

2 Short communication

3
4 **Enumerating actinomycetes in compost bioaerosols at source – use of soil**
5 **compost agar to address plate ‘masking’**

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13
14 **Abstract**

15 Actinomycetes are the dominant bacteria isolated from bioaerosols sampled at composting
16 facilities. Here, a novel method for the isolation of actinomycetes is reported, overcoming
17 masking of conventional agar plates, as well as reducing analysis time and costs. Repeatable
18 and reliable actinomycetes growth was best achieved using a soil compost media at an
19 incubation temperature of 44°C and 7 days' incubation. The results are of particular value to
20 waste management operators and their advisors undertaking regulatory risk assessments that
21 support environmental approvals for compost facilities.

22
23 **Keywords:** actinomycetes, bioaerosols, compost, enumeration, risk assessment
24

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1 **1 Introduction**

2 Actinomycetes are a heterogeneous group of filamentous bacteria resembling fungi.
3 They are a major component of bioaerosols emitted from composting facilities (Strom, 1985;
4 Lacey, 1997; Niemi *et al.*, 1982; Swan *et al.*, 2003). Mesophilic and thermophilic
5 actinomycetes have growth profiles of 20°C-50°C and 30°C -60°C, respectively. They grow
6 as branching microorganisms with short chains of spherical spores, 0.7–1.3 µm in diameter.
7 Prolonged inhalation of actinomycetes is linked to adverse human health effects (Douwes *et*
8 *al.*, 2003), including allergic alveolitis and other respiratory responses (Millner, 1982; Lacey
9 and Crook, 1988, Lacey and Dutkiewicz, 1994; Reboux *et al.*, 2001; Herr *et al.*, 2003), in
10 which inflammation of the lung is believed to be caused by glycopeptides and protein
11 allergens released from spores, as is the case for ‘farmer’s lung disease’ (Edwards, 1972).

12 Actinomycetes concentrations, reported in colony forming units per cubic metre of air
13 (cfu/m³), are among the micro-organisms that comprise the atmospheric bioaerosol burden
14 emitted from composting facilities (Composting Association, 1999; Taha *et al.*, 2005; 2006).
15 In the UK, a quantitative estimation of bioaerosol load is expected as part of risk assessments
16 for composting facilities within 250m of sensitive receptors, prior to the development or
17 extension of such facilities and to inform permitting of Waste Licences (Environment
18 Agency, 2001). Meaningful risk assessments rely on quality source term data (Pollard *et al.*,
19 2006); particularly at source, so that downwind concentrations may be reliably approximated.

20 In bioaerosol sampling methods based on the capture of culturable micro-organisms,
21 problems associated with the identification and quantification of micro-organisms include
22 colony ‘masking’, where colonies overlap (Chang *et al.*, 1994; Chang *et al.*, 1995; Chen *et*
23 *al.*, 1998; Kalogerakis *et al.*, 2005). Masking impedes the visual inspection of the media
24 plate and enumeration of individual species, which may lead to colony counting errors.
25 Lacey (1997) suggests that the method used to collect samples may influence the growth of
26 other bacteria, and that this problem is particularly associated with isolating actinomycetes

1 from compost samples. Researchers have also commented that selecting the appropriate
2 media for the environment in which the sample is collected may also improve the
3 enumeration results (Balestra and Misaghi, 1997).

4 Here, we introduce an alternative media, soil compost agar, for the improved enumeration
5 of actinomycetes from bioaerosols sampled close to source at two green waste composting
6 facilities. Source term samples, we have found, are especially prone to masking and yet are
7 most relevant to estimating the bioaerosol flux from compost facilities and so a reliable
8 enumeration method must be established. We believe that the method proposed here is of
9 timely importance due to the current regulatory interest in occupational and public exposures
10 to bioaerosol emissions (Swan *et al.*, 2003; Herr *et al.*, 2003).

11

12 **2 Materials and methods**

13 *2.1 Bioaerosol and compost sampling*

14 Bioaerosol sampling was performed at two green waste windrow composting facilities,
15 with capacities of 2000 m³ and 1000 m³ per annum respectively. Air and compost samples
16 were taken on nine separate occasions between October 2003 and March 2005, covering the
17 winter, spring and autumn seasons. The samples were taken (i) >15 m upwind of the facility
18 boundary; (ii) within the site; and (iii) at 15, 100 and 200 m downwind of the composting
19 facility site boundary. Further details and the background context for our bioaerosols studies
20 at these composting facilities have previously been reported (Taha *et al.*, 2005; 2006; 2007).

21 Sampling employed medium flow, personal aerosol filter samplers (SKC Universal dust
22 and vapour sampling pump; models SKC Aircheck™ 224-PCXR8 and 224-PCTX8). Pumps
23 operated at 2.0 ± 0.1 l/min and were fitted with SKC dust sampling IOM (Institute of
24 Occupational Medicine) heads connected using 10 mm i.d. Tygon™ tube. IOM heads were
25 loaded with 25 mm diameter, 0.8 µm MCE (mixed cellulose ester, SKC) filters. The IOM
26 samplers were attached to a stand, 1.8m above ground, **similar to the method used by**

1 Wheeler *et al.* (2001) and Environment Agency (2007). The sampling time was 30 minutes,
2 reduced to 10 minutes where the expected bioaerosol concentration was high. Two air
3 samples were taken simultaneously in each case using two samplers, with one pump kept as a
4 standby, as recommended by the Composting Association (1999). Both samples were treated
5 identically in further analyses and the collection of simultaneous samples was used to
6 increase the statistical significance of the results. All pumps were calibrated before use.

7 Compost grab samples (*ca.* 30 g) were also taken from the windrows associated with
8 bioaerosol release. The grab samples were taken from as close to the area where the air
9 samples were taken as possible, usually at a depth of 0-10cm into the compost pile, which
10 had already been shredded and turned so that there was homogenisation of the material. This
11 procedure was adopted so as to sample the area most closely representing the area where the
12 air samples were collected.

13

14 2.2 Isolation

15 Micro-organisms were quantified using the plate count analysis steps of the CAMNEA-
16 method (Collection of airborne microorganisms on nuclepore filters, estimation and analysis;
17 Palmgren *et al.*, 1986) with subsequent visual enumeration. Filters and compost grab
18 samples were placed, whilst at the site, into 30 ml vials containing 10 ml 0.05% ^{v/v} Tween-
19 80™ mixed with 0.1% w/v NaCl to prevent osmosis. Samples were transported at < 4°C and,
20 on return to the laboratory, re-suspended by separating the filter from the head and shaking in
21 a vial for 2 min. The solution was diluted to a common logarithm order (10⁻¹, 10⁻² and 10⁻³)
22 and inoculated onto both media plates. All equipment was sterilized before use.

23 2.3 Enumeration

24 Media preparation, inoculation, dilution and sterilization were performed in accordance
25 with BS 5763: Part 0:1996 (British Standards Institution, 1996). Two media were used: (a)
26 half strength nutrient agar (NA) (Oxoid CM3 nutrient agar); 14 g/l, in ionised water; and (b)

1 soil compost agar (SCA); a supernatant of 100g loam compost (John Innes™ N^o.1) with
2 1000ml deionised water was vigorously shaken for 2 min. and centrifuged at 500 rpm for 5
3 min. The SCA agar was prepared using 7g of agar-agar powder with 500ml deionised water,
4 with no need for pH adjustment. After preparation, both media were autoclaved (121°C for
5 15 minutes), left to cool to below 47°C and treated with 1% ^{w/v} antifungal cycloheximide,
6 dissolved in 2ml of HPLC grade ethanol. In each case, the agar medium (12ml) was poured
7 into petri dishes to a depth of *ca.* 2mm and allowed to cool on a horizontal surface before
8 being inoculated with the sample solution as described above. Colonies grown at an
9 incubation temperature of 44°C on both media were enumerated visually after 7 days,
10 employing the BS 5763 standard (British Standards Institution, 1996).

11

12 **3 Results and discussion**

13 Actinomycetes were enumerated by estimated count from 76 air samples inoculated on
14 NA plates. However, 60 of these samples showed either full or partial overgrowth of other
15 bacteria ('masking') that obscured actinomycetes growth and it was therefore difficult to
16 count the colonies of actinomycetes (Figure 1). This resulted in these plates being rejected.
17 From the 24 compost samples inoculated on NA plates, all showed 'masking' by overgrowth
18 of other bacteria (Table 1). Serial dilution of the samples was not able to address this
19 masking effect that we attribute to a very high bacterial burden in samples taken very close to
20 source.

21 In contrast, the SCA plates, incubated at a temperature of 44°C, showed no masking by
22 eubacteria or fungi (Table 1), after allowing 7 days for the actinomycetes to grow and
23 sporulate. In each case, the enumeration of actinomycetes colonies was proportionate to the
24 dilution factor applied in constructing the plates, improving the confidence interval limit of
25 the study. The actinomycetes grown on SCA were easily identified by the white powdery
26 colour of their spores and spider web-like formation appearing on the colonies (Figure 2). **Of**
27 **the 13 samples where no actinomycetes were found on the SCA plates, 1 of the NA plates**

1 showed bacteria growth with no actinomycetes, 7 of the NA plates showed no actinomycetes
2 and 5 NA plates showed masking by other bacteria.

3 Several media have previously been used for the identification and enumeration of
4 actinomycetes (Table 2). The Composting Association (1999) suggests the use of half-
5 strength nutrient agar. Most significantly, Crawford *et al.* (1993), during their experiments
6 on plant roots in British soils, secured optimum enumeration using a low nutrient organic
7 media and compost extract agar and using this, the overgrowth of media by eubacteria and
8 fungi was dramatically reduced. Curtis and Beuchat (1998) argue that a common problem
9 related to quality control of culture media is the non-inhibition and growth of unwanted
10 micro-organisms on selective media. High colony counts of unwanted micro-organisms may
11 lead to significant colony overlap on the growth media plate, 'masking' the growth of the
12 species of interest.

13 The enumeration methodology described here offers substantive methodological
14 improvements over existing approaches (Composting Association, 1999) and allows a
15 reliable estimation of the actinomycetes load, especially where concentrations are expected to
16 be high, such as at source. This method has proved useful in achieving consistent results. In
17 addition, it has proven to be cost effective and simple, as well as reducing analysis time. This
18 method is also inline with the findings of Griffiths and Stewart (1999), who suggest the
19 tailoring of micro-organism analysis for the application of interest, as it focuses on
20 composting. This is because no one single assay is suitable for the study of micro-organisms.

21 The results presented here are encouraging for the further development of this method.
22 Future work will include the analysis of the consistency of the extracted compost supernatant,
23 as well as testing batch to batch variation and the use of different types of compost.

24

25 **4 Conclusions**

26 A novel method for the enumeration of compost-derived actinomycetes close to source
27 and in bioaerosol samples has been supported by evidence from over 70 air and compost

1 samples taken over 9 separate sampling days. The method is suited to the enumeration of
2 actinomycetes sampled from green waste compost facilities and significantly reduces the
3 masking of actinomycetes by other bacteria. It provides substantive improvements over the
4 nutrient agar media (*e.g.* Nielsen *et al.*, 1997; Würtz and Breum, 1997; and Breum *et al.*,
5 1997) for source term samples. The method is cost effective, reduces analysis time and
6 produces replicable results. Its application is particularly significant for practitioners
7 enumerating bioaerosols in support of regulatory risk assessments for the waste management
8 sector.

9

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21 recyclable paper of different quality. *Annals of Agricultural and Environmental Medicine*,
22 4, 129-135.

23

24 **Figure captions**

25 Fig 1. Actinomycetes growth using half strength nutrient media (NA). Plates show clear
26 evidence of masking effect, as individual colonies of actinomycetes are difficult to
27 distinguish.

1

2 Fig 2. Actinomycetes growth using soil compost agar (SCA). Plates show clear evidence of
3 improved visual inspection using soil compost agar, as individual colonies of actinomycetes
4 are clearly visible.

5

6 **Table captions**

7 Table 1: Actinomycetes growth from compost and air samples for the soil compost agar
8 (SCA) and the half strength nutrient agar (NA) (Samples were taken on 9 separate occasions
9 between 26/10/2003 and 10/03/2005).

10

11 Table 2: Media used for the identification and enumeration of actinomycetes.

12

13

14

1 Table 1: Enumeration outcome for air and compost samples with actinomycetes growth
 2 using soil compost agar (SCA) and half strength nutrient agar (NA) (samples taken on 9
 3 occasions between 26/10/2003 and 10/03/2005).

enumeration outcome	air samples				compost samples			
	SCA		NA		SCA		NA	
	Samples ^a	% ^b	Samples ^a	% ^b	Samples ^a	% ^b	Samples ^a	% ^b
actinomycetes enumeration achieved	28	37	0	0	23	96	0	0
actinomycetes enumeration achieved by estimated count	33	44	6	8	1	4	0	0
full masking	0	0	48	63	0	0	22	92
partial masking	0	0	12	16	0	0	2	8
no actinomycetes	13	17	8	11	0	0	0	0
bacteria, no actinomycetes	1	1	2	3	0	0	0	0
total samples	75	100	76	100	24	100	24	100

4 ^aThe number of samples that achieved each enumeration outcome

5 ^bThe percentage of samples for each media that achieved each enumeration outcome

6

7 Table 2: Media used for the identification and enumeration of actinomycetes

medium	reference
nutrient Oxoid agar	Nielsen <i>et al.</i> (1997), Würtz and Breum (1997) and Breum <i>et al.</i> (1997)
half strength nutrient agar	Composting Association (1999)
10% ^{w/v} nutrient agar	Nielsen <i>et al.</i> (1997), Würtz and Breum (1997) and Breum <i>et al.</i> (1997)
starch-casein (CS) agar	Folmsbee and Strevett (1999)
Czapek's solution agar (CZ)	Asan <i>et al.</i> (2004)
starch-ammonia medium	Andreeva <i>et al.</i> (2001)
half-strength (50% ^{w/v}) nutrient	Lacey and Dutkiewicz (1976); Recer <i>et al.</i> (2001)
tryptone soya with casein hydrolysate agar	Lacey and Dutkiewicz (1976); Recer <i>et al.</i> (2001)
half-strength tryptic soya agar	Dutkiewicz <i>et al.</i> , (2001)
trypticase soya agar	Lavoie and Alie (1997)
low nutrient organic media and compost extract agar	Crawford <i>et al.</i> (1993)

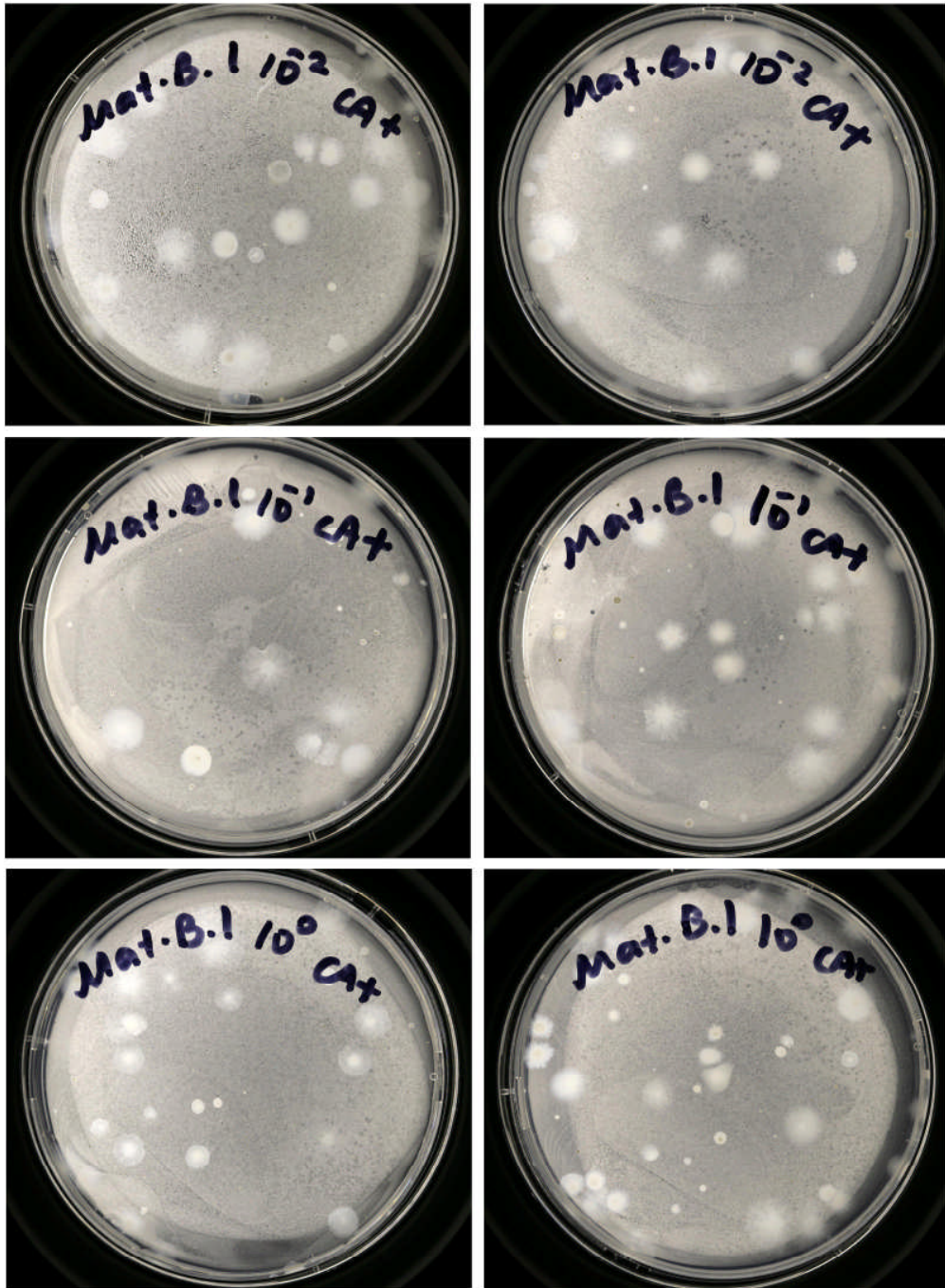
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