About the interest of a zooplankton compartment in pond systems: methodology to study the growth kinetic of *Daphnia pulex* on *Scenedesmus* sp.

M. N. D. Liady, T. T. Tangou, E. D. Fiogbe, H.-M. Cauchie and J.-L. Vasel

ABSTRACT

A reliable characterization of cladocerans' growth kinetic on their substrates is crucial for the estimation of their biochemical conversion rate in pond models. Although many studies reported cladocerans' growth inhibitions by high chlorophyceae contents, their growth kinetics had continued to be described in many pond system models by Monod-type kinetic, which describes growth saturation by high substrate contents, but fails to explain the disappearance of cladocerans observed during chlorophyceae's bloom periods. This study aimed to develop a methodology and assess whether growth-inhibition-type models used to describe microbial growth kinetics can be applicable to cladocerans. Experiments were carried out using *Daphnia pulex* populations and *Scenedesmus* sp. First, biomass of *D. pulex* was measured through digital image processing (DIP) during growth experiments. Then, three candidate models (i.e., Andrews, Edward and Haldane models), along with the Monod model, were fitted to the observed data and compared. The results showed that the DIP technique provided reliable results for estimating the biomass of *D. pulex*. Our findings show that the candidate growth inhibition-type models satisfactorily described *D. pulex's* growth kinetic (86% variance accounted for). *Scenesdemus* sp. were not strong inhibitors of the growth of *D. pulex* (high inhibition constant and low half-saturation constant found).

Key words | cladocerans, growth-kinetic, image processing, modeling, pond

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INTRODUCTION

Owing to the similarities of environmental factors in natural aquatic systems and stabilization ponds, cladocerans can grow in both systems. Cladocerans interact with water purification by grazing on the main contributors to water treatment: algae and bacteria (Canovas *et al.* 1991; Sevrin-Reyssac *et al.* 1994). Therefore, they play a key function in these bioreactors by decreasing the active biomass (algae and bacteria) and producing an interesting cladocerans biomass for receiving streams. Indeed, on the one hand, as primary consumers, cladocerans ensure matter and energy flows from primary producers toward the higher levels of the trophic chain. As a consequence, their biomasses can be valorized as fish food because of their nutritive value (Barnabe 1979, 1983; Guerrin 1988), or as pharmaceutical products due to their chitinous cuticle (Cauchie 2000). On the other hand, their biomass can be considered as invader (predator) species in the case of high-rate algal ponds and other systems aiming at algal production (Dabbadie 1992; Liady 2014). For these reasons, studies on cladocerans' biomass production and substrate conversion are still needed.

Although mathematical models may be useful to describe the interaction of cladocerans with the other compartments of waste stabilization pond systems (Hathaway & Stefan 1995 and references therein; Moreno-Grau *et al.* 1996; Reichert *et al.* 2001), experimental characterization of cladocerans' growth kinetics on their substrates was seldom carried out. Only assimilation was made (to our knowledge) in existing mathematical models on pond systems. For instance, Hathaway & Stefan (1995) assimilated their growth kinetic to a Monod-type model, and Moreno-Grau *et al.* (1996) assimilated their

doi: 10.2166/wst.2015.107

growth kinetic to a Monod-type model corrected by a term that represents the maximum attainable growth. Reichert *et al.* (2001) consider that zooplankton's growth is proportional to the product of two factors: a first-order kinetic relating to the substrate content, and a Monod-type equation relating to the oxygen content. However, from the literature (Ryther 1954; Ovie & Egborge 2002; Ovie & Ovie 2008), it seems intuitively obvious that cladocerans' growth on chlorophyceaes could be described by inhibition-type functions. Thus, growth-inhibition-type models generally used to describe microbial growth kinetics (e.g., Andrews model, Edwards model or Haldane model) might also be explored in the case of cladocerans' growth. The aim of this study was to check this possibility in order to improve the modeling of cladocerans' growth in pond systems.

This paper is considered as the first among a series dedicated to studies of growth-kinetic models of cladocerans, which take into account the effects of substrate types (bacteria, chlorophyceae and cyanophyceae) and possible substrates interactions. It is related to the growth-kinetic study of *Daphnia pulex* fed on *Scenedesmus* sp.

MATERIAL AND METHODS

To complete this work, we followed a methodology which comprises two main steps:

- Calibration of biomass estimates using digital image processing (DIP).
- Application of DIP biomass estimation to the characterization of the growth kinetic.

Calibration of biomass estimates by DIP

Two approaches are typically used to monitor the biomass of cladocerans at laboratory: one destructive, based on analyses of samples regularly taken from a large population; and the other, non-destructive, based on regular monitoring by image capture and processing of the whole culture (starting at low density). DIP makes it possible to follow the same population during the course of an experiment, starting with the desired individual composition (in terms of growth stage). It allows the counting of individuals and measurement of their size, while making it possible to return them back to their culture tank. As measurements are done on the whole population, no error is induced by the sampling process.

DIP was used in this study to estimate the biomass of *D. pulex*.

Image captures

The method adopted for digital-image capturing and processing was similar to that described in Færøvig *et al.* (2002). Individuals were kept free of movement on a sieve made of Plexiglas (Evonik Röhm GmbH, Darmstadt, Germany) and plankton net Dominique (100 μm; DUTSCHER SAS, Brumath, France), immersed in a Plexiglas tank containing the culture medium. For image captures, the tank was placed on a light table and illuminated from the top. Image captures consisted of two successive photographs in live-view using a Canon EOS 1000D camera (held on a tripod; Canon Inc., Tokyo, Japan), connected to a computer and equipped with an Ex Sigma macro zoom (focal 105 mm opening 1:2.8; Sigma France s.a., Lezennes, France).

DIP

DIP was performed using Image Proplus® software (Media Cybernetics, Inc., Rockville, MD, USA). An example of image processing using Image Proplus® is depicted in Figure 1.

A subtraction was achieved using two successive images (a and b in Figure 1). By this means, only living individuals were taken into account and shown on the resulting image (c in Figure 1) in their initial (in white) and final (in black) position. By choosing the appropriate background, it is also possible to show only final positions of individuals (d in Figure 1). Furthermore, measurements, data and total counts can be displayed (e and f in Figure 1).

Counts of *D. pulex* individuals by DIP have been calibrated by comparing them with manual counts carried out under a magnifying trinocular microscope (Jena, Citoval Carl Zeiss, GmbH, Oberkochen, Germany). Individuals of *D. pulex* were counted manually under the magnifying trinocular by groups of three to five individuals, then transferred to the counting chamber for image capture until 120 individuals were obtained ($n = 44$).

Calibration of size measurements by DIP

A total of 94 individuals were taken into account for the calibration of size measurements through DIP. These individuals included neonates (age <24 h) and adults (age >3 days). They were randomly selected with or without eggs. Each individual was photographed, then preserved in an aqueous solution of sucrose (60 g/l) and buffered formaldehyde (2%), and maintained at 4 °C in the darkness for future microscopic measurements (Prepas 1978; Mastail & Battaglia 1978).

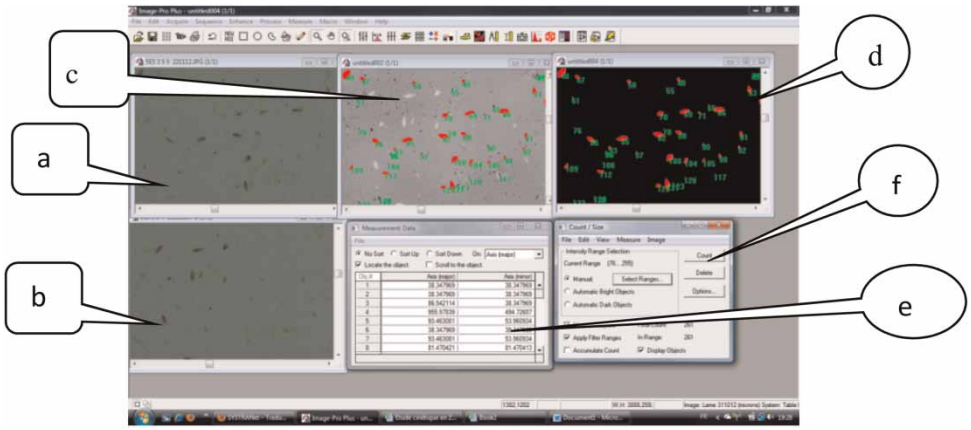

Figure 1 | Screenshot of the DIP using Image Proplus®: a and b = successive digital images used at the same time; c = result of the subtraction of the two images displaying the intial (white color) and final (black color) positions of the alive individuals; d = final position of alive individuals displayed on a black background; e = results of the measurements; f = total counts of individuals.

The dimensions (i.e., major axis, lateral minor axis and dorsal minor axis) of each individual were measured at X32 magnification with a micrometric eyepiece calibrated beforehand using a standard micrometric slide. Each measurement on a given individual was repeated three times. Note that the smallest graduation at X32 magnification corresponds to 30.303 μm.

Relationship between dimensions of an individual

Færøvig *et al.* (2002) reported that the bio-volume of *D. magna*, assimilated to a prolate spheroid, was the best predictor of their biomass. The bio-volume is determined as follows:

$$V = \frac{\pi}{6} * \text{Major axis} * (\text{Minor dorsal axis})^2.$$

This character (prolate spheroid) was checked for *D. pulex* in our study through regression analyses, including the three axes that define the dimensions of each individual. Indeed, *D. pulex* could be considered as a prolate spheroid if its lateral and dorsal minor axes were similar.

Given that the dorsal minor axis could not be assessed through DIP, it was determined using a regression analysis, including the major and lateral minor axes.

Dry weights measurements and estimates

Biomass (dry weight) estimation by DIP was calibrated using a regression equation between individuals' dry weights determined on a microbalance (accuracy ±1 μg; MX5 Balance, Mettler-Toledo SAS, Viroflay, France) and their dimensions measured by DIP. Fifteen size classes of individuals were considered. The number of individuals in each size class was defined taking into account the sensitivity of the microbalance. Dry weight determination was achieved according to McCauley (1984).

Growth kinetic characterization

Overview of cultivation methods implemented for growth-kinetic studies

Growth-kinetic studies are usually focused on microorganisms (bacteria, algae, etc.) and on inert substrates. Two culture methods are generally used: batch culture and continuous culture. In batch culture, measurements are carried out by simultaneously checking the increase in biomass of the studied organism and the depletion of each inert substrate at time intervals. In this case, the biodegradation caused by the studied organism is assumed to be the only factor in the consumption of substrates. In continuous cultures, the measurements of biomass and substrate concentrations are performed at steady state. In this case, the consumption of substrates corresponds to the balance between the substrate concentrations in the inlet and that in the outlet of the culture (chemostat); the biomass concentration does not vary since the growth rate and the dilution rate of the culture are balanced.

Adaptation of the method to cladocerans

The adaptation of the method to our objective took into account the living character of the substrates and the constraints related to the culture of cladocerans.

Aspect related to the living character of the substrate (*Scenedesmus* sp.)

In contrast to inert substrates, the concentration of living substrates may experience a decline that can be linked both to the death of the species involved and the consumption by the studied organism. To overcome this problem, the living substrate suspension is generally prepared daily (in order to avoid its fluctuations) and delivered continuously (Lampert 1976; Sterner 1993). Experimental conditions (light intensity = 25 μmol m^{-2} s^{-1}) are usually set up to avoid the growth of the living substrate (*Scenedesmus* sp. in our study) but still favorable only to *D. pulex*.

A two-stage experimental artificial food chain was set up to avoid the sedimentation and the variability in food quality and quantity (Lampert 1976; Sterner 1993). It consisted of the following:

(i) A 5-l chemostat, which ensures the production of *Scenedesmus* sp. fed at a dilution rate of 0.73 d^{-1} with a combo medium (Kilham *et al.* 1998) from a 5-l reservoir. The chemostat was illuminated with a 213 μmol m^{-2} s^{-1} light intensity.

(ii) Three 400 ml culture tanks of *D. pulex*, each one fed continuously with the culture of *Scenedesmus* sp. produced in the chemostat and diluted, if needed, in a mixture tank with a sterile combo medium, and three Plexiglas tanks receiving the outflow coming from each *Daphnia* culture tank. Each *D. pulex* reactor was fed with 800 ml/d and illuminated at 25 μmol m^{-2} s^{-1}.

The cultures were started with *D. pulex* neonates (less than 24 hours old) and were carried out over 2 weeks. The cultivations were performed in thermostated baths. Temperature, pH and dissolved oxygen in the experimental tanks with *D. pulex* were measured once a day around noon.

Aspect related to the constraints inherent to *Daphnia* cultivation

The cultures of *D. pulex* were performed continuously, not in the manner of a chemostat, as the outflows of *Daphnia* biomass were restricted by the plankton net placed at the bottom of the *Daphnia* culture vessel. Only biomass of *Scenedesmus* sp. were transported in the outflow. It was therefore not expected to reach a steady state for the determination of the growth rates of *D. pulex*. Accordingly, the biomass of *D. pulex* was determined every 2 days using DIP. The growth rate of *D. pulex* in each substrate (*Scenedesmus* sp.) concentration was then determined graphically.

Selection of *Scenedesmus* sp. (substrate) concentrations for the study

To cover the substrate concentrations, including half-saturation constant (K_X) and inhibition constant (K_I), *Scenedesmus* sp. concentrations were defined on the basis of the simulations performed with data from Ovie & Ovie (2008). The results of simulation analyses allow us to consider *Scenedesmus* sp. concentrations lower than 5×10^5 cells/ml, and those higher than 1.7×10^6 cells/ml in our experimental design. Practically, five concentrations of *Scenedesmus* sp. ranging from 0.18 (9.42×10^3 cells/ml) to 235.64 mg/l (1.21×10^7 cells/ml) were considered.

Because each of the five food concentrations feeding experiment tested was run for 2 weeks, the number of points used for fitting our data to existing kinetic models was limited to 10.

Application of growth kinetic characterization

For each experimental series, the logarithm of dry weight to the time (day) was plotted. The growth rate (r_{graphic}) was then deducted as the slope of the regression line.

Regarding the growth kinetic of *D. pulex*, it was assessed through the fitting of four models to the experimental data. The models included the Andrews model (Andrews 1968), the Edwards model (Edwards 1970), and the Haldane model (Briggs & Haldane 1925), along with the Monod model (Monod 1949).

Monod (1949)

$$r = \frac{r_{\max} X_{\text{substrat}}}{K_X + X_{\text{substrat}}};$$

Andrews (1968)

$$r = \frac{r_{\max} X_{\text{substrat}}}{(X_{\text{substrat}} + K_X)\left(1 + \frac{X_{\text{substrat}}}{K_I}\right)}$$

Edwards (1970)

$$r = \frac{r_{\max} X_{\text{substrat}}}{(K_X + X_{\text{substrat}})} \exp\left(\frac{-X_{\text{substrat}}}{K_{\text{I}}}\right)$$

Haldane (Briggs & Haldane 1925)

$$r = \frac{r_{\max} X_{\text{substrat}}}{K_X + X_{\text{substrat}} + (X^2_{\text{substrat}}/K_I)}$$

where r is the growth rate (d^{-1}); r_{max} is the maximum specific growth rate (d^{-1}); K_X and K_I refer to the half-saturation and inhibition constants, respectively (mg/l), and $X_{substrat}$ is the biomass of the substrate (mg/l).

K_X expresses the substrate content for which half of the maximum growth rate (r_{max}) is observed. Its value gives an indication of the affinity of the substrate for the studied organism: the higher the value, the lower the affinity between the substrate and the studied organism. The inhibition constant K_I traduces the affinity of the inhibitor for the studied organism: the lower its affinity with the studied organism, the higher its value.

Non-linear least-squares estimation method was used for data fittings.

RESULTS AND DISCUSSION

Counting calibration using image processing

A strong correlation was found between the results of the manual and the DIP-based counting ($R = 0.998$, $p < 0.05$; $n = 44$). DIP enables a counting of *D. pulex* individuals as reliable as manual counting, even at a density of 120 individuals/100 cm^2. Similar results have been obtained by Færøvig *et al.* (2002) on counts of *D. magna* by this same technique.

Relations between an individual's axes length

A *T*-student test involving the normalized lateral minor and dorsal minor axes showed a significant difference ($p < 0.05$), suggesting that *D. pulex* cannot be assimilated to a prolate spheroid for its bio-volume estimation. However, the three axes of the same individual present good linear correlation between their lengths ($0.89 < r < 0.95$)

The major axis was a better descriptor of each *D. pulex* than the two minor axes.

The lateral minor and the dorsal minor axes (determined using the microscope) of each *D. pulex* could be expressed as a function of the major axis. The equations were:

Lateral minor axis $= 0.570 \times$ Major axis (*F*-value $=$ 709.525, $p < 0.0001$; adjusted $R^2 = 0.884$);

Dorsal minor axis $= 0.316 \times$ Major axis (*F*-value $=$ 669.720, $p = 0.0001$; adjusted $R^2 = 0.878$).

All the obtained regressions for the two minor axes confirm that the major axis was the best descriptor of the individual compared to the other axes. This justifies why the major axis is always used for the biomass estimate of cladocerans species, based on dry weight-size relationships.

Size measurements calibration using image processing

Correlations analysis between size measurement by microscopy and DIP showed a correlation of 0.93 ($p = 0.0001$) for the major axis, compared to the dorsal minor axis ($R = 0.72$).

The comparison between DIP-based and microscopy-based measurements revealed a good correlation for the major axis (adjusted $R^2 = 0.852$, *F*-value $= 537.103$, $p < 0.0001$). This relationship was weak for the dorsal minor axis (adjusted $R^2 = 0.381$, *F*-value $= 57.331$, $p < 0.0001$).

Given that *D. pulex* individuals arise most often laterally rather than dorsally, the estimate of the major axis appears more realistic. But despite the good statistical indicators for the major axis, the measured values were under-estimated when using the DIP method (bias $\approx 21\%$). Our analysis showed that a correcting factor of 1.289 is required and must be applied. The relatively high bias in our case, compared with the 10% bias found by Færøvig *et al.* (2002), is probably due to the differences in size of the two species (*D. magna* being at least twice larger than *D. pulex* at the same age).

Calibration of biomass estimates by image analysis

High correlations ($R > 0.9$) were found between the logarithm of dry weight and that of each axis (i.e., major, lateral minor and dorsal minor axes). In agreement with Færøvig *et al.* (2002), a relatively better correlation was observed for the bio-volume.

The comparisons between the estimated and measured dry weights revealed that the regression-based model derived from the corrected major axis was more reliable than the bio-volume based estimation (Figure 2). The average bias of -4.325% was not significantly different from 0 ($p = 0.568$). Færøvig *et al.* (2002) did not mention if they compared estimated and measured values.

The orders of magnitude of our measured and estimated (regression model based on the major axis) dry weights are comparable to those from the literature. The differences between our findings and the conclusions from previous studies, namely the model of Dumont *et al.* (1975), could be explained by the differences in environmental conditions.

Characterization of the growth kinetic of *D. pulex*

The results of the fitting using the four models are given in Tables 1 and 2. Better fits were obtained by the inhibition-type models (Haldane, Andrews and Edwards models)

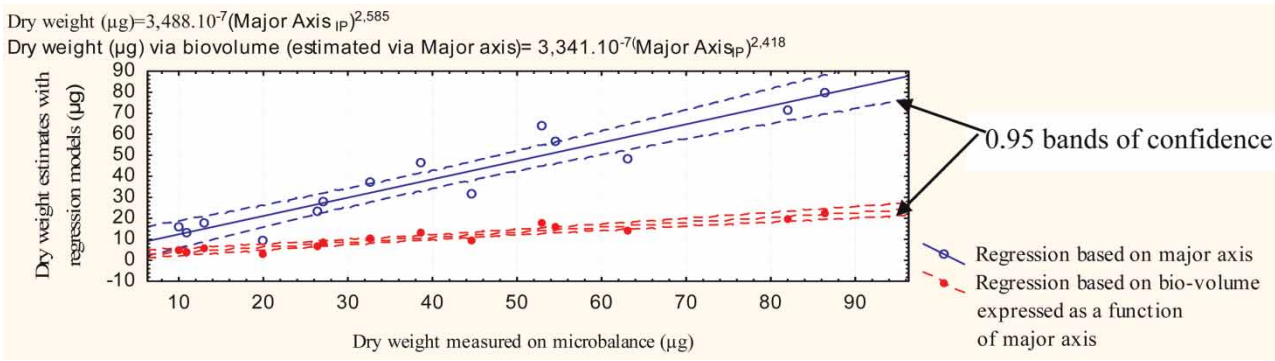

Figure 2 | Comparison of estimated and measured dry weights.

Table 1 | Comparative analysis of model variances

Model	Effect	Sum – squares	df	Least squares	*F*-value	*p*-value	*r*	% of VAF	RMSE	MAE
Monod	Regression	1.21	2	0.60	259.62	0.00	0.74	55	0.002	0.04
	Residues	0.03	11	0.00						
Haldane	Regression	1.23	3	0.41	557.51	0.00	0.93	87	0.001	0.02
	Residues	0.01	10	0.00						
Andrews	Regression	1.23	3	0.41	557.51	0.00	0.93	86	0.001	0.02
	Residues	0.01	10	0.00						
Edwards	Regression	1.23	3	0.41	545.07	0.00	0.93	87	0.001	0.02
	Residues	0.01	10	0.00						

df = degree of freedom; VAF = variance accounted for; RMSE = root-mean-square error; MAE = mean absolute error.

Table 2 | Comparative analysis of the estimates of kinetic parameters

Model		Param.	Estimate	Standard error	*t*-value df = 6	*p*-level	Lo. Conf. lim	Up. Conf. lim
Monod	$r=\frac{r_{\max}X}{K_X+X}$	$r_{\max}$ (d^{-1})	0.33	0.02	21.09	0.00	0.30	0.36
		K_X (mg/l)	0.10	0.04	2.52	0.03	0.01	0.19
Haldane	$r=\frac{r_{\max}X}{K_X+X+(X^2/K_I)}$	$r_{\max}$ (d^{-1})	0.38	0.01	26.17	0.00	0.35	0.41
		K_X (mg/l)	0.15	0.03	5.19	0.00	0.08	0.21
		K_I (mg/l)	459.43	123.08	3.73	0.00	185.2	733.66
Andrews	$r=\frac{r_{\max}X}{(K_X+X)(1+(X/K_I))}$	$r_{\max}$ (d^{-1})	0.38	0.01	26.17	0.00	0.35	0.41
		K_X (mg/l)	0.15	0.03	5.19	0.00	0.08	0.21
		K_I (mg/l)	459.43	123.08	3.73	0.00	185.2	733.66
Edwards	$r=\frac{r_{\max}X}{(K_X+X)}\mathrm{Exp}\left(\frac{-X}{K_I}\right)$	$r_{\max}$ (d^{-1})	0.37	0.01	28.15	0.00	0.35	0.4
		K_X (mg/l)	0.14	0.03	5.16	0.00	0.08	0.2
		K_I (mg/l)	577.36	128.95	4.48	0.00	290.04	864.68

with at least 86% of variance accounted for (VAF). The VAF using the Monod model was 55%. Overall, the kinetic parameters were well estimated ($p < 0.0001$, Table 2) with the inhibition type models.

Regardless of the model used, the growth rate of *D. pulex* was satisfactorily estimated ($p < <0.05$), with similar values for growth inhibition-type models ($r_{\max} = 0.38 \pm 0.02\ d^{-1}$) and different values for the model of Monod ($r_{\max} = 0.33 \pm 0.02\ d^{-1}$). The half-saturation constants (K_X) were slightly different between the models: $K_X = 0.15 \pm 0.03$ mg/l, that is about 7.71×10^3 cells/ml) for the growth-inhibition type models, and $K_X = 0.10 \pm 0.04$ mg/l, for the

Monod model (Table 2); whereas the value of the inhibition constant (K_I) did vary between the growth-inhibition type models: $K_I = 459.43 \pm 123.08$ mg/l (Haldane and Andrews models), and $K_I = 577.36 \pm 128.95$ mg/l (Edwards model), i.e., about 2.97×10^7 cells/ml.

The high value of the inhibition constant observed demonstrates that chlorophyceaes are not strong inhibitors for cladocerans. Likewise, the relatively low value obtained for the half-saturation constant reflects the high affinity of *Daphnia* to chlorophyceaes as substrates. Operatively, in our case, 2×10^6 cells/ml appears to be the optimum concentration of chlorphycaes that should not be exceeded for good productivity of cladocerans. That is also true in studies reported by Ovie & Ovie (2008).

The ingestion rate saturation is well known for occurring with cladocerans (Peters 1984 and references therein). It seems that this phenomenon has been considered in many modeling works by choosing Monod's model to describe the growth kinetics of daphnids (Hathaway & Stefan 1995).

However, the monod model does not give a good description of *Daphnia*'s growth kinetic in the presence of high chlorophyceae concentration, which rather results in a decrease of the growth rate (Ovie & Egborge 2002; Ovie & Ovie 2008) even in the presence of non-senescent chlorophyceae food (as is shown in this study).

CONCLUSION

Our results provide a contribution to the modeling of the functioning of pond systems by specifying that growth inhibition by high-substrate content-type models are the ones that adequately describe the growth kinetic of cladocerans on chlorophyceae. These results could explain why algal blooms, instead of increasing the production of cladocerans, gradually cause their disappearance, even in the absence of cyanobacteria. Developing this kind of research on other types of substrates, such as bacteria, would be helpful for improving the modeling of biochemical conversion processes that occur in pond systems.

ACKNOWLEGEMENTS

We are grateful to the Government of Belgium for granting financial support to the first author, M. N. D. Liady, for his Mixed PhD research. The authors would like to acknowledge the Belgian Technical Cooperation, which executes this financial support program on behalf of the Belgian Government.

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First received 8 October 2014; accepted in revised form 23 February 2015. Available online 12 March 2015