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Evaluation of manometric respiration tests to assess the effects of veterinary antibiotics in soil

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Abstract

Extensive use of antimicrobials in veterinary medicine results in environmental exposure. Of major concern are microbial effects; including effects on nutrient soil cycles and antibiotic resistance. There is a need to assess the effects of these compounds in the environment. The application of standardized guidelines is relevant in studying many compounds. However there is a lack of special test methods designed for antibiotics.

We validate manometric test flasks using glucose and a recalcitrant herbicide. The suitability of these tests for studying antibacterial agents is then investigated using two target functions (aerobic biodegradation and carbon transformation). Compound stability is quantified using HPLC techniques.

Effects on total soil respiration in the biodegradation test are immediate and differ significantly from background. We show that compounds do not function as substrates, so effects are due to other soil processes, correlate well to sorption characteristics and are not dose dependent. This test provides details of relative antimicrobial potency towards soil microorganisms and can be used to rank compounds. However the test does not provide details on the nature or extent of specific microbial effects. In contrast, the carbon transformation test is more specific and provides a reproducible indication of dose effect relationships, which is more suitable in assessing the effects of these compounds in the environment.

Presently, standard guidelines do not take into account the normal input of antibiotics into soils via contaminated sludge or manure. This should be corrected in future guidelines as these inputs alter microbial composition, organic matter, ionic strength and pH affect sorption and overall impact the test results.

Keywords: Antibiotics; Manometric respiration; Routine test suitability; Soil

1. Introduction

Antibiotics are extensively used in both veterinary and human medical treatments and have greatly contributed to the control of infectious diseases. These substances have until recently been considered environmentally harmless. However, their potential as environmental contaminants is now in focus, and there is increasing concern about their fate and long-term effects after excretion (Halling-Sørensen et al., 2001; Jjemba, 2002). Major current concerns of these compounds include the potential to induce resistance in bacterial strains in the environment. That resistance may be transferred to pathogens relevant for public or farming animal health (Bogaard and Stobberingh, 2000; Mateu and Martin, 2001). Furthermore, antibiotics can alter microbial diversity or function, which may indirectly affect soil fertility and nutrient balances.

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The use of antibiotics as either growth promoter or therapeutic agents for agricultural animals constitutes the major tonnage of antibiotic use in most countries. In Denmark alone, a total of over 94 tonnes of active ingredients (a.i.) were used in 2002, 80% of which was attributed to use in the therapeutic treatment of animals (Heuer et al., 2003). In the EU, more than 4700 tonnes were used annually in veterinary therapy in 1999 (EMEA—The European Agency for the Evaluation of Medicinal Products, 1999; FEDESA, 2003).

Due to inherent stability a substantial quantity of unchanged antibiotic residues and/or their metabolites may pass through target organisms and spread in terrestrial and aquatic environments (Halling-Sørensen et al., 1998a). The percentage of antibiotic excreted varies according to compound, target organism and administration method, however, up to 75% chlortetracycline CTC, 20% oxytetracycline OTC (Montforts et al., 1999), 72% tetracycline TC (Winckler and Grafe, 2001) and 78% tylosin TYL (Feinman and Matheson, 1978) of an administered dose may be excreted as the parent compound through the urine and faeces of agricultural animals. For animals on pasture, the excreta will be released directly to soil whereas for intensively reared animals, the main route of entry will, after a storage period in tanks, be through slurry as a component in fertiliser. According to degradation rates and sorption properties, the parent compounds and/or metabolites can in theory reach the aquatic environment through surface run-off or leaching through the soil profile.

The need to assess the stability and effects of antibiotics in the terrestrial environment has been identified. But research is still in its early stages and results on environmental fate and effects are therefore sparse. A number of review articles (Halling-Sørensen et al., 1998b; Tolls, 2001; Jjemba, 2002; Thiele-Bruhn, 2003) have compared pharmaceutical substances to other environmental pollutants such as pesticides. However, the routine tests used for pesticides may be unsuitable for assessing the effects of antibiotics, leading to erroneous results. There are presently no special test methods applicable for antibiotics as pollutants. It is, therefore, important to investigate the suitability of test methods such as assays for studying ecotoxicity and biodegradation (SETAC—Europe, 1995) that are routinely applied in the study of the environmental properties of other substances. Routine tests do not take into account standard addition of manure with related changes in soil pH, ionic strength, carbon content and microbial structure. Furthermore, in comparison to other chemicals, antibiotics have inherent stability and very specific modes of action and only influence the susceptible target fraction of total biomass. Therefore, the question of suitability of the methods used to assess effects of antibiotics with routine tests is important.

Total assessment of chemicals usually includes target parameters such as biodegradation tests (Miles and Doucette, 2001), toxicity tests (OECD, 1998a) and tests for other soil microbial parameters such as total soil biomass (International Organization of Standardization, 1997). This is carried out in closed systems and CO_2 is often used as the primary endpoint to assess biodegradation potential using ^{14}C-labelling, gas chromatography or some other techniques. A very similar concept for measuring respiration is manometric respirometry. Here, O_2-consumption/CO_2-production is measured indirectly as a pressure reduction in flasks where CO_2 is trapped in a sodium hydroxide absorber (Painter and King, 1985; Weytjens et al., 1994; Pagga et al., 2000). These systems are attractive due to their simplicity and low cost.

The objective of this laboratory study was to examine the applicability of routine test systems in the assessment of the effects of antimicrobial effects in the environment. Simple flask microcosms in which manometric respirometry is used to quantify respiration were used. Target functions used in this examination were carbon transformation and aerobic biodegradation, respectively, as to quantify dose–response relations and total microbial effects. The veterinary antibiotic substances tested (see Fig. 1) were selected from the tetracycline, sulfonamide and macrolide sub-groups which represent more than 60% of the antibiotics used for therapeutic purposes in animal production in Denmark in 2002 (Heuer et al., 2003). Names, molecular structures and physicochemical properties are shown in Fig. 1.

2. Materials and methods

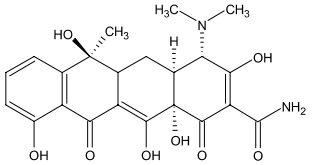
Chemicals: Tylosin, TYL was from Sigma (St. Louis, MO, USA) and used in the form tylosin tartrate (89.9% purity) consisting of two tylosin molecules. Oxytetracycline, OTC was from Unikem (Copenhagen, Denmark). *Iso*-chlorotetracycline, *iso*-CTC, was from Arcos-organics (New Jersey, USA). Tetracycline, TC (99.6% purity), chlorotetracycline hydrochloride, CTC (79% purity), erythromycin, ERY (99.9% purity) and sulfadiazin, SDZ sodium salt (99.0% purity) were from Sigma (St. Louis, MO, USA). Sulfachloropyridazin-NA, SCP was a gift from A.B.A. Boxall, Cranfield University (England).

D(+)-Glucose, sucrose, NaOH, and methanol, MeOH were all of analytical grade, formic acid (pro-analysis 98–100%) and 3,5-dichlophenol, 3,5-DCP were from Merck (Darmstadt, Germany). 2,4-Dichlorophenoxyacetic acid, 2,4-D from Acros-organics (New Jersey, USA).

Microcosms: The total microbial activity was assessed using the measured accumulative pressure changes over time in the gas phase of microcosms equipped with a CO_2 trap and containing soil and test chemicals.

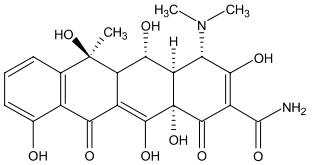
A. Tetracycline (TC)

M_w 444.45 g/mol; pK_a 3.3, 7.7, 9.7; log K_{ow} (-)1.3; K_d 1140 – 1620 l/kg



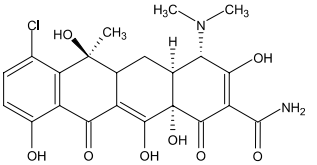
B. Oxytetracycline (OTC)

M_w 460.44 g/mol; pK_a 3.27, 7.32, 9.11; log K_{ow} (-) 0.9; K_d 680 - 1026 l/kg



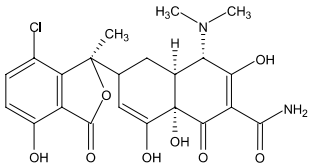
C Chlorotetracycline (CTC)

M_w 478.89 g/mol; pK_a 3.3; log K_{ow} (-)0.62



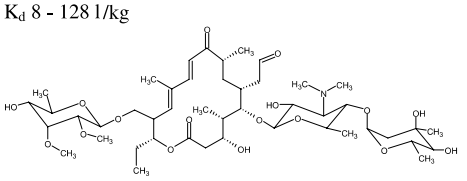
D Iso-chlorotetracycline (iso-CTC)

M_w 515.34 g/mol



E. Tylosin (TYL)

M_w 916.11g/mol; pK_a 7.1; log K_{ow} (+) 3.3; K_d 8 - 128 l/kg



F Sulfachloropyridazine (SCP)

M_w 284.7 g/mol; pK_a 1.76, 5.71; log K_{ow} (-)0.52; K_d 0.9 - 1.8 l/kg

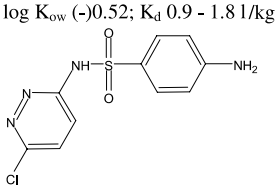


Fig. 1. Names, physicochemical characteristics and structures of the compounds used. Source for K_d values for TC, OTC and TYL (Tolls, 2001) and source for K_d value for SCP (Boxall et al., 2002). Other data for TC and CTC (SRC PhysProp database), OTC and SCP (Blackwell et al., submitted for publication) and TYL (Tolls, 2001).

A Danish agricultural sandy loam soil (Typic Hapludalf) from Askov Research Station (55°28′N 9°6′E Jutland, Denmark) was sampled by The Danish Institute of Agricultural Sciences. The soil (pH 6.1, CEC 10.0 meqv/100 g and organic carbon 1.6% (Danish Institute of Agricultural Sciences, 2003)) was refrigerated at 4 °C for a maximum of 1 week prior to the experiments. Before use, the soil was sifted through a 2 mm sieve removing all large stones and roots. Water content of the soil samples was determined and the remaining soil was transferred to test vessels in 60 ± 0.5 g portions. The test design was a simple adaptation of Organisation for Economic Co-operation and Development, OECD, Standard bottle test 304A. All tests were prepared in 115 ml opaque serum flasks with airtight butyl rubber septa (Apodan, France). Butyl stoppers were inserted in each flask and the bottles incubated at 20 ± 1 °C for 24 h to activate indigenous bacteria. The test compounds were then added as dry solids to the desired concentration, along with 1 ml MilliQ® water followed by vigorous shaking. An alkali trap containing 1 ml 1 M NaOH in a 5 ml test tube was then inserted. Finally, the flasks were sealed with butyl rubber airtight serum stoppers and metal clamps and incubated in the dark at 20 ± 1 °C for the duration of each experiment. Accumulated pressure changes were measured through the septum using a syringe needle mounted on a pressure gauge EPM 2023 from Ametek Denmark A/S (Allerød, Denmark). To ensure aerobic conditions, the serum stoppers and clamps were changed when the accumulated pressure indicated 35% oxygen depletion. Pressure loss resulting from perforations of bottle septa were avoided by regular septum changes.

Sterilisation of test series intended to study abiotic degradation was achieved by autoclaving the soil in the respective bottles at 121 °C for 45 min on three consecutive days. Thereafter, the test compounds were added and the bottles incubated as described above. Fluctuations in background atmospheric pressure were monitored in all experiments using empty flasks containing an alkali trap.

Using the generalised procedure described in the above section, the following experiments were performed.

Validation, assessment and optimisation of microcosms: the optimum gas phase volume was found by comparing the stability of results obtained in a series of three experiments, including the respective addition of 20, 40 or 60 g soil to the test vessels. Sucrose was then added to these flasks in duplicate concentrations of 0, 1, 5 and 10 mg/bottle at each soil mass.

The importance of applying the dry compounds was assessed. Carrier organic solvents were avoided as these could create artefacts or additional carbon sources. However, due to low water solubility, the antibiotics could not be added in aqueous concentrations high enough to give measurable pressure changes. We compared the respiration resulting from dry and dissolved applications using 0, 18, 90 and 180 mg glucose/kg dry soil (DS) added to triplicate flasks either as a 1 ml aqueous solution or as dry substance simultaneously with 1 ml of water.

The sensitivity of the method was verified in an experiment with a series of flasks containing sucrose in duplicate concentrations within the range of 18–180 mg/kg dry soil (DS).

The stability of biomass respiration and the long-term changes of the soil systems were assessed by exploring changes in glucose respiration-rates as a function of soil age. A total of 8 series of duplicate microcosms were prepared with fresh soil and alkali traps. Glucose (180 mg/kg DS) was then added to one pair of microcosms at a time after 0, 7, 21, 35, 49, 63 and 91 days respectively. No glucose was added to series 8 (control). Accumulated pressure changes were recorded in each microcosm for a 21-days duration after glucose addition. First-order rate constants were calculated and used to assess microbial stability as a function of soil age.

Applicability: The suitability of this method for assessing the stability of antibiotics in the environment was carried out by examining (1) the total effects antibiotics had on the respiration of the whole microbial population using a biodegradation test and (2) dose–response relations obtained in a carbon transformation test as described underneath.

(1) A biodegradation test was prepared as follows: TC, OTC, CTC, TYL, SCP were tested at concentrations of 60 and 600 mg/kg DS. *Iso*-CTC was tested at concentrations of 6 and 60 mg/kg DS, whilst the reference compound 2,4-D was tested at the sole concentration of 700 mg/kg DS. All test concentrations were replicatet for times. Controls including soil without any amendments and blanks (empty flasks) were each replicated six times. Sterile soil controls monitoring abiotic degradation for each antibiotic compound at 60 mg/kg DS were included. Tolerance and adaptation due to pre-exposure was investigated after applying all test compounds again at the same concentration as initially added 35 days after the initial exposure. At both the time of second addition and experimental termination, one bottle from each series containing antibiotics was sacrificed and the soil was immediately stored at -18 ± 1 °C for chemical analysis.

(2) An OECD Test Guideline 217 on carbon transformation (OECD, 1998b) test was set up to test one antibiotic (OTC, TYL, SCP) from each sub-group at five concentrations of 1, 10, 50, 100 and 1000 mg/kg DS. Flasks containing the benchmark chemical 3,5-dichlorophenol (3,5-DCP) were included at concentrations of 1, 10 and 100 mg/kg DS. Controls and blanks were included. All series were tested in four replicates and incubated for 28 days to carbon stress/deplete the soil. Glucose was added after 28 days at a concentration of 4000 mg/kg DS after which the respiration rates for both controls and amended soils were monitored and compared for a period of 24 h.

Chemical analysis: Antibiotics were extracted from 5 g soil portions using a method developed, validated and described by Blackwell et al. (submitted for publication). The following internal standards were used: ERY for analysis of TYL, SDZ for analysis of SCP, OTC for analysis of TC and finally TC for analysis of CTC, OTC and *iso*-CTC.

Chemical analysis and separation was achieved using a HPLC system (Waters 2690, Milford, MA) equipped with a photodiode array detector DAD (Model 996 from Waters). Data were acquired and treated using Millennium software version 3.0. The chromatographic column (Waters, Xterra C_{18} 2.1 mm × 100 mm) was maintained at room temperature and the sample was maintained at 4 °C. Injection volumes of 10.0 μl were used and the flow rate was 0.25 ml/min. The mobile phase was a two solvent gradient eluent of 20.0% MeOH and 95.0% MeOH respectively. Both mobile phases were prepared in 1 l flasks with the addition of 308 μl formic acid. The response at 350, 290 and 280 nm was used for the respective quantification of tetracyclines, macrolides and sulphonamides.

Data analysis: Average accumulated pressure was corrected for fluctuations in atmospheric pressure for each set of experimental data after which average background respiration measured in unamended flasks was subtracted.

The volume of the gas phase (headspace + soil pore volume) was determined by measuring the mass of the water used to replace the gas phase in flasks containing soil and alkali trap. In our test flasks V (gas phase volume = 0.078 l), R (gas constant = 0.0821 atm l/mol) and T (temperature 293.15 K) are constant. Thus, assuming ideal conditions the molar changes in oxygen (n = mole O_2) are proportional to pressure (p, atm) changes according to the ideal gas law ($pV = nRT$). We determined (1) theoretical maximum pressure changes resulting from the conversion of 100% of added carbon and (2) soil respiration (BA, mg O_2/kg dry soil) using: BA = 32 000 mg/mole $\frac{pV}{RTm_{DS}}$, where m_{DS} is the mass of dry soil in kg.

In the carbon transformation test, first-order respiration-rate constants were determined for the conversion of glucose at each concentration of antibiotic pre-exposure. These degradation rates were used to calculate the relative inhibitions, I, using:

$$I = \frac{k_{1,c} - k_{1,i}}{k_{1,i}} \times 100$$

where $k_{1,i}$ and $k_{1,c}$ are the first-order rate constant for glucose conversion in the flasks, with and without pre-exposure to antibiotics.

3. Results and discussion

3.1. Validation and assessment of microcosms

Based on the result of the optimisation of the gas phase to soil ratio, 60 g soil mass per test flask was selected for further studies.

A two tailed t-test ($p < 0.01$), revealed no significant differences in the average respiration found in flasks

containing either glucose applied as dry compound in association with 1 ml of water or in flasks with glucose applied in aqueous 1 ml solutions. The low variability between replicates indicates that the addition of dry substance addition does not lead to systematic experimental errors.

The sensitivity of the microcosms was tested using sucrose added in duplicate at concentrations within the range of 18–180 mg/kg DS each (Fig. 2A). These results show significant differences in pressure changes of the system with dependence of the concentrations of the substrate. Sensitivity was quantified using limits of detection ($\mathrm{LOD} = X + 3 \cdot S$) and limits of quantification ($\mathrm{LOQ} = X + 10 \cdot S$), where X is the lowest detectable average reading of replicate samples with no substance added. In our microcosms LOD and LOQ were respectively determined as equivalent to the respective oxidation of 4.1–12.5 and 13.78–41.1 mg glucose/kg DS. These data indicate that the sensitivity of the microcosms is satisfactory at the concentrations used for testing antibiotics. The variability between replicates was typically ±10% and therefore reasonably low throughout both of the preliminary tests for sucrose and in the final tests with antibiotics.

Changes in microbial composition and stability with time could easily change the biodegradation capacity of the soil thereby affecting reproducibility. These changes were investigated in a 112-day study where high concentrations of glucose were added to fresh and aged soil. Respiration rates, expressed as first-order rate constants, for the 21-day period after glucose addition are presented as a function of the time of additions in Fig. 2C. This figure also presents equivalent respiration rates determined in control soil flasks containing unamended soil (controls) of the same age. A decline in the respiration rates over time (Fig. 2C) is interpreted as carbon starvation. The initial increase in oxygen transformation rates seen from $k_{(7\,\mathrm{d\,old\,soil+GLU})}$ to $k_{(21\,\mathrm{d\,old\,soil+GLU})}$ could be attributed to increased respiration of the viable, carbon-starved bacteria that rapidly oxidise the glucose added on day 21.

The applicability of the system for investigating the biodegradability was tested using a readily available (glucose) and a more recalcitrant reference substance (2,4-D). Results for 2,4-D are shown in Fig. 2B. A sigmoid pattern, which is typical for biodegradation curves, was observed for both compounds. It was calculated that respiration reaches 80% and 40% of the theoretically maximum for glucose and 2,4-D after 35 and 55 days respectively. This is in accordance with figures previously reported for these compounds (Anderson and Domsch, 1978; McCall et al., 1981). In conclusion, these results indicate that the reference compounds function as substrates and that this system is applicable for both easily degradable and recalcitrant compounds.

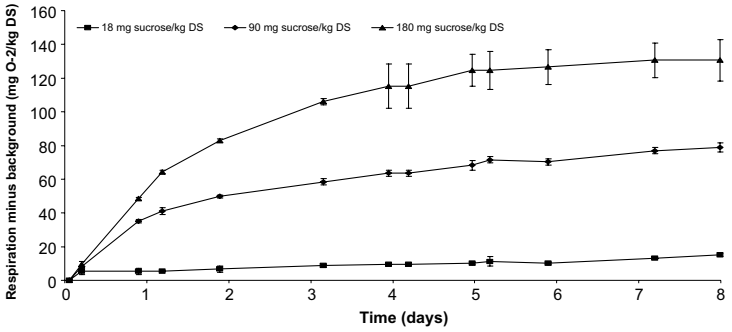
3.2. Applicability of the method for use in the assessment of antibiotics

3.2.1. Suitability in the biodegradation test

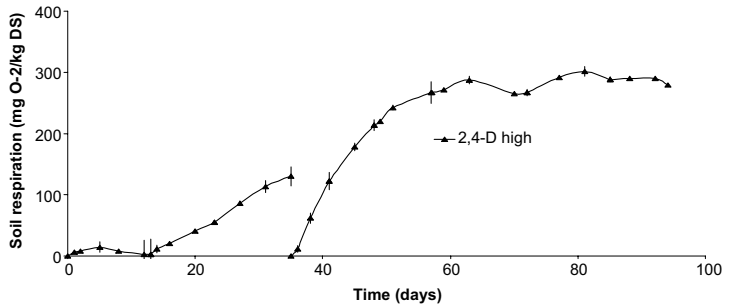
The impact on soil respiration of six antibiotics at two concentrations was studied in microcosms with re-addition of the antibiotics after 35 days. The concentration of antibiotics in the soil was determined on days 35 and 100 using chemical analysis whereas pressure development was followed over 100 days period (see results in Table 1 and Fig. 3 respectively). The graphs in Fig. 3 represent accumulated oxygen consumption in amended microcosms after subtraction of average background respiration measured in the unamended controls. With the exception of SCP, increased curve fluctuations and higher standard deviations was observed after 80 days. This may be due to reduced soil stability that also was observed in the 112-day experiment (Fig. 2C) and illustrates a limitation associated with running long-term experiments. No significant changes in pressure were observed in sterile controls indicating that abiotic processes were negligible.

The recovery (Table 1) of the compounds is in agreement with other works (Hamscher et al., 2002; Jacobsen et al., in press) in which it is concluded that losses of the compound are attributed to effects associated with irreversible sorption to the soil matrix rendering them non-extractable using the chemical methods employed. Respiration measured as mg oxygen consumed per kg dry soil (mg O_2/kg DS) varied with the antibiotic applied. In the first 35-day period of exposure, all the antibiotics tested activated respiration 1.3–1.7 times above background levels (flasks with no antibiotics). After the same test compound was re-added (days 36–100), all tetracyclines again activated respiration by a factor 1.5–2.0. In this same period, the effects of TYL fell and approached background levels whilst SCP reduced respiration by a factor 0.8 when compared to backgrounds. Assuming that the increased respiration during the first period of exposure results solely from mineralization of the antibiotics, the theoretical maximal oxygen consumption can be determined as described previously. According to these calculations the results represent concentrations significantly over 100% of the carbon applied for all compounds at their low concentrations. This combined with the absence of a lag phase in respiration curves (Fig. 3), the recoveries measured (Table 1), and the recovery of the antibiotics reveal that the antibiotics do not function as substrates and that other mechanisms are responsible for the respiration changes observed. Several explanations are possible: (I) Due to the specific modes of action, antibiotics may affect susceptible species and thereby increase the activity of competing organisms. (II) The presence of antibiotics induces stress on target microorganisms possibly increasing

A. Flask sensitivity using 18, 90 and 180 mg sucrose/ kg DS. Graph shows accumulated respiration (mg O_2/kg DS) as a function of time.



B. Suitability of test using reference compound 2,4-D. Graph shows respiration (mg O_2/kg DS). Start concentration was 700 mg/kg DS.



C. Soil stability and the effects of aging on the glucose degradation. Graph shows rate constants in fresh and aged soil with and without glucose additions. X-axis represents the age of soil at time of glucose addition.

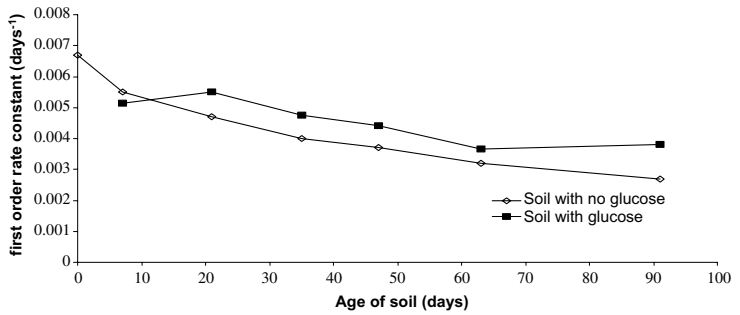


Fig. 2. Flask sensitivity, suitability of test using reference compounds and soil stability. (A) Flask sensitivity. Graph shows respiration in test flasks (mg O_2/kg DS) due to the addition of 18, 90 and 180 mg sucrose/kg DS. (B) Suitability of test using reference compound 2,4-dichlorophenoxyacetate. Graph shows respiration (mg O_2/kg DS). (C) Soil stability and the effects of aging on the glucose degradation. Graph shows rate constants in fresh and aged soil with and without glucose additions. *X*-axis represents the age of soil at time of glucose addition.

their respiration. (III) Bacteria that are killed by the antibiotics may constitute a carbon source for other microorganisms, which thereby increase their respiration.

The uncertainties and lack of information of specific effects on microorganisms associated with the observations in this test explain why manometric systems are unsuitable as biodegradation assays for antibiotics. However, the data indicate overall effects and can furthermore, provide the following substance specific information about the effects of antibiotics in soil environments:

(i) There was a steeper curve observed immediately after the second addition of tetracyclines indicating microbial tolerance or adaptation due to pre-exposure to these compounds. Tetracycline resistance exists amongst

Table 1
Soil compounds concentrations measured on days 35 and 100 using HPLC and presented as % start and including 95% confidence intervals

Start concentration (mg/kg)	Time of analysis (days)	% of start concentration					
		TC	CTC	OTC	*iso*-CTC	TYL	SCP
60	35	101 ± 7	46 ± 3	17 ± 6	52 ± 5	NA	137 ± 8
600	35	38 ± 4	25 ± 2	39 ± 6	11 ± 1	NA	122 ± 5
120	100	NA	26 ± 2	3 ± 2	26 ± 8	NA	100 ± 5
1200	100	26 ± 3	25 ± 6	29 ± 6	6 ± 3	NA	62 ± 9

NA—soil concentration was not quantified.

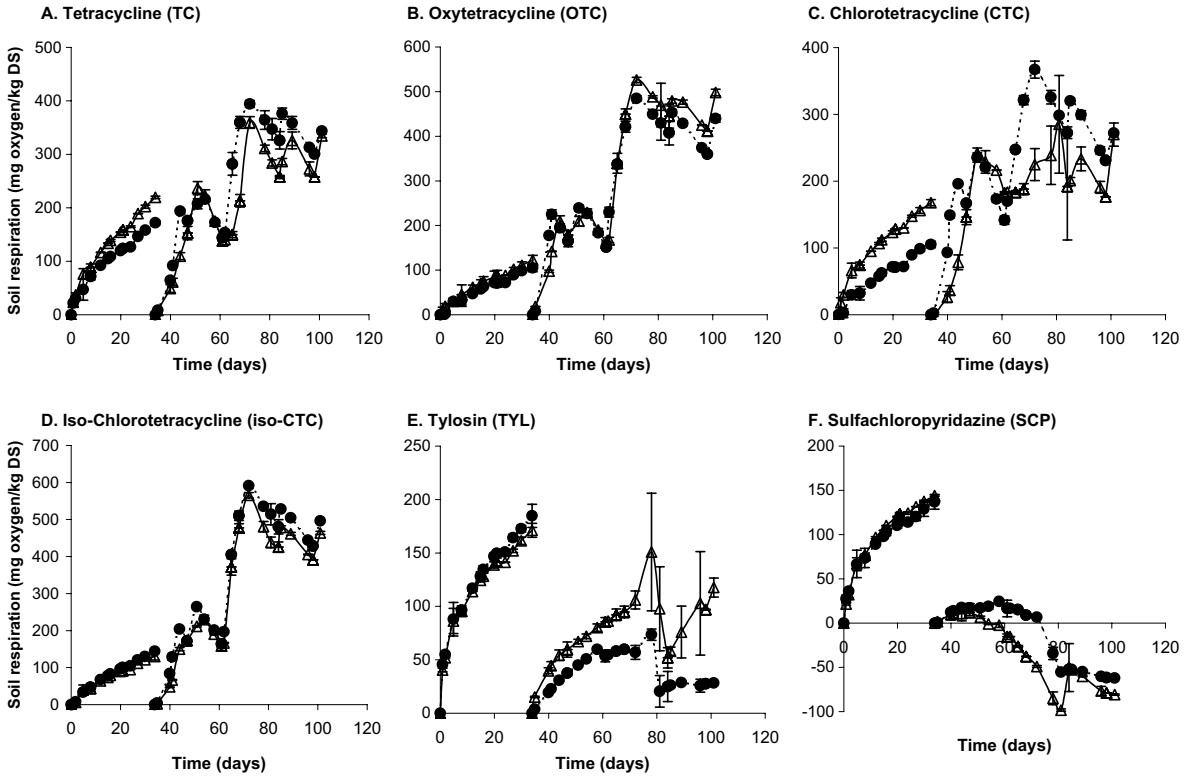


Fig. 3. Soil respiration (mg O_2/kg DS) after background controls are subtracted. The test substances are added at concentrations of 60 (- -●- -) and 600 (—△—) mg/kg DS. (A) Tetracycline (TC), (B) oxytetracycline (OTC), (C) chlorotetracycline (CTC), (D) *iso*-chlorotetracycline (*iso*-CTC), (E) tylosin (TYL), (F) sulfachloropyridazine (SCP).

most bacterial strains (Chopra and Roberts, 2001) and the steep curve observed after re-addition is probably the result of an increased number of resistant bacteria.

(ii) The long-term effects of SCP were a significant inhibition of respiration after day 50 when compared to background respiration. These pronounced long-term effects could be attributed to two factors. Firstly, SCP is more mobile and thus more bioavailable due to a low sorption coefficient (Boxall et al., 2002). Therefore, soil bacteria are sensitive to changes in concentrations of this compound. Secondly, SCP is a bacteriostatic broad spectrum agent, inhibiting DNA/RNA synthesis by competitively inhibiting the incorporation of *para*-aminobenzoic acid in the synthesis of dihydrofolic acid (Rang et al., 1995). Bacterial cells have reserves of growth factors such as folic acid. However, within the period of this experiment, these reserves may have been exhausted, causing cell death and a subsequent reduction in the total respiration.

(iii) The reduction in activation effects in flasks containing TYL could be due to a number of factors. Firstly, Westergaard et al. (2001) has previously shown that TYL is a narrow spectrum antibiotic effecting only gram-positive bacteria and thus creating selective pressure on bacteria. This group also proved that long-term exposure effects of TYL on soil biomass included permanent changes in bacterial diversity (number and abundance of colony morphotypes) and community structure (based on both colony morphology, DGGE and sole carbon utilisation) with immediate divergence in CFU (colony forming units) counts and simultaneous increase in tylosin resistant CFU. These changes may in our study, have created dominant bacterial strains with lower degradation abilities, thereby affecting total soil respiration. On the other hand, TYL has a relatively high sorption coefficient (see Fig. 1) and the decline in activity after re-introducing this compound in relation to unamended soil may be due to decline in bioavailability.

(iv) Respiration effects observed in this test were generally independent of the dosages used. However, TC and CTC did however illustrate slight dose dependency before 35 days. Furthermore, dose-dependent stimulation of respiration was observed when TYL and SCP were re-added to the test flasks. The reasons for the latter observations was discussed previously.

3.2.2. Suitability in the carbon transformation test

The results above indicate that the microcosms is unsuitable for studying biodegradation. However, the total effects were compound dependent and the question is whether a relative ranking of the degradability of the antibiotics could be applied? The aim of the second test was to establish whether microcosms could be used to quantify the effects of antibiotics on a specific target function using a more detailed dose–response approach. The impact of antibiotics was assessed using the OECD217 carbon transformation test (International Organization of Standardization, 1997). A known bacterial toxin 3,5-dichlorophenol (3,5-DCP) was included as a reference substance and the EC_{50}-value of 30 mg/kg DS confirmed that the microcosms were affected at a concentration that is typical for this compound. Each antibiotic was tested at five concentrations (ranging from 1 to 1000 mg/kg DS) and the results are presented in Fig. 4 as dose–response curves. In contrast to the results observed in the biodegradation test where respiration increased in the presence of antibiotics, the respiration of the glucose was negatively affected by the pre-exposure of the antibiotics. This inhibition was dose dependent and therefore, in comparison to the biodegradation test, the carbon transformation test gives more specific details. When testing the toxicity of common synthetic pollutants in soil (e.g., pesticides) the dose–response curves are used to calculate effect-concentrations (e.g., EC_{50}-values). In the current experiment data are unsuitable for obtaining such values. The main reason is that when testing antibiotics, only a fraction of the total biomass contributes to the observed inhibition, therefore, the 100% inhibition level of all organisms may never be reached and thus, dose–response curves relationships could not be established. With OTC the inhibition at the maximum concentration of 1000 mg/kg DS used was close to 100% reflecting broad spectered activity of this antibiotic. In contrast, SCP and TYL only affect the gram-positive fraction of the total biomass and this is reflected by the 35% and 70% plateau-levels observed for these substances. When assuming maximum inhibition at the plateau-levels for TYL and SCP the following EC_{50}

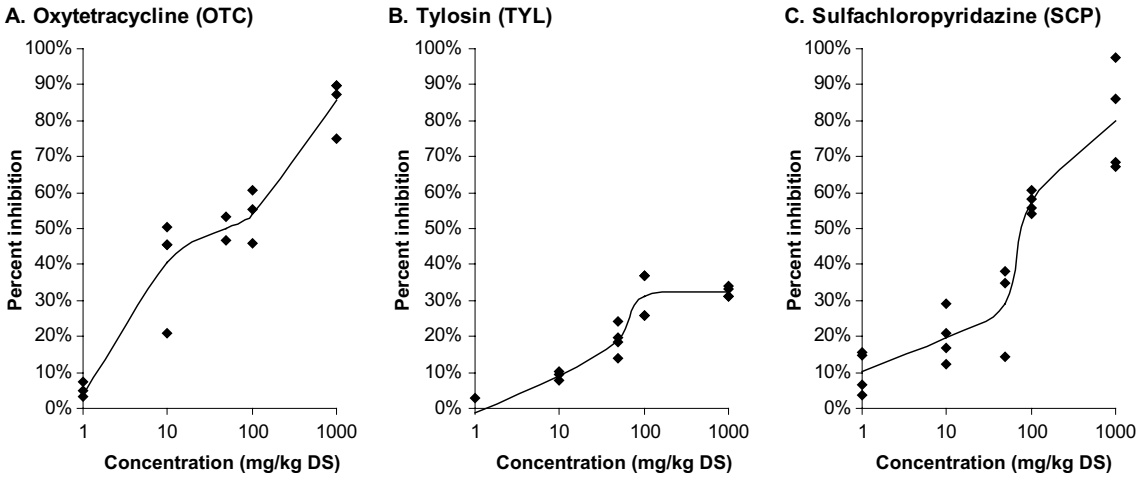


Fig. 4. Dose–response curves obtained with OECD test guideline 217. (A) Oxytetracycline (OTC), (B) tylosin (TYL), (C) sulfachloropyridazine (SCP).

values of 50, 30 and 72 mg/kg DS were respectively determined for OTC, TYL and SCP. These values are higher than previously reported soil concentrations (Winckler and Grafe, 2001; Hamscher et al., 2002) and worst case PEC's (Halling-Sørensen et al., 1998b) which were respectively in ranges of 1–900 μg/kg DS and 0.2–9 mg/kg DS. This means that the compounds would not be expected to effect respiration of the soil organisms at the concentrations found in the environment. However effects such as resistance have been reported at sub-lethal concentrations and this is not accounted for in determination of EC_{50} values for respiration.

The study of the effects of antibiotics on soil processes is very important. However, as we have shown here, routine tests and the experience gained from the study of pesticides may not be directly applicable to antibiotics raising the need to design new test systems that are specific for antibiotics. For example it is invalid to assume that changes in pressure resulting from the oxidation of carbon within the compounds are applicable to antibiotics. We establish that effects observed are an activation or inhibition of other soil mechanisms. Furthermore, the effects on microbial biodiversity should be included in the total assessment. In this study, total microbial biomass was examined but future tests should include effects on selected microbial consortia. The tests lacked realism; flasks were incubated at 20 ± 1 °C, which was higher than environmental soil temperatures and compounds were added as dry substances in the form of the parent compound at concentration above environmental concentrations, PEC's and trigger values (Thiele-Bruhn, 2003) previously reported for soils. Furthermore, these tests fail to take into account that antibacterial agents normally reach the environment after being partly metabolized or conjugated prior to excretion and reach soil environments as components in a matrix such as manure slurry. Manure additions change soil pH, carbon content, ionic strength, and microbial composition. It has previously been shown that pH (Sithole and Guy, 1987; Tolls, 2001; Boxall et al., 2002) and manure (Marengo et al., 1997; Tolls, 2001; Boxall et al., 2002) affect sorption patterns and thus the potency of the compounds and changes in microbial composition affect resistance development and degradation.

4. Conclusions

In this study we demonstrated the ineffectiveness of existing routine test approaches for assessing biodegradation and the effects of antibiotics in soil. The applicability of two routine tests based on manometry in the assessment and quantification of veterinary antibiotic impact on soil processes was examined. The tests were simple, fast, cost-effective and characterized as suitable and stable for use in testing biodegradable and recalcitrant compounds. Usage of the tests for antibiotic biodegradation assessment is limited and data can only be used to provide a relative rank of the influence of the antibiotics on soil respiration. Compounds exert an effect on respiration in this test without themselves functioning as a source of carbon and this effect is dose independent. The results are non-specific and associated with uncertainty as soil processes responsible for respiration and nutrient cycling are complex, further this test does not give any details on genetic changes or changes in microbial structure or function.

In the second OECD carbon transformation test we show a more specific dose related response and quantify EC_{50} values. However, these values were higher than ECs and PECs reported in literature.

Assessment of antimicrobial effects on soil fauna should include tests on specific bacterial sub-groups or effects on population diversity. Further research is needed for a detailed description of new tests that incorporate the effects of standard addition of manure.

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