

Full Length Research Paper

Optimization of *Paramecium tetraurelia* growth kinetics and its sensitivity to combined effects of azoxystrobin and cyproconazole

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Accepted 22 August, 2011

For using an organism for ecotoxicological studies, it is important to understand its model of growth under some physicochemical parameters that influence with a direct manner its development. The knowledge of the optimal parameters for the paramecia development leads to realize a model of growth with clear stages. For that, we tried to study the influence of temperature, pH, nutrient density in the culture medium and pollution on the growth of *Paramecium tetraurelia*. Obtained results showed that paramecia prefer definite conditions. However, the growth was better in temperatures included between 25 and 30 °C, with preference to increased temperatures and a slightly acidic pH. The culture medium was prepared from the mixture of several vegetables which the rates were studied depending to the growth. The combined effects of azoxystrobin and cyproconazole on the growth kinetics were studied. Results showed that treatment affected the population growth, the generation time and the velocity of generation.

Key words: *Paramecium tetraurelia*, growth kinetics, physicochemical parameters, azoxystrobin, cyproconazole.

INTRODUCTION

The research for study models for different disciplines of the applied biology becomes an imperative scientific requirement. The growth constitutes the basic criteria that can make from an organism a model of survey. Growth of microorganism can be quantified by an increase of the size, the weight or the number. Nevertheless, there are several agents limiting this important criterion. The limiting factor is the one that conditions the speed or the amplitude of a phenomenon that depends on several other parameters (Liebig, 1844). Generally, we use the ecological factors to describe, analyze or model an ecosystem or a physiological function of a given species. Among the ecological conditions influencing the growth, we find abiotic or physicochemical factors; they have direct and rapid effects on the living organism

development (Wehner and Gehring, 1999; Branger et al., 2007). The knowledge of growth parameters of a given species seems to be primordial for the experimental utilization (Schopfer, 1935; Ershov et al., 1999).

P. tetraurelia is a very large (120 µm) eukaryotic cell covered with vibrating cilia. It belongs to the Ciliate phylum (Ciliophora). The use of *Paramecium* species as a model of survey has been reported by several authors in some disciplines; in ecotoxicology, *Paramecium* species were used to study environmental qualities and toxic effects of industrial, agricultural and domestic chemicals (Edmiston et al., 1985; Madoni, 2000; Miyoshi et al., 2003; Takahashi et al., 2005; Venkateswara et al., 2006; Rouabhi et al., 2006; Mortuza et al., 2009, 2010; Amanchi, 2010); in genetic, because its sequencing genome is well known, researchers used *P. tetraurelia* for genetic analysis, gene expression and mutation (Houten et al, 1977; Brygoo, 1977; Mayer et al., 1998; Haynes et al., 2000; Vaysié et al., 2000). In physiology, paramecia

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Table 1. Different ingredient combinations of the *Paramecium* culture medium.

Quantity (g.L ⁻¹) \ Ingredient	Hay	Lettuce	Wheat plant	Cucumber rind	Potato rind	Yeast	Peanut
	2	2	2	1	1	0.5	0.5
Com. 1	+						
Com. 2		+					
Com. 3	+					+	+
Com. 4		+				+	+
Com. 5	+	+				+	+
Com. 6	+	+	+			+	+
Com. 7	+	+	+	+		+	+
Com. 8	+	+	+	+	+		
Com. 9	+	+	+	+	+	+	+

(Com.: combination).

are used in general for studying the role, the function and the cell organization. (Glaser, 1925; Majima et al., 1986; Preston and Usherwood, 1988; Stelly et al., 1991; Hemmersbach et al., 2001; Momayezi et al., 2004).

In the present study, we try to elucidate two main points; the first concerns the *P. tetraurelia* growth in a new synthetic culture medium, the second point focuses on the assessment of the *Paramecium* population responses towards combined effects of azoxystrobin and cyproconazole.

MATERIALS AND METHODS

Paramecium culture

The habitual culture of *P. tetraurelia* was done in the culture medium (com. 9) described in Table 1, at pH 6.5 and 25°C into the oven (memmert 400). Cells were transplanted each three days for keeping the youthful state of the culture. The bacterization of the medium was done with non specific manner from a culture medium contaminated previously.

Growth measurement

For growth experiments, the culture was done at 30°C in test tubes using 10 ml of the culture medium. For each tube we added 10 cells of paramecia. The growth kinetics study was realized by the daily cell counting, after fixation with formalin at 4%, under optic microscope using grooved blade. The count was repeated at least five times for each repetition. Growth assessment was determined by the following formulae:

$$n = \frac{\log N_t - \log N_0}{\log 2}$$

$$k = \frac{n}{t}$$

$$g = \frac{1}{k}$$

Where n is the number of generation, Nt is the population in time t , N_0 the initial number of cells, k is the generation velocity, and g is the generation time (time required for a population of cells doubles in number).

Impact of the culture medium on the *Paramecium* growth

To show the impact of the culture medium density on the *Paramecium* growth, we prepared a concentrated medium by mixing several dried vegetables (hay 7.5 g, wheat plant 7.5 g, lettuce 10 g, cucumber rind 5 g, potato rind 5 g, dash of yeast and source of sterol (2 g of peanut or almond)). Mixture was boiled throughout one hour in 1.5 liters of distilled water. The broth was filtered, sterilized by boiling at 100°C during 30 min in thermo-resistant bottle and conserved to the shelter of light. The medium must undergo several dilutions to determine the one that lead to better growth. These dilutions were measured as optic densities using a spectrophotometer (Jenway 6300). The tested densities were 0.100, 0.300, 0.500, 0.700 and 0.900 nm.

To test the intensity of *P. tetraurelia* growth according to the medium composition, several ingredient combinations were made in order to adopt the best synthetic formula. The ingredients used for these experiences are presented in Table 1.

Effects of physiochemical parameters on the *Paramecium* growth

To study the temperature effect on the *Paramecium* growth, different incubations were realized at 10, 20, 25, 30 and 35°C. Cultures were made in an oven Memmert type 400.

The previously pH of the culture medium was 6.8. The adjustment of pH, for each experience, was realized with pH-meter (professional pH 213 Hanna) by adding some drops of HCl (1 mol) and NaOH (1 mol). The studied pH values were 5, 6, 7, 8, and 9.

Chemicals

Amistar Xtra is a fungicide containing 200 g.L⁻¹ azoxystrobin and 80 g.L⁻¹ cyproconazole (Figure 1). It is used for protecting plants against fungal diseases. The azoxystrobin belongs to the chemical family of strobilurins and the cyproconazole to the family of triazoles. The association of these two active ingredients gives to

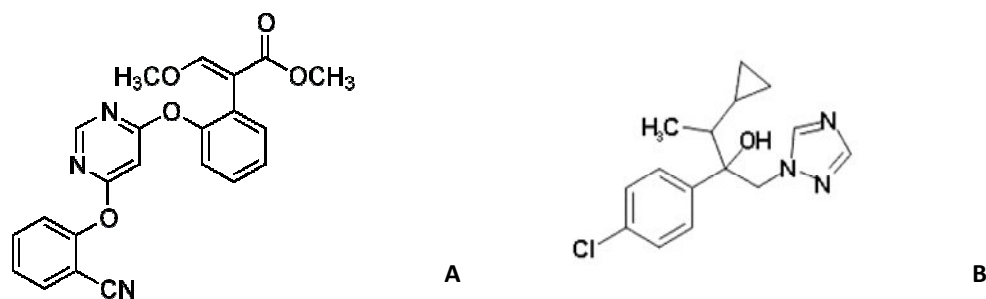


Figure 1. Chemical structure of the two active matters of Amistar Xtra. Azoxystrobin (A) and Cyproconazole (B).

Amistar Xtra a preventive and curative action, a high efficiency and a very long persistence (6 - 8 weeks) against many plant diseases (Syngenta, 2007).

Treatment with Amistar Xtra

Chosen concentrations were the result of several tests on different ranges of Amistar Xtra. The maintain concentrations were 1, 2, 4, 6 and 8 mg.L⁻¹. The treatment with fungicide was done at the beginning (at $t = 0$) before the transplantation of *Paramecium* cells. The growth monitoring lasts up to fifth day.

After exposition to the fungicide, *Paramecium* population responds in a dose-response relationship. The assessment of this response in percentage is calculated by the following formula:

$$\text{Response (\%)} = \frac{(N_c - N_E)}{N_c} * 100$$

Where N_c is the number of cells in the control treatment and N_E is the number of cell in the treatment that received the fungicide.

Statistics

The median inhibitory concentration (IC₅₀) required to reducing 50% the *Paramecium* population growth was calculated by the kinetic growth method using the linear regression analysis. To study effects of treatment on the paramecium growth, we applied a general statistical analysis based on the *Mood's median test*. To look for differences by pairs between samples treated and the control, we applied the *Mann Whitney* test. To evaluate the relationship between the concentrations of amistar Xtra and the effects on the growth we used the regression analysis. The software used for this survey was the Minitab software (15.0).

RESULTS

Growth study

Impact of the medium concentration on the *Paramecium* growth

Curves recorded in Figure 2 show that the concentration of the culture medium has an influence on the paramecia

number. A concentrated medium has a negative effect on the survival of cells, whereas a less concentrated medium produces a weak growth. The concentration that produces the maximal number of paramecia was 0.700 nm; however, in 0.900 nm the growth was null. Comparisons between densities were significantly different (Chi-Square = 16.00; DF = 4; P = 0.003; Overall median = 203).

Paramecium growth according to the culture medium composition

The combinations of culture medium ingredients led to different growth (Table 2). The medium containing all constituents (com. 9) was the more benefic, in which the number of cells at the fourth day exceeded 10⁴ cells per milliliter. Yeast and peanuts showed strong effect on *Paramecium* growth increasing it around 60%.

Influence of physicochemical parameters

The growth of *P. tetraurelia* is influenced distinctly by the temperature, if the temperature increases or decreases, the number of cells changes. According to Figure 3, temperatures that seem to be favorable were those included between 25 and 30°C. Below 25°C and more than 30°C the number of cells decreased and the growth rate changed with a remarkable manner. In low temperatures (< 20°C), although the number of cells was low, paramecia growth remained constant relatively for a long period; however, in high temperature, the number of cells was higher and the constancy phase decreased in according to the temperature variation. Statistical analysis showed significant differences between various temperatures (Chi-Square = 8.00; DF = 4; P = 0.092; overall median = 908).

Paramecia present an affinity to the slightly acidic medium where, the growth reaches a highest level with regard to the other values of pH (Figure 4). Statistical

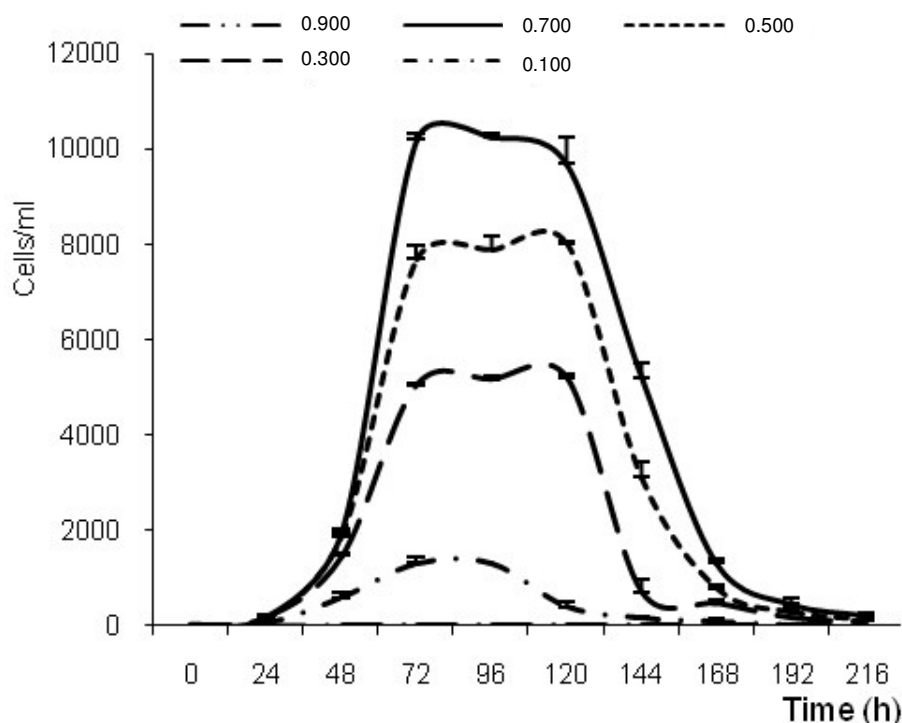


Figure 2. Impact of the culture medium concentration on the *Paramecium* growth (T°: 30°C, pH: 6.5).

Table 2. Growth of *P. tetraurelia* according to the composition of the culture medium.

Culture medium combination	Number of cells/ml/h			
	24	48	72	96
Com. 1	27	120	413	540
Com. 2	40	127	633	673
Com. 3	67	533	1133	1217
Com. 4	73	860	1183	1233
Com. 5	127	867	4067	4200
Com. 6	120	1250	5025	5117
Com. 7	147	1250	7500	8500
Com. 8	120	1250	4333	4300
Com. 9	167	1900	9975	10150

(T°: 30°C, pH: 6.5).

analysis reveals significant differences between different pH values (Chi-Square = 0; DF = 4; P = 1; Overall median = 4025). Pairwise comparisons between the growth at pH 6 and 7 have shown that the test was significant at $P = 0.63$.

From the previous results, it appeared that paramecia grow better in a temperature included between 25 and 30°C, in a slightly acidic pH (6 - 7) and in a very definite concentration of the culture medium. The meeting of all

these conditions leads to put a specific growth model with clear stages and a maximal number of cells (Figure 5).

The growth of paramecia follows a curve with four remarkable phases, a phase of latency in which the growth is hopeless; this phase lasts from 0 to 24 h; a second very fast phase which is characterized by an increase of the number of cells with an exponential manner lasts two days. The third day records the pick of the growth. The third one is a phase of constancy, in

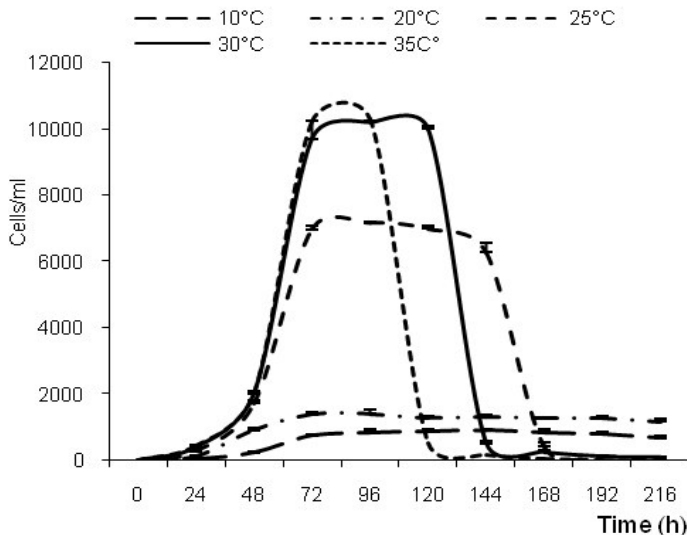


Figure 3. The effects of temperature on the *P. tetraurelia* growth (pH: 6.5; Culture medium: com. 9).

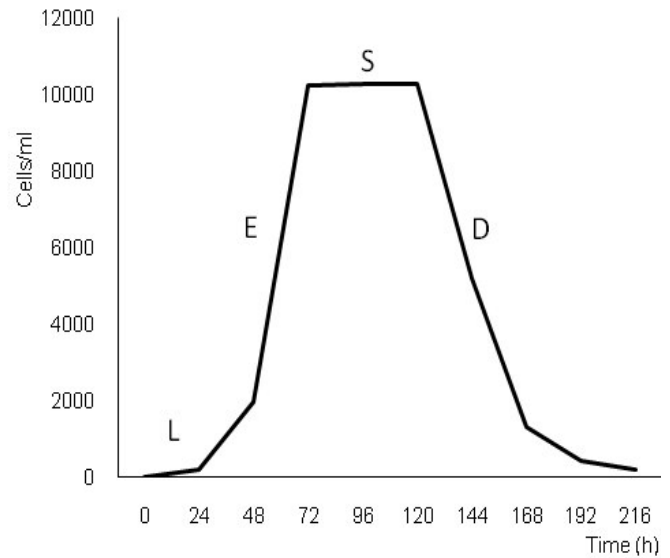


Figure 5. Different stages of the *P. tetraurelia* growth model. (T: 30°C; pH: 6.5; Culture medium: com. 9). L: latency phase, E: exponential phase, S: stationary phase, D: decline phase

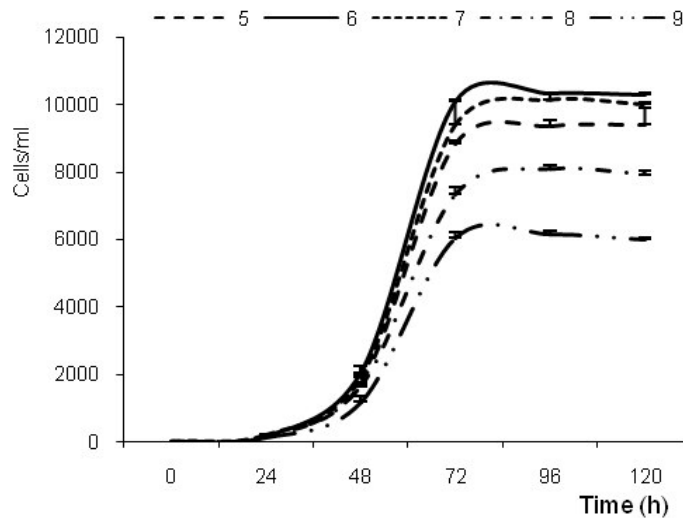


Figure 4. The effects of pH variation on the *P. tetraurelia* growth (T°: 30; Culture medium: com. 9).

which the number of cells remains constant. The duration of this phase depends on the richness of the culture medium and the temperature (Figure 5). The second phase is characterized by the intensity of the binary division; however, the stationary phase is characterized by a sexual reproduction. The fourth phase is marked by a progressive reduction of the number of cells until the disappearance of paramecia. The generation time was calculated to be 9.6 h/g. The number of generation at 96 h and the generation velocity were estimated to be 9.9 generation and 0.1g/h respectively.

Treatment with Amistar Xtra

Amistar Xtra has a negative effect on the paramecia growth; in the below of 1 mg.L⁻¹ there is no effect occurs with regard to the control; however, beyond of 8 mg.L⁻¹, the growth of paramecia is null (Figure 6a). Between these two limits, growth changes depending to the concentration. The rates of the growth inhibition were from 13 to 86%. The regression analysis shows that the concentrations explain more than 98% of the growth decrease (Figure 6b). The IC₅₀ was calculated from the linear equation of $y = 42.34x - 44.29$ to be 2.23 mg.L⁻¹ (Figure 6c).

The global statistical analysis by *Mood's median* test shows that there were significant differences relating to variations between the treated cells and the control (Chi-Square = 6.66; DF = 5; P=0.247; Overall median = 1367). Paired analysis by *Mann Whitney* test shows that there were significant differences between the control and each concentration.

Impact of Amistar Xtra on the time, the number and the velocity of generation

The generation time of the control was calculated to be in order of 9.63; it can spread until more than 13 h at paramecia treated with the concentration of 8 mg.L⁻¹ (Figure 7a); consequently, the number of generation and generation velocity know a progressive decrease along with the increasing concentrations (Figures 7b and c).

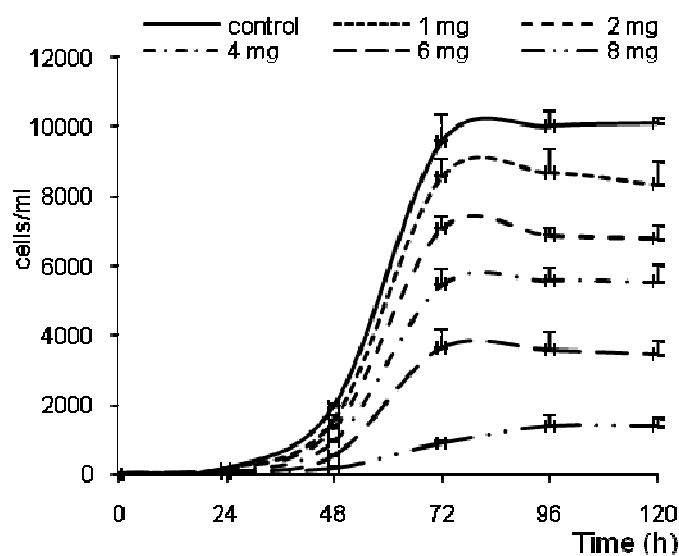


Figure 6a. Effects of Amistar Xtra on the *P. tetraurelia* population (T° : 30; pH: 6.5; culture medium: com. 9). Growth curves.

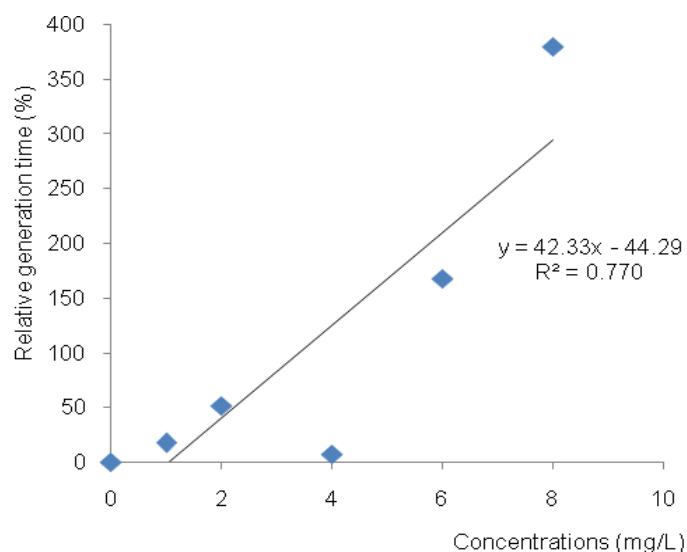


Figure 6c. Effects of Amistar Xtra on the *P. tetraurelia* population (T° : 30; pH: 6.5; culture medium: com. 9). Determination of the IC_{50} by the kinetic growth method.

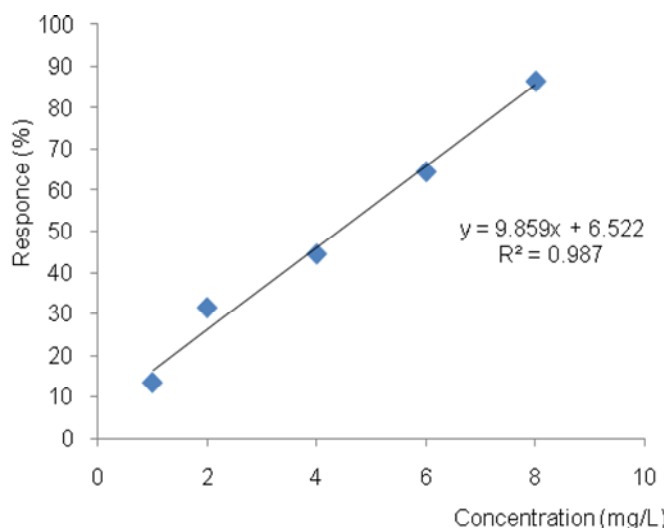


Figure 6b. Effects of Amistar Xtra on the *P. tetraurelia* population (T° : 30; pH: 6.5; culture medium: com. 9). Percentage of responses.

DISCUSSION

The effect of the culture medium on the growth results from the different interactions of its components; some of them stimulate the development process, others have an influence against the growth. The improvement of the culture medium for *Paramecium* species seems to be important for the laboratory studies for two reasons; firstly, that increases the capacity of growth compared to other media; secondly, that minimizes the experiment costs. From this vision, we tried, in the present study, to

develop a new synthetic culture medium from a simple preparation. Results showed that the improved medium increased the paramecia growth from 10^3 cells to more than 10^4 cells per milliliter.

The pace of the growth curve in the model established by our experiments is comparable with that of bacteria described by Prescott (2003). Stages of the growth curve are the same in both types of organisms. Although, bacterial growth is stronger and faster than the paramecia growth regarding to the number, the speed and the time of generation (Dreidt et al., 1994; Prescott, 2003). A lack of bacteria leads directly to the reduction of the number of paramecia. The intensity of the paramecia growth is strongly bound to the intensity of bacteria in the culture medium. As a result, a culture medium which produces more bacteria is very favorable medium for paramecia.

The effect of physicochemical parameters on *Paramecium* has been the subject of research by several authors on several levels. In 1924, Glaser studied the impact of the temperature variation (6-40°C) on the direct movements and the speed of the *Paramecium caudatum* displacement versus time. Experiences showed that the displacement of paramecia becomes faster with the temperature increase. Lee (1942) demonstrated that the digestive vacuoles formation was more important with elevated temperatures (from 25 to 40°C). The impact of the temperature change on the swimming behavior of *P. caudatum* was studied by Nakaoka and Oosawa (1977) who observed that cells change their directions and swim toward the optimal temperature. The temperature influenced also the bacteria growth in the culture

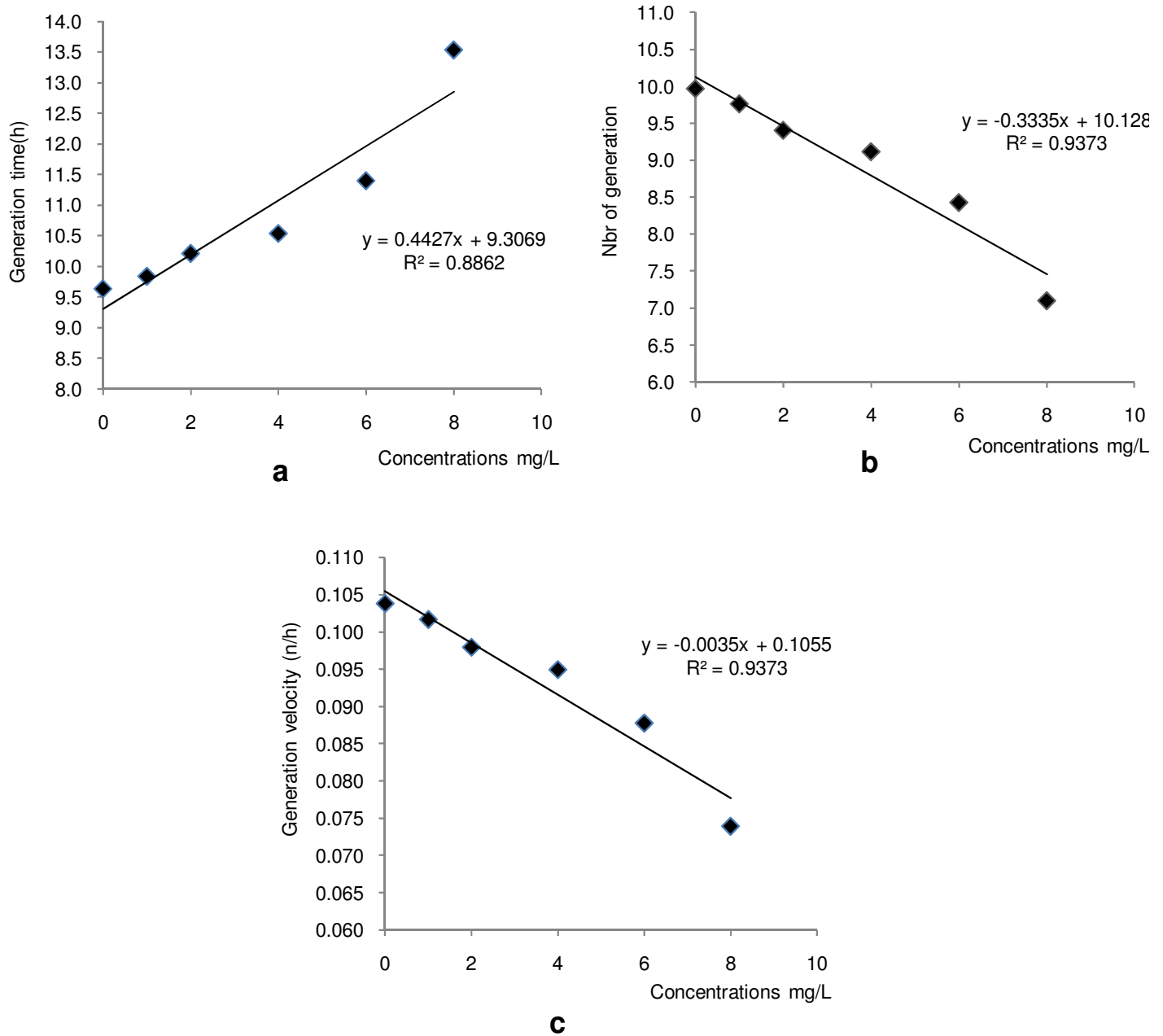


Figure 7. Effects of Amistar Xtra on the generation time (a), the number (b) and the velocity of generation(c) of *P. tetraurelia* (calculated for 96 h of growth).

medium. Karou et al. (2003) studied the influence of temperature on five species of bacteria of *Listeria* genus; they demonstrated that the growth was slow in a low temperature and that only stimulated from the 20°C. The growth reached its maximum in a temperature of 30°C. Doubtless this trend of bacterial growth explains the shape of the paramecium growth curve.

P. tetraurelia not showed high sensitivity to differences of pH values, but at pH around 6 showed better growth. According to Packroff (2000), freshwater protozoan

dominate in acidic pH. After testing a wide range of pH, Heydarnejad (2008) confirmed that the optimal pH for *P. caudatum* is located between 4.6 and 6.7. He observed an immobilization and a rapid mortality in a pH 4 and 10. In an acidic pH, displacements of paramecia are accelerated and the digestive vacuoles formation is intense (Mills, 1931).

Paramecium species are sensitive to industrial pollution (Matsubara et al., 2006; Mortuza et al., 2010). Several studies have shown that different types of biocides have

toxic effects on freshwater ciliates; these effects are perceptible at the population level by reducing the number of cells and to the cellular level by a structural, behavioral and physiological damages (Takiguchi, 2002; Garad et al., 2007; Venkateswara et al., 2006; Rouabhi et al., 2009; Sbartai et al., 2009).

ACKNOWLEDGMENTS

The authors are grateful to Professor J. Bohatier (from *Blaise Pascal* University, Clermont-Ferrand, France) who gave us *P. tetraurelia* and Dr. J. L. Bonnet for his help.

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