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# Development of a new ecotoxicological assay using the testate amoeba *Euglypha rotunda* (Rhizaria; Euglyphida) and assessment of the impact of the herbicide *S*-metolachlor

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## HIGHLIGHTS

- An ubiquitous soil testate amoeba as test organism in ecotoxicology.
- An ecotoxicological assay including a small microbial trophic chain.
- Direct observation of atypical behaviours and structures as potential endpoints.
- Non-linear effect of the herbicide *S*-metolachlor on *E. rotunda* growth.
- Strongest inhibition observed at exposure of 15 μg/L.

*Keywords:*
Soil
Microorganisms
Protist
Protozoa
*Escherichia coli*
Pesticide

## ABSTRACT

An ever-increasing diversity of potentially toxic chemical compounds are being developed and released into the environment as a result of human activities (e.g. agriculture, drugs, and cosmetics). Among these, pesticides have been shown to affect non-targeted wildlife since the 1960s. A range of ecotoxicological tests are used to assess the toxicity of pesticides on various model organisms. However most model organisms are metazoans, while the majority of Eukaryotes are unicellular microorganisms known as protists. Protists are ubiquitous organisms of key functional roles in all ecosystems but are so far little studied with respect to pesticide impact. To fill this gap, we developed a new ecotoxicological test based on *Euglypha rotunda*, a common soil amoeba, grown in culture flask with *Escherichia coli* as sole food source. We tested this assay with the herbicide *S*-metolachlor, which is known to affect cell division in seedling shoots and roots of weeds. Reproducible growth conditions were obtained for *E. rotunda*. The growth of *E. coli* was not affected by the herbicide. The growth of *E. rotunda* was affected by the herbicide in a non-linear way, growth being significantly reduced at ca. 15 μg/L, but not at 150 μg/L. Our results show the potential for using soil protists in ecotoxicology and adds to the growing body of evidence for non-linear impacts of pesticides on non-target organisms. With the acquisition of additional data, the protocol should be suitable for standard ecotoxicological tests.

## 1. Introduction

Various chemical compounds have become part of our modern societies and are spread intentionally (e.g. in agriculture) or not (e.g. drugs, cosmetics, etc.) in the environment (Schwarzenbach et al., 2006). Among these, pesticides are widely applied on crops with aim to protect them from pests and pathogens or to suppress competitive weeds. In addition, pesticides are used in public health program to control vectors of human diseases especially in the tropics (World Health Organization, 1990; Chen et al., 2010). However, the world-wide use of these substances has led to direct and indirect damages to non-target organisms, including humans and natural antagonists of pests (Carson, 1962; Wilson and Tisdell,

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2001; Pimentel, 2005; Moebus and Bödeker, 2015). In addition, only a small fraction of the applied pesticides typically reach the target pests, while most contaminates the soil, air or water compartments of ecosystems, thus presenting a potential risk to various organisms (van der Werf, 1996; Arias-Estévez et al., 2008). The need to assess the effects and impacts of pesticides and their metabolites led to the development of a broad range of ecotoxicological tests over the last 40 years (Férard and Blaise, 2013; Newman, 2015).

Internationally validated standard guidelines for assessing the impact of pesticides on the environment including soils are now provided by organizations like the Organization for Economic Cooperation and Development (OECD), the US Environmental Protection Agency (USEPA) and the International Organization for Standardisation (ISO). Current testing protocols however focus on a small number of species that cover only a fraction of the overall taxonomic and functional diversity existing in ecosystems (Ruden et al., 2016; Ockleford et al., 2017). This discrepancy is particularly obvious for the soil environment, which can thus be considered to represent the "extreme end of the RA (*Risk Assessment*) spectrum" (Ockleford et al., 2017). Existing relevant ecotoxicological assays for soils focus mainly on invertebrates including the compost earthworms *Eisenia fetida* or *E. andrei* (Lumbricidae) (OECD, 2004), the springtail *Folsomia candida* (Collembola) (OECD, 2009), and the mite *Hypoaspis aculeifer* (Acari) (OECD, 2008) (van Gestel, 2012; Ockleford et al., 2017). The bulk of eukaryotic diversity, and in particularly micro-Eukaryotes including protists is not represented among model organisms and hence we currently lack the necessary tools to assess the potential impact of pesticides on this highly diverse and functionally central component of soil biodiversity (Geisen et al., 2018).

Microorganisms including bacteria, fungi, micro-Metazoa and protists play many key roles in the maintenance of soil fertility by contributing to the stabilisation of soil aggregates, litter decomposition, and nutrient cycling (Barrios, 2007; Nielsen and Winding, 2002). Microorganisms are believed to be particularly sensitive to pollution (van Beelen and Doelman, 1997; Plaza et al., 2010), but existing knowledge on the effects of pesticides on soil microbial communities shows that it is highly variable, with both positive and negative impacts detected depending on the compound and the species investigated (Lo, 2010). These results call for an increased research effort to better understand the effects of pesticides on soil microorganisms. Currently, only one test is routinely done to assess the impact of pesticides on soil microbes, as soil microbial nitrogen transformation (OECD, 2000) (Ockleford et al., 2017). Most existing studies on microorganisms focus on bacteria, some on Archaea. Studies on soil micro-Eukaryotes have historically dealt primarily with fungi and nematodes, but focus now increasingly on protists (Geisen et al., 2018).

Besides representing the majority of the eukaryotic diversity, protists support essential ecological functions in soils. As predators of bacteria, fungi, other protists and micro-Metazoa, parasites, primary producers, they play central roles in many biogeochemical cycles and contribute to plant growth (Bonkowski, 2004; Coûteaux and Darbyshire, 1998; Xiaoyun et al., 2007; Geisen et al., 2018). Most protists are unicellular but nevertheless exhibit a high structural and physiological complexity; some are easy to cultivate in the laboratory and could thus potentially represent useful ecotoxicologial indicators (Foissner, 1999; Gerhardt et al., 2010; Gomiero et al., 2013; Mansano et al., 2016). Several studies reported adverse effects of pesticides on different protists, especially on ciliates: for example the concentrations of 50 and 100 ppm DDT inhibited the growth and cell division of *Stylonychia notophora* (Lal and Saxena, 1980); the toxicity of 39 pesticides was investigated on *Colpidium campylum* and could be to a certain extend related to the chemical structures of the compounds (Dive et al., 1980); the growth and respiratory metabolism of *Paramecium* spp. (Alveolata) was reduced when exposed to diflubenzuron or flucycloxuron (Rouhabi et al., 2006). Todorov and Golemansky reported in 1992 an overall strong toxicity of Lavendotricin (at 0,001%, 0,01, and 0,1%) on a ciliate (*Blepharisma japonicum*), an amoeba (*Amoeba proteus*) and a testate amoeba (*Arcella vulgaris*) but more species-specific response with Fuzamicin and Fundasol. The lowest concentration of Fuzamicin (0.001%) did not influence *B. japonicum* growth, stopped reproduction of *A. vulgaris* on the fifth or tenth day, while all *A. proteus* died on the first day. Fundasol was mostly toxic for *A. proteus* (suppression of reproduction at 0.001% and 0.01%, death on the first day at 0.1%) when only the highest concentration (0,1%) affected the two other protists (complete inhibition and reduced reproduction for resp. *B. japonicum* and *A. vulgaris*). More recently, the reproduction of the slime mould *Dictyostelium discoideum* (Amoebozoa) was reported to be inhibited when exposed to mercury (II) chloride (1 μM) or Diazinon (0.1 μM) at 26 °C, and to cadmium chloride (10 μM) or Dicofol (0.1 μM) at 28 °C (Amaroli, 2015). Despite these efforts supporting the relevance and sensitivity of protists, to our knowledge not a single model organism for ecotoxicology exists among the Rhizaria, a highly diverse group of terrestrial and aquatic protists (Burki and Keeling, 2014). The soil testate amoeba, *Euglypha rotunda*, is available from the culture collection of algae and protozoa (CCAP), is ubiquitous in soils and appears as an interesting candidate model organism for soil protists in general. Testate amoebae are good bioindicators of environmental quality (Chardez and Lambert, 1981; Clarke, 2003; Geisen et al., 2018) and were also reported to be more sensitive to pesticides than ciliates in agroecosystems (Foissner, 1997).

In this study, we aimed to develop a robust ecotoxicity test with *Euglypha rotunda.* In particular, we aimed to defining i) the factors that influence its growth in the laboratory, and ii) the conditions that support a reproducible growth allowing to testing the effects of chemicals. We then investigated the effects of the herbicide, *S*-metolachlor, on the growth of *E. rotunda* and *Escherichia coli*, co-inoculated to serve as carbon source. *S*-metolachlor is used on crops such as corn, soybeans, sorghum and beetroot and is among the top three most used chloroacetamides (Gutowski et al., 2015). It affects the production of very-long-chain-fatty acids, and is reported to inhibit cell division in the shoots and roots of weeds (Saitoh et al., 1996; Vallotton et al., 2008). Cell division being the most common reproduction strategy of testate amoebae (Meisterfeld, 2002), we expected the population growth of *Euglypha rotunda* to by inhibited by *S*-metolachlor.

## 2. Materiel and methods

### 2.1. Culture conditions

We used a stock culture of *Euglypha rotunda* CCAP 1520/1 grown on agar NCL/0.01% NPA from the Laboratory of Soil Biodiversity of the University of Neuchâtel. The amoebae were transferred into culture flasks (CELLSTAR Filter Cap Cell Culture Flasks with a growth area of 25 $cm^2$, Cat.-No 690 175) containing 10 ml of "Volvic growth medium". The protocol for the Volvic growth medium was initially developed by Ogden and Meisterfeld (1991) based on filtered pond water. The modified protocol used in this project was as follows: To 1,5 L of commercial Volvic water, 30 mg $KNO_3$, 15 mg $KH_2PO_4$, 6.5 mg $Na_2SiO_3$ (anhydrous), 13.3 mg $Na_2$-EDTA x $2H_2O$, and 11 ml of soil extract are added. The soil extract was obtained following the protocol of the Culture Collection of Algae and Protozoa (CCAP) Soil Extract 2 (SE2) (see Supplementary Materials). The soil was sampled in the Botanical Garden of Neuchâtel in a location free of pesticides and fertilizers. The amoebae were grown with *Escherichia coli* as carbon source. The gram-negative bacteria

*Escherichia coli* MG1655 grown on agar was obtained from the Department of Fundamental Microbiology of the University of Lausanne. The bacteria were transferred into 10 ml liquid growth medium in the same culture flask as the one used for the amoebae. All cultures were incubated at 12 °C, in the dark.

Cultures of *E. rotunda* with less than 100 initial individuals, 100−300, and more than 1000 were monitored over a maximum of five weeks (performed at least in duplicates). These cultures were all grown with low bacterial density (i.e. $OD_{630}$ ~0.08, $10^3$ cells/μl - estimation obtained with a hemocytometer (microscopic counting chamber type "Improved Neubauer cell") at a total magnification of 400×). Furthermore, two cultures with an initial density of 100−300 protists were grown with high bacterial density (i.e. $OD_{630}$ ~0.3, $10^5$ cells/μl) and monitored once a week for 42 days. One culture started with 100 individuals was weekly monitored for 114 days.

The bacterial growth was monitored directly from the protozoan culture flasks, and from pure bacterial cultures. Three cultures were started with similar bacterial density; two were inoculated in culture flasks that already contained 100−300 protists, while the third was inoculated in a culture flask free of protists. The bacterial growth was monitored once a week for five weeks. The bacterial growth was also monitored for 22 days in cultures containing initially more than 1000 protists. Counts under the microscope were done every two or three days.

### 2.2. Quantification

Different quantification methods were considered. Direct count of *E. rotunda* was performed using an inverted microscope (Leica DMI4000 B with a camera Leica DFC3000 G). The use of an inverted microscope was preferred to an upright microscope as the amoebae are lying on the surface of the culture flask. The number of *E. rotunda* was reported over twenty pre-defined lines per flask, according to one of the three categories: "alive" (i.e. nucleus, cytoplasm and/or pseudopods clearly visible), "dead/empty test", or "undefined". The phase contrast allowed to observe details like nucleus and pseudopods. All counts were done at a total magnification of 200× (400× in case of doubt). The dimensions for the visual square of the screen was given by the Leica Application Suite and estimation per flask was calculated as follows:

$$\text{Number } E.rotunda\ [\text{ind/flask}] = \frac{\text{Number } E.\ rotunda \text{ sample [ind.]x Total surface } [\mu m^2]}{\text{Sampled surface } [\mu m^2]} \quad (1)$$

Bacteria were monitored mainly using hemocytometers (Improved Neubauer cell) and a total magnification of 400× with the bright field (microscope Olympus BX53). Counts were always performed in duplicates: a volume of 10 μl was put on each side of the hemocytometer. All the cells observed over four lines of the central square were reported, covering a surface corresponding to a final volume of 0.02 μl.

The hemocytometer cell counts were confronted with the measures of Colony Forming Units (abbr. CFUs) during the definitive test to investigate the effects of the herbicide *S*-metolachlor (see paragraph 2.3). A serial dilution (500 μl from the culture in 4500 μl of 0.9% NaCl water) was performed up to $10^{-4}$. A volume of 100 μl from this last dilution was then homogenized on prepared Lyria-Bertani (LB) agar medium with a sterilized glass spreader to enable the growth of isolated colonies (performed in duplicate). The petri dishes were sealed with parafilm and left upside down at room temperature for 48 h. Colonies could then be counted. The dilution of $10^{-4}$ was identified as suitable based on preliminary tests.

### 2.3. Testing S-metolachlor on the growth of E. coli and E. rotunda

A stock solution of 1000 μg/L *S*-metolachlor (from Sigma Aldrich©; grade: analytical standard, product line: PESTANAL, product number: 33859, purity: assay (HPLC) 98.2%) was prepared and stored at 4 °C, in the dark. The solution was prepared by adding 1 μl from the pure solution (from Sigma Aldrich) to 1 L of MQ-water. The concentration of the stock solution was analysed with a Liquid Chromatography coupled to Mass Spectrometry (LC-MS) at the Central Environmental Laboratory (CEL) of the Swiss Federal Institute of Technology Lausanne (EPFL). The stability over time of *S*-metolachlor in the Volvic growth medium was evaluated by comparing this first measurement with a second one, performed four months later. Solutions containing the theoretical concentrations of 4.5 μg/L and 121.5 μg/L of *S*-metolachlor were analysed twice with an interval of 35 days. Inoculated cultures (co-culture and bacterial culture) exposed to 121.5 μg/L of *S*-metolachlor were analysed as well after 35 days. The latter were prepared using a Whatman GD/X filter with a pore size of 0.2 μm before analyses with the LC-MS.

The evaluation of the effect of *S*-metolachlor on the organisms was performed in a two-steps approach. The organisms, in co-culture with *E. coli*, were first exposed to 5 concentrations of *S*-metolachlor (1.5 μg/L, 4.5 μg/L, 13.5 μg/L, 40.5 μg/L, and 121.5 μg/L; Volvic growth medium) and monitored during four weeks. The pesticide, being water soluble, was directly added to the liquid growth medium. This preliminary test (also referred as pre-test in the present study) aimed to provide a first overview of concentrations to test, and was done therefore without any replicates. The definitive test followed almost the exact same procedure than the pre-test but in triplicates and with the following four concentrations: 0.1 μg/L, 1.5 μg/L, 15 μg/L, and 150 μg/L. The amoebae were monitored once a week using the inverted microscope. A test with the bacteria *E. coli* alone were also performed, with the similar concentrations, and monitored once a week using the microscopic counting chamber. *Escherichia coli* was monitored in addition using serial dilution and CFUs at the beginning and at the end of the definitive test. Furthermore, abiotic cultures, with identic *S*-metolachlor concentrations, served to monitor the effect of time on the environmental variables (pH, dissolved oxygen and conductivity).

Evaporation was monitored by weighing the flasks each week. The evaporated volume constituted of maximum 16% during the whole test duration and was counterbalanced with the appropriate volume of Volvic growth medium.

### 2.4. Ancillary parameters and data analyses

Ancillary parameters (pH, dissolved oxygen and conductivity) were measured in all tests with a pH Meter (Model 350, JENWAY)

and a Multi portable Meter (for dissolved oxygen and conductivity; Model 350i, WTW). All data analyses were carried out on the open source statistical software R (R Core Team, 2016). A first descriptive analysis was performed by plotting the estimated population versus time. The growth of amoebae being exponential, the response variable (i.e. estimated number of individuals) was transformed using the natural logarithm to allow better comparison among samples. The data transformation was computed using the R package base (R Core Team, 2016). A least-square regression was performed on the ln-transformed data to obtain a linear model using the R package stats (R Core Team, 2016). The slope value was used as proxy for the growth rate. Six growth rates from cultures grown in similar conditions (low bacterial density, monitored over 28 days, no pesticide exposure) were used to draw a control chart. The minimum and maximum values were taken to define an interval in which growth rates could be expected under standard conditions. The growth rates obtained from the first test and the definitive test were compared to the control chart to evaluate an effect of the pesticide. When mean values are reported, the standard error around the mean is displayed as well.

## 3. Results & discussion

### 3.1. Finding the best conditions for reproducible growth

We tested the influence of three factors expected to affect the growth of *Euglypha rotunda* using different set-ups (Fig. 1a): (1) starting population of *E. rotunda*, (2) starting bacterial density, and (3) test duration. The growth of *Escherichia coli* was monitored as well (Fig. 1b), following different set-ups to investigate the influence of: (1) the starting bacterial density, (2) the presence and density of the bacterivorous amoeba, and (3) the duration.

The starting number of amoebae clearly influenced the growth rate of the population (Fig. 2a, b and c). An initial density of 100–300 amoebae resulted in an exponential growth (i.e. with constant growth rate as shown in Fig. 2b) while the multiplication rate was slower and linear for lower (<100) (Fig. 2a) and higher initial density (>1000) (Fig. 2c). With an initial density of 100–300, one population of amoebae showed a possible lag phase up to seven days before entering the exponential phase (Fig. 2b dark points), while the two other populations seemed to enter the exponential phase earlier (Fig. 2b grey and white points). Special care is therefore required when inoculating to achieve similar initial density of 100–300 amoebae per flask to allow exponential growth over the 28 days of the test, and thus to ensure the reproducibility of the results. By contrast, the bacterial density had no significant effect after four weeks (data not shown). The population growth of *E. rotunda* was exponential for up to 91 days when started with 100–300 amoebae (Fig. 2d), and then collapsed. The generation time was observed of being circa seven days. In a recent study, Wanner et al. (2016) investigated the growth of testate amoebae (including *E. rotunda*) under different silicon concentrations (50, 100, 150 μmoL/L). They reported that a population started with a single *E. rotunda* reached the exponential phase as early as after 15 days (with a silicon concentration of 150 μmoL/L) to the latest after 20 days (with a silicon concentration of 50 μmoL/L). Moreover, after exponential growth the population (started with six individuals) reached a stationary phase after 28 days which remained until the end of their experiment without death phase. Despite the different experimental conditions (initial density (100–300 ind. vs 1–6 ind.), carbon source (*E. coli* vs. *Saccharomyces cerevisiae*), silicon concentration (1,5 μmoL/L vs 50, 100, 150 μmoL/L)), a duration of 15–28 days seems appropriate to obtain a population in exponential phase in both setups (i.e. the present study and the study of Wanner et al., 2016). We therefore choose a duration of 28 days for the ecotoxicological test. Indeed, the effect of a chemical is best observed during the exponential phase, where the organisms are not limited by nutrients or their growth hindered by the accumulation of waste materials (Newman and Unger, 2002; Madigan et al., 2011). Several ecotoxicological tests on invertebrates also use a period of 28 days (van Gestel, 2012), including earthworms (*Eisenia fetida*/*E. andrei*, ISO, 1998 and OECD, 2004), the mollusca *Helix aspera* (ISO, 2006), or the collembola *Folsomia candida*/*F. fimetaria* (ISO, 1999; OECD, 2009).

The experimental conditions did not support *E. coli* growth. This is not surprising as *E. coli* is mesophilic (Kumar and Libchaber, 2013)

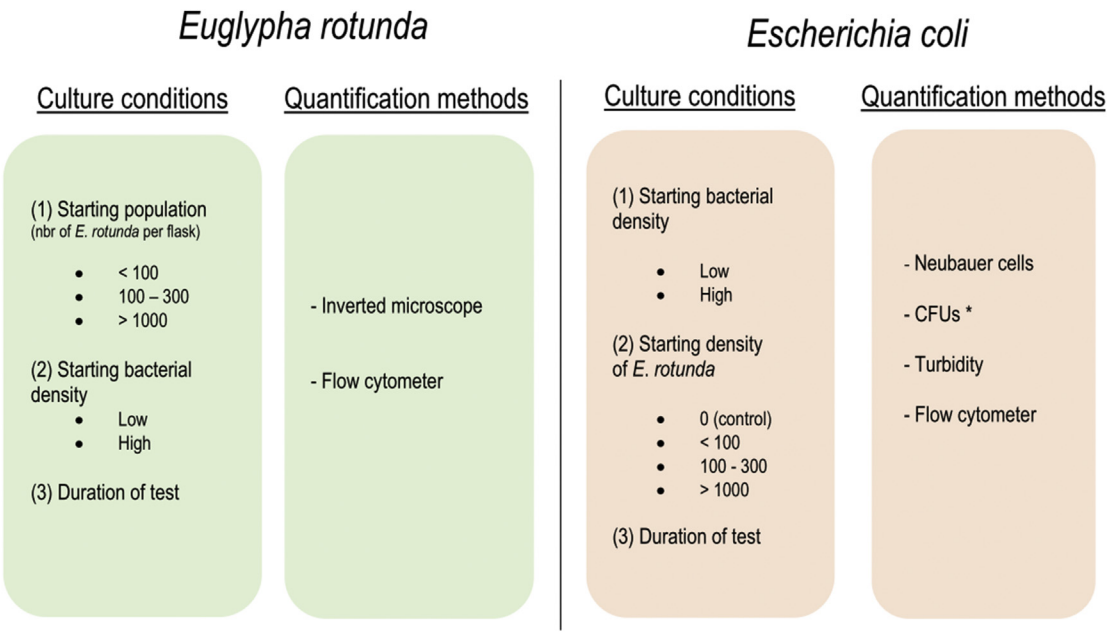


**Fig. 1.** Synoptical diagram of the performed experiences to investigate the parameters influencing the growth of *E. rotunda* (a) and *E. coli* (b) and to define the conditions allowing reproducible growth. The colony forming units assay (CFUs; signalized with a star) were only performed completely during the definitive test.

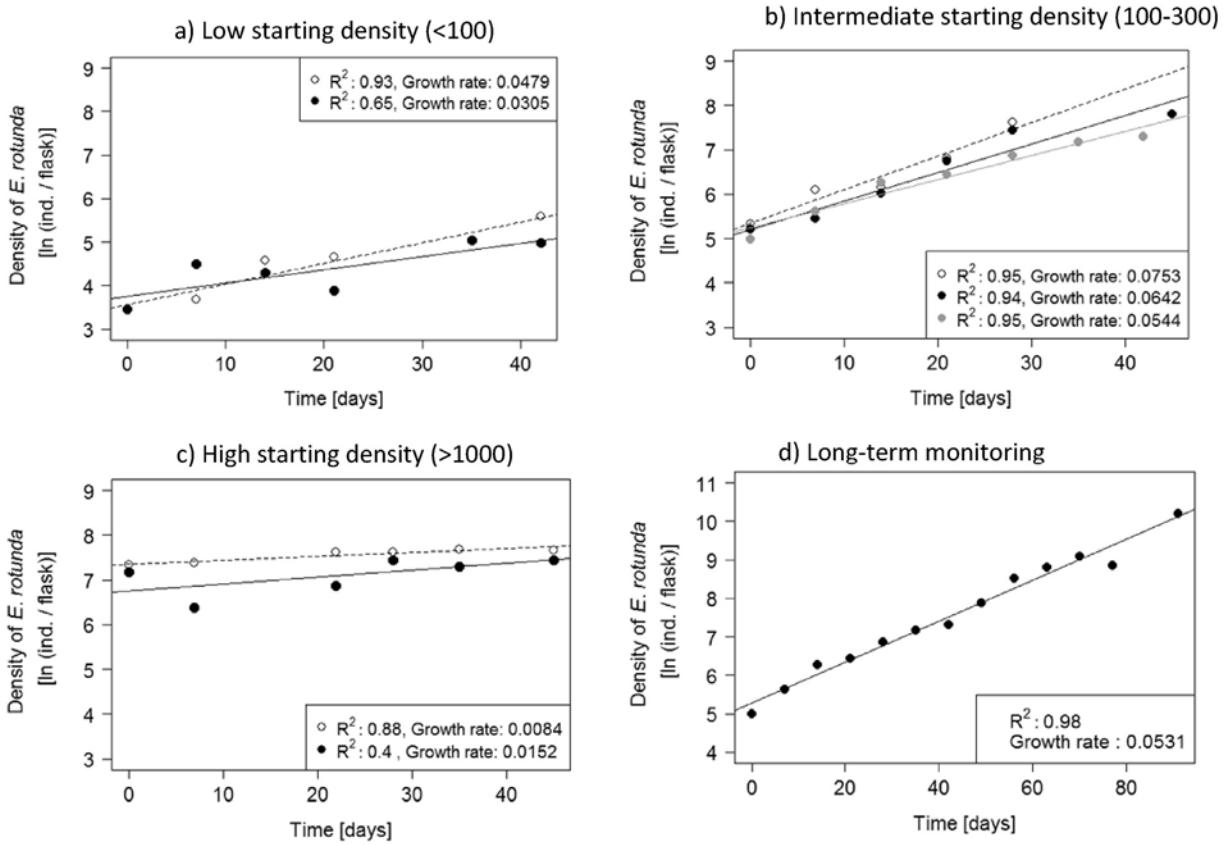


**Fig. 2.** Temporal pattern of different culture of *Euglypha rotunda*. The graphs show different patterns depending of the starting density of amoebae: lower than 100 (a), 100–300 (b), and more than 1000 (c). The last graph (d) shows a culture started with 100 individuals and monitored over 91 days; the values of the density are ln-transformed. The growth rates correspond to the slope of the linear models.

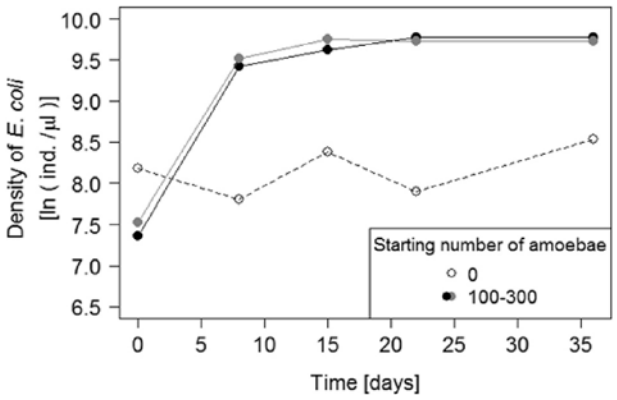


**Fig. 3.** Estimated population density of *Escherichia coli* over time in the control and in presence of the predator *Euglypha rotunda* at two initial densities. The estimation was done with the hemocytometer (Improved Neubauer cell).

and the growth medium contained a limited carbon source from the soil extract. However, when grown with an initial number of 100–300 amoebae, the bacteria showed an increased cell density after four days (Fig. 3). Bacteria may indeed directly profit from nutrients released by their predators (Hahn and Höfle, 2001).

Even if the bacteria could only grow and divide in a very restrictive way, their abundance was still sufficient to allow the amoebae to grow for several weeks, as shown by their exponential growth rate. The amoebae also showed low activity and only a slight increase in growth when grown with high bacterial density. *Euglypha rotunda* occurs in a broad range of rather nutrient poor conditions, being very common in forest and grassland litter and is rare in suboptimal habitats such as skeletal or saline soils (Bonnet, 1992); it may therefore not be adapted to growing fast on an unlimited supply of bacterial prey, as also illustrated by the total disappearance of all testate amoebae under actively decomposing pig cadavers (Szelecz et al., 2014). High bacterial population may build biofilms that would enhance the complexity of the system. Being specialized surface grazers, amoebae would probably not be much affected (Zhang et al., 2014). However, the tested chemical could be adsorbed to the biofilms, thus modifying the experimental conditions (Behra et al., 2002; Guasch et al., 2010). The choice of a low initial bacterial density is therefore preferred.

### 3.2. Effect of S-metolachlor on the growth of E. coli and E. rotunda

The evaluation of the effect of *S*-metolachlor on the organisms was performed in a two-steps approach (Fig. 4). The organisms (*E. rotunda* in co-culture with *E. coli* (Fig. 4a) and *E. coli* alone (Fig. 4b)), were first exposed to five concentrations without any replicates, to define a range of concentration to be tested. They were then exposed following almost the exact same procedure to four concentrations, in triplicates. The experimental conditions were chosen to support reproducible growth (see paragraph 3.1): 100–300 amoebae, low bacterial density and monitoring over four to five weeks.

The mean growth rate over 28 days was 0.057 (range: 0.018–0.082). A control chart (represented on Fig. 5a and c) is obtained by the data collected from six cultures grown in similar

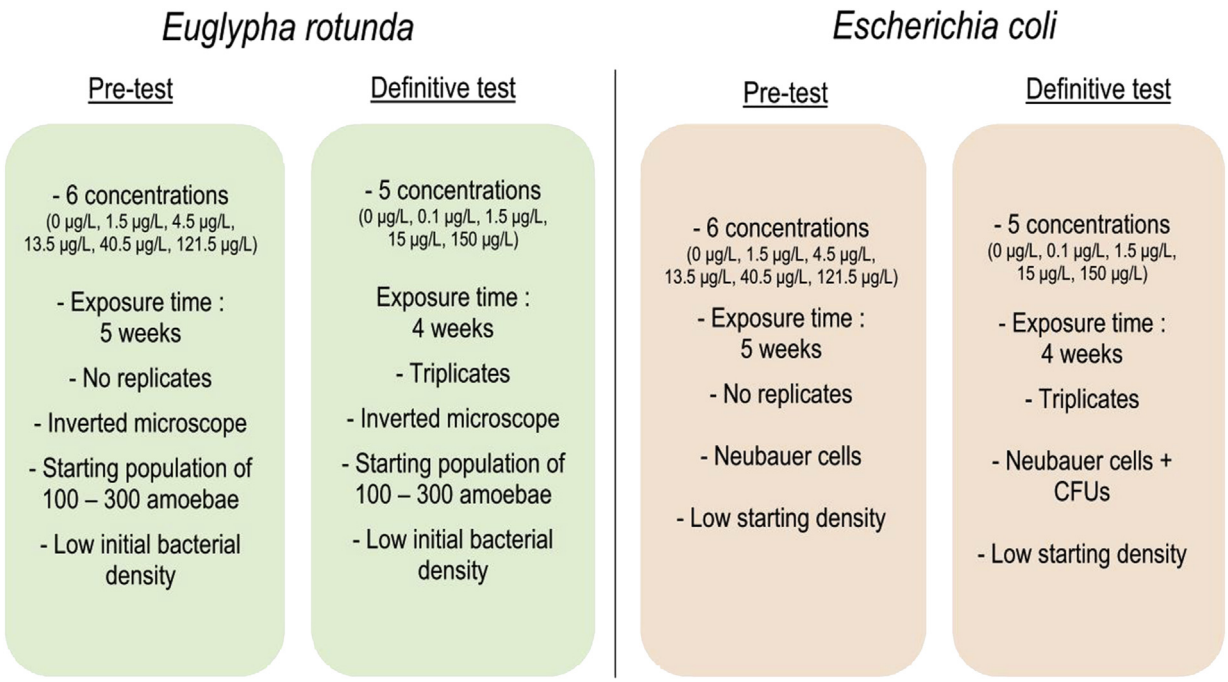


**Fig. 4.** Synoptical diagram of the tests and chosen conditions to investigate effects of the herbicide *S*-metolachlor on *E. rotunda* (a) and *E. coli* (b). The effect of *S*-metolachlor was investigated in a two-steps approach including a preliminary test (also referred as pre-test in the present study) and a definitive test.

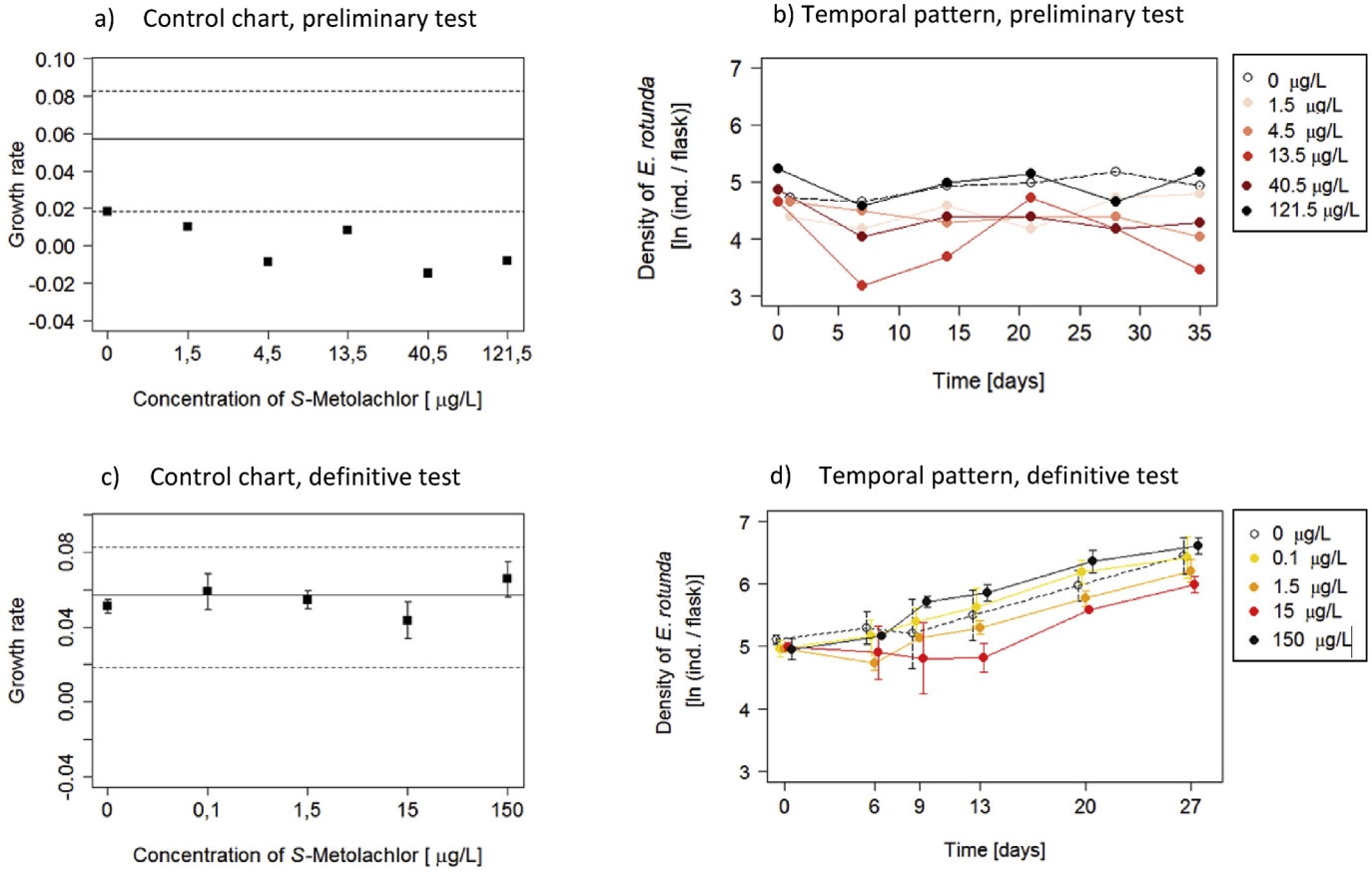


**Fig. 5.** Effects of *S*-metolachlor at different concentration on *Euglypha rotunda* cultures. Left (a, c), growth rates obtained using linear models. Lines present the values obtained from cultures non-exposed to the pesticide, and are used as control; the black line represents the mean, while the dashed lines represent the maximum and the minimum value obtained. Right (b, d), the temporal patterns of the cultures are shown; different colours stand for different concentration of *S*-metolachlor. The pre-test (upper graphs) was done without replicates, while the definitive test (inferior graphs) was done in triplicates. The inferior graphs show thus the mean and the standard error around the means (error bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conditions (initial amoeba density 100−300, low bacterial density, monitored over 28 days, no pesticide exposure). The minimum and maximum values are taken to define an interval in which growth rates could be expected under standard conditions. The standard deviation of the growth rate was 0.023 and the coefficient of variance (abbr. CV) was 40%, thus higher than the 30% maximum limit recommended for collembolan (ISO, 1999; Krogh, 2008), earthworms and draft mite (ISO, 1998; OECD, 2004, 2007). In order to diminish the CV, we propose that stock cultures should be inoculated and kept, at least one week, at 12 °C in the dark to let the amoebae adapt to the new conditions before starting the experiment. Further measurements would be useful to define more precisely the normal growth rate and thus to decrease the uncertainty of the control chart.

The representation of results as temporal patterns and growth rates complete each other (Fig. 5): while the growth rates give an overview, the temporal patterns allow a more precise observation of an effect (if any) of the compound of interest on the population. The growth rates obtained from the first test of all five cultures exposed to the pesticide were lower than the values of the controls (Fig. 5a), supporting a negative effect of *S*-metolachlor on the growth of *E. rotunda*. The temporal pattern (Fig. 5b) revealed a more complex pattern, not reflected by the growth rates. The population decreased after six to seven days when exposed to *S*-metolachlor and then increased. A final reduction of the population was observed in the last days of the test for the concentrations 4.5 μg/L, 13.5 μg/L, and 40.5 μg/L, with the overall most pronounced effect recorded at around 13.5 μg/L.

The effects observed during the preliminary test were not confirmed by the definitive test. However, the cultures (incl. the control) did not grow well during the preliminary test; the state of the stock culture may have been less active at the time point of the preliminary test inoculation. It is therefore important for the stock culture to be in an exponential phase (and thus active state) in order for the start population to quickly enter the exponential phase. The growth rates all fitted in the interval for the standard growth of the control chart (Fig. 5c). The temporal patterns did not show any decrease in the first days of the test (Fig. 5d). Only the concentration of 15 μg/L caused a delayed growth of the population. By contrast, the highest concentrations of pesticide used in the present work (i.e. 121.5 μg/L and 150 μg/L) did not cause any decrease; the population reached similar or even higher densities than the control after 28 days. The discordance between the two tests calls for further experimentation.

The pesticide was expected to influence cellular division (Götz and Böger, 2014; Matthes and Böger, 2002), but *E. rotunda* was frequently observed to divide during microscopic observations. The pesticide could also have affected the amoebae in a way that could not be detected with the measurements chosen in our setup, e.g. by affecting other health parameter than the growth rate.

We could not find any report of similar, non-linear effect on protist growth due to the exposure to chemicals. However, as Todorov and Golemansky (1992) reported, different protists can be affected in very different ways, making comparison between distantly related species difficult. This further stresses the need to better understand how pesticides are affecting the high diversity of protists belonging to different major phylogenetic groups. It would indeed be ideal to have several model test organisms with contrasted responses to better document how pesticides affect the diversity of soil organisms.

The bacterial density was not affected by the pesticide. Inhibition of the bacteria may however only occur at much higher concentrations (the highest concentration being 150 μg/L). Pereira et al. (2009) reported for example the half maximal effective concentration ($EC_{50}$; for increased lag phase, reduced bacterial yield, and reduced growth rate) of metolachlor (a mixture of the enantiomers *R*- and *S*-metolachlor) to range between 113′600−142′000 μg/L for the gram-positive bacteria *Bacillus stearothermophilus*.

### 3.3. Ancillary parameters and S-metolachlor stability

The ancillary parameters measured during the definitive test (dissolved oxygen, pH and conductivity) followed similar patterns independently of the *S*-metolachlor concentrations and thus cannot explain the observed differences in growth rate (see Supplementary materials). The pH increased with higher *S*-metolachlor concentrations and with time in presence of *E. rotunda* and/or *E. coli*. The concentration of dissolved oxygen decreased over time (by ca. 1 mg/L) in presence of *E. rotunda* and/or *E. coli*, while conductivity increased (by ca. 60 μS/cm).

After four months stored at 4 °C, a small increase in the concentration of *S*-metolachor was reported. The two stock solutions of the preliminary test analysed at day 0 and day 36 showed similar increases, while the two solutions filtered from bacterial and amoeba cultures presented lower concentrations than the abiotic solutions after 36 days (Table 1). The fate of the pesticide may thus have varied over time in the cultures, leading to different level of exposure, and hence possibly influencing the observed results. The pesticide in the biotic solutions (bacterial culture and co-culture) may have been degraded similarly to the bacterial degradation of acetolachlor reported by Xu et al. (2008) or adsorbed on the walls of the flask as reported by Coquillé et al. (2015). Biodegradation of *S*-metolachlor is however mainly performed by fungi according to Vryzas et al. (2012) and Zemolin et al. (2014). Adsorption cannot be ruled out: the stock solutions were held in glass bottles while the biotic cultures were in culture flasks manufactured with polystyrene and polyethylene terephthalate, adsorption may thus have occurred for the latter but not in the glass bottles. Some photodegradation could also have occurred during the weekly 20−30 min required to quantify the individuals when the flask remained exposed to light; *S*-metolachlor was reported to be degraded at 50% in eight days if located at the soil surface (Rivard, 2003). The fate of the toxic compound need to be investigated as part of the test to correctly evaluate the concentration faced by the organisms.

### 3.4. Measurements and proposed improvements

From the three categories distinguished during counting (i.e. “alive”, “dead/empty test”, and “undefined”), only the category “alive” was used to monitor the population. Interestingly, the abundance of empty tests followed a similar temporal pattern than the density of living individuals, but reduced by a factor of 10 compared to the living population. The number of “undefined” individuals was low (0−3 individuals per flask), and they were therefore ignored in the data analyses.

**Table 1**
Measured concentrations of *S*-metolachlor to investigate its stability over time. C2 and C5 correspond to the theoretical concentrations of 4.5 μg/L and 121.5 μg/L, respectively. The concentration for the inoculated cultures were not analysed at t0, thus the theoretical values are given here (in quotes). The measurements were performed with Liquid Chromatography coupled with Mass Spectrometry.

| Sample | Concentration at $t_0$ [μg/L] | Concentration at $t_{final}$ [μg/L] |
|---|---|---|
| Stock solution | 333 | 346.9 |
| C2 | 4.4 | 4.8 |
| C5 | 123.1 | 128.9 |
| *E. rotunda* C5 | “121.5″ | 82.2 |
| *E. coli* C5 | “121.5″ | 78.3 |

The use of microscopic observation, although relatively time-consuming, allows direct observation and may provide additional information on potential impacts of the exposition to chemicals. Sub-lethal effects such as behavioural or structural changes can be observed under the microscope. For example, Coquillé et al. (2015) showed that although the growth of the diatom *Gomphonema gracile* was not affected by the pesticide *S*-metolachlor, it allocated its energy *in situ* to resist the pollutant at low doses as an increase in fluorescence was measured. Furthermore, exposed to higher concentrations, the algal motility increased, suggesting an escape behaviour.

Some specific behaviours or shell forms were observed during the present work (however without correlation to the *S*-metolachlor concentration). We observed: plasmogamy (i.e. cytoplasm of two cells fusing into a third shell, which agrees with previous observations of sexual cycles in euglyphid testate amoebae – Lahr et al., 2011); individuals with two-three nuclei-like structures; potential kleptosquamy (i.e. use by living individuals of other individuals' plates scavenged on empty tests – Lahr et al., 2015); cluster of individuals in high density cultures; curved shells (may be referred as *E. rotunda* var. *obliqua* described by Decloitre (1962)), small shells or some other unusual shells. Aberrant forms in amoeba cultures have been previously reported (Cowling, 1986). Although not linked to the tested pesticide, these variations illustrate the morphological variability that can be observed for *E. rotunda* growing in culture, which could possibly be influenced by growth conditions, including exposure to pesticides.

As mentioned earlier, the bacterial growth was enhanced by the presence of the amoebae in the controls (Fig. 3). However, although the preliminary test results were supported by a similar pattern during the definitive test using the same method (i.e. microscopic counting chamber), this was not confirmed by the Colony Forming Units (CFUs) results (Fig. 6). The higher values obtained by the microscopic counting chamber may be due to the inability to distinguish between dead and living bacteria, while the CFUs only report living individuals. The difference between the two methods is, however, only reported in the co-cultures, i.e. in presence of amoebae. The presence of the amoebae may have provoked the death of bacteria in an additional, indirect way beside predation, such as the accumulation of metabolic waste or other chemical compounds. In any case, we believe that the two methods should be used while performing the test: the microscopic chamber method is less invasive, requires less material and allows a weekly monitoring while the CFUs is more precise regarding viable cells and can reveal contaminations. The latter is however based on larger volumes restricting the quantification at the beginning and end of the test duration.

### 3.5. Other points of interest

Turbidity was also considered to monitor bacterial growth. Measurements were done in triplicates using a spectrophotometer (BioTek, EL800) at 630 nm in preliminary tests. Limitations of this method for our experimental design were however met and the method was not further used. Low bacterial density resulted in low optical density that could not be distinguished from growth medium values.

A pilot assay was done using a flow cytometer to investigate if one could distinguish between bacteria and amoebae, as this could be a powerful tool to quantify the two species at the same time. Despites the high potential of this method, limitations were met due to our experimental design: the flow cytometer is more suitable for cultures displaying higher density than ours and the use of a dye was not suitable in our set-up.

## 4. Conclusion

We believe that the experimental set-up proposed in the present study yields reproducible results and could be developed as an additional, accurate tool to assess the effect of pesticides and other pollutants on the soil ecosystem. The set-up we used allows the appreciation of a small trophic system and is based on direct observation of individuals allowing the integration of behavioural characteristics. The interaction between predator and prey could be modified due to the exposure of a pollutant and such information could inform on trophic effects of pesticides. Moreover, the chosen organism, *E. rotunda*, is proposed as first representative of the Rhizaria. Very little is known about the potential impacts of pesticides on this highly diverse and ubiquitous super-group of Eukaryotes and this represents a major knowledge gap. More generally, despite their major functional roles in terrestrial ecosystem, soil protists are only marginally used in ecotoxicological

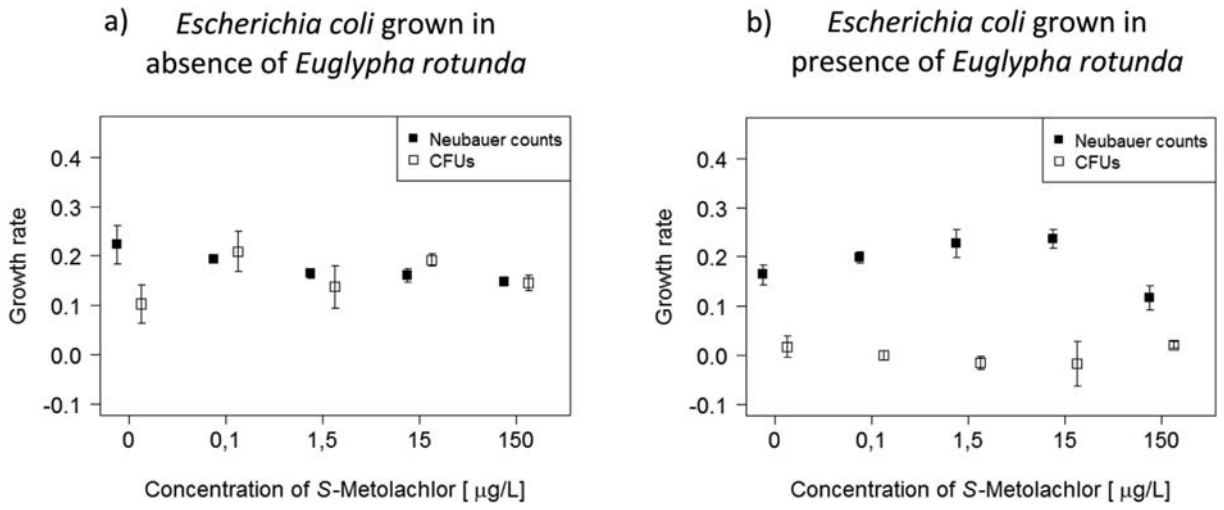


**Fig. 6.** Growth rates of *E. coli* exposed to different concentration of *S*-metolachlor, and in absence (a) or presence (b) of the *E. rotunda*. The squares present the mean of calculated growth rates and the vertical bars the standard error around the mean. The results from two quantification methods (hemocytometer; Improved Neubauer cell), and the Colony Forming Units (CFUs)) are shown.

studies but as many species can be grown in the laboratory there is no *a priori* reason why this should be the case.

*Euglypha rotunda* is a promising model organism for ecotoxicology. The biological variability of population growth however needs to be further defined. Additional data are clearly needed to better-define the control reference growth curves and hence better assess the impact of pesticides. To this aim, we propose to add an adaptation time of one week for the stock cultures before doing the inoculation for the test. The fate of the toxic compounds also needs to be carefully monitored during the test; reaction of the toxic compounds (including degradation and possible toxicity of metabolites) could change the effective concentration at which the organisms are exposed.

The growth of *E. rotunda* was not affected in a linear way by *S*-metolachlor; the strongest effect was recorded at ca. 15 μg/L, while the bacteria were not affected at all. If confirmed, these results would add to the growing body of literature showing non-linear effects of pesticides on living organisms and often significant lethal or sub-lethal effects at concentrations below the maximum residue levels (MRL). A concentration of 15 μg/L is indeed within the EU MRL authorised for fruit, vegetables and meat (0.01–0.05 mg/kg) and 15 times higher than the value for milk (0.001 mg/kg) (European Food Safety Authority, 2012). This study therefore shows that additional tools to study different trophic levels and various phylogenetic groups are required to accurately assess the impact of pesticides on the environment. Due to their abundance and ecological relevance in soil ecosystem, we believe that soil protists should be included in the toolbox of ecotoxicological model organisms.

## Conflict of interest

None.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.03.001.

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