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Title

Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate

Running Title

Mathematical model of antimicrobial resistance

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Abstract

Antimicrobial resistance is of global concern. Most antimicrobial use is in agriculture; manures and slurry are especially important because they contain a mix of bacteria, including potential pathogens, antimicrobial resistance genes and antimicrobials. In many countries, manures and slurry are stored, especially over winter, before spreading onto fields as organic fertilizer. Thus these are a potential location for gene exchange and selection for resistance. We develop and analyze a mathematical model to quantify the spread of antimicrobial resistance in stored agricultural waste. We use parameters from a slurry tank on a UK dairy

farm as an exemplar. We show that the spread of resistance depends in a subtle way on the rates of gene transfer and antibiotic inflow. If the gene transfer rate is high, then its reduction controls resistance, while cutting antibiotic inflow has little impact. If the gene transfer rate is low, then reducing antibiotic inflow controls resistance. Reducing length of storage can also control spread of resistance. Bacterial growth rate, fitness costs of carrying antimicrobial resistance and proportion of resistant bacteria in animal faeces have little impact on spread of resistance. Therefore effective treatment strategies depend critically on knowledge of gene transfer rates.

Introduction

Antimicrobial resistance is of growing global concern (Wise *et al.* 1998, Tenover 2006, Ashbolt *et al.* 2013). While much research has concentrated on resistance arising in humans as a result of antibiotic usage, it is widely acknowledged that resistance in agriculture is a major challenge (Khachatourians 1998, Allen *et al.* 2010, Heuer *et al.* 2011, Ashbolt *et al.* 2013). Veterinary use of antimicrobials, especially in swine, poultry, beef and dairy production, has led to increased levels of resistance to such antimicrobials, as detected in manures, slurries, and soil to which these have been applied (Khachatourians 1998, Teuber 2001, Byrne-Bailey *et al*. 2009, Cook *et al.* 2014, Fahrenfeld *et al.* 2014). The resulting risks are the emergence, selection for and exposure to multiple antimicrobial resistant human and animal pathogens, with considerable medical and economic consequences (Ashbolt *et al.* 2013).

Farm slurry tanks are of particular interest because they contain a mix of fecal bacteria, (including potential pathogens), antibiotics and other antimicrobials, which are then stored for considerable periods of time. Analyses of correlation between presence of some resistance genes (*tet*, *sul*, *erm*) and presence of corresponding antibiotics in both slurry lagoons (Zhang *et al.* 2013) and laboratory stored pig-manure (Joy *et al*. 2014) have shown varied results, with

both positive (Zhang *et al.* 2013, Joy *et al*. 2014) and negative (Zhang *et al.* 2013) correlations between presence of different resistance genes and their corresponding antibiotics under different conditions. This variation might reflect the wide range of environments, bacterial species and mobile genetic elements involved. Our own microbiological studies have shown considerable resistance to antibiotics, both currently and previously used on the farm, with at least two thirds of cultured *E. coli* strains demonstrating multiple antibiotic resistance (Ibrahim *et al.* 2016), including to beta lactamase antibiotics. These led us to hypothesise that the combination of fresh fecal matter, antibiotics and storage time within the slurry tank could provide an ideal environment for the emergence of antimicrobial resistant populations of bacteria. Moreover, because of the mechanism of action of beta lactamase antibiotics, the observed genetic resistance could suggest that it is selected for because the cells are growing.

Mathematical models for spread of antimicrobial resistance in bacterial populations have successfully explored the balance between the fitness advantage to hosts of resistance against the cost to hosts of plasmid carriage (Levin *et al.* 1997, Stewart *et al.* 1998, Bootsma *et al.* 2012). While models have mainly been applied in clinical or community settings (Levin *et al*. 2014), some modelling has been carried out for waste water (Sharifi *et al.* 2013), survival of resistant bacteria in slurry-amended soils (reviewed in Ongeng *et al* 2014), and, of particular relevance for this study, for selection for plasmid-mediated cephalosporin resistance in *E. coli* in cattle gut (Volkova *et al.* 2012, Volkova *et al.* 2013). The latter have shown persistence of resistance in these environments driven by horizontal and vertical gene transfer.

In this work we describe and analyze a mathematical model for the spread and selection of antimicrobial resistant bacteria in a slurry tank of a typical high performance UK dairy farm. In common with Volkova *et al.* (2012), we focus on spread of resistance genes through mobile genetic elements such as plasmids (Davies 1997, Krone *et al.* 2007). These pose a greater environmental risk than chromosomal resistance because resistance can spread between organisms,

between species, and from a non-pathogenic reservoir to potential pathogens (Heuer *et al.* 2011, Jechalke *et al.* 2014a, Jechalke *et al.* 2014b).

The main purpose of this model is to identify the factors to which emergence of resistance is most sensitive, and thus inform future research studies and potential interventions. We use parameters taken from a dairy farm in the East Midlands of the UK as a model system for this work, although the mathematical model is developed in a way to be generally applicable. We choose default parameter values from both literature and farm conditions, which are relevant to *E. coli* populations, since these are a sentinel species for antimicrobial resistance (AMR), a major source of mastitic infection in dairy cattle on the studied farm, and of particular potential concern to human health (Pfeifer *et al.* 2010, Liu *et al.* 2015). However, we expect there to be considerable microbial diversity within the slurry tank and the model is applicable to any bacterial species, with appropriate parameter values. An important part of the analysis is to explore behaviour of the model to a wide range of possible parameter values, which could represent different bacterial species or mobile genetic elements. Moreover, the model could be applied to different dairy farms by using different parameter values, and could be adapted to study stored manures from other farm animal species.

We simulate how the population of resistant bacteria changes over realistic timescales, and consider how variations in the parameter values may alter these time courses. Through parameter variation and sensitivity analysis we are able to draw conclusions about the importance of the model parameters, which could potentially be used in identification of control measures to limit emerging antimicrobial resistance. We conclude by discussing the significance of this model and implications for future research and analysis in this area.

Materials and methods

Dairy Farm Background

The farm is a typical high performance dairy farm in the UK. It has been chosen for study because it holds detailed veterinary records of every dose of antibiotic treatment given to each and every animal. It has a herd of *circa* 200 dairy cows; because milking is done by an automated milking system ('AMS') the animals are housed indoors for the majority of the year. Annual average milk yields per cow in milk vary depending on a number of factors, including forage quality; current (2015) average yield is 10,700 litres per year. Each cow produces approximately 63kg of waste per day. To reduce the quantity of slurry requiring storage, solids are mechanically separated and the remaining liquid, containing only about 5% solids, is then pumped into an on-site slurry tank and stored for field spreading. The slurry tank has a capacity of 3 million litres and is generally emptied after *circa* 90 days, either into a slurry lagoon by means of a pipeline, or taken directly to fields for spreading. Cattle slurries are useful as a source of Nitrogen, Phosphate and Potash: standard figures for these nutrients for mechanically separated slurries are given by Chambers *et al.* (2001) as 3.0, 1.2 and 3.5 kg per m^3 respectively.

Mathematical model

The mathematical model describes homogeneous populations of antimicrobial resistant (R) and antimicrobial sensitive (S) bacteria, in the host range of a single type of plasmid that transfers resistance. It is based upon that of Volkova *et al.* (2012) for antibiotic resistance in the cattle gut. As in that model, it uses two ordinary differential equations (ODEs) to describe the dynamics of growth, gene transfer and selection of antimicrobial resistance in these two populations. While this model is necessarily a simplification, we demonstrate that it is extremely useful for identifying the key factors behind emergence of resistant populations, and the model's simplicity also makes it more readily generalizable to other systems, or extendable to models with different types of bacterial hosts, antibiotics or plasmids.

As in Volkova *et al.* (2012) we assume that the sensitive and resistant populations can grow within the tank, and for large populations, could grow to a carrying capacity (N_{max}) associated with the availability of nutrients; when the population reaches the carrying capacity there is no net growth. We use a standard logistic growth term which combines both slowing/cessation of growth and cell death into a single coefficient. The effect of antibiotic on the bacteria is modelled as a reduction in the growth rate, with Hill-function dependence on the concentration of antibiotic. Carriage of antibiotic resistance incurs a fitness cost (α) on the host bacteria. Sensitive bacteria may become resistant to antibiotics in the presence of resistant bacteria by means of horizontal gene transfer. Since the tank receives a constant inflow of fresh slurry each day, including bacteria, there is an inflow term of both sensitive and resistant bacteria. Our model differs from that of Volkova *et al.* in several important ways. We eliminate the outflow term, since there is no outflow from the slurry tank; the tank is emptied when the slurry is spread on the fields, and this is not included in our model. Instead, we explicitly model the increasing volume of the slurry in the tank. We include a model for the amount of antibiotic in the tank, with constant in-flow with the slurry, and first order degradation kinetics, where the degradation rate would depend upon the type of antibiotic. This gives an exponential function describing antibiotic concentration in time. Finally, we use parameter values more relevant to our system, as will be described in subsequent sections. Thus the model equations are:

$$\frac{dS}{dt} = r\left(1-\frac{N}{N_{\max}}\right)E_S S - \frac{\beta SR}{N} + \lambda(1-\rho)\nu \qquad (1)$$

$$\frac{dR}{dt} = r(1-\alpha)\left(1-\frac{N}{N_{\max}}\right)E_R R + \frac{\beta SR}{N} + \lambda\rho\nu \qquad (2)$$

where

$$N = S + R \quad (3)$$

$$V(t) = V_I + \lambda t \quad (4)$$

$$N_{\max}(t) = \mu V(t) \quad (5)$$

$$A(t) = (A_I - \frac{\theta}{\gamma})\mathrm{e}^{-\gamma t} + \frac{\theta}{\gamma} \quad (6)$$

$$E_S = 1 - \frac{E_{\max}\left(\frac{A}{V}\right)^H}{MIC_S^H + \left(\frac{A}{V}\right)^H} \quad (7)$$

$$E_R = 1 - \frac{E_{\max}\left(\frac{A}{V}\right)^H}{MIC_R^H + \left(\frac{A}{V}\right)^H} \quad (8)$$

The meaning of each of the parameters is summarised in Table 1. In the next sections, we describe how we obtain values or realistic ranges of values for each of the parameters. As described in the Results, we carry out sensitivity analysis for many parameters to check how sensitive the model is to realistic variation.

Bacterial parameters

The model considers homogeneous populations of unspecified bacteria that would be within the host range of the plasmid transferring resistance. Generally, we use default parameter values relevant for *E. coli*, because our experimental work has focussed on identifying resistance in *E. coli* populations as a sentinel species (Ibrahim *et al.* 2016). However, the model would be applicable to any bacterial population capable of growing under these conditions, which

undergoes conjugative plasmid transfer and where there are no barriers to the transmission of plasmids, e.g. other *Enterobacteriaceae*. by using different parameter values.

In the model we have a parameter for the maximal specific growth rate of the bacteria (r). Typical generation times for *E. coli* in optimal laboratory conditions are around 20 to 30 minutes. In slurry systems these are likely to be considerably longer, although the specific growth rate, r, of *E. coli* in dairy slurry has not been published in the literature. Volkova *et al.* (2012) use a slower growth rate (0.17 hr^{-1} equivalent to a generation time of 4.16 hours) than in laboratory conditions to account for competition within the gut. This is based on an experimental model of *E. coli* growth in the large intestine of a mouse, and is commensurate with measurements of growth rate of *E. coli* O157:H7 in low carbon fresh water of 0.19 hr^{-1} (Vital *et al.* 2008). Godwin and Slater (1979) and Levin *et al.* (1979), both studying antibiotic resistance, found faster growth rates in laboratory conditions, 0.69-0.9 hr^{-1} and 0.86 hr^{-1} respectively. In earlier work, Curds (1971) used a growth rate of 0.5 hr^{-1} for modelling sewage bacteria in an activated-sludge process. We choose to use the same growth rate (0.5 hr^{-1}) in this work as this appears to be an appropriate compromise between the rates seen in ideal conditions and those seen in very low carbon or highly competitive environments. As will be seen later, the modelling results are not sensitive to the value of this parameter, justifying this (or any other suitable) choice of this parameter value.

In addition to the proliferation described above, horizontal gene transfer is a major source of antibiotic resistance in bacteria. In the dairy slurry tank we expect to find a diverse range of bacteria, and gene transfer on a range of plasmids between different bacterial types is well documented. Hence, we would expect to find significant variation in the rate of horizontal gene transfer. Subbiah *et al.* (2011) reported experimental work looking at *E. coli bla*-CMY2 plasmids from dairy cattle. In this work, they found that the transfer rate varied significantly depending on the plasmid considered. The Volkova model uses a gene transfer rate, β, of 0.004 hr^{-1} based on this work and we start with a rate of

similar magnitude to Volkova (β=0.001) and then explore model behaviour for a wide range of variation in this parameter, which could be thought of as representative of different plasmid types.

Several sources have measured the fitness cost of resistance to a range of antibiotics in *E. coli* (Godwin and Slater 1979, McDermott *et al.* 1993, Subbiah *et al.* 2011). The range of fitness costs spans 0-30%. There is also a growing consensus that initial fitness costs evolve to reduce over time by compensatory mechanisms as discussed in Andersson and Levin (1999). In line with our stated objective to keep the model simple, we use a constant value of the fitness cost. Given that we are considering relatively long time scales, compared to many laboratory experiments, we choose to use a fitness cost, α, at the lower end of the range, and choose a value of 0.1 (10%) to allow for compensatory mutations over the long time scales. We also consider in later sections how changes to the fitness cost affect the model results.

Slurry tank parameters

We calculate an estimate for the rate of slurry inflow, λ, based on estimates of slurry production and dairy wash volumes given by the Agriculture and Horticulture Development Board (DairyCo. 2010, cost effective slurry storage strategies on dairy farms, Kenilworth, UK) and farm specific data. An adult dairy cow deposits approximately 63 litres of faecal/urinary waste per day. Removal of solid waste from this can reduce the volume by up to 15%. We also account for an additional 20 litres of water per cow per day from washing, that also enters the slurry system. Hence based on the 200 cow herd we estimate an inflow of 14710 litres of slurry per day, which we assume is pumped in continuously through the day giving an hourly rate of 613 l hr^{-1}.

Our data shows the levels of E. coli in the slurry tank are consistently in the same range of 2–6 x 10^4 per mL as Reithaler et al. (2003) found for sewage. Reithaler *et al.* (2003) also reported approximately 40% of *E. coli* strains resistant to at

least one antibiotic. This range is in line with the *E. coli* concentrations detected in cattle slurry in work by Fenlon *et al.* (2000) where an *E. coli* count of 5.3 x 10^4 CFU ml^{-1} was found in slurry that was to be spread on land, and Sawant *et al.* (2007) who found multi-drug resistance in 40% of *E. coli* isolates from healthy lactating dairy cattle. Many other papers reference bacterial loads of specific strains of *E. coli* (particularly pathogenic strains), however here we wish to consider the total *E. coli* count, since non-pathogenic strains may provide a reservoir for resistance genes. Additionally we often find co-selection of antibiotics due to genetically linked elements (Herrick *et al.* 2014). Here we use the parameters from Reithaler *et al.* (2003) of $\nu = 6 \times 10^4$ CFU ml^{-1} and $\rho = 0.4$ (i.e. 40%); although the value of 40% appears to be high, we later consider sensitivity of the model to wide variation of this value and it turns out to have very little impact on the results.

The capacity of the slurry tank on this dairy farm is 3 million litres. When this is emptied there is always a small amount of residue left in the tank. We estimate this to be 5% of the total capacity, hence we assume an initial slurry volume of 1.5 x 10^5 litres. The initial concentration of antibiotics in the slurry tank is relatively unknown. For simplicity we assume that the initial concentration of antibiotic in the tank at the beginning of the simulations is zero.

In the farm under study, the overwhelming majority of antibiotic treatment is for mastitis, and is injected directly to the udder. As is common practise in the UK, milk from mastitic udders is discarded into the slurry, and this is the main source of antibiotics in slurry. Therefore we calculate the rate of antibiotic inflow, θ, using the amount of waste milk we expect to be entering the slurry tank and published data on antibiotic residues found in waste milk. Brunton *et al.* (2014) tested for antibiotic residues in waste milk, after Cefquinome treatment, destined to be fed to calves from a single UK dairy, with 550 cows. They found Cefquinome in the waste milk at an average concentration of 0.746 mg l^{-1}. In a wider study by Randall *et al.* (2014), 103 UK dairy farms were sampled, with an average Cefquinome concentration of 1.4 mg l^{-1} and a range of 0.006 - 4.6 mg l^{-1}. Since Cefquinome has been one of the main antibiotics used to treat mastitis on

the farm in this model, we use the mean from the wider study (1.4 mg l^{-1}) and multiply this by the amount of waste milk we expect per hour to give a rate of antibiotic inflow. We assume that during treatment for mastitis, milk is withdrawn from supply for a period of 5 days, as per treatment guidelines, resulting in 176 litres of waste milk per case of mastitis. A case of mastitis occurs on average every three days on this particular farm, giving a rate of milk waste as 2.4 l hr^{-1}. Hence the rate of antibiotic inflow is 3422 μg hr^{-1}. This parameter value could be modified to take into account different sources of antibiotics, e.g. through faeces or urine, associated with different farming practises, and disease burden.

We assume that antibiotics will degrade though a natural decay process within the slurry tank, hence we model this with an exponential decay term. Dolliver (2008) found degradation rates of antibiotics in composting conditions to vary between 1 and 23 days, depending upon the antibiotic type, although cephalosporins were not studied. Wang and Yates (2008) reported half-lives for Oxytetracycline, a different type of antibiotic, to range between 8 (relatively short timescales) and 56 (relatively long timescales) days in laboratory experiments depending on moisture content. Jaing *et al.* (2010) found cephalosporins to degrade in lake surface water with half lives of 2.7 to 18.7 days. Here we use an antibiotic half life of 10 days, equivalent to a decay constant of 0.0029 hr^{-1}.

We have no data at present on the carrying capacity for bacteria in the slurry tank. However, given the amount of nutrients in the slurry we expect it to be large, and not a limiting factor in the model. For this reason we use a value of 10^{10} CFU l^{-1} for all bacteria based on the typical stationary phase populations of *E. coli* in laboratory conditions. We multiply this value by the tank slurry volume to give the total carrying capacity at any time *t*. In effect, this means that the bacterial population is free to proliferate.

Emax model parameters

The Emax model determines the effect of the antibiotics in the tank on the growth of bacteria. Thus the model is relevant to beta lactamase type antibiotics and other classes of antibiotics that impact upon cell growth. We use an Emax parameter of 2 and a Hill coefficient of 2 as in the Volkova model. We take the MIC values from published product information for Cobactan (cefquinome), which is commonly used on the study farm. Hence we choose MIC_S to be 0.008 μg ml^{-1} and MIC_R to be 2 μg ml^{-1} (http://www.vmd.defra.gov.uk/ProductInformationDatabase/Default.aspx). The model could be used for other antibiotics by varying the values of the Emax and MIC parameters.

Simulations

We simulate the model using the ODE45 solver in Matlab v7.12.0 for our default parameter values (Table 1) to produce time courses of the model variables, slurry tank volume and amount of antibiotics in the tank over time. We also calculate the proportion of resistant bacteria in the model as R/N. For all simulations we initialise the model with an effectively sterile tank (R = S = 1 to avoid division by zero errors in the gene transfer term), however the model is relatively insensitive to the initial amount of bacteria in the tank.

We produce both single- and two-parameter variation plots by conducting multiple simulations as described above. For each simulation we vary either one or two parameters within the range given in Table 1, and record the variable values at t = 90 days. For each parameter we run between 50 and 100 simulations, with a uniform distribution of parameter values.

Sensitivity analysis

We conduct a global sensitivity analysis of four of the model parameters (α, β, θ, ρ) as well as the length of time that slurry is stored. We take 3000 randomly chosen points in parameter space, within the feasible range (see Table 1) varying the five parameters of interest but keeping the other parameters

fixed. We conduct a local one-at-a-time sensitivity analysis of the model at each of the 3000 points. We measure the relative sensitivity using the function

$$S_k^{\phi} = \frac{\delta\phi / \phi}{\delta k / k}$$

where ϕ is the feature being measured and k is the parameter being changed. We measure the proportions of sensitive and resistant bacteria in the slurry tank and plot these as box plots.

Results

Slurry tank conditions increase absolute and relative numbers of antimicrobial resistant bacteria

For our default parameters, we see that initially the numbers of both resistant and sensitive bacteria increase (Figure 1B and C). Whilst the resistant bacterial population continues to grow, the sensitive population reaches a peak (at t=74 days) then declines rapidly. This is also reflected in the proportion of resistant bacteria in the tank, which increases from 0.2 to 1 (Figure 1D). At t=90 days approximately 62% of the bacterial population is resistant to antimicrobials, which is significantly greater than the 40% resistance at inflow. This is an important time point since we expect the slurry tank to be emptied after 90 days and put to agricultural use.

With these parameters, if the slurry tank is allowed to fill to maximum capacity (3 million litres), which we expect to take approximately 200 days, 94% of the bacterial population is modelled to be resistant. These proportions of resistant bacteria are far in excess of the proportion present in the slurry inflow, hence the conditions in the slurry tank can potentially exert a selective pressure on the bacterial populations' increasing antimicrobial resistance.

Rate of horizontal gene transfer determines effectiveness of preventative measures to control resistance

Increased antimicrobial resistance is seen when β, the rate of horizontal gene transfer, is set at 0.001 hr^{-1}. In reality, this rate is likely to vary considerably, between different bacteria, different mobile genetic elements, and in different conditions, e.g. suspension vs biofilm, different temperatures or pH. Hence we considered what happens to the proportion of bacterial resistance as β is altered within a realistic range. Figure 2A shows proportion of resistant bacteria at 90 days as the rate of horizontal gene transfer β varies. The rate of horizontal gene transfer makes no significant difference to the population size. For small rates ($\beta < 10^{-4}$ hr^{-1}) the proportion of resistant bacteria is lower than that at inflow (20%). However, further reductions to β result in no further reduction to the amount of resistance seen. If β is increased above 10^{-4} hr^{-1} the amount of resistance increases to a maximum of 100%.

The different behaviours of the system as β changes affect the types of behaviour we see as we also vary other parameters. Figure 2B shows that as we vary the antibiotic inflow parameter, θ, we see decreased antimicrobial resistance. However, this is highly dependent upon the rate of horizontal gene transfer. We have two clear regions of different behaviours as we vary the rate of antibiotic inflow (θ) together with β (Figure 2C). Where we have a high value of β, reducing gene transfer rate has a large impact on the level of antibiotic resistance in the tank, while changing the rate of antibiotic inflow in the slurry tank makes little difference. At lower β ($\beta < 10^{-4}$ hr^{-1}) reducing antibiotic inflow has a large impact on the proportion of resistant bacteria, while reducing gene transfer has little impact. Whilst we have only considered changes of β and θ here, parameter

variations for some of the other parameters can be found in the Appendix, and show a similar dependence on the value of β.

Resistance control measures should focus on horizontal gene transfer, antibiotic inflow and the length of time the slurry is stored

A global sensitivity analysis of the realistic parameter space shows the importance of key parameters in the model (Figure 3). The most sensitive parameter is the length of time that slurry is stored in the slurry tank.

The rate of horizontal gene transfer is also a very sensitive parameter in the model, as expected from parameter variation. Figure 3 shows it is the second most sensitive model parameter, both in its median sensitivity gain and also in the range of sensitivity it exhibits. Since this parameter is also one of the most uncertain in the model it would be of critical importance to get a better measure of this parameter, through experimental measures, before any resistance control measures were recommended or implemented. Figure 2 showed that the value of the horizontal gene transfer rate could, in some parameter regimes, make a large difference to the amount of resistance seen in the slurry tank. Hence, changes to this rate could be an extremely effective way of reducing antimicrobial resistance seen in the slurry.

The sensitivity analysis shows that the model is relatively insensitive to the fitness cost, the proportion of resistant bacteria in the slurry inflow and the growth rate of the bacteria. Hence, measures aimed at changing these parameters are unlikely to be as effective as changes to the rates of horizontal gene transfer or antibiotic inflow. This would also suggest that, when devising a more sophisticated model, experimentally derived estimates of these parameters are less crucial and estimates from literature may suffice.

Discussion

We have developed a model to describe populations of antimicrobial sensitive and resistant bacteria in a slurry tank on a typical high performance UK dairy farm. We include terms for population growth, slurry inflow, fitness costs, horizontal gene transfer and selective pressure due to antibiotic use. The parameter values we use for the model are derived mainly from published literature, with a small number based on specific details from the dairy farm studied. The farm specific values (herd numbers, milk volume, mastitic incidence rates) are fairly typical of UK high performance dairy farms (Langford *et al.* 2009), and the model could be readily adapted to other dairy farms with stored slurry through changing parameter values. Moreover, the model could also be adapted for other farm animal species where manure is stored, for example swine or poultry. The model predicts that the proportion of bacteria showing antimicrobial resistance increases during the three month storage period. This increase is driven partly by horizontal gene transfer and partly by selection, as evidenced in Figure 2B. Even with no antibiotic inflow, the proportion of antibiotic resistant bacteria is predicted to be as high as 60%. As the flow of antibiotic into the tank is increased, so too does the proportion of resistant bacteria, indicative of selection for resistance. This result is concordant with other models associated with experimental data (Bootsma *et al.* 2012), including that of Volkova *et al.* (2012), whose model matches the experimentally observed rise in the proportion of Ceftiofur-resistant *E. coli* in cattle gut during treatment.

Through analysis of one- and two-parameter variations in the model we have shown that the rate of horizontal gene transfer is of critical importance to both the amount of resistance seen in the slurry tank and also to the effectiveness of changes to other parameters. An unexpected outcome of the model is that two distinct behaviours emerge for different potential values of gene transfer rate, consistent with other reported rates (Zhong *et al.* 2010, Subbiah *et al.* 2011, Volkova *et al.* 2012). If gene transfer rate is high, then resistance is best controlled through its reduction, and reducing selection through antibiotic inflow has little impact. However, if gene transfer rate is low, then resistance is best controlled by reducing antibiotic inflow, and reducing gene transfer has

little impact. Due to limited experimental research and the inherent variability of the gene transfer rate, this parameter is one of the least certain parameter values within this model, and therefore it is not clear which of these two behaviours is realistic. In particular, the first of these behaviours is unexpected and challenges the current view that resistance is primarily driven by the level of antimicrobial exposure. This warrants experimental study to investigate this further. Moreover, there is little published on how best to reduce the gene transfer rate in practise. Measures might include physical measures, such as increased stirring of the slurry tank or more efficient filter pressing to remove a greater proportion of solids so that there would be less substrate for biofilm formation. Alternatively, chemical measures, such as the addition of additives that might reduce plasmid spread or biofilm production, could be employed.

On a technical note, the value of the horizontal transfer parameter is model-dependent. Our model, following Volkova *et al.* (2012), has a saturating term for plasmid transfer, with the total population in the denominator. Other models, for example as used by Zhong *et al.* (2010), use a mass action term. These authors report a range of transfer rates between 10^{-8} and 10^{-15} hr^{-1}. However, to compare these rates with ours, it is necessary to multiply them by the total bacterial population density, and thus the transfer rates used are indeed comparable.

That said, gene transfer is likely to be extremely complex, with variations between different species, mobile genetic elements, bacteriophage, bacteria found in biofilm or suspension, as well as variability due to environmental factors such as temperature, pH and eukaryotic predation (Johnsen and Kroer 2007, Subbiah *et al.* 2011, Bellanger *et al.* 2013). We anticipate that more detailed modelling that includes biological, environmental and spatial complexity would be warranted and give results with greater predictive value (Krone *et al.* 2007, Hellweger and Bucci 2009, Merkey *et al.* 2011).

A global sensitivity analysis confirmed the importance of an accurate estimate for the gene transfer rate parameter, showing it to be one of the most sensitive model parameters. It also showed that the length of time that slurry is stored in

the slurry tank is also of utmost importance. While there may be changes in slurry storage that could reduce gene transfer rate, changing storage times may be difficult in practise. EU legislation requires storage from September to January, depending on soil type, to mitigate against environmental loss of nutrients. However, in all countries, it is not possible to apply manure or slurry to frozen ground, so storage over winter is likely to remain an essential practise.

The model outputs are not sensitive to the proportion of resistant bacteria entering the tank, the fitness cost of carrying resistance or the growth rate of the bacteria. This confirms the importance of a model at the level of the whole slurry tank, rather than studies focusing on antimicrobial resistance at individual cow level. Control measures at the individual cow level would likely be ineffective at changing the amount of resistance emerging from the slurry tank after storage periods of several months. This also suggests that measuring resistance at the individual cow level, or indeed changes in fitness due to carriage of antibiotic resistance genes, may be less important than, say, measuring rates of horizontal gene transfer. Growth rate is known to be affected by factors, including strain, temperature and pH (Johnsen and Kroer 2007, Bellanger *et al.* 2013); indeed there is conflicting evidence as to whether *E. coli* strains can survive in the open environment (reviewed in Fremaux *et al.* 2008, van Elsas *et al.* 2011), grow (Vital *et al.* 2008, Sharifi *et al.* 2014), or decline (Semenov *et al.* 2008, Ongeng *et al.* 2014). These studies are further compounded by the fact that cells could enter a viable but nonculturable state (Na *et al.* 2005). In the case of the slurry tank in this study, we are consistently able to isolate *E. coli* bacteria (Ibrahim *et al.* 2016), with widespread resistance to beta lactamase antibiotics, suggesting some level of survival or growth. The model itself in fact includes both cell growth and death, and it is possible that alternative parameter values may be more relevant for different environmental conditions. Moreover, environmental factors (Johnsen and Kroer 2007, Bellanger *et al.* 2013), segregation loss, growth rate (Merkey *et al.* 2011) and antibiotic concentration (Jeters *et al.* 2009), may all impact upon horizontal gene transfer rates. These factors are not included in the model, and their inclusion could lead to increased importance of both growth rate and antibiotic inflow to spread of resistance.

The model assumption of spatial homogeneity in the slurry tank is unlikely to hold in the real system. A more realistic, spatially accurate model would be needed to make testable predictions of the impact of gene transfer on antimicrobial resistance and possible measures to counter this. A more complex model could also include biofilms, pH gradients and temperature variations as these have been shown to be important considerations in determining spread of resistance (Johnsen and Kroer 2007, Bellanger 2013). At present, we also have little data on the rate at which degradation of cephalosporins may take place in these conditions, and the literature search revealed a wide range of degradation rates for other antibiotic groups. For this reason it is important that future modelling takes this into account, and experimental measures of cephalosporin (and other veterinary antibiotics) degradation in the slurry tank would be particularly useful. Other antibiotics, e.g. sulfonomides or tetracycline, can be sequestered in organic matter and slowly released. These processes could be included in more detailed models (Müller *et al.* 2013).
The model also assumes that the only source of antibiotics is from discarded milk from antibiotic treated mastitic udders. While this assumption is reasonable for the farm under study, the value of the antibiotic inflow parameter would need to be different for the model to be applied to farms with different veterinary practises, for example to take into account antibiotic inflow from faeces or urine.

In the model we present here we neglect the microbial biodiversity within the slurry tank. We assume that the bacteria are all of the same type, and select parameters relating to *E. coli* since we know this is a major cause of environmental mastitis in UK dairy cattle (Bradley 2002). However, *Streptococcus uberis* is another major cause of contagious mastitis and a wide range of different bacteria can be found in the faecal matter of dairy cattle. Additionally the slurry tank is open to the environment and could contain bacteria from other sources. Some of these bacteria will be better suited to the slurry tank conditions, hence competition will exist between different bacteria, as well as the transfer of resistance between different types and strains of bacteria via mobile genetic elements. Bacterial population dynamics will also be

impacted on through phage infections and predation by protozoa, nematodes and other occupants of the slurry. For this reason it is essential to build an accurate model of the population dynamics of bacteria within stored manures and slurries and to include such complexities in order to develop effective control measures.

This theoretical model of the slurry tank dynamics shows that emerging antimicrobial resistance in agricultural manures and slurries is a legitimate and well-founded concern. Despite the simplifying assumptions, the model is able to point to key parameters which should be given extensive consideration both in experimental studies and in a fuller, more realistic and predictive model. Further research in the area is crucial to prevent new antimicrobial resistant pathogens entering the human food supply chain, soil and water supplies.

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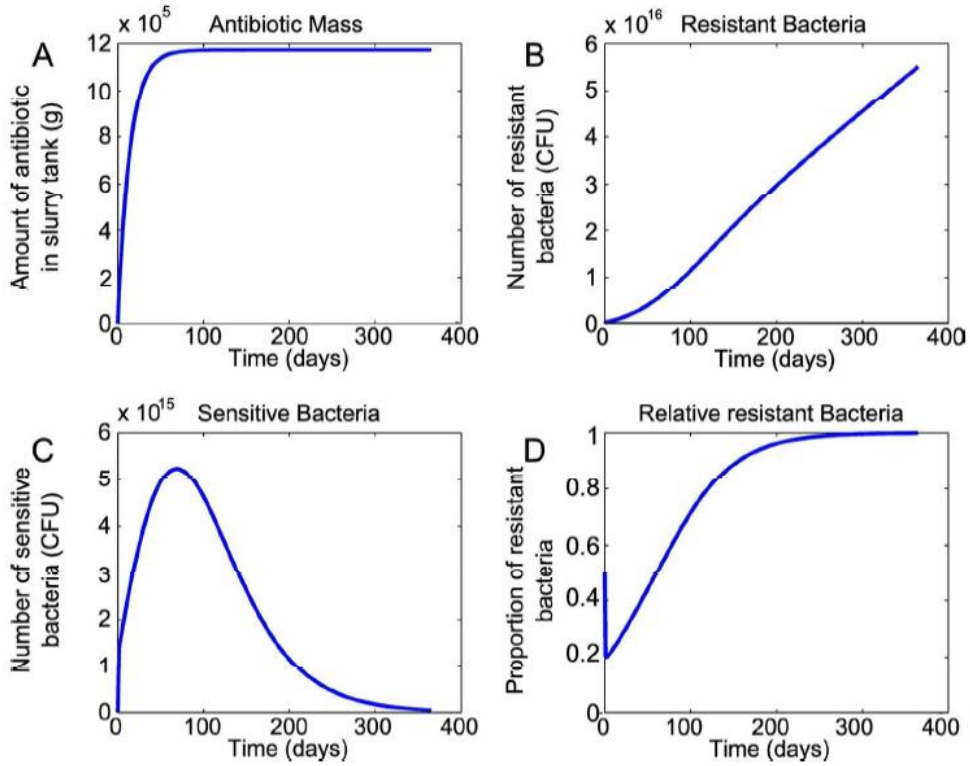

Figure 1 (A) Antibiotic mass, (B) number of resistant bacteria, (C) number of sensitive bacteria, and (D) number of resistant bacteria relative to total bacteria, all against time for parameter values specified in Table 1. We assume constant increase in slurry volume and antibiotic amount. The numbers of resistant bacteria increase and dominate the bacterial population in the tank. The tank is normally emptied after 90 days so the longer time scale would not normally be observed.

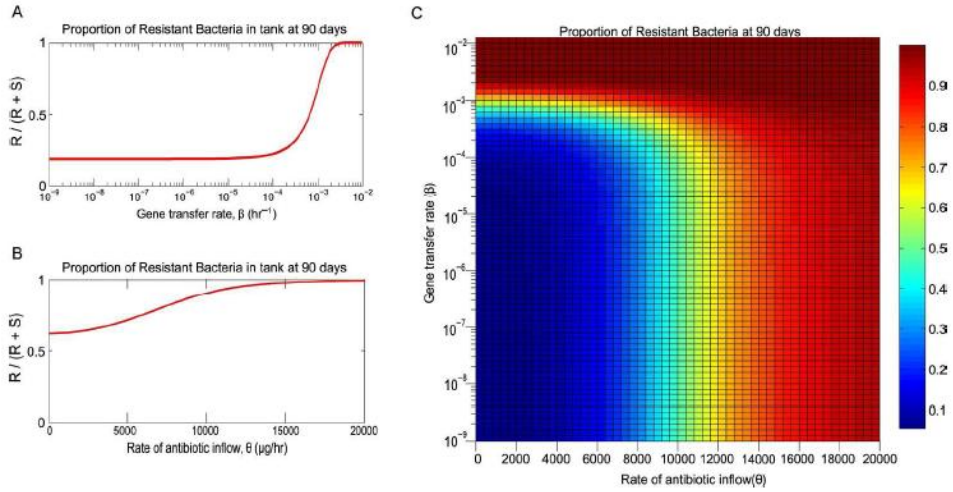

Figure 2 (A) Total number of bacteria and number of resistant bacteria relative to total bacteria against differing values of the gene transfer rate β. Whilst the overall number of bacteria remains the same, the amount of resistance increases with increasing gene transfer. (B) Total number of bacteria and number of resistant bacteria relative to total bacteria against differing rates of antibiotic inflow θ. The total number of bacteria remains constant whilst the proportion of resistant bacteria decreases with decreasing antibiotic inflow. (C) Two parameter variation plot showing the number of resistant bacteria relative to total bacteria against variations in gene transfer rates and amount of antibiotic inflow. The white dashed lines show the parameter values at which β and θ are fixed in A and B. In all plots the other parameter values are specified in Table 1 and results are plotted at t = 90 days. The two parameter plot clearly shows two regions of different behaviour depending on β. For a high β we have a region where resistance is best controlled by reducing gene transfer, while changes to antibiotic inflow make no difference to the level of resistance. For low β we have a region where reducing the rate of antibiotic entering the slurry tank would reduce the amount of antimicrobial resistance, while changing gene transfer rate has little impact.

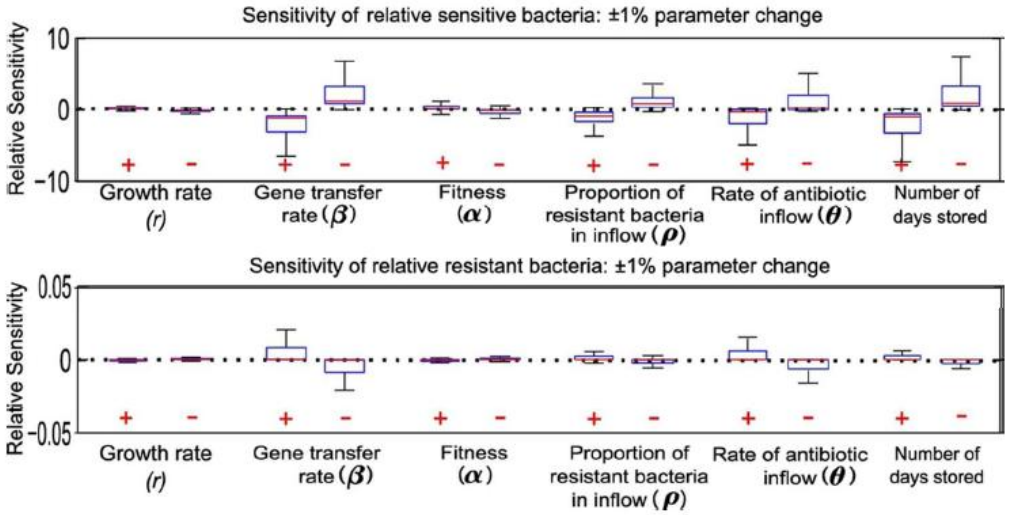

Figure 3 Boxplots of a global one-at-a-time sensitivity analysis of the relative sensitive and resistant bacteria numbers to a ±1% change in the parameters: growth rate (r), gene transfer rate (β), fitness (α), rate of antibiotic inflow (θ), proportion of resistant bacteria in inflow (ρ) and length of slurry storage. The length of slurry storage and gene transfer rate are consistently the most sensitive parameters, both in terms of the median sensitivity value and in the range of sensitivities seen as we vary the nominal parameter set. Rate of antibiotic inflow is also a relatively sensitive model parameter. The proportion of resistance is insensitive to growth rate, fitness cost and the proportion of resistant bacteria in the slurry inflow.

Table 1: The parameters in the system given by Equations 1-8, their interpretation and typical values.

Parameter	Description	Typical value (and range)	Source
r	Specific growth rate of *E. coli*	0.5 (0.17 – 0.9) hr^{-1}	Curds(1971),Godwin and Slater (1979),Levin *et al.* (1979)
β	Gene transfer term	0.001 (10^{-9} – 10^{-2}) hr^{-1}	Subbiah *et al.* (2011),Volkova *et al.* (2012)
λ	Rate of slurry inflow	613 l hr^{-1}	DairyCo (2010)
ν	Concentration of bacteria in slurry inflow	2 x 10^7 CFU l^{-1}	Reinthaler *et al.* (2003)
ρ	Proportion of resistant bacteria in inflow	0.4	Reinthaler *et al.* (2003)
α	Resistance fitness cost as fraction of r	0.1 (0 – 0.3)	Godwin and Slater (1979), McDermott *et al.* (1993), Subbiah *et al.* (2011)
V_I	Initial volume in slurry tank	150000 l	
A_I	Initial concentration of antibiotics in tank	0 μg	
θ	Rate of antibiotic inflow	3422 μg hr^{-1}	Randall *et al.* (2014)
E_{max}	Maximum effect of antibiotics on bacterial growth	2	Volkova *et al.* (2012)
H	Hill coefficient in E_{max}	2	Volkova *et al.* (2012)

	model		
MIC_S	MIC for sensitive bacteria	8 μg l^{-1}	VMD-DEFRA (2012)
MIC_R	MIC for resistant bacteria	2000 μg l^{-1}	VMD-DEFRA (2012)
μ	Carrying capacity of liquid slurry	10^{10} CFU l^{-1}	
γ	Decay rate of antibiotics	0.0029 hr^{-1}	Dolliver *et al.* (2007), Jiang *et al.* (2010)