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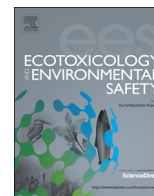
Microcalorimetry: A powerful and original tool for tracking the toxicity of a xenobiotic on *Tetrahymena pyriformis*

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ABSTRACT

Fighting against water pollution requires the ability to evaluate the toxicity of pollutants such as herbicides. *Tetrahymena pyriformis* are ubiquitous ciliated protozoans commonly used in ecotoxicological research. Microcalorimetry can be used in many biological investigations as a universal, non-destructive and highly sensitive tool that provides a continuous real-time monitoring of the metabolic activity. This technique based on the thermal power output was applied to evaluate the influence of herbicide diuron on cultures of *T. pyriformis*. The heat flux produced upon addition of 0, 3.5, 7.0, 14.0, 28.0, and 56.0 $\mu g \, mL^{-1}$ of diuron was monitored. The biomass change during the growth was also determined by flow cytometry. The results confirmed that the growth of *T. pyriformis* is progressively inhibited as the concentration of diuron increases and revealed that the state of the living system is severely altered at a concentration of 56.0 $\mu g \, mL^{-1}$. The IC_{50} was estimated at 13.8 $\mu g \, mL^{-1}$ by microcalorimetry and at 18.6 $\mu g \, mL^{-1}$ by flow cytometry. It was shown that microcalorimetry is not only a very effective tool for the determination of the growth rate constant but that it is also a valuable probe for a rapid detection of the metabolic perturbations and, in ultimate cases, of the critical alterations of the living system under the action of a toxic agent.

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1. Introduction

Tetrahymena pyriformis are ubiquitous ciliated protozoans belonging to a free-living, fresh-water genus that is commonly used in ecotoxicological research (Bogaerts et al., 1998, 2001; Bonnet et al., 2008; Láng and Kőhidai, 2012; Nilsson, 1989; Sauvant et al., 1999). *Tetrahymena spp.* combine traits of a single cell with traits of a whole organism and population, thus allowing to study complete metabolic pathways and its consequences on population growth and genetic adaptation (Gerhardt et al., 2010). For assessing the impact on viability and cytotoxicity of xenobiotics towards eukaryotic cells, the applied cell models have to meet some important requirements: their biology and general responses should be well known, laboratory handling should be relatively easy, and it is desirable that they have a short time generation whenever studies of long term effects are necessary (Nilsson, 1989). Tetrahymena possesses all these qualities.

Diuron, N-(3,4-dichlorophenyl)-N,N-dimethyl-urea, is an herbicide belonging to the phenylamide family and the subclass of phenylurea. This substituted urea herbicide inhibits photosynthesis by preventing oxygen production and blocks the electron transfer at the level of photosystem II in photosynthetic microorganisms and plants (Giacomazzi and Cochet, 2004). It is extensively used on many agricultural crops and non-crop areas and its dispersion leads to the pollution of the aquatic environment by soil leaching. According to the French Environmental Institute (IFEN, 2013), diuron was detected in 23% of the samples from rivers in the national basin. Diuron, which is known to be slightly toxic to mammals and birds as well as moderately toxic to aquatic invertebrates (http://toxnet.nlm.nih.gov)(Giacomazzi and Cochet, 2004), was placed on the Priority Substance list established by the European Commission (Directive 2008/105/EC).

Population level test methods are often used to classify the effect of toxic substances. The population growth can be determined by different methods (Gerhardt et al., 2010). Most of them rely on direct cell count (electronic particle counter, hemocytometer) or indirect turbidity measurements. Cytometry is a valuable tool in aquatic and environmental microbiology that combines direct and rapid assays to determine numbers, cell size distribution and physiological characteristics of individual cells, revealing the

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heterogeneity present in a population or community. Its major advantage over the colter counter is that it simultaneously records several parameters for each event, allowing for the discrimination between cells and detritus. Cytometry was used, for instance, to estimate the effect of heavy metals Cd, Zn and Cu on populations of *T. thermophila* (Gallego et al., 2007) but also to study the effect of serotonin, histamine or insulin on *T. pyriformis* cells (Csaba et al., 2011).

The growth rate constant can also be determined by microcalorimetry. The heat produced by a living system gives an overall estimation of the metabolic activity (Belaich, 1980; Gnaiger, 1989; Gustafsson, 1991). It is made up of the heat evolved by each catabolic reaction, the heat absorbed by each anabolic reaction, and the heat associated to any other chemical or physical process involved in the microorganism activity. In most cases, the heat absorbed by anabolism is negligible compared to the heat produced by the catabolic processes. The heat associated to other biological processes such as motility and transport can also be neglected. The microcalorimetrically-measured heat production thus mainly reflects the catabolic reactions, which can be modified by presence of toxic molecules. Forrest (1961) first showed that the heat produced by a growing culture under limiting energy source follows the same kinetics as the biomass increase. After this pioneering work, several papers did confirm that the rate of synthesis of new cell material, the degradation of energy source, the appearance of catabolic products and the heat evolution are all described by the same exponential function (references cited in the following reviews: Battley, 1987; Belaich, 1980; Braissant et al., 2010, 2013). The growth kinetic parameters can thus be obtained from microcalorimetry, which has the advantage of enabling metabolic activities to be monitored in real-time, continuously, and at constant temperature.

With sensitivity on the order of 0.2 μW, modern isothermal heat-conduction microcalorimeters allow the investigation of slow processes generating small heat effects. Assuming that the heat evolved by a single active bacterium is on the order of 2 pW (Higuera-Guisset et al., 2005), it can be inferred that the microcalorimeters of today can detect the heat flux produced by only 10^5 bacteria (Braissant et al., 2010; Higuera-Guisset et al., 2005). The detectable concentration of active bacteria in a typical 1–4 mL microcalorimetric vessel is thus expected to be on the order of 10^4–10^5 bacteria mL^{-1}. Metabolic activities of larger microorganisms that produce more heat, like eukaryotic protozoa for instance, could be studied at lower concentrations. Microcalorimetry thus provides a highly sensitive and non-invasive mean to assess toxicity effects in population growth. It was successfully used with *T. thermophila* (Chen et al., 2008; Kong et al., 2009; Zheng et al., 2006).

In the present study, microcalorimetry has been used to track the toxicity of diuron on *T. pyriformis*. The toxic action has been assessed by analyzing the response of *T. pyriformis* to increasing concentration of the herbicide in terms of heat production and growth rate constant. In parallel, cytometry has been used to follow the impact on the evolution of the treated populations versus controls.

2. Material and methods

2.1. Chemicals compounds

Diuron [N-(3,4-dichlorophenyl)-N,N-dimethyl-urea] was purchased from Riedel-de Haën (Pestanal 99%, Laboratory Chemicals). For all bioassays diuron was dissolved in DMSO. Working concentrations were prepared by dilution of a stock solution of diuron of 11.2 g L^{-1}. As we want to compare the methods, the same dilutions were used for all experiments; the concentrations were not

analytically verified. The amount of solvent added to the cultures was always equal to 0.5%

2.2. Cell culture

The ciliated protozoan *T. pyriformis* (amicronucleate strain GL), was from the Biology Institute of the Carlsberg Foundation (Copenhagen, Denmark). It was grown axenically without shaking or stirring, at 28 °C, in a proteose-peptone yeast salt (PPYS) complex medium (pH 6.8–7.0) containing 0.75% proteose-peptone (Difco, Detroit, MI, USA), 0.75% yeast extracts (Difco) and inorganic salts (Plesner et al., 1964). The cells were maintained in exponential growth phase by reseeding in PPYS medium weekly. For testing purposes logarithmically growing pre-cultures were required. These pre-cultures were set up one day prior to the test using cells from experimental stock cultures and kept under the exact same culture conditions as the test.

2.3. Microcalorimetry

Isothermal microcalorimeters of the heat conduction type were used to monitor the metabolic activity of the living cells. Instruments of this type differ from classical calorimeters in the sense that they directly record the heat flux dQ/dt, also called the thermal power output P, evolved by the cellular culture. A TAM III (Thermometric – TA Instruments, France) equipped with a multicalorimeter holding six independent minicalorimeters allowed continuous and simultaneous recording, as a function of time, of the heat flux produced by six different samples, with a precision greater than 200 nW. The minicalorimeters were electrically calibrated and the heat Q values were obtained by integration of the thermal power output curves at different times using the TAM Assistant software (Thermometric – TA Instruments, France). The TAM III temperature was set at 28 °C; its long-term stability was better than $\pm 1 \times 10^{-4}$ °C over 24 h.

Specific disposable 4 mL sterilized glass ampoules, capped with butyl rubber stoppers and sealed with aluminum crimps, were used in each channel. These are referred to as 'microcalorimetric ampoules' throughout the text. In order to check the impact of the air volume into the ampoule, preliminary tests were conducted by introducing, respectively, 1, 2 and 3 mL of growth medium inoculated with *T. pyriformis*. The results showed that the total heat (obtained by integration of the whole power–time curve) increases with the amount of air indicating that, under the present conditions (unstirred batch incubation), oxygen is the limiting factor. But the results also showed that the amount of air does not affect the growth kinetics, the exponential part of the three test curves yielding strictly identical growth rates. So, to simplify, it was decided to conduct all the experiments in microcalorimetric ampoules half-filled with 2 mL of medium containing 1250 cells mL^{-1} at the beginning. The experiments were duplicated.

Since the bioassays were carried out in medium containing 0.5% DMSO, it was important to check whether or not this solvent does affect the heat production of *T. pyriformis*. The control experiment relative to the growth of *T. pyriformis* on PPYS in the absence of diuron was thus performed both in the presence and absence of DMSO. The same heat curve was obtained in both cases, which confirmed that DMSO does not have any significant influence on the metabolic activity of *T. pyriformis*.

2.4. Flow cytometry

The cell counting was done by cytometry. The fluorochrome SYBR® Green I was used to stain the cells because it is a nucleic acid stain well suited for excitation by the argon laser blue line at 488 nm. It was added to 10^{-5} dilution of commercial stock (ref S7563, Molecular Probes, Invitrogen). The samples were stained for 30 min at room temperature before being analyzed with the cytometer. The number of cells was expressed per unit volume of suspension.

All experiments were performed with a FACSCalibur® flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser beam providing 15 mW at 488 nm. Green fluorescent cells stained by SYBR® Green I were collected in FL1 channel (530 ± 15 nm). All analyses were performed at a high rate setting, and acquisition was performed over a 1-min period or 10,000 events. Cells were detected in a plot of 90° side scatter (SSC) or forward scatter (FSC) versus green fluorescence (FL1). The measurements were repeated in duplicate. Analysis was done with Cytowin (Vaulot, 1989).

2.5. Toxicity assays

An experimental scheme of the experiments is presented in Supporting information 1. Test cultures were prepared by inoculating axenically cultivated *T. pyriformis* from a stock culture into 50 mL of PPYS in 100 mL glass conical flask. The day after, *T. pyriformis* were counted and used to inoculate 160 mL of PPYS at a concentration of 1250 cells mL^{-1}. This culture was then divided up by 25 ml in 50 mL flasks, one for each concentration of diuron (0, 3.5, 7.0, 14.0, 28.0, and 56.0 μg mL^{-1}). The chemical was added at initial time (T_0) and each culture