

# The development of a rapid screening technique to measure antibiotic activity in effluents and surface water samples

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## Abstract

Use of antibiotics in medicine, farming and aquaculture results in a continual supply of these pharmaceuticals and their breakdown products reaching the environment. A method has been developed to use for screening environmental samples to establish whether they contain antibiotics. The assay is based on a commercially available test kit and uses some modifications of the test procedure and SPE techniques to deliver a fast, semi-quantifiable result. Development of the assay and results from spiked and environmental samples are discussed.

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## 1. Introduction

Antibiotics are prescribed to both the human and farm animal populations of many countries in both Europe and the rest of the world. In 1999, 13,288 tons of antibiotics were used in the EU and Switzerland, 65% was used in human medicine, 29% in veterinary medicine and 6% as growth promoters in animals (FEDESA, 2001). Up to 50% of the antimicrobial use in humans is considered inappropriate (Paskovaty et al., 2005) and, like many other chemicals that are heavily utilised in modern culture, they are finding their way back into the environment. This may be in their original state e.g. up to 90% of veterinary antibiotics may pass through the animals unchanged (Carpagnolo et al., 2002) or as metabolic breakdown products with unknown activity. High environmental concentrations are likely to occur in sewage treatment works, which rely on their bacterial populations to convert the raw sewage into a treated effluent. High concentrations may also be found in streams with large amounts of agricultural run-

off from livestock areas (Sarmah et al., 2006; Kumar et al., 2005) or in the vicinity of large aquaculture units (Bjorklund et al., 1991; Kerry et al., 1996; Hirsch et al., 1999; Lalumera et al., 2004). If antibiotics are present in these or any other environmental compartment at active concentrations, a wide range of bacterially mediated processes may be affected due to a reduction in the bacterial biomass available e.g. the rate at which organic matter in the environment is broken down.

A different aspect of the problem, but one that is perhaps of more immediate concern from a human health perspective is that, at low concentrations, the presence of antibiotics can lead to the development of antibiotic resistant strains of bacteria known to cause diseases which afflict humans (Guillemot et al., 1998; Yoneyama and Katsumata, 2006; Drlica, 2003). The resistant families of microbes that develop in hospitals, aquaculture and agriculture are proving to be a major problem, despite large amounts of research, primarily in hospitals, to identify means to control them. The identification of potential new breeding grounds for these ‘superbugs’ in areas that are much more difficult to control, is therefore a cause for concern.

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Apart from the risks associated with antibiotic activity on populations of microbes, they are also toxic to higher organisms (Kummerer, 2003). Surprisingly low concentrations of antibiotics in the range of 5–100 µg/l have been shown to be toxic to algae and to the freshwater crustacean, *Daphnia* (Holten-Lutzhoft et al., 1999; Wollenberger et al., 2000). When environmental samples are tested, however, the use of such organisms gives very little information about antibiotic effects, as the organisms tend to be sensitive to a wide range of toxicants and the methodology does not select for antibiotics. The aim of this research was to establish a method for identifying and measuring antibiotic activity in the environment. It is anticipated that the activity could arise not only from antibiotic parent compounds but also from products of their metabolism. Although this study refers mainly to antibiotics, it would be more accurate to describe the chemicals we are seeking to identify as antimicrobials. Chemicals of interest are pharmaceutical products used as human or animal medicines, or as live-stock growth promoters that subsequently find their way into effluents, waterways or the sea. This will include man-made antibiotics such as the sulfa drugs, but exclude chemicals such as Triclosan, Chloramine T and other toxic antimicrobials used in surface treatments and other applications.

## 2. Materials and methods

### 2.1. Premitest

In Europe, the meat and dairy industry have their products regularly tested for antibiotic residues to demonstrate compliance with EU Directive EC 96/23(1996). The testing protocols in the UK have recently been revised to allow the use of a commercially available kit, which had been validated for testing antibiotics in meat and dairy products. The method has been shown to be responsive to all antibiotics tested to date (42 in number, Stead et al., 2004) and provides a simple yes/no response to the presence of antibiotics. The product, called Premitest (DSM food industries, Netherlands), is commercially available in kit form. The test uses a species of thermophilic bacteria, *Bacillus stearothermophilus*, which is responsive to all of the most commonly used antibiotics and provides a measurable colour change in the agar substrate when exposed to samples of contaminated meat or a similar sample matrix.

### 2.2. Method development

A Premitest start up kit was obtained from DSM. This contained a batch of test vials; pre-inoculated by DSM with *B. stearothermophilus*, breathable membranes for the tops of the vials, and a solid-state incubator block for 10 vials. This block was pre-set to 64 °C, and was found to be accurate to within ±1 °C using a calibrated and certified thermometer.

Premitest was designed for testing meat, urine and dairy products rather than extracted water samples. Test vials contain agar impregnated with spores of *B. stearothermophilus* and a pH indicator dye, Bromocresol purple. Vials are purple at the start of the test. As the spores germinate and grow they produce acid which changes the colour of the test medium to yellow. Samples with active concentrations of antibiotics prevent the spores from germinating and the agar stays purple.

Following the manufacturer's method, 200 µl of sample was added to each vial. The vials were capped with breathable foil. Sample vials were then placed in the preheated incubating block and incubated for approximately 3 h at 64 °C. Later in the development of the assay, samples were run in an incubator set at 64 °C (±1 °C). Tests were found to develop slightly slower in the incubator therefore samples were read when the inoculated agar in the blank vials changed from the 'positive' purple colouration, to the 'negative' yellow colour.

Some adaptations of the technique recommended by the manufacturer were required to produce results from environmental samples. Initial tests showed no response for either the samples or the associated blanks in several tests. Further studies established that the samples would need to be transferred into a nutrient media to provide the bacterial spores with the requirements for rapid growth and respiration. A new test method was developed using Lab Lemco (Oxoid), a meat-extract based media component used in the preparation of agars and broths. Addition of 200 µl of Lab Lemco to the vials before incubation enabled the spores to grow rapidly after germination, causing the acidity change required for a successful test thus producing a 'negative' result. Lab Lemco was used as a blank, and as a carrier for the extracted samples in all subsequent tests.

### 2.3. Direct application

Testing was carried out using standards directly applied to the vials. Standards were made up in Lab Lemco where possible, and solvent exchanged into Lab Lemco where necessary. Two antibiotics were tested using this methodology at a range of dilutions to produce simple dose–response curves. We also screened a selection of representative anthropogenic toxicants to establish whether they could cause false positives. Triclosan, a broad-spectrum biocide used in a large number of healthcare and consumer products, was selected due to its ubiquitous use and its apparent antibiotic action (Schweizer, 2001; Slater-Radosti et al., 2001). Zinc, used to represent the action of various toxic metals, was selected due to its use as a reference chemical in many standard toxicity tests. Diuron, a booster biocide which, has been used in antifouling paints and also as a broad-spectrum agricultural herbicide was used as it has been found in environmental samples (Comber et al., 2002; Giacomazzi and Cochet, 2004; Thomas et al., 2001a).

## 2.4. Extracted spiked samples

Antibiotic spiked samples were made up in deionised water and extracted using Strata X (Phenomenex) solid-phase extraction cartridges that have been found to have excellent recovery rates for many pharmaceuticals in environmental samples. Extracted samples were eluted with methanol and then solvent transferred back into Lab Lemco giving a 1000:1 concentration factor. Reference antibiotics erythromycin and sulfamethoxazole were spiked at a range of concentrations, and in simple binary mixtures. Some samples were also spiked with either zinc, for reasons already discussed, or 4,4'-DDT to establish whether they would cause false positive results after extraction. 4,4'-DDT was selected as a representative organochlorine pesticide which is still found in environmental samples (Na et al., 2006).

## 2.5. Environmental samples assay procedure

To demonstrate the use of this assay as an effective screen for antibiotic activity, selected environmental samples were run at high concentrations. Those samples that showed strong activity were made into a dilution series and repeated alongside an erythromycin dilution series for comparison. The end point for the assay was determined as the time at which the blanks turned yellow. All other vials were read for colour at this time. Vial colour at the end of each test was recorded on a five-point scale from purple through to yellow. The whole assay procedure was developed as an AntiBiotic Challenge assay and is referred to as the ABC assay.

## 3. Results

To calculate some nominal EC<sub>50</sub> data (i.e. a concentration which will hypothetically affect 50% of the population) from the results, the colour development was given a numerical value. These values can be seen in Table 1. Comparative values for erythromycin concentrations have been included in the table to put the colour development into context.

Table 1  
Assay colour scale and associated percentage score

Colour	Yellow	Yellow with some purple	50/50	Purple with some yellow	Purple
Antibiotic effect (%)	0	25	50	75	100
Comparative erythromycin potency (µg/l)	<25	40	50.7	63	>100
Comparative sulfamethoxazole potency (µg/l)	<40	40	100	155	>250

0% = No antibiotic activity detected. Erythromycin and sulfamethoxazole values are for direct application.

Table 2  
Assay results from directly applied spiked stocks

Chemical	NOEC (h)	LOEC (h)
Sulfamethoxazole	440 µg/l (3)	132.5 µg/l (3)
Erythromycin	11 µg/l (3) 50 µg/l (3.5)	23 µg/l (3) 110 µg/l (3.5)
Zinc	5600 µg/l (3)	10,000 µg/l (3)
Diuron	33,000 µg/l (3) 100,000 µg/l (4)	56,000 µg/l (3) –
Triclosan (Irgasan)	7000 µg/l (3) 10,000 µg/l (3.5) 15,000 µg/l (4)	10,000 µg/l (3) 15,000 µg/l (3.5) 20,000 µg/l (4)
Lab Lemco	All	–

The number in brackets indicates the number of hours incubation. NOEC = No observable effect concentration. LOEC = Lowest observable effect concentration.

## 3.1. Direct application

Table 2 shows the responses for directly applied samples. First readings were taken at the point where the blank samples became yellow. In early trials, samples were read at several time intervals and some positive samples became negative over time. These clear changes in the NOEC and LOEC over time allow the time-series data to provide information about which of the positive samples were most antibiologically potent according to this assay.

The assay responds to zinc and other toxicants, but at concentrations higher and less environmentally relevant than those at which it responds to antibiotics. Most metals and some other potential toxicants will be removed by the sample extraction process. Lab Lemco blanks produced a negative response as expected.

## 3.2. Extracted spiked samples

For extracted spiked samples the likelihood that common environmental contaminants other than antibiotics will produce a false positive result in this assay is dependent on two variables. One is whether the chemical in question has the potential to kill the *B. stearothermophilus* in the test media. The second is whether the contaminants are retained on the solid-phase columns and then elute at sufficient concentrations to affect the response. The use of the Strata X sorbent provided dual benefits to the method by concentrating the antibiotics in the sample and also excluding most metals due to their chemical nature. Data from the extracted samples in Table 3 showed that spiked zinc samples produced no measurable effect in the assay. 4,4'-DDT also elicited no response, so is either inactive in the assay, or not extracted by this method.

Using extracted, concentrated samples increases the sensitivity of the assay and thus reduces its detection limit. A 1000:1 concentration factor enables detection of selected antibiotics in environmental samples in the ng/l range.

Table 3  
Assay results from extracted spiked stocks

Chemical	NOEC	LOEC
Sulfamethoxazole	–	0.1 µg/l (3)
	0.25 µg/l (4.3)	0.8 µg/l (4.3)
Erythromycin	–	0.25 µg/l (4.3)
SM/EM mix	–	0.25 µg/l (4.3)
Zinc	15,000 µg/l (3)	–
4,4'-DDT	100,000 µg/l (3.5)	–
H20, Lab Lemco	All tests	–

The number in brackets indicates the number of hours incubation.

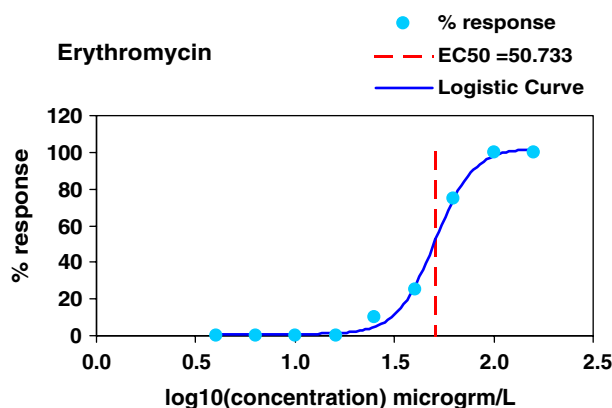


Fig. 1. Dose–response curve for erythromycin using the ABC assay. Response is measured using the scale from Table 1.

### 3.3. Environmental samples

With each batch of samples, EC<sub>50</sub> data for erythromycin and sulfamethoxazole were generated from two replicate dose–response series. Erythromycin is an antibiotic that is commonly found in environmental samples (Roberts and Thomas, 2006; Hirsch et al., 1999) and sulfamethoxazole is representative of a second group of widely used antibiotics that have also been detected in environmental samples (Hirsch et al., 1999). Fig. 1 shows the dose–response curve

and associated EC<sub>50</sub> generated for a set of erythromycin replicates. The same procedure was carried out with the sulfamethoxazole replicates. The EC<sub>50</sub> values were used to convert data from extracted water samples into erythromycin or sulfamethoxazole equivalents. This relative potency calculation simplifies the comparison of data from different batches of samples and areas of sampling. Responses are plotted against the curve in Fig. 1, and the concentration of either antibiotic needed to produce that response is calculated. This concentration is the relative potency figure used in the tables.

Table 4 shows data from water samples taken in three heavily industrialised river and estuary systems in Europe; the Elbe in Germany, the Schelde in the Netherlands and the Llobregat in Spain. Figures shown with a ‘greater than’ EC<sub>50</sub> produced no response on the assay. The corresponding ‘less than’ ng/l figure is the limit of detection for that sample taking into account the concentration factor.

The data shows a range of results from below the limit of detection in the assay, to over 6 µg/l in the most active sample. It is possible from these results to sort the samples by antibiotic concentration, with the highest Elbe samples appearing to have a lower antibiotic concentration than those from the Llobregat or the Schelde. The Elbe sites were associated with industrial effluents, which would not be expected to contain high concentrations of antibiotics. On the Llobregat sites, the reference site at Jorba actually had higher antibiotic equivalent activity than the most contaminated Elbe site. The Schelde samples range from sites with no obvious antibiotic concentration to others which are the highest we measured. Reference sites such as Rundvoortbrug and Halle produced results lower than their associated polluted sites at Eenhoorn and Weerde, respectively. These differences could be due to a number of variables e.g. proximity of samples to point source contamination, and mixing and dilution characteristics of the waterbody. The results of the assay provide a means of focussing further sampling efforts to establish more information about areas with high antibiotic concentration.

Table 4  
Water samples

Sample site	Sample concentration factor producing EC <sub>50</sub>	Erythromycin equivalent (ng/l)	Sulfamethoxazole equivalent (ng/l)
Negative control (Lab Lemco)	–	0	0
Elbe, Pardubice	>386	<131	<259
Elbe, Prelouc	>357	<141	<280
Elbe, Litvinov	386	131	259
Llobregat, Jorba, Anoaia	144	352	697
Llobregat, Sant Joan Despi	21	2383	4717
Schelde, Eenhoorn	158	319	632
Schelde, Rundvoortbrug	>625	<81	<160
Schelde, Weerde	16	3207	6349
Schelde, Halle	81	624	1235
Schelde, Geel	>750	<67	<133
Schelde, Westerlo	95	534	1057

Results from the ABC assay. Samples with no activity in the assay at the concentration tested are qualified with > and < symbols.

#### 4. Discussion

The prevalence of antibiotics use in the developed world combined with the unresolved problems associated with antibiotic resistance amongst many pathogens suggest a current need to monitor antibiotics in the environment. In areas with a lot of antibiotic usage, such as hospitals or intensive animal rearing facilities, the continuous presence of antibiotics at low concentrations can lead to the development of resistant sub-strains of organisms. Chemical analysis of various environmental samples has shown that sub-lethal concentrations of antibiotics can be found in both natural surface waters and at the point of use (Roberts and Thomas, 2006). Even if these low environmental concentrations are insufficient to kill microorganisms they may result in selection for resistance to the antibiotics in question. Antibiotics are biologically active and may have the capability, even at low concentrations, of inducing effects. Chronic exposures may affect populations exposed across multiple generations (Halling-Sørensen et al., 1998). If any of the environmental concentrations prove to be high enough to affect survival in bacterial populations (Drlica, 2003) then environmental populations could develop the same resistance characteristics shown by hospital based populations. This has direct implications on the resistance status of bacterial populations in areas such as recreational beaches, where human populations may come into contact with them.

This assay is being developed as a screen to be used in the rapid identification of sites where there is antibiotic activity and to establish relative levels of the activity that is present. Once key sites have been identified in this way, it will be possible to apply targeted chemical analysis in a cost effective way to identify the specific antibiotics present. The screen is comparatively rapid, inexpensive and capable of a very high throughput. Results are easy to interpret and can even be read at the field sampling location. The assay uses living bacteria, which means that it will respond not only to known antibiotic chemicals, but also to the presence of other antibiologically active compounds formed during the metabolic breakdown of parent chemicals: the assay therefore provides an indication of the total antibiotic activity in a sample. Specific chemical analysis of individual antibiotics is valuable to identify sources of contamination but used in isolation it may fail to identify sites with high antibiotic contamination from unexpected sources.

The results from this first round of tests suggest that the method outlined in this paper is capable of detecting antibiotic activity in extracted environmental water samples. Once the active samples are identified, samples can be serially diluted and re-tested to provide dose–response data. This will give important information about which samples in a batch are the most active and should therefore be the focus of more detailed study. To generate semi-quantitative results the assay needs to have antibiotic standards run at the same time. Table 4 shows the kind of data that can be generated using this approach.

Tables 2 and 3 show the distinct differences in LOEC and NOEC which are generated if the test is allowed to run beyond the development of the negative controls, where the antibiotic is present at a concentration that does not kill all the bacteria, enough may be killed to prevent colour change in the media. This can be followed by a slow recovery in bacterial numbers until enough respiring bacteria are present to affect the indicator dye. To standardise results they all need to be read at the point at which the blanks turn yellow, however, by allowing the assay to over-run after the initial reading further information can be developed. It can provide an early indication of which samples are most active out of several that were positive at the initial read-point.

Although the assay has been shown to work well with spiked extracts or direct application of antibiotics, one of the development issues with this assay has been the potential for false positives. The assay uses an indicator dye to detect change in acidity caused by bacterial respiration so in the presence of antibiotics, the bacteria respire less or not at all and no change in colour occurs. Obviously a compound that is toxic to the bacteria will also cause these effects, leading to a false result. In this study, tests on chemicals representative of those likely to be found in environmental samples suggest that some of the major contaminant groups, e.g. metals, will be selectively excluded due to the solid-phase extraction step. The presence of other common contaminants of water samples can also be relatively easily screened for. Of selected chemicals tested so far (Tables 1 and 2), all of them either exhibited no activity, or were only active at concentrations hundreds to thousands of times higher than those of the antibiotics. Diuron, Irgasan (Triclosan) and 4,4'-DDT all proved to have little or no toxic effect at environmentally relevant concentrations and further testing is ongoing to establish if there are groups of chemicals that may affect the results with environmental samples.

False positives can also be reduced when this assay is run in parallel with other bioassays. Intelligent use of cytotoxicity measurements from complementary assays such as Microtox, Green screen or the YES/YAS assay would discriminate samples or concentrations of samples where the bacteria are likely to be killed by toxicity. Elimination of these results would allow antibiotic activity to be identified with greater certainty. In some cases, it is possible that TIE techniques (e.g. Thomas et al., 2001b) or effects directed analysis (EDA) will be used on particular samples to demonstrate that it is antibiotics causing the response. Selective use of targeted chemical analysis could also identify the specific antibiotics involved, which may help to establish their most likely source.

The environmental samples tested produced several positives at the maximum sample concentration tested. These samples were then made into dilution series and re-tested. The results allowed the production of EC<sub>50</sub> data (Table 4) showing the concentration of sample required to produce a 50% inhibition of response in the test. A high value

is therefore associated with very low antibiotic activity which could lead to misinterpretation of the results. By applying a relative potency calculation based on measured erythromycin or sulfamethoxazole response, the results are easier to interpret. Table 4 demonstrates the high (equivalent) concentrations of antibiotics which may be present at heavily affected sites. The data shows the ability of the assay to differentiate between sites on the same river, and to indicate where hot spots of activity might be. This demonstrates the value of this assay as a tool for screening samples to establish where the highest concentrations occur and to direct further research into specific areas. It is not certain whether the concentrations of antibiotics found would be within the ‘mutant selection window’ (Drlica, 2003) required for inducing antibiotic resistance in populations. The induction of antibiotic resistant microbes in various environmental niches may be more detrimental to the longevity of currently available antibiotics than any of the populations found in hospitals around the world. This assay would be a useful technique to establish how many areas have antibiotic concentrations at critical levels and would enable positive action to regulate specific inputs.

## 5. Conclusions

1. The ABC assay works as a rapid, inexpensive yes/no test for antibiotic activity in environmental water samples.
2. False positives can be avoided by incorporating cytotoxicity data from other bioassays and by further research into assay behaviour in the presence of non-antibiotic contaminants.
3. Most toxic metals which could lead to false positives will not be retained during the water sample extraction process, further reducing the likelihood of a false positive response.
4. More statistically useful responses, including EC<sub>50</sub>s and antibiotic equivalents can be determined by using a ‘score’ for the colour change.
5. Directly applied standards and extracted water samples can be assessed using this assay.

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