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# Biological control of ciliate contamination in *Chlamydomonas* culture using the predatory copepod *Acanthocyclops robustus*

Nguyen Thi Kim Hue [1,2,*], Bert Deruyck[1], Ellen Decaestecker[1], Dries Vandamme[1, 3], Koenraad Muylaert[1]

[1] Lab. Aquatic Biology, Department of Biology, Campus Kulak Kortrijk, KU Leuven University, Etienne Sabbelaan 54, 8500 Kortrijk, Belgium.

[2] Department of Biology, College of Natural Sciences, Can Tho University, Vietnam.

[3] UHasselt – Hasselt University, B 3590 Diepenbeek, Belgium

* Corresponding author: Nguyen Thi Kim Hue at Laboratory of Aquatic Biology, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium. E-mail address: thikimhue.nguyen@kuleuven.be (N.T. Kim Hue).

**Abstract**

Ciliates are a common but less-explored group of contaminants in microalgal cultures that feed on microalgae and can cause severe losses in productivity of cultures. The aim of this study was to evaluate the potential of biological control to eradicate ciliates from microalgal cultures. In lab-scale experiments, we used the carnivorous cyclopoid copepod *Acanthocyclops robustus* as a biological control agent to eliminate the ciliate *Sterkiella* from cultures of the microalga *Chlamydomonas*. Our experiments showed that the copepod *Acanthocyclops robustus* can consume up to 400 ciliates individual$^{-1}$ day$^{-1}$. Addition of 0.07 copepods mL$^{-1}$ to a culture that was contaminated with 10 ciliates mL$^{-1}$ resulted in a complete elimination of ciliates from the culture within 1 day and restored the algal biomass production at the level of a non-contaminated culture. Addition of copepods to a fresh *Chlamydomonas* culture did not cause a reduction in the microalgal biomass concentration, indicating that this copepod does not feed on *Chlamydomonas*. These laboratory-scale experiments indicate that copepods have potential to be used as a biological control agent to address the problem of contamination of large-scale microalgal cultures by ciliates.

**Keywords:** ciliate contamination, culture crash, biological control, *Acanthocyclops robustus*, algal biofuels, *Chlamydomonas*.

## 1. Introduction

Microalgae have been attracting interest over the past decade as a promising source of biomass for the production of biofuels and valuable bioproducts [1]. Microalgae are cultivated either in open raceway ponds or in closed photobioreactors [2]. These systems often suffer losses in production due to contamination of cultures by predators, parasites and competing microalgae [3, 4]. Among these contaminants, ciliate predators are a common problem [5]. Ciliates can achieve very high growth rates (about 0.77 - 1.01 $day^{-1}$, [6]) and can therefore cause a crash of the microalgae culture within a matter of days [7, 8]. Preventing contamination of microalgal cultures by ciliates is difficult as ciliates form dormant resting cysts that are dispersed through air, rainfall, wind or dust as well as water [9]. Since open pond cultivation systems are exposed to the atmosphere they are highly susceptible to contamination [5]. Even though photobioreactors are closed to the atmosphere, preventing contamination in these systems is also a challenge considering the need to sterilize large volumes of water (to prepare the culture medium) and air (to sparge the culture) [10].

There is need for sustainable strategies to control contamination of microalgal cultures by ciliates in order to avoid losses in productivity [11]. A few studies have proposed chemical control of ciliates. Moreno-Garrido and Canavate [8] used quinine sulphate to control contamination of a *Dunaliella* culture by hypotrich ciliates. More recently, Xu et al. [12] demonstrated the use of the natural plant chemical toosendanin to control contamination by the ciliate *Stylonychia* in a *Chlorella* culture. In a previous study, we used natural chemicals produced by marine microalgae as a defense against predators to control contamination of *Chlamydomonas* cultures by the ciliate *Sterkiella* [13].

As an alternative to chemical control, several authors have advocated the use of biological control of biological contaminants in microalgal cultures [5, 14]. In biological control, the predator of a pest species is introduced to control this pest species. Biological control is based on the 'trophic cascade' principle, which states that biomass of primary producers can be controlled by introduction of a top-predator that feeds on the predator of the primary producers [15] (Fig. 1). Since trophic cascades are often strong in aquatic food webs [16], biological control has a lot of potential to be used as a contamination control strategy in microalgae cultivation [5]. Biological control is increasingly used in agriculture to protect crops [17]. It is assumed to be more sustainable than chemical control because pests cannot evolve resistance to a biological control agent as rapidly as against chemicals [18]. Recently, Montemezzani et al. [19] proposed to control the rotifer *Brachionus* in high rate algal ponds by introducing the cladoceran *Moina tenuicornis* or the ostracod *Heterocypris incongruens*. Thom et al. [20] proposed to introduce aquatic insects into microalgal cultures to control *Daphnia* invasions.

To our knowledge, however, no study has so far attempted to implement a biocontrol method to control ciliate contamination in microalgal culture. Copepods are important predators of ciliates both in freshwater and marine environments [21]. Cyclopoid copepods are particularly common in freshwater while calanoid copepods are more common in seawater [21]. Cyclopoid copepods are ambush predators that hang motionless in the water and detect movements of ciliates using sensory setae on their antennae [22]. When a ciliate is detected, the copepod carries out an attack strike to catch and consumes the ciliate [23]. Smith and Crews [5] proposed the idea to introduce copepods into microalgal cultures to control contamination by ciliates. Biological control will not work when the copepods not only feed on the ciliates but also on the microalgae [11]. Although some cyclopoid copepods may also feed on microalgae, they generally prefer ciliates over microalgae [24].

The aim of this study was to test whether a cyclopoid copepod can be used in a biological control strategy to control ciliate contamination in microalgal cultures. As a model system, we used a culture of the microalga *Chlamydomonas* that was deliberately contaminated with the ciliate *Sterkiella*. We isolated a cyclopoid copepod from a freshwater lake and tested whether this copepod was capable of eradicating the ciliate *Sterkiella* from a contaminated *Chlamydomonas* culture and maintaining the microalgal biomass production of that culture (Fig. 1). We also evaluated whether the copepod itself impact the microalgal biomass by direct consumption of microalgae.

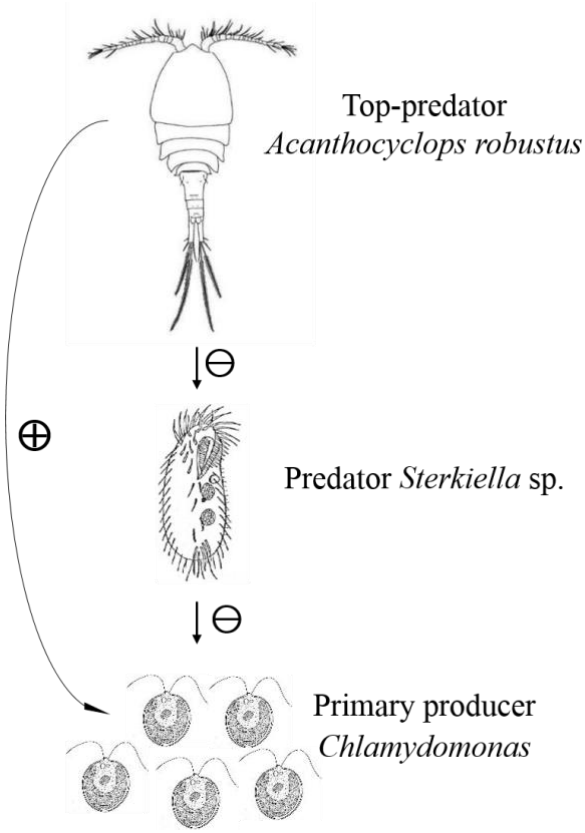


**Figure 1:** Illustration of the trophic cascade in which the cyclopoid copepod *Acanthocyclops robustus* is used to control contamination by the predatory ciliate *Sterkiella* sp. in cultures of the microalga *Chlamydomonas* (⊖). The top-predator *Acanthocyclops robustus* has an indirect positive effect on the primary producer *Chlamydomonas* by controlling the predator *Sterkiella* (⊕).

## 2. Materials and methods

### *2.1 Isolation and cultivation of the copepod*

Copepods were collected in June 2017 from a eutrophic freshwater pond near the KU Leuven campus in Kortrijk, Belgium, using a zooplankton net (nylon mesh size 180 µm). At that moment, a dense population of cyclopoid copepods was present in the pond (about 500 individuals $L^{-1}$). The concentrate containing copepods was gently rinsed over a 100 µm nylon mesh using sterile distilled water to remove as much microalgae and other microorganisms as possible. A few adult copepods were transferred to 300 mL of sterile distilled water and were fed every 3 d with a suspension containing about 1000 cells $mL^{-1}$ of the ciliate *Sterkiella* sp. The copepod cultures were subcultured once in every 3 weeks by collecting the copepods on a 30 µm nylon mesh. The copepod species was identified based on morphological criteria using detailed microscopic observations (Olympus SZX10 stereo microscope).

Like other crustaceans, copepods have a metamorphic life cycle and go through a nauplius and a copepodite phase before reaching the adult phase. Only adult copepods and copepodites were used in the experiments. Adult copepods and copepodites were separated from nauplius stages by filtration over a 180 µm nylon mesh. Dead individuals were removed by pipette to ensure that only live copepods and copepodites were used in the experiments. Egg sacs-carrying females were also removed to avoid the emergence of large numbers of nauplii during the course of the experiment.

*2.2 Ciliates and microalgae preparation*

The chlorophyte microalga *Chlamydomonas reinhardtii* SAG 77.81 and the hypotrich ciliate *Sterkiella* sp. were used as model systems to evaluate the control of ciliate contamination in microalgal cultures by a copepod. *Chlamydomonas reinhardtii* is a widely studied model microalgal species. The ciliate *Sterkiella* was isolated from a rainwater storage reservoir in Belgium. Identification of the ciliate *Sterkiella* sp. was based on morphological criteria (Fig. 2) and was confirmed by partial 18S ribosomal DNA sequencing (GenBank accession number: MF375457; more information in [13]). *Sterkiella* belongs to the hypotrich ciliates, a group of ciliates that commonly invade microalgal cultures. *Sterkiella* has been reported as a contaminant in a large-scale *Scenedesmus* culture [25].


**Figure 2.** Microphotograph of the hypotrich ciliate *Sterkiella* and *Chlamydomonas* on which it feeds.

*Chlamydomonas* cultures were maintained in 2 L batch cultures in Wright's Cryptophyte (WC) medium [26] in a temperature-controlled room (20 ± 2 ºC) at a light intensity of 80 µmol $m^{-2}$ $s^{-1}$ and a light-dark cycle of 16:8 h. Growth of *Chlamydomonas* was monitored spectrophotometrically at optical density 750 nm ($OD_{750}$). $OD_{750}$ was linearly related to cell density (determined microscopically) as well as dry weight biomass concentration (determined gravimetrically) [27]. Exponential phase (7 d old) *Chlamydomonas* cultures were used in all the experiments.

A *Sterkiella* culture was maintained in 6-well plates and was fed with a suspension of microalga *Chlorogonium elongatum* SAG 30.98, cultured in sterile WC medium. Large numbers of ciliates needed for the experiments were grown in glass bottles (350 mL) using the same food source. Abundance of ciliates was determined using a Sedgewick-Rafter counting chamber under Olympus SZX10 stereo microscope (32 x magnification). Exponentially growing *Sterkiella* cultures with an abundance of about 1000 cells $mL^{-1}$ were further concentrated on a 10 µm nylon mesh to obtain a density of 4000 cells $mL^{-1}$. This dense suspension was added to the *Chlamydomonas* cultures in controlled contamination experiments.

*2.3 Biological control of ciliates by copepods*

A series of experiments was carried out to investigate whether the cyclopoid copepod that was isolated was capable of biological control of the ciliate *Sterkiella* in a *Chlamydomonas* culture. All experiments were carried out in 350 mL glass jars with 150 mL of exponential phase *Chlamydomonas* culture (initial $OD_{750}$ 0.15 - 0.3). The cultures were gently mixed by bubbling with filtered air from the bottom of each jar. The experimental treatments were incubated in a temperature-controlled room (20 ± 2 ºC) and exposed to a light intensity of 80

µmol $m^{-2}$ $s^{-1}$ and a light-dark cycle of 16:8 h. Ciliates were added from a concentrated stock culture. Copepods and copepodites (further referred simply as 'copepods') were added immediately after addition of the ciliates. The copepods were starved for 3 d before addition. In all experiments, dead copepods were removed routinely and were replaced with living copepods. Mortality of copepods was always below 10%. Each experimental treatment was executed in triplicate for all experiments. Abundance of ciliates was monitored using a Sedgewick-Rafter counting chamber and an Olympus SZX10 stereo microscope (32 x magnification) while biomass production of microalgae was monitored as $OD_{750}$. Copepods (at < 0.4 ind. $mL^{-1}$) were not present in the samples collected for optical density measurements. Whereas, ciliates even when present, their influence on $OD_{750}$ is negligible (ca. 1%).

Firstly, the predation impact of copepods on the ciliate population in a contaminated *Chlamydomonas* culture was determined. In this experiment, a fixed number of copepods (0.2 ind. $mL^{-1}$) was added to a *Chlamydomonas* culture to which varying number of the ciliate *Sterkiella* had been added (1, 10, 30 and 60 $mL^{-1}$). *Chlamydomonas* cultures without *Sterkiella* and without copepods were used as controls. Ciliate abundance in the experimental treatments was monitored every day for 3 d.

In the second experiment the capability of copepods to maintain the biomass production of ciliate-contaminated microalgal culture to the level of a non-contaminated culture was evaluated. This experiment consisted of 4 treatments. The first treatment served as a positive control and consisted of a *Chlamydomonas* culture that was not contaminated with ciliates and to which no copepods were added. The second treatment was a negative control and consisted of a *Chlamydomonas* culture that was contaminated with 10 ciliates cells $mL^{-1}$ and to which no

copepods were added. The third treatment consisted of a *Chlamydomonas* culture that was contaminated with 10 ciliates $mL^{-1}$ and to which 0.2 copepods $mL^{-1}$ were added. The final treatment served to evaluate whether copepods have a direct negative effect on *Chlamydomonas* and consisted of a *Chlamydomonas* culture that was not contaminated with ciliates but to which 0.2 copepods $mL^{-1}$ were added. Ciliate abundance and microalgal biomass ($OD_{750}$) in the experimental treatments was monitored every day for 4 d. As an add-on to this experiment, we tested whether the nauplii of copepods might have a negative impact on microalgal biomass concentration of the *Chlamydomonas* culture. To test this, three different densities of nauplius stage 6 (1, 5, 10 nauplii $mL^{-1}$) were added to *Chlamydomonas* cultures and *Chlamydomonas* culture without nauplii served as control. Microalgal biomass ($OD_{750}$) in the experimental treatments was monitored every day for 4 d. The density of nauplii was determined after 4 d to determine the survival rate.

In the third experiment the minimal density of copepods required to control ciliates in a microalgal culture was determined. This experiment had the same positive and negative control as in the second experiment. Six different experimental treatments were prepared with different densities of copepods added (0.007, 0.03, 0.07, 0.1, 0.2, 0.4 $mL^{-1}$) in the beginning of the experiment. Following 2 d of introduction of copepods,10 ciliate cells $mL^{-1}$ were added to the culture. Ciliate abundance and microalgal biomass ($OD_{750}$) were monitored every day for 4 d.

*2.4 Statistical analysis*

For the first experiment, two-factor analysis of variance (two-way ANOVA) was used to test for the independent and interacting effects of ciliate concentration and the presence/absence of copepod *Acanthocyclops robustus* on the biomass production of *Chlamydomonas* in the cultures. One-way ANOVA was used to evaluate the predation effect of copepods on the biomass production of *Chlamydomonas* and on ciliate density in the second experiment. In the third experiment, the effect of copepods on ciliate density and *Chlamydomonas* biomass production was also analyzed in a one-way ANOVA. Tukey's HSD post hoc test was used for multiple pair-wise comparisons between samples. All statistical analyses were carried out using R.

To determine the maximum consumption rate of ciliates by the copepod, the relation between consumption of ciliates and ciliate abundance was investigated using data from experiments 1 and 3. For each one-day time interval of each experimental treatment (n = 30), the number of *Sterkiella* that were consumed by *Acanthocyclops robustus* was estimated from the difference between the observed and expected abundance of ciliates at the end of a one-day time interval. The expected number of ciliates and the end of the one-day time interval was estimated from the initial abundance at the start of the time interval assuming that the ciliate population experienced exponential growth. A constant exponential growth rate was used, which was the average of the growth rates calculated in the experimental control treatments to which no copepods were added ($0.874 \pm 0.06$ day$^{-1}$; n = 12).

## 3. Results and discussion

### *3.1 Copepod isolation and cultivation*

A cyclopoid copepod species was collected in a eutrophic pond and transferred to the laboratory where it was fed with a suspension of the ciliate *Sterkiella*. The cyclopoid copepod was identified as *Acanthocyclops robustus* (G.O. Sars) [28]. Adults of *Acanthocyclops robustus* are 1-1.3 mm in size. *Acanthocyclops robustus* closely resembles *Acanthocyclops vernalis* but has a round (as opposed to pointed) genital segment, a convoluted (as opposed to smooth) inner edge on the first segment of the endopod (E1) of the fourth leg, and an extra growth at the base of forked caudal rami (Fig. 3D-E). *Acanthocyclops robustus* is common in eutrophic freshwater environments, where it can achieve abundances of several hundred individuals $L^{-1}$ in summer [29]. Like other copepods, *Acanthocyclops robustus* has a metamorphic life cycle that consists of 4 stages: egg, nauplius, copepodite and adult. In our cultures, adult females produced two egg sacs containing 40 to 75 eggs in every 2 to 5 d. These eggs required 2 d to mature. From the eggs nauplius larvae emerged that consist of a head and a small tail but that lack a thorax and abdomen. After 5 d and 6 rounds of molting, the nauplius transformed into a copepodite. The copepodite is similar to an adult state except that the tail is not segmented. After another 6 d and 5 rounds of molting the copepodite transformed into an adult copepod. In our cultures, a single adult female copepod produced more than 400 offspring (about 6 clutches) within a time-frame of 3 weeks. The life expectancy of an adult copepod is about 3 months [30].

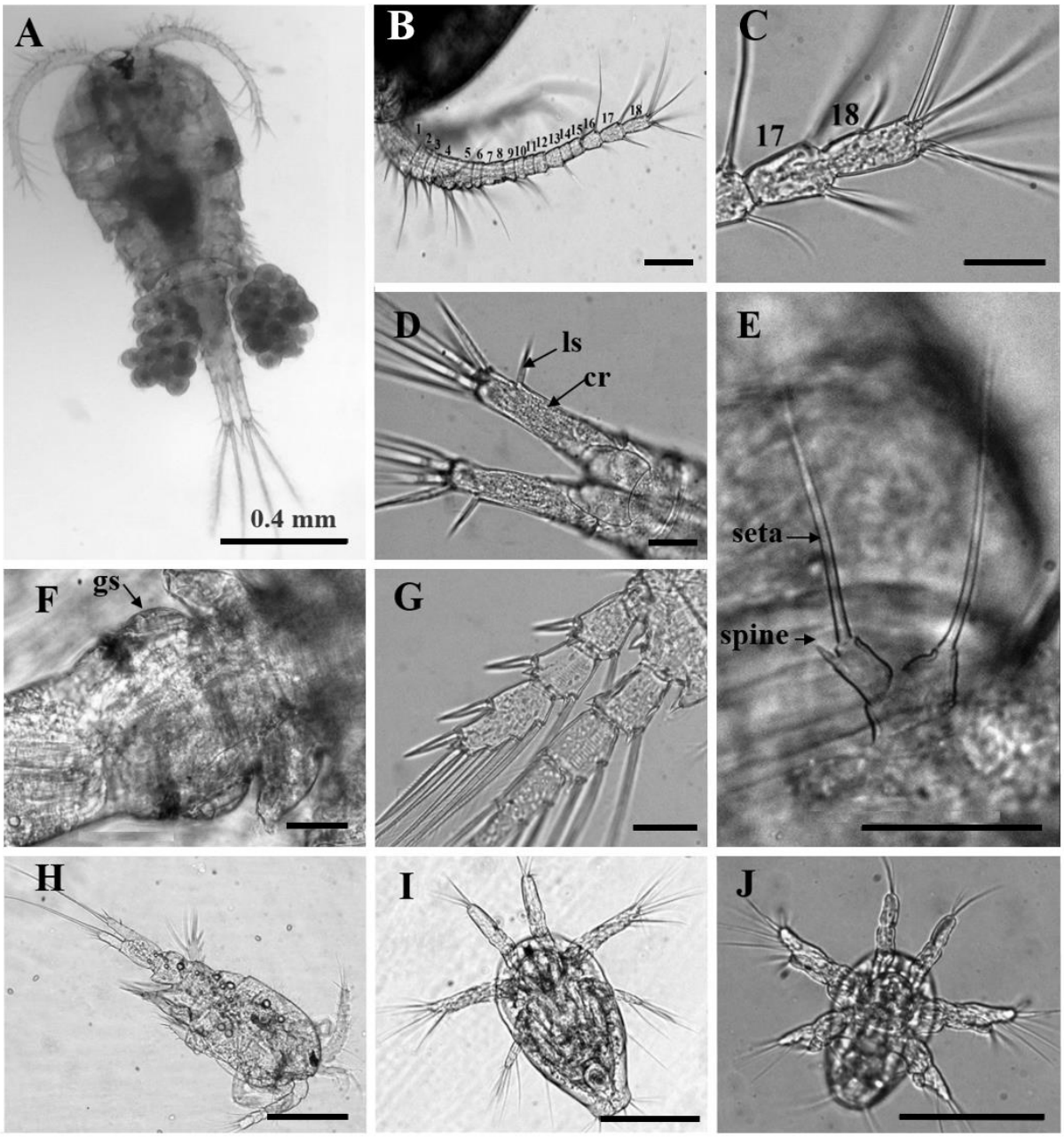


**Figure 3**: Microscopic picture of a female adult *Acanthocyclops robustus* (A). Detailed pictures of the first antennae (B), the 18th segment of the first antennae (C), the caudal rami (cr) and lateral setae (ls) (D), the terminal segment of the 5th leg (E), the genital segment (gs) (F) and the rectangular endopite 1 (E1) of 4th leg (G). Pictures of copepodite stage 1 (H), nauplius stage 6 (I) and the nauplius stage 1 (J). Scale bar 30 µm in B-E, 100 µm in H-J.

*3.2 Predation impact of copepods on ciliates*

The first experiment aimed to quantify the predation rate of the copepod *Acanthocyclops robustus* on the ciliate *Sterkiella*. 0.2 copepods $mL^{-1}$ were introduced into *Chlamydomonas* cultures that were inoculated with varying densities of the ciliate *Sterkiella* (1, 10, 30, and 60 ciliates $mL^{-1}$). In the contaminated control cultures that received no copepods the number of ciliates increased exponentially over time (Fig. 4A, Table 1). When copepods were added, the number of ciliates was reduced. In cultures that were inoculated with 1 and 10 ciliates $mL^{-1}$, the copepods eliminated all ciliates from the culture within 1 d (Fig. 4B). In cultures that were inoculated with 30 ciliates $mL^{-1}$, the copepods eliminated all ciliates from the culture after 2 d (Fig. 4B). In cultures that were inoculated with 60 ciliates $mL^{-1}$, the copepods reduced the ciliate population to 20 ciliates $mL^{-1}$ after 2 d but were not capable of completely eliminating all ciliates during the course of the experiment. These results indicate that the copepod *Acanthocyclops robustus* is a voracious predator of the ciliate *Sterkiella*.

In this study biocontrol was only tested on the ciliate *Sterkiella*. Cyclopoid copepods are known to feed on a wide range of ciliate species [31]. It is therefore likely that cyclopoid copepods like *Acanthocyclops* are also capable of controlling ciliates other than *Sterkiella*. It should be noted, however, that some ciliates are immune to copepod predation. Some ciliate species can detect a copepod attack and have a 'jumping' response that allows them to avoid predation (e.g. *Strombilidium*, [31]). Other species possess a calcium carbonate 'shell', a lorica or spines that provide protection against copepod predation (e.g. tintinnid *Metacylis* sp. [36, 39]). So far, however, ciliates that can escape predation using a jumping response or species that possess a lorica, shell or spines have not been reported as contaminants in large-scale

microalgal cultures. Further research needed to determine the range of ciliate species than can be controlled by cyclopoid or other copepods.

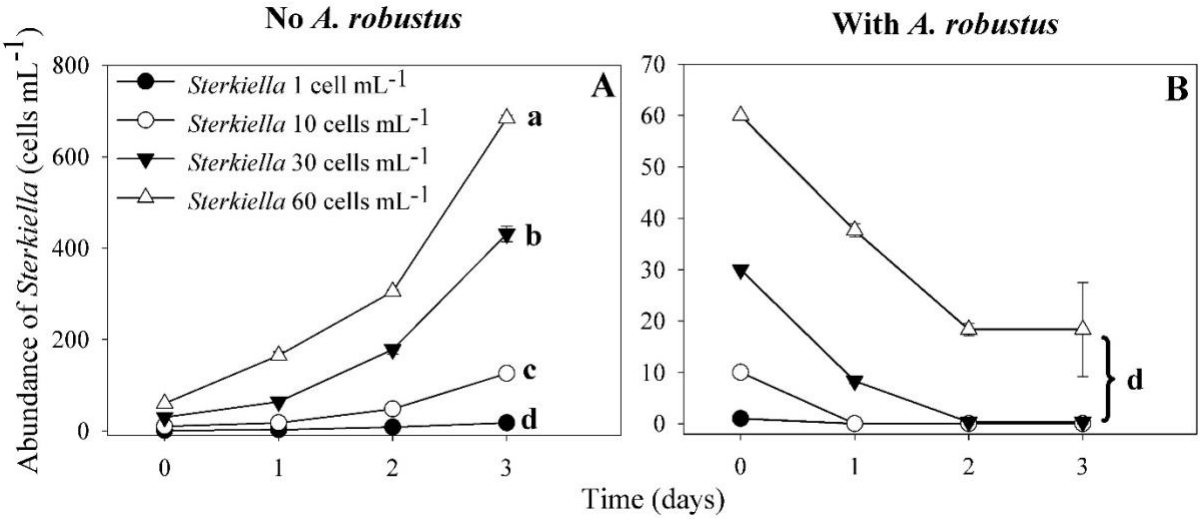


**Figure 4:** Abundance of the ciliate *Sterkiella* in *Chlamydomonas* cultures in the absence (A) and presence (B) of the copepod *Acanthocyclops robustus*. The *Chlamydomonas* cultures were inoculated with varying numbers of ciliates. The error bars correspond to one standard deviation of the means. The different letters "a-d" in panel A indicate that all treatments are significantly different from each other, while the letter "d" in panel B indicates no significant differences between different treatments according to Tukey's HSD post-hoc tests.

| Experimental treatment | d.o.f. | F | *p* |
|---|---|---|---|
| Copepods | 1 | 6268 | < 0.0001 |
| Initial ciliate abundance | 3 | 1564 | < 0.0001 |
| Copepods x Initial ciliate abundance | 3 | 1416 | < 0.0001 |

**Table 1.** Results of two-way ANOVA testing for the effects of initial ciliate abundance and presence/absence of copepods determined on day 3 in experiment 1 (d.o.f.: degrees of freedom).

### *3.3 Copepods increase microalgal biomass concentration in a microalgal culture contaminated with ciliates*

The aim of the second experiment was to evaluate whether the copepod *Acanthocyclops robustus* is capable of maintaining the microalgal biomass production in a contaminated *Chlamydomonas* culture to the same level as a non-contaminated culture. In a non-contaminated *Chlamydomonas* culture, the biomass concentration as estimated from $OD_{750}$ increased more or less linearly over time (Fig. 5A). Contamination of the culture with 10 *Sterkiella* $mL^{-1}$ caused a crash of the culture between 2 and 4 d (Fig. 5B). During the crash, *Sterkiella* abundance had increased to about 120 ciliates $mL^{-1}$ and the microalgal biomass concentration was reduced 75% compared to the non-contaminated culture. Addition of *Acanthocyclops robustus* (0.2 copepods $mL^{-1}$) to a non-contaminated *Chlamydomonas* culture had no significant effect on the biomass concentration of *Chlamydomonas* in the culture (Fig. 5A). When *Acanthocyclops robustus* was added to a *Chlamydomonas* culture that was contaminated with the ciliate *Sterkiella*, all ciliates were eliminated from the culture within 1 d (Fig. 5B, Table 2) and the biomass concentration of *Chlamydomonas* was not significantly different from the non-contaminated culture. These results indicate that the copepod *Acanthocyclops robustus* can be used as a biological control agent to eliminate the ciliate *Sterkiella* from cultures of the microalga *Chlamydomonas* and maintain the biomass production of the culture to the same level as a non-contaminated culture.

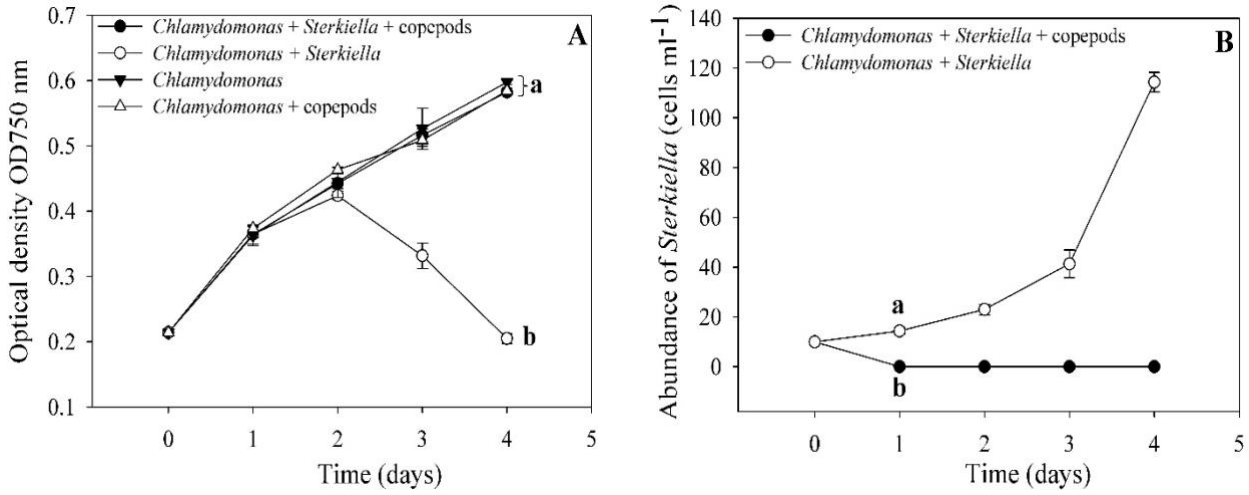


**Figure 5:** Comparison of the microalgal biomass concentration (as estimated from $OD_{750}$, A) and ciliate abundance (B) in a non-contaminated *Chlamydomonas* culture (black triangle-down), a *Chlamydomonas* culture to which the copepod *Acanthocyclops robustus* was added (0.2 individuals $mL^{-1}$) (white triangle-up), a *Chlamydomonas* culture that was contaminated with the ciliate *Sterkiella* (10 cells $mL^{-1}$) (white circle) and a contaminated *Chlaymodomonas* culture to which the copepod *Acanthocyclops* was added (black circle). The error bar corresponds to the standard deviation of means. The letter a in panel A groups treatments that are not significantly different from each other according to Tukey's HSD post-hoc test, while the letter b indicates that this treatment is different from the treatments grouped under letter a. The letters a and b in panel B indicate that the two treatments are significantly different.

| Experimental treatment | d.o.f. | F | *p* |
|---|---|---|---|
| Microalgal biomass (day 4) | 3 | 3001 | < 0.0001 |
| Ciliate abundance (day 1) | 1 | 264 | < 0.0001 |

**Table 2.** Results of two one-way ANOVA tests comparing microalgal biomass concentration (as estimated from $OD_{750}$) and ciliate abundance between the different treatments in experiment 2. (d.o.f.: degrees of freedom).

The experiment also indicated that *Acanthocyclops robustus* does not feed directly on *Chlamydomonas*, at least not to a degree that microalgal biomass concentration is reduced. This is important, as introduction of copepods into microalgal cultures should not cause a reduction in microalgal biomass concentration [11]. Like most other copepods, *Acanthocyclops robustus* is a carnivorous predator that feeds on relatively large organisms that range in size from 100 to 1000 µm [33]. *Sterkiella* (100 µm) fits perfectly within this size range but *Chlamydomonas* is too small (7 µm). It is known that most copepods have difficulty in handling small food items in the size range of unicellular microalgae like *Chlamydomonas* [34]. Hopp et al. [35] confirmed that *Acanthocyclops* cannot be raised on microalgal alone and reproduce only when its diet contained animal feed. Not only size of the feed may play a role, but also the high nitrogen content and nutritional value in ciliates than in microalgae [36]. Further research, however, is needed to evaluate whether *Acanthocyclops* feeds on species of microalgae other than *Chlamydomonas*.

While adults and copepodite copepods may not feed on microalgae, it is possible that the naupliar stages of *Acanthocyclops robustus* may feed on microalgae. Nauplii of some copepod species have been reported to feed on *Chlamydomonas* (e.g. *Mesocyclops leukartii* and *Cyclops vicinus* [37]). To test this, we incubated *Chlamydomonas* cultures with varying densities of nauplii from the copepod *Acanthocyclops robustus* (1, 5 and 10 nauplii $mL^{-1}$). As a result, no significant effect of addition of the nauplii on the biomass concentration of the *Chlamydomonas* culture could be observed (one-way ANOVA, $F = 0.227$, $p = 0.875$) (Fig. 6). Despite the fact that few *Chlamydomonas* cells could be observed in the gut of the nauplii (Fig. 3I), the impact of the nauplii on the biomass concentration of the microalgae was negligible.

Moreover, the survival rate of nauplii in the cultures without ciliate feed was poor: no live nauplii nor copepodite stages remained in the cultures to which 1 nauplius $mL^{-1}$ was added, while in the treatments with 5 and 10 nauplii $mL^{-1}$ only 15% of the nauplii survived, out of which only 12% had transformed into the copepodite stage after 4 d. This low survival of the nauplii may have been due to a lack of animal feed [35]. Most likely, the copepod nauplii have a similar diet as the adult copepods and also feed on animal prey such as ciliates [30, 31].

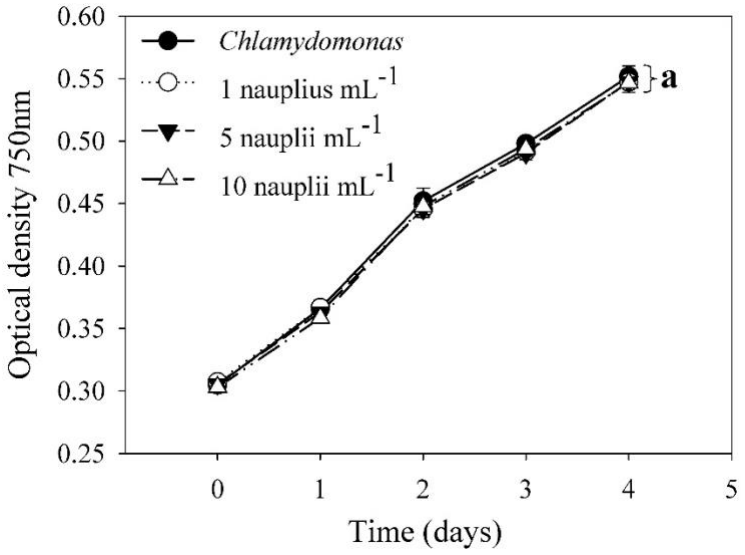


**Figure 6**: The effect of nauplii of the copepod *Acanthocyclops robustus* on the biomass of *Chlamydomonas* cultures. The error bars correspond to one standard deviation of the means. The letter a indicates that all treatments are not significantly different from each other according to Tukey's HSD post-hoc test.

### *3.4 Minimal number of copepods needed to avoid a culture crash in contaminated Chlamydomonas cultures*

In a third experiment we investigated the minimal number of copepods needed to avoid a crash of a *Chlamydomonas* culture that was contaminated with 10 *Sterkiella* cells $mL^{-1}$. We used 10 ciliates $mL^{-1}$ as a contamination load in this experiment as this is the minimum abundance at which ciliates can be detected during routine culture monitoring and hence the minimum abundance at which corrective action can be undertaken [11]. When no *Acanthocyclops robustus* were added to the contaminated culture, the number of ciliates increased to 80 ciliates $mL^{-1}$ between 3 and 5 d and the microalgal biomass concentration decreased by 73% compared to a non-contaminated culture (Fig. 7B). When copepods were added to the contaminated cultures, the number of ciliates was reduced and the biomass concentration of *Chlamydomonas* was higher than in the contaminated cultures to which no copepods were added (Fig. 7A). Addition of 0.03 to 0.4 copepods $mL^{-1}$ resulted in elimination of most ciliates from the culture within 1 d and resulted in *Chlamydomonas* biomass concentration of that was not significantly different from that of a non-contaminated culture (Fig. 7A). When only 0.007 copepods $mL^{-1}$ were added, however, the number of ciliates increased over time and the biomass of *Chlamydomonas* was significantly lower than in a non-contaminated culture ($p < 0.0001$, Table 3). This indicates that addition of 0.03 or more copepods $mL^{-1}$ is needed to prevent a culture crash. In a previous study where the ostracod *Heterocypris incongruens* or the cladoceran *Moina tenuicornis* were used to control rotifer contamination in high rate algal ponds, much higher predator densities ranging from 1.5 to 2.5 individuals $mL^{-1}$ were required to achieve a reduction of only ~23 - 27% in the rotifer population [19].

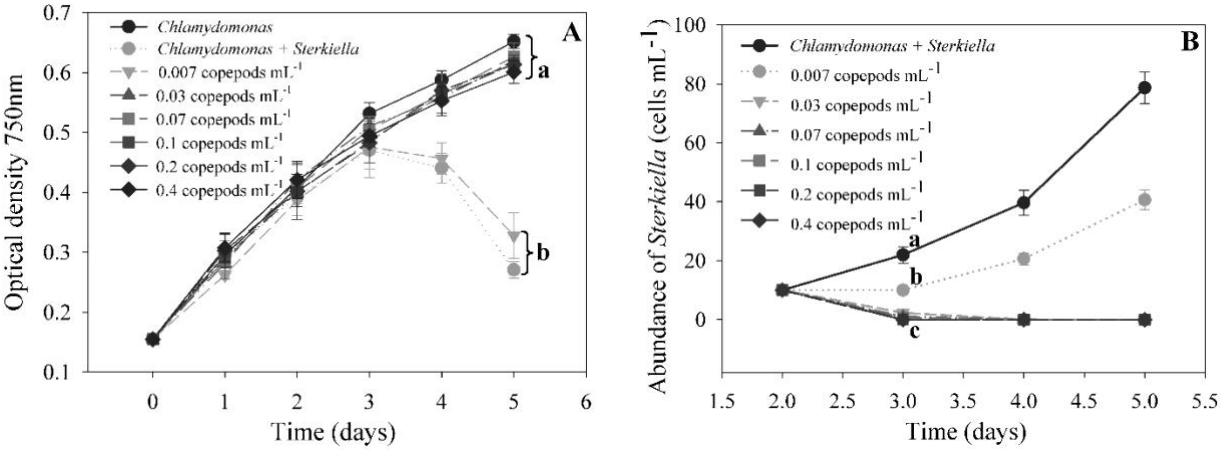


**Figure 7**: A) Influence of different densities of the copepod *Acanthocyclops robustus* to a *Chlamydomonas* cultures that was contaminated 10 *Sterkiella* cells $mL^{-1}$ on (A) the microalgal biomass concentration (as estimated from $OD_{750}$) and (B) the abundance of the ciliate *Sterkiella*. The copepod *Acanthocyclops robustus* was added in different densities and the effect of the copepod addition was compared to a positive (no copepods, no ciliate contamination) and negative (no copepods, with ciliate contamination) control. The error bars correspond to one standard deviation of the means. The letters a and b in panel A and the letter a, b and c in panel B group treatments that are not significantly different from each other according to Tukey's HSD post-hoc test.

| Experimental treatment | d.o.f. | F | *p* |
|---|---|---|---|
| Microalgal biomass (day 5) | 7 | 115 | < 0.0001 |
| Ciliate abundance (day 1) | 6 | 91 | < 0.0001 |

**Table 3.** Results of two one-way ANOVA tests comparing microalgal biomass concentration (as estimated from $OD_{750}$) and ciliate abundance between the different treatments in experiment 3. (d.o.f.: degrees of freedom).

### *3.5 Practical implications for the use of cyclopoid copepods for biocontrol of ciliate contamination in large-scale microalgal cultures*

To determine how many copepods should be added to microalgal cultures, it is important to know the maximum rate of consumption of ciliates by the copepods. The rate of consumption of the ciliate *Sterkiella* by the copepod *Acanthocyclops robustus* was fitted in a hyperbolic curve to the number of ciliates available per copepod (Fig. 8). This relation between food consumption and food abundance corresponds to a Type II functional response, and is the result of satiation of the copepods at high food abundance [40]. Previous studies of consumption rates of ciliates by copepods also reported a type II functional response [21]. The maximum ingestion rate that was estimated from the functional response was in excess of 400 ciliates copepod$^{-1}$ day$^{-1}$. This is comparable to ingestion rates reported for the closely related copepod species *Acanthocyclops vernalis* (312 ciliates day$^{-1}$; [41]).

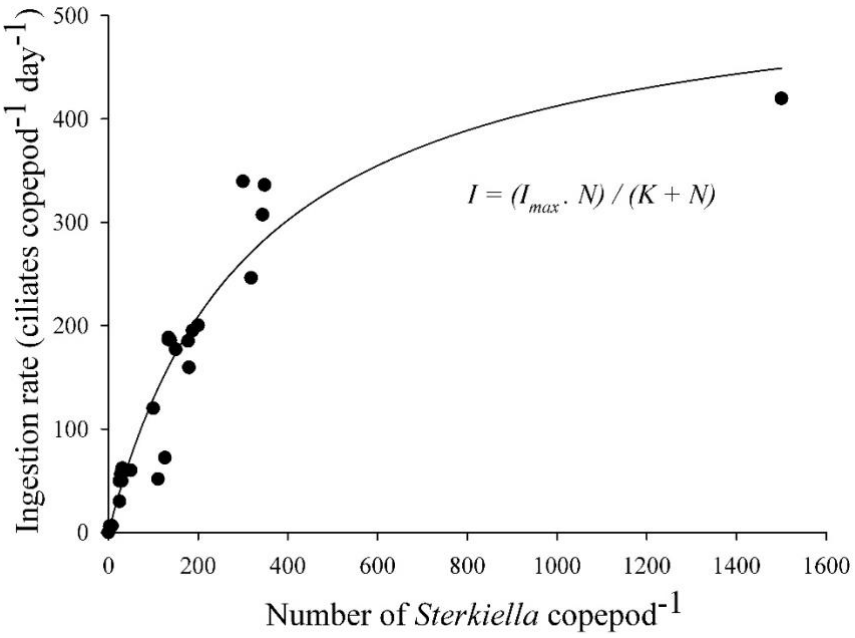

**Figure 8:** Consumption rate of *Sterkiella* by *Acanthocyclops robustus* as a function of ciliate availability per copepod. The ingestion rate ($I$) versus prey abundance (N) fits to Holling type II functional response ($I = (I_{max}.N)/(K + N)$), with maximal ingestion rate ($I_{max}$ = 545 ciliates copepod$^{-1}$ day$^{-1}$) and the prey abundance (K = 322) at which half the maximal ingestion rate occurs. ($R^2$ = 0.921, $p < 0.0001$).

Ciliate contamination in microalgal cultures can be detected when the ciliate density exceeds about 10 organisms mL$^{-1}$ [11]. Therefore, corrective action can be taken only when ciliate contamination load is 10 ciliates mL$^{-1}$ or more. At this level of contamination, addition of 0.07 copepods mL$^{-1}$ was sufficient to control the ciliate contamination (experiment 3). When this is extrapolated to large-scale cultures, biological control of ciliates would require sufficient numbers of copepods (thousands per m$^3$). This will require cultivation of copepods specifically for biocontrol of ciliates in microalgal cultures. Large-scale cultivation of copepods has not yet been demonstrated for the species *Acanthocyclops robustus* used in this study, but marine copepods are already cultivated for use as live feed for fish larvae in aquaculture [37, 38].

The cyclopoid copepod *Acanthocyclops robustus* is a freshwater species and can therefore only be used to control ciliate contamination in cultures of freshwater microalgae. In marine ecosystems calanoid copepods are important predators of ciliates [38]. Calanoid copepods that live in seawater achieve similar ingestion rates on ciliates as the cyclopoid copepod *Acanthocyclops robustus* [38]. Therefore, calanoid may have potential to be used to control ciliate contamination in cultures of marine microalgae. This, however, remains to be tested.

## 4. Conclusions

Our experiments demonstrate that the copepod *Acanthocyclops robustus* has potential to be used as a biological control mechanism to eliminate the ciliate *Sterkiella* from contaminated *Chlamydomonas* cultures and can prevent a decrease in biomass concentration of a ciliate-contaminated culture. A single copepod individual can consume about 400 ciliates per day. The copepod or its naupliar stages did not have any measurable impact on the biomass of the *Chlamydomonas* culture.

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**Conflict of interest statement**

All the authors declare that there are no conflicts of interest.

**Declaration of authors' agreement**

The authors agree to authorship and agree to submit the manuscript for peer review.

**Statement of informed consent**

No informed consent or human or animal rights are applicable.

**Declaration of author's contributions**

Nguyen Thi Kim Hue designed and performed the experiments, did the molecular identification of predator, collected and combined data, analysed statistics and interpreted the data, wrote the whole manuscript, reviewed and edited the manuscript for important intellectual contents. Koenraad Muylaert obtained the funding, conceived the research and designed the experiments, checked statistical analysis and interpreted the data, reviewed and developed the whole manuscript for important intellectual contents. All authors read, reviewed and approved the final manuscript.

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