Biofilms are a protective microbial growth strategy (Hall-Stoodley et al. 2004) with an improved resistance to antimicrobial agents (Mah and O'Toole 2001) and as we saw in our preliminary molecular work-ups, molecular assessments in biofilter systems have already shown extraction of microbial DNA from

active biofilms to be problematic (Cabrol et al. 2010). With DNA and biomarker extraction techniques utilizing different hot solvents, fumigants, and cell disruption steps, their abilities to overcome biofilm protection and access wood microbiota is highly unpredictable, justifying biofilm removal prior to analysis.

Sample storage further affected biomarker analysis, particularly for ergosterol. In an accompanying methods development experiment, extractable ergosterol from *attached whole* fractions increased with length of storage time, eventually reaching levels not statistically different from *attached ground* fractions (Figure S4 in the Supplementary Material). This additional source of variability might explain the unique result of greater measurement variability for *attached whole* fractions than for *attached ground* fractions. In the accompanying experiment, where degradation was standardized by use of a single fungal strain, milled sample variability was greater than whole sample variability. It is possible that the biofilm and bait test material conditions generated here were more varied, as a consortium inoculum was used. Consequently, inconsistent solvent diffusivity through *attached whole* samples may have contributed more to measurement variability than milling where a well-mixed, uniform sample enabled uniform biomarker extraction. Selecting storage time or extraction conditions that would limit over-estimation of biofilm biomarkers would be difficult with variable biofilm and bait field conditions. Biofilm scraping (facilitated by the use of baits), is therefore a more standardizable approach for targeting biofilms and is suitable for various biomarkers, environmental condition, and

avoids the limitations encountered when directly sampling bulk, heterogeneous field biofilter media.

4.2. The effect of biofilm sample weight on the extraction of microbial biomarkers.

Sample size relationships indicated a threshold requirement of ≥ 100 mg of biofilm for stable, low variability measurement of biomarkers. Based on biofilter sampling experiences from this study, approximately 1 mg of wet biomass was harvested on average from each 1 cm^2 of wood bait surface. Thus, at least 100 cm^2 of surface area was required per microbial measure. This sample requirement may be particularly helpful when employing molecular approaches, to avoid under-sampling biofilm surface areas.

In our study, adequate sample size was achieved by pooling scrapes from smaller baits, as baits with a 100 cm^2 surface area would have misrepresented actual biofilter media dimensions and airflow exposures. Pooling also ensured sample representativeness, as some collected biofilm growths had wet weights an order of magnitude smaller (desiccated and early time collections) or larger (wetted and later collections) than the mean biofilm size. High variability of biofilm thickness in response to localized abiotic conditions is not uncommon (Deshusses 1997; Wäsche et al. 2002). Although pooling may mask some microbial populations in soil (Manter et al. 2010) and biofilm microbial communities (Nyvad et al. 2013), no significant difference was seen between microbial populations of individual and pooled composite soil samples (Osborne et al. 2011), and bioreactor studies have shown pooling reduces sampling bias and variability (McIlroy et al. 2009). With biofilm growths variable within and across systems,

we report our findings on a mass basis to facilitate adaptation and pooling optimization for other pollution control bioreactor study systems. Once optimized for a new system, the use of baits allows microbial biomarkers to be normalized more relevantly by surface area.

4.3. The fungal: total microbial ratio was greatest in biofilms harvested from drier biofilter media.

As could be done for emissions reductions, the optimized monitoring approach successfully correlated a full-scale biofilm community shift across a small spatial scale to abiotic conditions. Specifically, microbial biomass inside the wood was shown to remain stable across sample locations while fungi dominated the biofilms in drier areas of the biofilter. The greatest microbial biomass, fungal and total, was in the far-deep location, an inlet area of the biofilter less affected by solar and wind drying. More stable temperature and moisture conditions support vigorous microbial growth, and reported cell densities in the laboratory are usually greatest at biofilter inlets where emissions loading is highest (Cabrol and Malhautier 2011). The use of baits corroborated those findings in a field-scale biofilter. When the proportions of biomass were investigated, however, fungi were found to dominate biofilms from drier samples, such as those collected in the more exposed far-shallow location. Biomarker data suggested that greater total fungal: total microbial ratios in these biofilms were the result of significant losses in total microbial biomass and not additional fungal biomass. It is plausible that bacterial susceptibility and fungal tolerance to the rapid and frequent drying drove these shifts, supporting other lab-scale

observations of fungi better tolerating periodic biofilter desiccation (Kennes and Veiga 2004).

The potential tolerance of fungi to drier media observed in this study may be explained by the fungal production of hydrophobins and to their unique hyphal growth form. Once secreted, hydrophobins assemble into surface membranes on hyphal and spore surfaces (Linder 2009). Work with a pathogenic fungal system has shown this protein coating facilitates spore desiccation tolerance (Klimes and Dobinson 2006). Perhaps hydrophobin assembly on biofilter hyphae also creates an impervious barrier to moisture loss, in addition to their known facilitation of hyphal protrusion into the air from aqueous biofilms (Wosten 2001). Fungal morphology may also help explain tolerance to biofilter media desiccation. As shown in our confocal micrograph, there is connectivity between the biofilm and hyphal reserves inside the wood. This connectivity may allow fungi to conduit water from the wood to drying surface growths, in-line with their well-documented capacities to translocate water and nutrients (Cairney, 1992). Additionally, this “harbor” of inoculum may enable fungi to quickly redevelop biofilms when media moisture returns.

5. Conclusions

Here we have detailed an optimized wood ‘bait and scrape’ monitoring technique that can be used to explore the patterns and potentials of bacterial and fungal biofilm growths in gas-phase biofilters. A sampling threshold of ≥ 100 mg of microbial biofilm was identified for repeatable, low variability biomarker measurement. Through field testing,

we have evidence of fungal tolerance to media moisture stress, and suggest further investigation of fungi for their role in biofilter desiccation tolerance. Overall, this targeted biofilm sampling approach enabled full-scale microbial monitoring of biofilters and can be optimized to facilitate more detailed downstream analyses.

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Figure Captions

Figure 1 The effect of sample fractioning on extraction of microbial biomarkers from baits incubated 8 mo. in a biofilter microcosm. For each biomarker (n=10) tested subsamples included baits with an attached biofilm left whole (*attached whole*), or milled (*attached ground*), biofilms (*detached biofilm*), biofilm-free wood left whole (*wood whole*), or milled

(*wood ground*), and the sum of paired biofilm and wood fractions (*sum*). Non-incubated treatments (*controls*) ($n=5$) were also tested. Means with different letters are significantly different ($\alpha = 0.05$). Boxplots depict the media, 1st and 3rd quartiles, and quartile values plus or minus the interquartile range times 1.5. Circles represent outliers.

Figure 2 Regressions of the average coefficient of variation (CV) for dry weight normalized total microbial C (CFE) or total ergosterol (ERG) ($n=10$) and the biofilm sample weight.

Figure 3 Boxplots depicting total fungal:total microbial biomass ratios (measured as mass normalized total ergosterol to total microbial C) of biofilter sampling locations for biofilm (light grey) and wood substrates (dark grey). On the second axis is the mean sample moisture content (% dry weight) of biofilter sampling locations (black diamonds), standard deviation represented by the error bars ($n=6$). Means with different letters are significantly different ($\alpha = 0.05$).

Figure 4 a. Photograph of a bait from the far-shallow location after the moisture content subsection was removed. A fungal dominant biofilm can be seen growing on the bait surface. **b.** Cross section of the bait showing drill holes used to sample internal microbial biomass. Aerial hyphae can be seen extending out from the biofilm and wood degradation is evident below the biofilm. The black box indicates the approximate location of the confocal image. **c.** An unmixed, stitched confocal micrograph of biofilm and bait section showing fungal hyphae (stained with WGA-TMR, excited with a 561 laser) growing aerially (white arrow) and connected to mycelial growths in and across wood cells. The dashed line indicates the surface of the bait.

Figure 1

Figure 2

Figure 3

Figure 4