University of Bielefeld

Faculty of Science

Department of Animal Ecology

Academic year 2010-2012

Effects of the antibiotic Tetracycline: sublethal nematode toxicity tests

Matthew Vangheel

Promoter: Prof. Dr. Walter Traunspurger
Co-promoter: Dr. Nicole Spann

Thesis submitted to obtain the degree of European Master of Science in Nematology
Effects of the antibiotic Tetracycline: sublethal nematode toxicity tests

Matthew VANGHEEL\textsuperscript{1,2}\* 

\textsuperscript{1} University Gent, Department of Biology, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium  
\textsuperscript{2} University Bielefeld, Department of Animal Ecology, Morgenbreede 45, 33615 Bielefeld, Germany  

\* Corresponding author email: matthewvangheel@gmail.com
**Summary** - Modified ISO tests were used to examine the effects of the antibiotic Tetracycline on the reproduction, growth and population growth rate of the free-living nematode *Caerorhabditis elegans*. Tetracycline has been observed in open waters and soils today, as a consequence of inappropriate usage. In a comparison of two different media, one liquid, one more viscous (gellan gum), *C. elegans* was found to be more sensitive in the latter. This was determined from a decrease in the animals’ reproduction over a range of different concentrations. In the liquid medium, growth was found to be more tolerant to the toxic effects of the antibiotic than reproduction. Lower inhibition concentrations (IC) were recorded for reproduction. The nematode’s intrinsic growth rate was estimated at the end of a prolonged ISO test, conducted in the more viscous medium. After 15 days, the population growth rate of *C. elegans* was estimated and a decrease was noted correlated with increasing concentrations of Tetracycline. For concentrations higher than 3mg/L, the carrying capacity was reached 3 days later than seen in the control. A food attraction experiment to examine the nematode’s appeal to Tetracycline contaminated food was unsuccessful in drawing unambiguous conclusions. Finally, it was obvious that the Lowest Observed Effect Concentrations (LOEC) for different endpoints, examined in both media, were far beyond those concentrations found in the environment.

**Keywords** - *Caerorhabditis elegans*, ISO test, intrinsic growth rate, ecotoxicology, veterinary medicine, risk assessment, CNGG
Today’s livestock farming requires the usage of many pharmaceuticals, in order to keep the animals sound and to increase the production rate. Products are used to stimulate growth, to induce ovulation, but more important in prevention or treatment of parasites and bacterial diseases (Boxall et al., 2003). These farm animals are often bred on a large scale and as a consequence animal medicine or other drugs are applied in large quantities. These amounts are catching up with those of typical agrochemicals (Jones et al., 2001; Benbrook, 2002). Only in some situations the dosage is specifically adapted to the needs of one or a small group of animals. In most cases the entire livestock is treated even if the majority does not show any symptoms. Unadjusted dosages lead to incomplete metabolisation and not metabolised and degradation products are excreted by the animal (Boxall et al., 2003). The excessive use of pharmaceuticals has led to an increase in concentrations of these potentially harmful compounds in natural soils and aquatic systems (Hamscher et al., 2002; Kolpin et al., 2002). Active components can form a serious risk for both public health and natural ecosystems (Boxall et al., 2003; Kümmerer, 2008; Sibley & Hanson, 2011).

Luckily degradation of pharmaceuticals can occur under natural conditions, resulting in abiotic products (Oka & Ikai, 1989). Once parental compounds and their derivates (biotic or abiotic degradation products) are leached into the environment, they can disperse in many ways. During their journey they are subjected to different environmental conditions, possibly enhancing the degradation process. As noted by Boxall et al. (2003), animal manure is often collected and stored for a long period before application. Storage keeps the pharmaceuticals out of the environment, until the manure is used to fertilise the fields. This extra storage time may contribute in decreasing the effectiveness of the pharmaceutical, but there is a lot of variation in degradation times between chemical compounds (Dolliver et al., 2008). Furthermore, under the right conditions storage of manure may lead to regenerated parental compounds (Langhammer & Buening-Pfaue,
and degenerated products are not always less harmful (Boxall et al., 2003). The activity of these compounds depends a lot on their chemical structure and the surrounding physical conditions. Variations in pH may alter binding properties and isoelectric points. Hydrophobic structures can reduce their solubility. Cation exchange capacity may result in captured molecules. All these physico-chemical properties may reduce the chemical’s bioavailability (Le-Minh et al., 2010; Yang et al., 2010; Zhou et al., 2011). Sorption to soils or sediments may even completely inhibit any effect. Although usually only found in very low concentrations, the continuous supply through various ways makes pharmaceuticals and their degradation products pseudo-persistent in the environment (Koschorreck & de Kecht, 2008) and therefore still potentially dangerous.

Antibiotics and other pharmaceuticals bring forth biological effects, even at low concentrations. They are specifically constructed for this purpose, making them potentially more harmful than other chemical compounds (Williams, 2005). According to Boatman (1998) more than 2500 tons of the antibiotic tetracyclines are used every year in veterinary therapy, of which tetracycline (TC), chlortetracycline (CTC), and oxytetracycline (OTC) are applied the most. This group of antibiotics are broad-spectrum agents, active against a wide range of microorganisms. For example, different types of gram-positive and species of gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, rickettsiae and protozoan parasites are affected. The octahydronaphthalene ring, which is the backbone structure of this group of antibiotics, binds to 30S ribosomal RNA. This binding prevents translation and hence inhibits the synthesis of bacterial proteins (Chopra & Roberts, 2001). As mentioned above, some of the antibiotic may be excreted by the animal after treatment. Between 50 - 80% (of the applied dosage) of not metabolised material can be found in animal urine. The variance is due to different factors such as age, route of administration, pH of urine, glomerular filtration rate and the particular tetracycline used (Aiello, 1998).
Table 1 - Concentrations TC found in the environment, as reported in recent literature

<table>
<thead>
<tr>
<th>Environment</th>
<th>Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil fertilised with animal manure:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swine slurry</td>
<td>295 ± 128a1</td>
<td>Hamscher et al. (2005)</td>
</tr>
<tr>
<td>Livestock</td>
<td>20.9 - 105a2</td>
<td>Hu et al. (2010)</td>
</tr>
<tr>
<td>Swine</td>
<td>90.0 ± 14.7a1</td>
<td>Qiao et al. (2012)</td>
</tr>
<tr>
<td>Fish</td>
<td>86.2a</td>
<td>Hamscher et al. (2002)</td>
</tr>
<tr>
<td>Dairy cattle</td>
<td>55a</td>
<td>Watanabe et al. (2010)</td>
</tr>
<tr>
<td>Swine</td>
<td>9a</td>
<td>Zilles et al. (2005)</td>
</tr>
<tr>
<td>River sediment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearl Rivers (CHN)c</td>
<td>40.9a</td>
<td>Yang et al. (2010)</td>
</tr>
<tr>
<td>Cache la Poudre River (USA)</td>
<td>32.8 ± 0.3a1</td>
<td>Kim &amp; Carlson (2007)</td>
</tr>
<tr>
<td>Surface water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jiangsu Province (CHN)</td>
<td>0.81b</td>
<td>Wei et al. (2011)</td>
</tr>
<tr>
<td>Queensland (AUS)</td>
<td>0.080b</td>
<td>Watkinson et al. (2009)</td>
</tr>
<tr>
<td>Jiulongjiang River, Fujian (CHN)</td>
<td>0.050b</td>
<td>Zhang et al. (2012)</td>
</tr>
<tr>
<td>Haihe River (CHN)</td>
<td>0.027b</td>
<td>Luo et al. (2011)</td>
</tr>
<tr>
<td>Cache la Poudre River (USA)</td>
<td>0.02 ± 0.01b1</td>
<td>Kim &amp; Carlson (2007)</td>
</tr>
<tr>
<td>Mess River (LUX)</td>
<td>0.017 ± 0.003b1</td>
<td>Meyer et al. (2011)</td>
</tr>
<tr>
<td>Atibaia River (BRA)</td>
<td>0.011b</td>
<td>Locatelli et al. (2011)</td>
</tr>
<tr>
<td>Choptank River (USA)</td>
<td>0.005b</td>
<td>Arik et al. (2008)</td>
</tr>
<tr>
<td>WWTPd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>effluent (PRT)</td>
<td>≤ 6b</td>
<td>Cavnati et al. (2012)</td>
</tr>
<tr>
<td>effluent (CAN)</td>
<td>1.0b</td>
<td>Boussu et al. (2007)</td>
</tr>
<tr>
<td>influent (USA)</td>
<td>0.1 - 0.6b2</td>
<td>Kim et al. (2005)</td>
</tr>
<tr>
<td>influent (NLD)</td>
<td>&lt; 0.1b</td>
<td>Schrap et al. (2003)</td>
</tr>
</tbody>
</table>

1 values represent the means ± standard deviations
2 values represent a range
a µg/kg
b µg/L
ISO 3166 Codes (Countries)
wastewater treatment plants
A high quantity of tetracyclines leach into the environment, forming a potential danger for all sorts of different ecosystems. Hamscher et al. (2005) sampled farmed soils that were fertilised using animal manure and found a maximum concentration of 0.30 mg Tetracycline (TC) per kilogram of soil. Table 1 summarises concentrations found in recent literature. A clear distinction between aquatic and sediment samples is due to sorption onto sediment particles (Le-Minh et al., 2010; Yang et al., 2010).

Because of its abundance in the environment, researchers have been aware of the potential problem and tried to assess the environmental risks. Many different approaches, using all sorts of organisms, help us to evaluate the problem. Different tests have been done, mostly on bacteria -as they are the main target of the antibiotic (Chopra & Roberts, 2001; Christensen et al., 2006; Halling-Sørensen et al., 2002)- but also on phytoplankton (Wilson et al., 2004), periphyton (Quinlan et al., 2011) and water plants (Brain et al. 2005; Pomati et al., 2004). Araujo & McNair (2007) tested the effects of TC on the reproduction and lifespan of two species of rotifers: *Brachionus calyciflorus* Pallas (freshwater) and *B. plicatilis* Müller (brackish water). In general reproduction was reduced and shifted to an earlier age. Furthermore the lifespan was shortened. The 1% Inhibition Concentration (IC) for lifespan ranged between 31.5 and 32.43 mg/L for *B. calyciflorus* and for *B. plicatilis* between 28.11 and 28.80 mg/L. The IC50 was noted between 171.21-172.74 mg/L and 329.69-331.31 mg/L respectively. Considering the effects on life time reproduction IC1-values were much lower: between 3.53-4.11 mg/L (*B. calyciflorus*) and 8.60-9.07 mg/L (*B. plicatilis*). The IC50 was estimated between 78.81 - 79.56 mg/L and 119.41-121.47 mg/L respectively. Wollenberger et al. (2000) assessed the acute and chronic toxicity on *Daphnia magna*. Acute effects were only recorded in dosages far beyond ecological relevance (no effect was shown below a 100 mg/L concentration). The IC10 of reproduction was estimated at 29.4 (24.6-35.2) mg/L.
Within this research nematodes will be used in the experimental setup, because they are ubiquitous and highly abundant animals (Bongers & Bongers, 1998) and according to Höss et al. (2009) essential in ecotoxicological assessments. The soil free-living nematode *Caenorhabditis elegans* Maupas (1899), was proposed by Brenner (1974) as an “easy-to-work-with-organism”. Its ease of cultivation in the lab, short generation times and hermaphrodite reproduction made it excellent for genetic testing. Ever since Brenner suggested *C. elegans*, many other scientists have seen its potential in many different fields. The nematode has crawled into the world of ecotoxicology and has been used in numerous experiments. Höss & Williams (2009) summarised the benefits of using nematodes and in particular *C. elegans*, in assessing the effects of potential toxins on the environment.

To assess the possible environmental impacts of specific chemicals, an integration of different disciplines is absolutely essential (Caswell, 1996; Van den Brink, 2008). Only by combining the data and findings of different experimental approaches, effects can be evaluated. A simple standard test may yield some results and be the foundation of more in-depth research, such as life cycle, behaviour, mesocosm studies and eventually molecular research. Although more relevant than biochemical, molecular or cellular testing, the life cycle tests of individuals are still far off from the environmental effects. Assessments of changes in demographic structures within a population or micro- and mesocosm experiments (that involve different organisms), are more pertinent to the investigation, but naturally have a high complexity (Höss & Williams, 2009). The ultimate experimental setup with the highest relevance are of course field experiments, conducted *in situ*. However, it is hard to standardise or eliminate parameters, leaving it very difficult to interpret the results. This trade-off forces the investigators to integrate different approaches, in order to make a good environmental assessment (Van den Brink, 2008; Sibley & Hanson, 2011). In this research, it was chosen to test the effects on a single
organism *in vitro*. This approach can be found somewhere in the middle between clear results and ecological relevance (Höss & Williams, 2009).

To test the effects of Tetracycline on *C. elegans*, different experimental endpoints were taken into account. Alterations in growth (Kim *et al.*, 2012; Dhawan *et al.*, 1999), reproduction (Kim *et al.*, 2012; Anderson *et al.*, 2001) and population growth rate (Kim *et al.*, 2012 Ohba & Ishibashi, 1984) were examined. These parameters are of great ecological relevance and can clearly indicate how an organism responds to a potential toxin. The aim of this study is to assess the toxicity of Tetracycline, by observing the effect on those different endpoints. Possible alterations of reproduction were examined in two different media: K-medium and CNGG (Brinke, Heininger & Traunspurger, 2011). Both differ in their viscosity and resemble different environments in which *C. elegans* can confront the pharmaceutical. Furthermore, behavioural effects were examined by tracking the nematode’s movement towards a food source (Dhawan *et al.*, 2000; Boyd & Williams, 2003; Ma *et al.*, 2009). If hampered, the animal may not find enough nutrients to reproduce or grow, indicating a secondary effect of the antibiotic. Expectations were that Tetracycline will affect all endpoints negatively. The question however was to what extent.
Material & Methods

Preparation of the food source

The bacterium *Escherichia coli* strain OP50 was chosen as the food source in all the conducted experiments. Stock cultures were prepared and stored in agreement with ISO 10872:2010. Prior to experimental setup, 25mL of autoclaved LB-medium (10g/L NaCl, 10g/L peptone, 5g/L yeast extract) was inoculated with 30µL of thawed stock culture in an Erlenmeyer flask. After 17h in an incubator (37°C) on a shaker (200rpm), the medium was washed (using K-medium: 3,1g/L NaCl, 2,4 g/L KCl) in order to remove excretion and waste products. Therefore the medium was first centrifuged (2000g for 20min) without addition of K-medium. After removal of the supernatants the bacterial pellet was resuspended in K-medium and centrifuged again. The resulting much clearer supernatant was again discarded and the bacterial pellet resuspended in K-medium in order to measure the bacterial density (photo-spectrometer: 600nm). Bacterial densities were different for each experimental setup and will therefore be discussed further in detail below.

Test organism

*Caenorhabditis elegans* Maupas (1899) strain N2 (Bristol, UK) was selected to be an appropriate test organism. It was cultured in agreement with ISO 10872:2010 on nutrient agar plates (15g/L agar, 0,5 g/L peptone, 0,3g/L yeast extract, 0,1g/L NaCl and 5mg/L cholesterol). Cultures were prepared 4 to 7 days before experimental setup. To obtain first stage juveniles (J1), the plates were rinsed (K-medium) above stacked nylon mesh sieves (10µm and 5µm pore size) and captured in a beaker.
Tetracycline

The Tetracycline hydrochloride (TC) (Molecular Weight 480.90; PubChem Substance ID 24900468; ≥95% CELLPURE®) was purchased from Roth as a powder. Stock solutions of 4g/L were prepared, by dissolving the powder in de-ionised water. They were kept in glass flasks, wrapped in tinfoil at 7°C for no longer than 12 days.

ISO test (K-medium)

The ISO test was conducted according to ISO 10872:2010, with only few modifications. The bacterial density was adjusted to (1000±50) FAU (ISO 7027) and cholesterol stock solution was added (0,2% of the total volume of the bacterial suspension in K-medium). From the TC stock solution, a series of concentrations was prepared by dilution with K-medium (0,1,5,10,20,50,100,150,200 mg/L). Five replicas of each concentration (plus K-medium as control), were set up in 12-well plates. Therefore 0,5 mL of TC-solution (K-medium for control) was transferred to each well and in addition, 0,5 mL of well-stirred homogenised bacterial suspension, resulting in 45 wells of different TC concentrations and a bacterial density of 500 FAU. Ten moving J1 juveniles were transferred to each well, with a glass pasture pipet from which the tip had been narrowed down. Furthermore 30 J1 juveniles, were transferred to a K-medium solution in a Petri dish, stained using Rose Bengal stock solution and put in an drying oven for 15 min at 80°C. The 12-well plates were sealed using Parafilm® and incubated in the dark (at 20°C ± 0,5) for 96h. Since the generation time of *C. elegans* is around 2,5 days, a 4 day runtime would yield 10 adults and their progeny. To determine the test, Rose Bengal stock solution was added to each well and the plates were placed in a drying oven for 20 min at 80°C. Samples that were not analysed immediately, were stored in a fridge (app. 7°C).

To analyse the samples, the content of each well was transferred to a counting dish. The well and pipet were both checked thoroughly after transition to make sure all
nematodes were transferred. Adults were picked out using a metal fishing needle and stored in K-medium. Juveniles (progeny) were counted under a dissecting microscope (Leica S6E) at 10x3.2 magnification. The effects of different concentrations TC on reproduction were assessed by dividing the total number of juveniles by the total number of adult females. Samples containing adult males were checked if they were in agreement with the restrictions found in ISO 10872:2010.

The 30 juveniles that were isolated before the start of the experiment, were transferred to a slide and their length was measured (10x10 Zeiss A-plan; Microscope: AX10). The adults were also transferred to slides and measured (10x5 Zeiss N-achroplan; Microscope: AX10). To determine growth, the average length of 30 juveniles was subtracted form the measured length of the adult females.

Inhibition of reproduction and growth was calculated in reference to the average number of juveniles per females and the average growth of the control samples. The yielded percentages were then fed into the statistical program R (R Core Team, 2012) and dose-response curves (non-linear regression: \(1/(1+(x/\alpha_2)^{\alpha_1})\) with \(\alpha_1\) and \(\alpha_2\) as parameters) were calculated using the package ‘drc’, self starter ‘LL.2’ (Ritz & Streibig, 2005). Furthermore, estimates of Inhibition Concentrations (IC) were calculated.

The data were checked for significant differences between treatments by running an ANOVA in R. To satisfy the assumptions, all data (reproduction and growth) were log(1+x) transformed. Subsequently the significance level between control and treatments were checked by a post hoc Dunnett-test, with the ‘multcomp’ package (Hothorn, Bretz & Westfall, 2008) in R.

**ISO test (CNGG)**

The ISO test was conducted according to ISO 10872:2010, with only few modifications. The bacterial density was set to 5E9 cells/mL, therefore a corresponding
amount of suspended bacterial solution was transferred to 15mL plastic centrifugal tubes. After centrifugation (2000g for 20min) the supernatant was removed and 8mL of sterile Gelrite® solution (1,876g/L Gelrite®, 1,250mL/L cholesterol) was added to each tube. In total 9 tubes were prepared: control plus 8 different concentrations (1,5,10,20,50, 100,150,200 mg/L) of TC. To each tube, 1mL of corresponding concentration TC (diluted from stock using sterile de-ionised water) and 1mL of sterile de-ionised water for the control was added, followed by 1mL of sterile salt solution (1M CaCL₂, 1M MgSO₄). After each addition the tubes were mixed thoroughly (Vortex) to guarantee equal distribution. 1mL of each concentration (5 replicas) was pipetted into a well of a 12-well plate. Afterwards 10 J1 juveniles were transferred to each well using a glass pipet. The 12-well plates were sealed using Parafilm® and incubated in the dark (at 20°C ± 0,5) for 96h. To determine the test, 4 mL EDTA-Rose Bengal solution (0,04M EDTA, Rose Bengal stock solution) was added to each well and the plates were placed in a drying oven for 20 min at 80°C. Samples that were not analysed immediately, were stored in a fridge (approx. 7°C). Sample processing was similar to the K-medium ISO test described above, with the exception of measurements being taken. Inhibition of reproduction was calculated in reference to the average number of juveniles per female of the control samples. The data were analysed using the statistical program R (‘drc‘ package, non-linear regression: 1/(1+(x/α₂)α₁) with α₁ and α₂ as parameters), yielding dose-response curves and IC-estimates. An ANOVA was ran to check significant differences between treatments. The assumptions were met after log(1+x) transforming all data. Furthermore the significance level between control and treatments were checked by a post hoc Dunnett-test, with the ‘multcomp’ package in R.
Population growth rate

The CNGG ISO test’s yielded EC-values were the starting point for the population growth experiment, which is in many ways similar to the ISO test (CNGG) but for an extended period of time. Seven concentrations (5 replicas) of TC were tested (0, 1, 3, 7, 17, 68 mg/L), corresponding with control, IC10, IC30, IC50, IC70 and IC90 of the ISO test performed in CNGG medium. At the start of the experiment, 1mL of each solution was again transferred to a well within a 12-well plate and stored in the dark at 20°C ± 0,5. After 3 days a subsample was taken from each well, by pipetting 1/5 of the total volume (200µL) into a clean Petri dish and adding 3,8 mL of EDTA (0,02 M) and Rose Bengal. These Petri dishes were placed in a drying oven for 20 min at 80°C and stored in a fridge (approx. 7°C) until examination. Individual nematodes (adults and juveniles) were counted in each Petri dish, using a dissection microscope. The wells were refilled up to 1mL by adding 200µL of corresponding solution (correct concentration of TC and bacteria), which was prepared the same day from resuspended bacterial pellets. By adding fresh medium after 3 days, the degradation of TC and loss of bacteria through consumption was partially countered. The procedure was repeated every 3 days and after 15 days the experiment was determined.

To estimate the total number present in each replica at each sampling day, the counted numbers of individuals from the subsample were multiplied by 5.

The intrinsic population growth rate (PGR, r), defined as the growth rate of an increasing population (Lotka, 1913), was estimated for each replica and each concentration (grouped replicas) in SigmaPlot v11.0 by means of a non-linear regression:

\[
N_t = \frac{K}{1 + \left( \frac{K-N_0}{N_0} \right)e^{-rt}}
\]

N\_t: number of individuals per day
N\_0: initial number of individuals
r: population growth rate
t: time (day)
K: carrying capacity
This formula generated an approximation of the different PGRs under test conditions. However these PGRs have to be corrected because of the interference each 3 days (subsampling), which leads to an artificially higher mortality rate, estimated by:

\[ m = \frac{0.2 \cdot n}{Tn - T1} \]

\( m \): artificial mortality
\( n \): times sampled
\( Tn \): last sample day (days)
\( T1 \): first sample day (days)

Therefore the corrected growth rate \( (r') \) is calculated by adding \( r \) and \( m \). \( r' \) reflects the PGR under undisturbed conditions.

To check if there were significant differences in PGRs between treatments, an ANOVA was run in R after the data had been log(1+x) transformed, followed by a post hoc Dunnett-test. In order to construct a dose-response curve in R (‘drc’ package, non-linear regression: \[ 1/(1+(x/\alpha_2)\alpha_1) \] with \( \alpha_1 \) and \( \alpha_2 \) as parameters), percentages of inhibition were calculated in reference to the average PGR under control conditions. Furthermore estimates of IC-values were calculated.

**Food attraction**

To see if food attraction is hampered by TC, an additional experiment was set up. Firstly Volvic agar (Volvic water, 3g/L NaCl, 17g/L agar) was prepared, of which 25mL was transferred to 8.5cm Petri dishes after sterilisation. After solidification, the agar was perforated using a specially designed stencil, leaving 8 similar sized holes located at the same distance form the centre of the dish, approx. 3cm (figure 1). The holes were refilled with 150µL of Volvic agar, forming cavities in which the test solutions could be pipetted. All procedures were conducted under sterile conditions.

*Figure 1: Food attraction test plates*
Three different experimental setups (8 replicas) were carried out. In the first one 4 cavities were filled with a TC-contaminated bacterial solution (5E9 cells/mL, K-medium, 38mg/L TC) and 4 with K-medium. The cavities were filled with 350 µL of a solution in an alternating pattern, so that each contaminated sample was surrounded by 2 non-contaminated samples. Twenty mobile adult female nematodes were transferred to the centre of each plate (minimum 2 cm away from the cavities). They were allocated from a 7-days-old culture plate using an eyelash mounted on a toothpick or glass pipet. Every 30 min the dishes were checked using a dissecting microscope, to see if the nematodes had moved and the observations were noted down. This procedure was repeated 6 times and once more after 24h, assuming that most worms had made their final decision after one day. The experiment was repeated twice more, with different solutions: contaminated bacterial solution (5E9 cells/mL) versus uncontaminated bacterial solution (5E9 cells/mL) and contaminated bacterial solution (5E9 cells/mL) versus uncontaminated low density bacterial solution (2E8 cells/mL).

The data were combined per replica for each time interval, thereby neglecting intra-replica variation, leading to only 2 data points per replica per time. To see if these were significantly different, a likelihood G-test was run in R (Deducer package; Fellows, 2012). A difference would indicate a preferred food choice.
Results

ISO test (K-medium)

A decrease in both growth and reproduction (juveniles per female) corresponding to increased concentrations of TC is described in figure 2. A clear effect on reproduction (ANOVA: F-value: 472.77; p < 2.2e-16) and on growth (ANOVA: F-value: 245.64; p < 2.2e-16) is proven to be significant.

Figure 2: Test conducted in K-medium, 96h, N=5. 2A: representation of the average reproduction (juveniles/female) per concentration of TC. Error bars indicate standard deviation. 2B: representation of the average growth (mm) per concentration of TC. Error bars indicate standard deviation. Significant differences between treatment and control are indicated: ‘*’ : p <0.05 ; ‘**’ : p <0.01; ‘***’ : p <0.001
**Figure 3:** Dose-response curves, K-medium, 96h, N=5. Replicas are displayed as °. Inhibition is plotted in ratios (e.g. 0.5 = 50% inhibition). The concentrations of TC are plotted on a logarithmic scale, hence the broken axis for the control (0mg). **3A:** Inhibition of reproduction (in reference to the control average). **3B:** Inhibition of growth (in reference to the control average).
The decrease in progeny (figure 2A) starts to differ significantly from the control from 20mg/L TC onwards. 20mg/L can therefore be referred to as the Lowest Observed Effect Concentration (LOEC), considering reproduction in K-medium. However, the lowest observed effect of TC on growth is significantly different from the control at a much lower concentration of 5 mg/L. This indicates that the LOECs are dissimilar for different parameters. Nevertheless both reproduction and growth show a negative trend with increasing concentration higher than 1mg/L.

The dose-response curves (figure 3) plot the inhibition ratios in relation to the concentrations of TC. It is found that the inter-replica variation is small and extreme measurements are absent. In both fig. 3A and fig 3B, the data points approach the plotted curve within acceptable distances.

Table 2 - Calculated Inhibition Concentrations (mg/L) of TC

<table>
<thead>
<tr>
<th></th>
<th>K-medium</th>
<th>CNNG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reproduction</td>
<td>Growth</td>
</tr>
<tr>
<td>IC10</td>
<td>8.7 ± 1.1</td>
<td>13.6 ± 1.3</td>
</tr>
<tr>
<td>IC30</td>
<td>19.5 ± 1.5</td>
<td>81.1 ± 3.0</td>
</tr>
<tr>
<td>IC50</td>
<td>32.4 ± 1.9</td>
<td>249.0 ± 13.4 §</td>
</tr>
<tr>
<td>IC70</td>
<td>53.8 ± 3.2</td>
<td>764.1 ± 74.7 §</td>
</tr>
<tr>
<td>IC90</td>
<td>120.6 ± 12.1</td>
<td>4561.7 ± 798.9 §</td>
</tr>
</tbody>
</table>

Values represent arithmetic means ± SD.

‘§’ Indicates concentrations beyond the scope of the experiment and are therefore not considered to be of any value.

Although not found significant, a 10% inhibition of reproduction is seen in figure 3A between the 5mg/L and 10mg/L concentration data points. Reproduction is inhibited almost entirely at the highest concentrations, where growth is not. Reproduction is inhibited by 50% in K-medium at a calculated concentration of 32.391 ± 1.8520 mg/L TC. Growth however, is not inhibited by 50% or more in any of the samples. Estimations of 50-percent inhibitory concentrations for growth, are much higher than the IC50 of reproduction.
(Table 2). Figure 3B does not show a sigmoid curve, because absolute inhibition of growth is not reached given the test concentrations. Although shown significantly that growth is affected by TC, it appears not to be inhibited as much as reproduction.

**ISO test (CNGG)**

Figure 4 shows a clear effect of TC on the reproduction in CNGG (F-value: 337.22; p < 2.2e-16). The average progeny per female is affected negatively with increasing concentrations. The treatments with concentrations of 5mg/L and higher are significantly different from the control.

![Figure 4: Test conducted in CNGG, 96h, N=5. Representation of the average reproduction (juveniles/female) per concentration of TC. Error bars indicate standard deviation. Significant differences treatment control are indicated: ‘*’ : p <0.05 ; ‘**’ : p <0.01; ‘***’ : p <0.001](image)

Remarkably the effect of TC on reproduction seems to be more pronounced in CNGG than in K-medium (lower IC values; table 2). The IC50 in K-medium is around 4 times higher, than in CNGG, even though the bacterial concentration is 25 times higher in CNGG. The higher concentration is found to be necessary in CNGG to allow *C. elegans* to reproduce, after preliminary experiments (data not shown). Other than the bacterial density...
and the composition of the two different media, the experimental setups do not differ. It was thrived to keep differences to the minimum, to make comparison possible.

The variance between replicas is larger in figure 5 at low concentration, but still within acceptable ranges. This variance decreases with increasing concentration. The data points line up with the plotted curve and no outliers were found.

**Population Growth Rate (PGR)**

Figure 6 shows the increase in absolute numbers over a period of time (15d). Due to limited resources, a carrying capacity (K) was reached after 9 days in the control and 1mg/L setup. It took 3 days longer in the 3, 7 and 17mg/L treatments to reach K and for the 68mg/L treatment K was barely reached after 15 days. TC seemed to effect the growing abilities of a *C. elegans* population negatively, requiring more generations to reach K.
Especially in the control and 1mg/L samples, the carrying capacity was surpassed after day 9, followed by a collapse in numbers (recorded at day 12). The same event was seen in the 3, 7 and 17mg/L treatments, were it 3 days later. No such high peaks could be confirmed in the 68mg/L samples, given the huge variation between them. However, the average values never seemed to transcend the plotted curve.

Figure 6: Population Growth Rate. Calculated non-linear regression curve. CNGG, 15 days, N=5. Data indicated with an ‘A’ refers to the average number of individuals counted per sampling day. Error bars indicate standard deviation. Data points are slightly shifted to the right (in reference to the control data points) for reading convenience. Both control and 1mg-treatment data points are displayed in figure 6A and 6B, to make comparison between figures possible.
The calculated PGR (r) was corrected and is shown in figure 7 as r'. This r'-value is an estimation of the actual PGR under unlimited conditions. The PGR differs significantly from the control in all treatments with exception of the first, indicating an effect of TC on C. elegans's growth rate in CNGG. The effect seemed to be negative for concentrations higher than 1mg/L. One exception was noted for 17 mg/L, which seemed to indicate a higher growth rate than the 7mg/L samples, but the difference between these treatments was found not to be significant (t-value: 0.663; p-value: 0.738), nor was there any difference between 3mg/L and 17mg/L treatments (t-value: -1.407; p-value: 0.384).

The dose-responsive curve (figure 8), shows an incomplete sigmoid curve. The used concentrations seem not to effect the PGR more than 50%. In comparison to the reproduction (table 2), PGR seems to be a more rigid parameter (e.g. IC50 is 10 times higher).

![Figure 7: Test conducted in CNGG, 15 days, N=5. Representation of the corrected calculated population growth rate r' per concentration of TC. Error bars indicate standard deviation. Significant differences between treatment and control are indicated: ‘‘‘‘: p <0.05 ; ‘‘‘‘‘: p <0.01; ‘‘‘‘‘‘‘: p <0.001. Test concentration are related to IC-values of the ISO-test (CNGG): IC10:1mg; IC30:3mg; IC50:7mg; IC70:17mg; IC90:68mg](image-url)
Food attraction

The checks after 24h yielded the most nematodes on average in each experimental setup (figure 9). Numbers increased over time, indicating that once a spot was reached nematodes probably stayed and only in few occasions a second choice was made. Remarkable is that in none of the replicas (data not shown), the total number (summation of both treatments) exceeded 15. On average not even 10 of the 20 nematodes transferred to the plates, made a final choice after one day. Most of them were located in the centre of the Petri dishes, but at a certain distance from where they were initially placed. These low numbers indicate a low attractiveness in general. Comparing treatments, figure 9A shows a small preference of contaminated food over the nutrition low K-medium after 24h. However, this difference is not significant (table 3). Figure 9B shows
fluctuating preferences over time, but a higher appetite for the uncontaminated food source after one day. The food preference was nonetheless not found to be significant (table 3). In the third experimental setup, both food sources were contaminated but treatments differed in their bacterial concentration. On average 7 nematodes made a choice in each replica (figure 9C), but without any clear fondness for one of the food sources (table 3). In summary, there seems to be no significant preference in any of the setups after 24h.

Figure 9: Food attraction, 24h, agar, N=8. The average number (grouping intra- and inter-replica data) of nematodes counted in each treatment at a certain time point. Error bars indicate standard deviation. 9A: TC-contaminated food source\textsuperscript{a} vs. K-medium. 9B: TC-contaminated food source\textsuperscript{a} vs. Uncontaminated food source\textsuperscript{b}. 9C: TC-contaminated food source\textsuperscript{a} vs. TC-contaminated food source\textsuperscript{b}

\textsuperscript{a} bacterial density: 5e9 cells/mL

\textsuperscript{b} bacterial density: 2e8 cells/mL
### Table 3 - Summarised results of the food attraction experiment after 24h

<table>
<thead>
<tr>
<th>Medium</th>
<th>G-value</th>
<th>DF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC-contaminated food source&lt;sup&gt;b&lt;/sup&gt; vs. K-medium</td>
<td>13.9</td>
<td>12</td>
<td>3.10</td>
</tr>
<tr>
<td>TC-contaminated food source&lt;sup&gt;b&lt;/sup&gt; vs. Uncontaminated&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.8</td>
<td>10</td>
<td>0.30</td>
</tr>
<tr>
<td>TC-contaminated food source&lt;sup&gt;b&lt;/sup&gt; vs. TC-contaminated food source&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.4</td>
<td>20</td>
<td>0.56</td>
</tr>
</tbody>
</table>

A G-test was used to assess likelihood between experimental setups.

- <sup>a</sup> degrees of freedom
- <sup>b</sup> bacterial density: 5e9 cells/mL
- <sup>c</sup> bacterial density: 2e8 cells/mL
Discussion

Material and Methods

The salt solution (K-medium) used in the liquid ISO tests, was found to be an easy to work with medium. It requires little preparation time and is easy to transfer. Furthermore it is a very clear solution, making observations possible. On the downside, the lack of an internal structure (matrix) or viscosity restricts the free movement of the nematodes in all dimensions. As a consequence all action happens at the floor of the wells as both bacteria and nematodes sink to the bottom, where gas exchange can be limited. This may induce stress, leading to a higher energy requirement (Williams & Dusenbery, 1990). However, given the small volume (1mL) and the relatively large surface area, gas exchange was not considered to be a problem. The competition for space however was. Given the short runtime of the experiment (4 days) and only 10 nematodes in each well at the start, the effects of space limitations could be cancelled out by the results of the control.

The more viscous CNGG medium brought solution because of its internal structure and allowed the nematodes to fully benefit from the 1mL volume. However, this meant increasing the bacterial density 25 times, because the bacteria would also disperse, leading to a relatively lower density. The medium had proven to be a good alternative (Muschiol & Traunspurger, 2007) and provides the possibility to accurately subsample. This was absolute necessity in the population growth experimental setup. However, the presence of an organic compound (Gelrite®), may have interacted with TC, resulting in bio-availability differences between the two test media. In a comparison study of both hydrophobic (ivermectin) and soluble chemicals (aldicarb and cadmium), Brinke, Heininger and Traunspurger (2011), concluded that the results between experiments conducted in liquid and CNGG test medium, only differed significantly in the case of cadmium. This effect was dedicated to the chemical properties of the divalent cation. As a consequence
the bio-availability was reduced, as cadmium might have been incorporated into the medium. CNGG was therefore not recommended for assessing heavy metal toxicity.

**ISO test (K-medium)**

As seen in figure 2, TC affects both reproduction and growth negatively. The LOECs (20mg/L and 5mg/L, respectively) however differ between parameters. This is not unusual as previous studies have shown that the correlation is test specific. Although reproduction is generally considered to be the more sensitive of the two (Traunspurger et al., 1997; Höss et al., 2009; Hao et al., 2010), differences are not always as pronounced (Höss et al., 1999). Although the variance between treatments and control were only to be found significant at 20mg/L when considering reproduction, the dose-responsive curves and the calculated IC-values proved differently. The contrasting significant levels, are possibly due to the high variability in progeny at low concentrations. The variance between growth is less pronounced. Figure 3 clearly shows how reproduction decreases more than growth under the experimental conditions. Therefore it can be said that *C. elegans* subjected to the test environment, reduces investments in progeny first. The decrease in growth follows only when the organism is exposed to much higher concentrations of TC (table 2). With this strategy, *C. elegans* favours its own survival over the production of progeny, possibly saving its energy for when conditions are more favourable.

**ISO test (CNGG)**

Different from the ISO test conducted in K-medium, the LOEC (reproduction) in CNGG is 5mg/L. A clear decrease in average progeny in reference to the control was highly significant. The media seems to differ even more in the calculate IC-values (table 2), leading to the conclusion that *C. elegans* is far more sensitive to the applied chemical in CNGG than in K-medium. These findings are in particular remarkable, given the fact that
the food available in the former was 25 times higher than in the latter. Furthermore, pH-tests showed no differences in acidity (both pH=5). It is unlikely that the Gelrite® present in the CNGG can be held responsible, because interaction between TC and the organic compound would have led to a smaller bio-availability, hence lower sensitivity. This is even more the case, because the presence of divalent cations (Mg$^{2+}$ and Ca$^{2+}$) could also reduce the bio-availability of TC. TC has been proven to be less toxic in soils or sediments with a high concentration of divalent metals, since tetracyclines are able to chelate with metal cations (Halling-Sørensen et al., 2002). Though presumably present, it is clear that these interactions were overwhelmed by another event.

Possible flaws in the experimental setup, may explain the apparently larger sensitivity in CNGG. The order and the recipient in which the liquid test were prepared, may be key to the noted differences. In preparation of the ISO test (K-medium), a dilution series of Tetracycline HCl dissolved in K-medium was prepared, of which 0.5mL was pipetted into plastic 12-well plates. This was followed by 0.5mL of bacteria suspended in K-medium, but with an inevitable time gap in between. Tetracycline HCl in solution breaks down to Tetracycline molecules and H$^+$ and Cl$^-$-ions. The hydrophobic TC molecules are likely to adhere to the plastic walls of the wells during the time gap and furthermore during the complete run time of the experiment, leaving little of the antibiotic to interact with both bacteria and nematodes. In preparation of the ISO test (CNGG), the different compounds were added in a different order. Firstly, 8mL Gelrite® and a bacterial pellet were mixed to a homogeneous suspension in a small centrifugal tube. Secondly, Tetracycline HCl diluted to the appropriate concentration with de-ionised water, was added to the suspension, followed by the salt solution that started the solidification of the CNGG medium. In a final step 1mL of the prepared CNGG was transferred to each well in a plastic 12-well plate, but by the time the medium was transferred, a matrix of compounds and bacteria had been established with more or less a homogeneous distribution. Even though the medium was
stirred right before pipetting, causing it to liquify again, the CNGG solidified rapidly afterwards. The chemical properties of the medium, possible prevented sorption of TC to the plastic walls to some extent, leaving more to interact with the present organisms. In addition Chen et al. (2011) concluded that the presence of Mg\(^{2+}\) - ions, may slow down the normal degradation by the formation of metal-ion complexes, leading to a relatively higher concentration of TC present in the medium.

At the time, there was no notion that the preparation order and recipients used may have possibly influenced the results, simply because of alterations in bio-availability. Similar problems were countered by Brinke, Heininger and Traunspurger (2011) by the usage of glass wells. Glass or inert recipients and different preparation methods could reduce the variation in results between the tests run in the two different media.

As stated by Brinke, Heininger and Traunspurger (2011), the sorption of an antibiotic to food bacteria is most likely to happen, however this must not be considered as an experimental error. Free-living nematodes, such as *C. elegans*, can be subjected to contamination in many ways. Next to dissolved toxins in their surrounding aqueous environment, particle-bound and bacteria-bound chemicals are plausible to increase contact with a certain compound (Chandler et al., 1994; Höss et al., 2001; Offerman et al., 2009).

It is clear that the medium composition influenced both the toxicity and the bio-availability of TC in the performed tests. Similar problems have been addressed more in depth by Brinke, Heininger and Traunspurger (2011), Donkin and Williams (1995) and Khanna *et al.* (1997).

**Population Growth Rate (PGR)**

Figure 7 shows a significant decrease in *C. elegans*’ growth rate from 3mg/L onwards. This concentration corresponds with the IC30 (reproduction) found in the ISO
test (CNGG). Looking at the dose-responsive curve (figure 8), 3mg/L correlates with a 10% inhibition of the nematodes growth rate. Furthermore, table 2 provides a clear overview on how IC-values variate between different characteristics. The relatively high toxic effects of TC seen as low IC-values for reproduction, are not reflected in those for the PGRs. This is remarkable, because the effect on individual reproduction seems to be buffered by the population. Especially an estimated IC50-value for PGR 10 times higher than its counterpart for reproduction, is worth mentioning. These findings might question the ecological relevance of endpoints such as reproduction and growth, tested in ISO tests (Forbes & Calow, 2002; Höss & Williams, 2009). Nonetheless, it can be considered a good thing that the population might survive a greater toxic stress than could be expected from ISO test derived results. Although, one should be careful in reflecting conclusions drawn from experiments conducted in the lab to the natural world. As seen in figure 6, concentrations of 3mg/L and higher, result in a 3 day delay of two events (in reference to the control), both in reaching the highest peaks as in reaching the carrying capacity. This may seem insignificant at first, but given the fact that C. elegans’ generation time is approximately 2.5 days, a whole generation is lost. After an ecological catastrophe, this delay may hamper recovery of the system to a great extent.

Furthermore it has to be considered that the concentration during the test was never consistent. Even though it was thrived to keep the concentration within certain limits by adding fresh medium every 3 days, this could not counteract degradation of TC. Tetracyclines tend to degrade easily under intense light (photolysis: Oka et al., 1989, Chen et al., 2011) and therefore the wells were kept in the dark at a constant temperature. However, the plates were inevitable exposed to light every 3 days (subsampling). Even under complete darkness tetracyclines degrade (Mitscher, 1978; Halling-Sørensen et al., 2002), leaving a lot of degradation products to interact with both nematodes and bacteria. Given the runtime of the prolonged experiment, it is highly plausible that concentrations of
certain compounds at the end were very different from the start of the experiment. Not knowing the consistence of the medium at given time points, it is hard to make any conclusion strong. Furthermore, by disturbing the medium every 3 days and thereby liquifying it, chances are that sorption of TC to the walls of the well was unintentionally stimulated. Additionally the competition for space in a limited volume (1mL), might have brought about extra stress and bias the results.

Taking all this into account, it is most likely that the effects of TC on C. elegans’ PGR were underestimated, but the continuous supply (addition of TC every 3 days) may reflect an antibiotic’s pseudo-persistency in the environment (Koschorreck & de Kecht, 2008). Therefore this experimental setup may be considered ecologically relevant.

The growth rate of C. elegans N2 (Bristol, UK) was estimated by Muschiol et al. (2009) to be 1.375, which is slightly higher than the estimations of r’ in this study (1.24 ± 0.075). In a different lab estimates of the population growth rate were around 1.5 (Anderson et al., 2011). Given the variance between labs, probably due to overaged stocks, the estimated growth rate of this study can be accepted. Because of its close approximation, the experimental conditions in the control are likely to produce good estimates of PGR.

Recent studies on Daphnia magna Straus showed a similar negative effect of TC on the population growth rate (Kim et al., 2012). However, the effect concentrations were much lower. 0.1mg/L effected the PGR by 30% and concentration up to 5mg/L reduced the growth rate by 60%. Kim et al. (2012) described a slight increase in body mass, correlated with the loss of PGR. Even though this correlation was not checked in this study and given the experimental differences, it can be carefully suggested that C.elegans’ PGR is less sensitive to TC than the PGR of D. magna.
**Food attraction**

Although a trend towards uncontaminated food was seen in figure 9, the statistical analysis showed no significant differences in any of the experimental setups. The distribution of the nematodes seemed to have occurred at random. This conclusion is strengthened by the fact that an apparently high number was attracted to the K-medium. Since attraction towards contaminated food (Kenney et al., 2006) and nematodes feeding behaviour after exposure to contaminants have been described (Dhawan et al., 1999; Dhawan et al., 2000; Anderson et al., 2001; Boyd et al., 2003; Anderson et al., 2004), it is imaginable that the seemingly randomness is a result of a bad experimental setup. Even though nematodes possess an arsenal of sensors (Lee, 2002), it can be that the presence of certain chemical compounds limits their abilities to locate food (Riga, 2004; Donkin & Williams, 2000). In those cases an unambiguous experimental setup is the key to drawing conclusions. Unfortunately, the high number of cavities (8) in the food attraction test plates (figure 1) and the limited number of replicas (N=8) may not have been a proper experimental design. It is plausible that nematodes attracted to a food source of their choice, fell by accident in the wrong cavity. Furthermore, given the tension forces between liquid K-medium and solid agar, it might have been hard for the nematodes to escape these forces. Although a different experimental setup may yield better results, the experiment is not considered to be a total failure. If TC-contaminated manure enters the environment of a nematode population, it is likely to be dispersed in a very heterogeneous manner on micro scale. In this context the TC present may affect the nematodes ability to track food, to date this has not been proven. Given the fact that the nematodes survived for 15 days, with only contaminated food at their disposal in the PGR-experiments, it is likely that C. elegans feeds even upon TC-contaminated E. coli.
**C. elegans** among others

It has been shown that Tetracycline affects non-target organisms to some extend. Wilson et al., (2004) tested the effects of 4 antibiotics (tetracycline, TC; chlortetracycline, CTC; oxytetracycline, OTC and doxycycline, DC, mixed in different concentrations) on the structure, function and interactions of the plankton community. Wilson and his team recorded a significant reduction in abundance of phytoplankton, even at weighted concentrations as low as 0.218 μM (24.9μg/L TC, 25.1μg/L OTC, 29.4μg/L, CTC and 24.9μg/L DC). However the communities recovered after the treatment was ended. A more significant decrease was recorded when the concentrations were higher than 0.662 and 2.29 μM (72.0 μg/L TC, 79.9 μg/L OTC, 75.9 μg/L CTC, 76.1μg/L DC and 260 μg/L TC, 296 μg/L OTC, 208μg/L CTC, 281μg/L DC, respectively). The effects were strong in all tested groups, but more expressed in the cyanobacteria, cryptophyta and dinophyta groups. The effect concentrations are relatively low in comparison with those found in this study. Plankton seems to be affected in a range of concentrations one magnitude lower than C. elegans, but like C. elegans, plankton may not be at risk, given the relatively low concentrations found in surface waters today (table 1).

Araujo and McNair (2007) described a reduced lifespan and reproduction among rotifers. Lifespan as such was not used as an endpoint in this study, but reproduction was. IC10-values were found between 26.10-28.34mg/L (B. calyciflorus) and 28.89-29.85mg/L (B. plicatilis) and IC50-values were estimated between 78.81 - 79.56mg/L and 119.41 - 121.47 mg/L, respectively. Given the differences in experimental setups, comparisons should be made carefully, but both IC-values are much higher than those found for C. elegans in both test media (table 2). This may indicate a higher tolerance of rotifers for TC-contamination in comparison with C. elegans. Moreover, rotifers are unlikely to be affected, because the concentrations discovered so far in their habitats are significantly lower than the described IC-values.
The reproduction of *D. magna* has been tested by Wollenberger *et al.* (2000) and effect concentrations (EC) were calculated. EC10 (29.4 mg/L) and EC50 (44.8 mg/L) are higher than those found in table 2 for both test media. But again it has to be stressed that different experimental setups yield results that are hard to compare. Remarkable is the relatively small difference between EC10 and EC50, which is approximately 15mg/L. Although both ICs are lower for *C. elegans* in both experimental setups, the comparative differences between IC10 and IC50 are bigger. This indicates that *D. magna* tolerates relatively high dosages of TC, but once a certain concentration has been reached, its reproduction is affected strongly. *C. elegans* is less robust and its reproduction is inhibited gradually with increasing concentrations. However as discussed above, Kim *et al.* (2012) described an earlier effect on *D. magna*'s PGR. The growth rate decreased up to 30% for concentrations of 0.1 mg/L and up to 60% for 5.0 mg/L TC. In comparison, *C. elegans*’ growth rates are affected less severely. These interesting differences between individual reproduction and population growth reflect different ecological strategies and make it even harder to assess possible toxic effect of TC in different environments. It is plausible that *D. magna* tolerates TC better than *C. elegans* if contact with the antibiotic is limited in time. Nonetheless, populations of *D. magna* may find it harder to survive if TC is (pseudo)-persistent in the environment, even at low concentrations.

Although dissimilar inhibition concentrations were found between different media and endpoints, none of them seemed to be in the range of concentrations found in the environment today. The apparently high tolerance of *C. elegans*, may indicate that accidental leaches of TC in the environment cause little harm. Nevertheless one should keep in mind that ecosystems are much more complicated than single-species tests, because of the complex interactions that exist between different organisms. Consequently the addition of indirect and direct effects of chemical compounds may lead to a far greater effect. Only through integration of different disciplines and more in depth research, a
proper risk assessment can be made. This study is just one of the many pieces of the puzzle, and in itself it has little meaning.
Acknowledgement

First and foremost I want to thank Dr. Nicole Spann for her guidance and support throughout the entire process. Without her help I would not have been able to complete this thesis successfully. I express my gratitude to my promotor Prof. Dr. Walter Traunspurger for his expertise and advise. Special thanks to Mrs. Stefanie Gehner for providing me with all the means to run my experiments and her assistance in the lab. I thank Dr. Kai Ristau and Sebastian Weber for helping me with the experimental setup. In extension, all members of the Department of Animal Ecology (University of Bielefeld), made me feel welcome and created a nice working atmosphere. Let it be clear that without the financial support from the European Master of Science in Nematology (EUMAINE) programme, this work would not have been possible. I express my gratitude for providing me with this wonderful opportunity. I want to thank all the interesting people I have met along the way, especially those who provided me with new scientific insights. I extend my thanks to all professors and people working hard in and around the nematology labs at Ghent University and the University of Jaén, in particular Mrs. Nicole Smol and Prof. Dr. R. Peña-Santiago. Last, but certainly not least, I want to thank my parents and girlfriend for supporting me in all my endeavours.
References


HALLING-SØRENSEN, B., SENGELØV, G., & TJORNELUND, J. (2002). Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria, including
selected tetracycline-resistant bacteria. *Archives of environmental contamination and toxicology*, 42(3), 263-71.


Declaration

I, Vangheel Matthew, hereby confirm that I have independently composed this Master thesis and that no other than the indicated aid and sources have been used. This work has not been presented to any other examination board.

Bielefeld, 20th August, 2012

Vangheel Matthew