Zooplankton Dynamics in Wastewater Treatment High Rate Algal Ponds and development of effective control methods

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ABSTRACT

High Rate Algal Ponds (HRAPs) are effective and economical open pond to provide near tertiary-level wastewater (WW) treatment, with the nutrients recovered as algal biomass. However, WW treatment performance can be reduced by the establishment of zooplankton grazers which can consume much of the algal biomass within a few days. The high food availability and the near neutral pH in HRAPs offer optimal conditions for the establishment of zooplankton taxa including cladocerans and rotifers. The control of these zooplankton species is essential for the effective operation of HRAPs. This thesis research was aimed at designing cost-effective treatments to control zooplankton population densities applicable to full-scale HRAPs. In this thesis, I: 1) reviewed existing and potential methods for zooplankton control in HRAPs; 2) assessed environmental and biological parameters for two wastewater HRAPs with CO_2 addition using a 14 month large-scale field experiment; 3) assessed chemical CO_2 asphyxiation, biological control of rotifers using competitor species, mechanical disruption of zooplankton using hydrodynamic shear stress, and the mechanical removal of zooplankton using filtration, using controlled laboratory conditions; 4) validated chronic CO_2 asphyxiation of zooplankton, the biocontrol of rotifers using the cladoceran *M. tenuicornis* and the ostracod *H. incongruens*, the control of *M. tenuicornis* using filtration and the hydrodynamic disruption of zooplankton, using outdoor mesocosms with physicochemical and operational conditions similar to those of full scale HRAPs; 5) assessed night time CO_2 asphyxiation to control zooplankton densities in an 8 m^3 HRAP over 14 months.

During the 14 months of the field experiment, zooplankton dynamics in paired 8 m^3 HRAPs eight species of zooplankton established, with higher water temperatures and longer detention times promoting larger populations. Grazing pressure was associated with changes in the dominance of microalgal species; large and rapid reductions of productivity; reduced nutrient removal; increased colony size, number of cells in colonies and formation of protective spines in microalgae; and higher biomass settleability. Maintaining a dominance of colonial microalgae, operating

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HRAPs with short retention times, and facilitating competition among zooplankton species showed to potentially reduce grazer populations. Promising options for zooplankton control included physical methods such as filtration, hydrodynamic cavitation, shear, bead mills; chemical methods such as increase of HRAP night-time CO_2 concentration, promotion of the lethal un-ionized ammonia toxicity, use of biocides, and the chitinase inhibitor chitosan; biocontrol using competitor and predatory organisms. In the laboratory tests CO_2 asphyxiation caused acute mortality of all zooplankton species (t<10 min). Increasing the cladoceran *Moina tenuicornis* to densities >2,500 individuals/L was associated with a decrease in rotifer populations that were ~23% of the population in the control. The ostracod *Heterocypris incongruens* at densities >1,000 individuals/L were also associated with a decrease in rotifer densities that were ~27% of the population in the control. Hydrodynamic shear stress killed 100% of *M. tenuicornis* and ~80% of the rotifer *Brachionus calyciflorus* after a single pass. In outdoor mesocosms a continuous CO_2 concentration of ~100 mg/L maintained low pond water zooplankton densities, while a continuous concentration of ~180 mg/L killed all microcrustaceans and rotifers present. As biocontrol agents, *M. tenuicornis* at ~2,000 individuals/L reduced average rotifer densities by 90%, and *H. incongruens* at ~1,000 individuals/L removed all rotifers. Mechanical filtration using 300 μm and 500 μm filters eradicated *M. tenuicornis* after one and four filtration periods, respectively. Mechanical hydrodynamic stress killed up to 100% of microcrustaceans, and ~50% of larger rotifers. Zooplankton control using night time CO_2 asphyxiation in an 8 m^3 HRAP reduced the average population densities of some zooplankton species over the experimental period: *M. tenuicornis* (41.3%), the copepod *Paracyclops fimbriatus* (43.9%), the rotifer *Filinia longiseta* (59.8%), but was associated with higher average population densities of others: *H. incongruens* (174.4%), the rotifers *Asplanchna sieboldi* (177.8%), *Cephalodella catellina* (200.0%), and *B. calyciflorus* (234.9%). However, the population densities of *B. calyciflorus* and *C. catellina* were always reduced after CO_2 treatments with flow rates ≥2 L/min were applied. The cladoceran *Daphnia thomsoni* and the rotifer *Brachionus urceolaris* established only in the control HRAP. Zooplankton

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control by CO_2 asphyxiation improved the overall performance of the treated WW HRAP compared to the control in several ways, including increasing algal biomass (VSS) (150.8%), productivity (151.4%), chlorophyll-*a* concentration (161.8%), particle size (MCSA) (115.8%), and average settleability efficiency (189.2%). Furthermore, *M. tenuicornis* was concentrated in the upper 50 mm of a 300 mm deep water column using vertical migration induced by CO_2 concentrations of between 25-180 mg/L in laboratory experiments, and by phototaxis-induced migration in an 8 m^3 HRAP. This suggested that mechanical treatments such as filtration and hydrodynamic stress could be performed to the upper layer of the pond water, reducing the amount of water processed and the overall treatment costs. Overall, CO_2 asphyxiation appeared to be the most reliable, versatile, and effective zooplankton control treatment.

All treatments reduced or eradicated zooplankton populations and promoted higher productivity of microalgae cultures. However, the efficacy of treatments to control diverse zooplankton species differed, and the implementation of any control strategy in a given system requires a preliminary assessment of zooplankton succession to identify the species able to consume the dominant microalgae that could establish in the system. Treatments can be dosed and combined to selectively kill specific zooplankton species. Moreover, zooplankton at controlled densities could potentially be used as a bio tool to improve biomass settleability or to consume unwanted microalgal species.

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CHAPTER 1

INTRODUCTION

Microalgal cultivation for the production of biofuels such as biodiesel and methane has been previously studied, as such microalgae have a higher biomass yield compared to that achieved using higher plants [1], [2]. However, the high cost of biofuel production using microalgae relative to the low cost of fossil fuels has encouraged the study of alternative uses for microalgal biomass, including for food, nutraceuticals and bioactive compounds production [3], [4]. A key factor for the economics of microalgal cultivation is the availability of a cheap growth media, and municipal WW can offer virtually unlimited growth media wherever human settlements are present. Wastewater is typically the major waste stream by volume in developed countries, and requires the application of technological and economic resources for effective treatment prior to being discharged into receiving waters [5]. Numerous WW treatment systems exist [6] and generally include primary treatment, to remove the solids, secondary treatment, to clarify the effluent via aerobic degradation of soluble organic compounds, and tertiary treatment, to remove dissolved nutrients (nitrogen, phosphorus). Coupling the nutrient removal process (tertiary WW treatment) with microalgal cultivation utilizing microalgae ponds is a cost effective option [5], [7], [8]. Microalgae can be cultivated in different systems including open ponds, vertical column photo-bioreactors, flat-plate photo bioreactors, and tubular photo bioreactors. Of these, open ponds are generally the cheapest solution wherever the land cost is low. Nevertheless, the unmixed, high depth (around 1 m) facultative ponds that are most commonly used around the world have low microalgal biomass productivity (10 t/ha/year) as well as low nutrient removal [8].

High Rate Algal Ponds (HRAPs) for WW purification (Figure 1) are shallow, paddlewheel-mixed ponds that have been measured to productivities up to ~30 t/ha/year, which may increase to ~60 t/ha/year when CO_2 is artificially added for extra carbon supply and pH regulation [5]. The photosynthetic metabolism of algae aerates the HRAP water, enhancing the aerobic bacteria degradation of organic compounds and their partial conversion into nutrients, which in turn can be used by algae and converted into biomass. HRAPs are particularly interesting because they have been constructed and operated at scales of up to ~1 hectare [9], [10], and can offer economic

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nutrient removal. The microalgae are recovered by the natural settling of algal-bacterial flocs and can be used as a soil amendment for agriculture, a protein-rich feed supplement or a substrate for biofuel conversion. Before being discharged into the environment, the algal settling pond effluent is further treated in a series of maturation ponds where zooplankton graze on any remaining microalgae and the solar UV radiation disinfects the water reducing bacteria and viruses. In WW HRAPs the main indicators of performance are the biomass productivity and settleability, and the nutrients removal from WW. High microalgae and bacteria biomass is required to sequestrate and remove the WW nutrients. Settleability efficiency of the biomass is required to remove the nutrients from the system.

The productivity and wastewater treatment efficiency of HRAP systems is dependent on various chemical, biological, physical and mechanical parameters.

Figure 1 One hectare High Rate Algal Ponds at the Cambridge Enhanced Pond System (EPS). Photo Rupert Craggs (NIWA)

One parameter that can seriously reduce the performance of HRAPs is biological contamination from free swimming zooplankton, such as rotifers and small crustaceans (copepods and cladocerans), which are introduced by wind or by birds and graze on the resident algae [11], [12]. Blooms of these organisms can deplete the HRAP algal biomass in a few days. High

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food concentrations and the lack of predators allow for the development of large populations of zooplankton species able to withstand the HRAP WW environment, especially when pH is controlled to a pH ~8, providing favourable growth conditions [13], [14], [15]. Zooplankton control is widely recognized as necessary for efficient and consistent WW nutrient removal, algal productivity, and HRAP stability [16], [17], [18], [19], [20]. HRAPs for microalgae cultivation have been used for over 70 years. However, zooplankton contamination still limits their extensive use worldwide, and to date, the availability of treatments options for zooplankton control in hectare scale WW HRAPs is scarce. For example, filtration, un-ionized ammonia toxicity, and use of biocides [21], [11], [22] have been used in pilot scale HRAPs with only moderate success. Options have been used to control zooplankton in smaller systems, or different pond system than HRAPs. For example acute application of CO_2 was used to inactivate zooplankton in experimental enclosures [23], and in 1.5 m^3 microalgae cultures [24]. The use of chemical substances to control zooplankton can also be effective, and have been previously reported [25], [26], [27], [28], [29], [19], [30]. However, chemicals are not usually applicable in WW HRAP systems because the beneficial zooplankton established in the maturation ponds could be killed by the residual toxic substances in the water that flows from the treated HRAPs. Other treatment strategies are not practical due to the high cost required to treat large volumes of water and the detrimental effect that treatments have on microalgae. For example, moderate heating [31] kills zooplankton species [32], [33], [31], but also a large portion of the microalgae. Moreover, the energy cost required to increase the temperature of large amounts of water (3,000-5,000 m^3) of hectare scale HRAPs makes this treatment costly. Potential options for zooplankton control such as centrifugation [34], cavitation [35], [36], UV radiation [37], deoxygenation [38], and biocontrol using competing or carnivorous zooplankton [39], [40], have been previously proposed, although never assessed, in WW HRAPs or in microalgae cultures with conditions typical of WW HRAPs.

The need for low-cost and effective zooplankton control treatments applicable to hectare-scale HRAPs and the lack of available options provide a wide and interesting spectrum for fundamental and field-scale research,

particularly in WW HRAP systems where a pH close to neutrality enhances zooplankton growth and makes their control more difficult and energy intensive. My PhD research aimed to measure the invertebrate dynamics in WW HRAPs with focus on the interaction between zooplankton grazers and microalgae, and to develop and validate cost effective and environmentally sound zooplankton control methods applicable to hectare scale HRAPs. Generally, algal biomass reduction depends on "what" and "how much" the zooplankton can ingest, and "how fast" they can reproduce. For my thesis research, existing technologies to control zooplankton were examined with an extensive review of published studies. The most promising options used to control zooplankton in laboratory cultures, experimental ponds, ballast waters, and aquaculture systems were used as a starting point to design and propose novel zooplankton control treatments applicable to hectare scale HRAPs and algal production raceways (Chapter 2). Then, a 14 month monitoring program of zooplankton dynamics in two pilot scale WW HRAPs was performed to: 1) determine zooplankton succession over different seasons; 2) measure the zooplankton densities able to reduce microalgal productivity or promote pond crashes; and 3) determine the effect of grazing pressure on microalgal dominance and structure (Chapter 3). Next, the most promising treatments were tested at a laboratory scale (Chapter 4), and then validated using outdoor mesocosms with physicochemical conditions typical of HRAPs (Chapter 5), and finally in two 8 m^3 HRAPs (Chapter 6). The experimental work of this study is based on the microalgae reduction caused by the zooplankton grazing activity. Hence, the main indicators of HRAPs performance are the concentration of total organic biomass (as volatile suspended solids, VSS) and of microalgae (as chlorophyll-a, Chl-a).

A zooplankton control treatment for hectare scale WW HRAPs should reduce the density of zooplankton species that are detrimental for the dominant microalgae species without negatively affecting the growth and structure of microalgae. Zooplankton control to low densities is preferred to eradication as moderate zooplankton populations are expected to promote a stable community and prevent the ecological imbalance resulting from eradication. Colonial microalgae and algae-bacterial flocs should not be disrupted by the treatment because large particles are

essential for a good settleability of the suspended biomass [41]. Moreover, the reduction of zooplankton should be limited to the HRAP, because zooplankton established in maturation ponds provide an important function in further polishing the HRAP effluent.

The thesis is composed of five research chapters and is concluded with summary including future research directions. The five research chapters continue as follows:

Chapter 2 reviews the ecology of zooplankton in polluted environments and WW pond systems, and potential methods for zooplankton control in these systems. Technologies used for the treatment of ballast water tanks in transoceanic ships, aquaculture ponds, and for the management of ecosystem which may potentially be applicable to HRAP systems are also reviewed. Finally, novel treatments based on modifications of existing methods are proposed.

Chapter 3 examines the physicochemical and biological parameters of two replicate 8 m^3 WW HRAPs with CO_2 addition over 14 months. In particular, the assessment is focused on the dynamics of the zooplankton community, and the biotic interactions between grazers and microalgae, in terms of HRAP performance. Recommendations and a general strategy for zooplankton management in HRAP environments are provided.

Chapter 4 assesses the efficacies of CO_2 asphyxiation, biocontrol using the cladoceran *M. tenuicornis*, biocontrol using the ostracod *H. incongruens*, and hydrodynamic stress, using controlled laboratory experiments conducted using batch microalgae and zooplankton cultures. Cladoceran and rotifer populations were exposed to increasing CO_2 concentrations and hydrodynamic stress intensities with the aim of enhancing death rates. Rotifer populations were incubated with given densities of *M. tenuicornis* and *H. incongruens* to assess the rotifer growth dynamics and the reductions in population densities.

Chapter 5 assesses the chronic (1-2 month) CO_2 asphyxiation of zooplankton, the biocontrol of rotifers using the cladoceran *M. tenuicornis* and the ostracod *H. incongruens*, the mechanical control of *M. tenuicornis* using filtration and the hydrodynamic disruption of zooplankton, using outdoor pilot mesocosms operated with typical WW HRAP conditions.

Phototaxis-induced vertical migration of *M. tenuicornis* is demonstrated in the water column of an 8 m^3 HRAP with the aim of only applying mechanical treatments to the surface (zooplankton dense) portion of the pond and reduce the treatment time and cost.

Chapter 6 compares the performance and zooplankton dynamics in paired 8 m^3 HRAPs, where one HRAP is treated with night time (acute) injection of CO_2 to control zooplankton density, and the other HRAP is untreated as a control, over a period of 14 months. The zooplankton community, the biotic interactions between grazers and microalgae, and the performance of the HRAPs in terms of biomass productivity are monitored. A protocol for zooplankton management in WW HRAPs is proposed based on the overall experimental work of the study.

Chapter 2 has been published as: V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, A review of potential methods for zooplankton control in wastewater treatment High Rate Algal Ponds and algal production raceways, Algal Research, 11 (2015) 211-226.

Chapter 3 has been published as: V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, Zooplankton community influence on seasonal performance and microalgal dominance in wastewater treatment High Rate Algal Ponds, Algal Research, 17 (2016) 168-184.

Chapter 4 has been published as: V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, Screening of potential zooplankton control technologies for wastewater treatment High Rate Algal Ponds, Algal Research, 22 (2017) 1-13.

Chapter 5 has been published as: V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, Assessment of potential zooplankton control treatments for wastewater treatment High Rate Algal Ponds, Algal Research, 24, Part A (2017) 40-63.

Chapter 6 has been published as: V. Montemezzani, I.C. Duggan, I.D. Hogg, R.J. Craggs, Control of zooplankton populations in a wastewater treatment High Rate Algal Pond using overnight CO_2 asphyxiation, Algal Research, 26 (2017), 250-264.

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CHAPTER 2

A REVIEW OF POTENTIAL METHODS FOR ZOOPLANKTON CONTROL IN WASTEWATER TREATMENT HIGH RATE ALGAL PONDS AND ALGAL PRODUCTION RACEWAYS

ABSTRACT

High Rate Algal Ponds (HRAPs) can provide economical and efficient near tertiary-level wastewater (WW) treatment, with the nutrients recovered as algal biomass. HRAP performance can be negatively affected by the establishment of zooplankton grazers that can consume much of the algal biomass within a few days. Zooplankton management is therefore essential for maintaining WW treatment performance and algal productivity. This paper reviews zooplankton ecology in WW systems and eutrophic environments, and potential methods for zooplankton control in HRAPs. Promising options for zooplankton control include physical methods such as filtration, hydrodynamic cavitation, shear, bead mills; chemical methods such as increase of HRAP night-time CO_2 concentration, promotion of the lethal un-ionized ammonia toxicity, use of biocides, and the chitinase inhibitor chitosan; biocontrol using competitor and predatory organisms. CO_2 and phototactic induced migration are proposed to concentrate zooplankton in specific areas to reduce the amount of pond water requiring treatment. Based on this review, we suggest that it may be most beneficial to maintain zooplankton grazer populations at low levels as part of a stable community, rather than to totally eradicate them. This will prevent the ecological imbalance of total control that could result in the establishment of other zooplankton species that are less easy to control.

Keywords: Wastewater treatment high rate algal pond, Zooplankton, Grazers, Microalgae.

INTRODUCTION

Traditional wastewater (WW) treatment has three common fundamental stages; 1) primary treatment, to remove the solids, 2) secondary treatment, to clarify the effluent via aerobic degradation of soluble organic compounds, and 3) tertiary treatment, to remove dissolved nutrients (nitrogen, phosphorus) so as to reduce the potential for eutrophication of receiving waters [1]. High Rate Algal Ponds (HRAPs) are 0.3-0.5 m deep closed-loop, paddlewheel-mixed ponds (Figure 1), and can be up to a few hectares in area. Such ponds are able to provide economical and efficient near tertiary-level WW treatment with the nutrients recovered as algal biomass [2], [3]. HRAPs are considered to be effective reactors to reclaim water, nutrients and energy from organic wastewaters [4], [5], [6], [7]. Algal productivity in HRAPs can be up to ~30 tonnes/ha/year and may increase to ~60 tonnes/ha/year when CO_2 is artificially added for extra carbon supply [8]. By comparison, algal productivity in traditional WW facultative ponds is only ~10 tonnes/ha/year [9]. The algal biomass grown in HRAP is usually composed of colonial microalgae, and is harvested from the effluent by natural settling of algal-bacterial flocs in Algal Harvest Ponds (conical tanks or ponds). The harvested algal biomass can be periodically removed for use as a fertiliser, protein-rich animal feed or for conversion into biofuel: biogas via anaerobic digestion; bioethanol via carbohydrate fermentation; bio-crude oil via high temperature liquefaction; or biodiesel via lipid trans-esterification [10], [11]. Before being discharged into the environment, the algal settling pond effluent is further treated in a series of maturation ponds where zooplankton graze on any remaining microalgae.
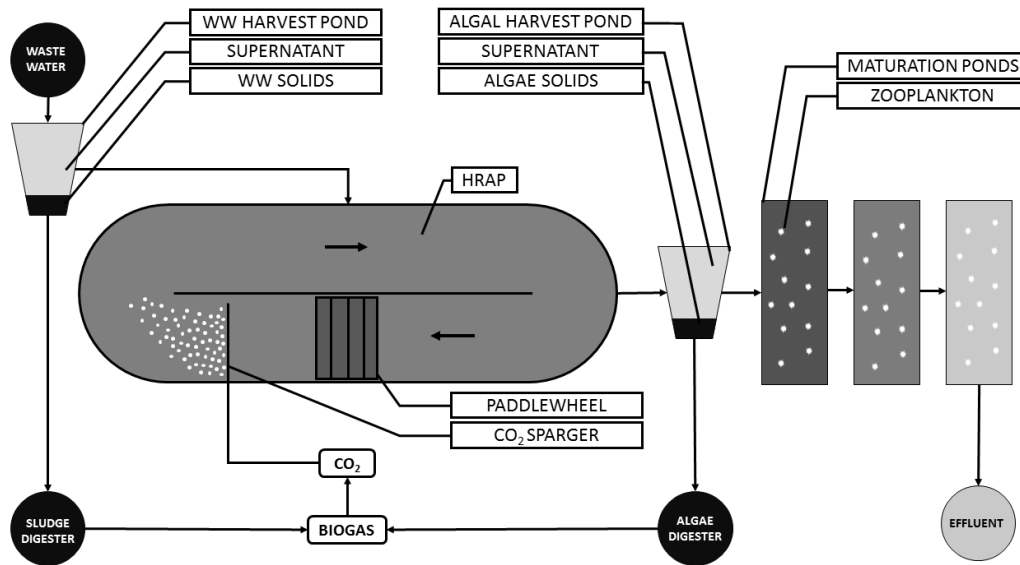
Figure 1. Schematic diagram of a HRAP, algal harvest pond, maturation ponds and anaerobic digesters for biogas production

HRAP performance depends on climatic (light, temperature), operational (pH, CO_2 concentration), water depth, dissolved oxygen (DO), nutrients, hydraulic retention time (HRT) and biological variables (parasites, fungi, zooplankton grazers) [12], [13], [14], [8]. However, establishment of zooplankton grazers, which enter as contaminants from the surrounding environment, is one of the greatest challenges for HRAP performance and management [15]. Zooplankton can consume microalgae biomass with negative effects on the WW treatment performance [16]. Moreover, artificial control of HRAP water pH to close to neutral (~7-8) by CO_2 addition to promote wastewater nutrient removal and algal production also promotes zooplankton survival [17], [18].

Herbivorous zooplankton grazers in HRAPs include ciliates, rotifers, cladocerans, copepods and ostracods. Of these, cladocerans (Subphylum Crustacea; Order Cladocera) and rotifers (Phylum Rotifera) constitute the greatest problem for both unicellular and colonial microalgae. These zooplankton have short generation times compared to copepods and ostracods resulting in rapid (within a week) depletion of algal biomass in both mass algal cultures [19], [20], [21], [22] and WW treatment HRAPs [23], [24], [25], [26]. Microalgae and bacteria are the main food source for zooplankton grazers. Grazing magnitude depends on the size of the grazer,

abundance, grazing mechanisms (e.g., filtering or grasping), water temperature, food particle shape, size and availability [27], [28], [29], [30], [31]. Generally, algal biomass reduction depends to "what" and "how much" the zooplankton can ingest, and "how fast" it can reproduce. For example, outdoor algal cultures of the small (~10 µm) unicellular microalga *Dunaliella salina,* have been depleted within two days by small unicellular, fast growing ciliates [32]. However, ciliates are not expected to be of concern in WW HRAPs microalgae as they are single celled organisms that can only consume food particles that are smaller than unicellular and colonial microalgae [33], [34]. Due to larger size, single cladocerans can typically filter larger amounts of biomass compared to the smaller rotifers [35], [36], [37]. However, rotifers have higher growth and reproduction rates, and can quickly reach large population densities that consume a greater amount of algal biomass [38]. The need for zooplankton management in large and open algal cultures is clearly recognized, both for efficient and consistent WW nutrient removal, as well as algal productivity [39], [40]. Furthermore, zooplankton grazing of microalgae is a primary reason for the unpredictability of HRAP performance that makes effective management a challenge [41].

Here, we review zooplankton ecology in polluted environments and WW pond systems, and potential methods for zooplankton control in these systems. Since literature specifically related to HRAPs is limited, we review methods used for the treatment of ballast water tanks, aquaculture ponds and ecosystem management which may potentially be applicable to HRAP systems. Finally, we propose novel treatments based on modifications of existing methods.

ZOOPLANKTON OCCURRING IN NATURAL AND POLLUTED ENVIRONMENTS, AND WASTEWATER PONDS

General ecology of freshwater zooplankton in natural waters
Zooplankton dynamics in natural ecosystems can be similar to those of HRAPs [24], and provides useful information on growth dynamics and

interactions with microalgae. Zooplankton are heterotrophic organisms that occur in lakes, rivers and swamps, and have high abundances in ponds with high organic matter. Zooplankton include small (<20 μm) unicellular (protozoa) and larger multicellular rotifers and micro-crustaceans (>200 μm), including copepods and cladocerans (up to 5 mm). Zooplankton provide the main trophic connection between bacteria, algae and higher consumers [42], with smaller organisms (bacteria, algae) eaten by the zooplankton, which, in turn, are food for aquatic insects and small fish. Zooplankton communities within any natural pond can be very complex, with hundreds of protozoan, 30-100 rotifer and 10-30 micro-crustacean species typically encountered in any one year [43], [44], [45], [46]. Zooplankton are dispersed among natural habitats through reproduction via parthenogenetic or resting eggs, and transport of individuals by both natural (e.g., birds, animals, surface waters) and artificial (e.g., equipment) vectors [47]. Movement within habitats occurs by floating, drifting or swimming [43]. Species composition within habitats is influenced by local climate, diurnal cycles, water quality, trophic state, pH, direct predation, competition among zooplankton for shared food, competition through mechanical interference, crowding, food composition and size, and physiological modifications induced by infochemicals [48], [49], [50], [51], [52], [53], [54], [55], [56], [57], [58]. Moreover, microalgal species and abundance are directly controlled by zooplankton, resulting in complex mutual interactions [59], [60], [61].

Types of zooplankton occurring in HRAP systems
Wastewater has extreme physicochemical characteristics compared to natural pond and lake water [62], and the HRAP environment in particular is only favourable to certain zooplankton species [63], [64]. High nutrient concentrations, diurnal variation of temperature, dissolved oxygen (DO) (daytime supersaturation), pH (daytime >10 without CO_2 addition), and consequently toxicity from free ammonia, coupled with short (2-8 days depending on season), hydraulic retention time (HRT), fast flowing water (~0.15 ms^{-1}) and low light penetration, all provide a strong selective pressures which limit the zooplankton species inhabiting these systems [17], [65], [12], [6]. Furthermore, the large diameter (50-200 μm) of colonial

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microalgae typically found in HRAPs (e.g., *Desmodesmus* spp., *Micractinium* spp., *Pediastrum* spp., *Actinastrum* spp., *Dictyosphaerium* spp.) [5], [66], [67], [68] limits the food available to most zooplankton species. However, for some species capable of withstanding the HRAP environment, the high food availability as well as the lack of interspecific competition and predation (e.g., fish) can promote high densities for these species. In natural systems food availability is the main driver of zooplankton blooms, but for wastewater treatment ponds operational parameters are also important. In long (50-200 d) HRT waste stabilization ponds, zooplankton occurrence depends mainly on pond temperature, food abundance and quality [69]. In HRAPs the combination of temperature and short HRT (2-8 d) are likely to be major factors affecting zooplankton composition and dynamics. Zooplankton generation times depend on temperature (Table 1 and Table 2) and individual species' life histories. Species with a generation time longer than the HRT are unlikely to establish populations in HRAPs. For example, in a HRAP with a HRT of eight days located in southern France, low temperatures promoted the dominance of zooplankton with short generation times, such as protozoa. In contrast, high temperatures reduced the development times and promoted the dominance of metazoans zooplankton with longer generation times [24]. Zooplankton blooms in four 12 m^2 HRAPs located in California, U.S.A., severely reduced algal densities during experiments using longer HRT (>4 days), while experiments with shorter HRT (2-3 days) did not experience zooplankton blooms or corresponding reductions in algal densities [70]. To help design zooplankton control methods that target particular zooplankton species that are found in HRAPs, a fundamental understanding of their life history and physiology is necessary.

Rotifera

Rotifers are small zooplankton, usually less than 200 μm in size and often the most abundant organisms in fresh water bodies, with densities ranging between 1,000 to 500,000 individuals/L in highly eutrophic environments such as sewage ponds [43], [71], [72], [73]. Rotifers are omnivorous, filter feeders eating small (1-20 μm) organic particles, including bacteria, small

microalgae (e.g. *Chlorella* spp.) and protozoa using rapidly moving cilia located in the corona to collect food particles [74]. Some rotifer species (e.g., *Brachionus calyciflorus*) prefer to graze on the largest particles available in their food size range and selectively graze large elongated unicellular microalgae such as *Ankistrodesmus* spp. (~40 by 5 μm) [36], [52], [75]. However, other species can target colonial microalgae. For example, rotifer species that possess a protrudable grasping mastax (e.g., *Cephalodella* spp.) can graze the individual cells of small-celled colonies (e.g., *Dictyosphaerium* spp.) [76], [77], [78]. The species composition of rotifers in natural systems is commonly associated with the concentration of nutrients (i.e., trophic state) [79], [80]. Accordingly, common rotifers in WW ponds are similar to those found in lakes with high nutrient inputs: *Filinia longiseta, Brachionus budapestinensis, B. calyciflorus B. angularis*, *B. rubens, B. patulus, Keratella tropica, K. slacki, K. tecta, K. cochlearis, K. quadrata, Polyarthra longiremis* and Bdelloids [17], [64], [71], [72], [73], [81], [82], [83], [84]. Rotifers have the fastest reproduction of metazoan zooplankton, and in optimal growth conditions such as high nutrients, neutral pH and high temperature (typical of HRAPs with artificial CO_2 addition) they reproduce asexually with doubling times sometimes less than one day [18], [85]. In response to adverse conditions, rotifers can use sexual reproduction to generate resting eggs that lie dormant in the pond sediment [86], [87]. The lifespan of rotifers can be up to 30-40 days [88], mainly depending on pond water temperature (Table 1).

Cladocera

Cladocerans are small crustaceans between 0.2 mm and 5.0 mm in length, which swim with discontinuous and sudden movements using their antennae. Cladocerans can graze on larger food particles than rotifers, including mature colonial microalgae. Representatives of the family Daphniidae are typical inhabitants of ponds, with *Daphnia* and *Moina* species commonly found in hypereutrophic environments [64], [81], [89]. *Moina* species are typically found in puddles and temporary ponds with decomposing organic matter although rarely in other permanent water bodies [43], [90], [91], and are particularly well adapted for living in WW

systems. *Daphnia* and *Moina* growth depends primarily on temperature (Table 2) and the availability of food. Peaks in population density typically follow a period of high algal concentration and are followed by a rapid decline when most of the algae have been consumed [94], [91]. In laboratory experiments, *Moina micrura* consumed microalgae in the range of 2-40 µm width, with preference for particular cell shape. For example, needle-like cells or *Monoraphidium contortum* were preferred to spherical *Chlorella vulgaris* [38]. Although *Daphnia* species can graze on particles in the range of 1-80 µm [36], and potentially consume larger colonial microalgae, they occur less commonly in HRAPs than *Moina* species. In laboratory cultures of *C. vulgaris* grown on WW, *Moina macrocopa* had longer lifespans, reproductive and neonate survival rates than in controls without WW addition, while the life span of *Daphnia pulex* was shorter than in the control [63], [92]. *Moina* species are also particularly resistant to DO variation and are capable of withstanding the typical DO variation of HRAPs (from almost zero to super saturation), due to their ability to increase their haemoglobin content. In contrast, *Daphnia magna* are less tolerant to low DO, surviving in small laboratory WW cultures with high atmospheric gaseous exchange but not in ponds with the same WW and less favourable gas exchange with the atmosphere [93]. Like rotifers, cladocerans typically reproduce asexually, and can also undergo seasonal sexual reproduction to produce resting eggs [91].

Ostracoda

Ostracods are small crustaceans, 0.3-5.0 mm in length, protected by a hard shell composed of two calcareous half valves, which can be tightly closed. They have eight pairs of appendages that are used to swim, sense, crawl, mate and feed [91]. Freshwater ostracods can be found in temporary water bodies such as puddles, where they live and crawl on, or in, the sediments, with species composition dependant on food abundance and water permanence [94]. Little is known about their occurrence in HRAPs and WW environments, although ponds with abundant sediment generally provide the most suitable habitat. Species such as *Cypridopsis vidua, Herpetocypris virens* and *H. incongruens* can tolerate moderately to highly polluted water

and survive very low DOs and high concentrations of organic matter [95], [96]. Ostracods are omnivorous scavengers, feeding on organic detritus, bacteria, protozoa, plant material, dead animal material and algae, with a preference for small food particles found in sediments. As ostracod generation time (up to three months) is much longer than HRAP HRT (2-8 d), only bottom dwelling species are expected, with little effect on microalgae suspended in the HRAP water column. Ostracods can also prey on rotifers and produce kairomones, chemicals that can induce morphological changes in rotifers (e.g., shortening of spines that reduces the chance of capture and retention by the ostracod's feeding appendages) [97]. Reproduction is mainly asexual and they can survive adverse conditions such as seasonal dry periods by producing resting eggs or by enclosing themselves within their valves [91].

Copepoda

Copepods are a group of small planktonic or benthic crustaceans, ~1-5 mm long, which swim with fast, erratic movements. They are divided into three orders, Calanoida, Cyclopoida and Harpacticoida. In general, calanoids are gliders and sporadic swimmers found in the open water; cyclopoids are constant swimmers, with a bouncing motion, and can be found in the plankton or associated with bottom sediments; and harpacticoids are found in bottom detritus moving with a wavy motion [43], [91]. Some species of cyclopoids (e.g., *Paracyclops fimbriatus* and *Mesocyclops thermocyclopoides*) have been found in sewage treatment ponds [98], [99]. Planktonic copepods are generally filter-feeders, forcing the water along their body to actively catch food particles in suspension [100]. Copepods primarily feed on algae [101], and also prey on ciliates, small rotifers and bacteria [98], [102], [103]. Cyclopoids can also prey on cladocerans, small copepods and chironomids (midge larvae). Copepods typically have only sexual reproduction [91], and as the reproductive age (7-30 d) is generally longer than the HRAP HRT (2-8 d), planktonic copepods are not found in large numbers in HRAPs.

Table 1 Comparison of length of life (days), reproductive age (days) and total amictic eggs (eggs that develop without fertilization) spawned during the life of different zooplankton groups at different temperatures [104][1], [88][2], [91][3], [74][4], [105][5].

Zooplankton Type	Rotifers			Cladocerans			Ostracods				
Temperature (°C)	10	20	25	10	20	25	10	17	20	23	25
Length of life (d) [1,2,5]	20	12	5	85	50	40	85	79-88	50	38-40	40
Reproductive age (d) [3,4,1,5]	5-7	2-3	1.25-1.75	20-24	7-8	5.5-6.5	28-32	48-54	13-15	22-27	7-11
Amictic eggs spawned during life [1]	15-25	15-25	15-25	400-600	400-600	500-700	250-500		250-500	500-750	

Table 2 Comparison of lifecycle parameters at different temperatures of zooplankton species typical of WW environments [18][6], [106][7], [71][8], [90][9], [54][10], [107][11], [108][12].

Zooplankton Type	Rotifera						Cladocera					
Species	*B. plicatilis*			*B. calyciflorus*			*Daphnia* spp.				*Moina* spp.	
Temperature (°C)	15	20	25	20	25	28	10	15	20	25	25	Optimal
Length of life (d) [6,7,11]	15	10	7			6-8		38-69	35-42			
Reproductive age (d) [6,7,9,10]	3	1.9	1.3					12-18	5-10	7-8	2.2	4.7
Interval between spawning (h) [6,9,11]	7	5.3	4			3.5-3.8						1.5-2
Amictic eggs spawned during life (n°) [6,9,11]	23	23	20			16-20						8-130
Embryonic development (d) [6,9,8,10]	1.3	1	0.6	1.2	0.8	0.4	11		1.9-2.2	2	1	
Doubling time (d) [2,12]			0.9-2.9								2.4	

Resting (diapausing) egg production, hatching and dispersal

A potential barrier for permanent eradication of zooplankton grazers from HRAPs are diapausing eggs. These resistant, dormant eggs are produced by sexual reproduction in response to a range of unfavourable conditions including physical (e.g. low daylight, extreme temperatures, drought) chemical (e.g. low pH, low DO) or biological (e.g. lack of food, high population density, genetic factors) or a combination of these [90], [109], [110], [111], [112], [113]. Zooplankton diapausing eggs are dispersed by wind, and via birds (e.g. ducks) with eggs attaching to their feathers and feet or passing through digestive tracts intact [114], [115]. Diapausing eggs

usually hatch when environmental conditions or food availability become favourable for a sufficient period of time [116], [117]. A resting "egg bank" is typically always present in the sediments although eggs (especially when buried) tend to lose their viability over time [118], and a seasonal production is essential to keep a viable pool [111]. In eddies within HRAPs, sediment accumulation is expected to promote the formation of an "egg bank". HRAPs are usually in close proximity to other ponds (including maturation ponds), and birds visiting the ponds are the most likely vector of contamination. Man-made water bodies such as wastewater ponds are more readily invaded by zooplankton than natural water bodies, which already have established species capable of efficiently consuming available resources [119], [120], [121]. A greater understanding of the factors controlling the production and hatching of diapausing eggs is therefore important to design effective zooplankton control strategies.

Migration in the water column and phototaxis

Understanding the natural distributions of zooplankton within the water column of HRAPs may be used to identify areas with high concentrations of zooplankton grazers, and perform any zooplankton control treatment only in these areas, reducing the amount of pond water treated, and the energy required. Zooplankton, especially cladocerans, commonly undertake diurnal vertical and horizontal migrations through the water column [122]. Migration is typically in response to changes in temperature, DO, pH, carbonate and bicarbonate ions, photosynthetic activity, predators and light, the latter of which is considered the most important, both for avoidance of visual predators and UV radiation [123], [124], [125]. Zooplankton often move to the warmer, food-rich surface layer after sunset and then move back into deeper water before sunrise to avoid visual predators [126], [127]. Laboratory experiments have shown that daphniids are able to orientate themselves in response to light, moving vertically toward the source (positive phototaxis) for light wavelengths between 480 nm (blue) and 735 nm (red), and horizontally in search of food-rich shaded areas at 440 nm (violet) and white light [128]. *Daphnia magna* has demonstrated negative phototaxis to ultraviolet light (260-380 nm) and positive phototaxis to white

light (420-600 nm) [129]. Rotifers usually have positive phototaxis (coming up to the surface during the day) with migration triggered by photoperiod, algal biomass, DO, and avoidance of predatory or competing zooplankton [130], [131]. For example, *Brachionus calyciflorus* has a positive phototactic response between 350 nm and 420 nm (ultraviolet) and between 500 nm and 650 nm (wavelengths least absorbed by algae) [132], whereas *Filinia longiseta* has a maximum positive phototactic response to 460 nm (blue light) [133].

Beneficial aspects of zooplankton in WW treatment ponds

Zooplankton grazing activity can be beneficial for WW treatment pond performance, biomass production and recovery, thus the control of desirable zooplankton species to below particular threshold levels may be preferable to complete eradication. For example, the settling of algae can be enhanced by selective grazing of undesirable buoyant, small diameter (1-10 µm) single celled microalgae [70], shifting algal dominance to heavier, colonial microalgae (e.g. *Micractinium*), and promoting large and stable algal/bacterial flocs [65], [134]. In laboratory experiments, chemicals released by the rotifer *B. calyciflorus* induced colony formation by the single-celled *Scenedesmus obliquus* [60], and the development of bristles by cells of *Micractinium pusillum* [135]. Bdelloid rotifers release chemicals that promote aggregation of suspended particles in WW stabilization ponds [83]. In 8 m^3 HRAPs and 20 L mesocosms in New Zealand, the rotifer *B. calyciflorus* quickly consumed single cells of *Ankistrodesmus,* while *Cephalodella catellina* depleted colonial *Dictyosphaerium* sp. and *Micractinium* sp. without spines, although not *Pediastrum* sp. with its armoured silica structure (V. Montemezzani unpublished data). When the effect of specific zooplankton grazers on different microalgae is known, manipulation of zooplankton species could be used to promote the dominance of desired microalgae. Although the use of a rapid zooplankton removal method is the priority in the event of blooms, the regular use of a non-disruptive removal and recovery method is a preferred approach. Zooplankton have high nutritional value and if harvested from the HRAP system could be used for feed in freshwater aquaculture [136], [137], or as

a source of chitin, proteins and oil for cosmetic and food industries [138], [139], [140]. The production of valuable zooplankton biomass from HRAP systems could provide a further benefit to the process.

Zooplankton control methods with potential application to HRAPs

Since the 1940s, a variety of zooplankton control methods including heating, centrifugation and application of chemicals, have been tested in open ponds to prevent loss of microalgal biomass [141]. However, these have generally given poor results and to date, there are no reliable zooplankton control methods that are applicable to full-scale HRAPs. Some ballast water treatment and aquaculture water recirculation treatment technologies may provide opportunities, while other treatments may be inferred from physiological and lifecycle studies. Some control methods are not applicable to hectare-scale HRAPs. Some of the main constraints on effective zooplankton control in large-scale HRAPs are the large volumes of water involved [142], the presence of zooplankton resting eggs and juveniles which are usually more resistant to treatments [39], the necessity to minimize capital and operational costs [143], and the need to preserve the zooplankton community of the following maturation ponds.

Potential HRAP zooplankton control methods that are cost effective, selective, and do not have detrimental effects on the microalgae, water quality or beneficial zooplankton are reviewed below. These can be divided into physical, chemical, biological and enzymatic methods. Novel strategies to both control and concentrate zooplankton in specific areas of the pond will also be discussed further. All methods focus on removal of the zooplankton adult stages, and when routinely applied for a short duration will also deplete the resting egg bank present in the pond.

Physical control methods

Physical control methods are generally effective although often have high capital and operation costs, and they can also remove or disrupt the microalgae-bacterial flocs reducing harvest efficiency. Furthermore, some technologies cannot be applied due to HRAP operational and physical constraints. For example, ultraviolet (UV) radiation has been successfully

used to eliminate zooplankton both in laboratory experiments and ballast water trials [144], [145]. However, effective UV penetration requires clear water, which is not the case with algal laden HRAP water. Another example is deoxygenation, which has the potential to inactivate zooplankton without harming microalgae. Deoxygenation may be achieved by gas sparging, adding reducing agents or increasing the organic loading of the HRAP to enhance bacterial growth and respiration (O_2 depletion). A low (0.27-0.87 ppm) O_2 concentration is required for 48 h to asphyxiate 99% of zooplankton [146], [147], but this is not achievable in HRAPs. During the daytime DO concentration is often supersaturated due to intense photosynthesis, while at night the constant water movement and the high surface area to depth ratio of HRAP promotes reaeration by O_2 diffusion from the air.

Several potential physical methods for zooplankton control include mortality by high temperature, hydrodynamic cavitation, solid shear stress, and removal of zooplankton grazers using filtration or a hydrocyclone.

Temperature

High temperatures (>35 °C) can drastically reduce the survival of zooplankton and could be used to depress their populations in HRAPs. Very high temperatures (60-100 °C) could be applied for short time periods, but would require large amounts of energy [148], and would also have a detrimental effect on the algal community. However, moderate heat treatment (35-45 °C) could be feasible, particularly when waste heat (e.g., coolant fluid from engines, power generators, or turbines) is available. However, while heat treatment (35-38 C° for few hours) has been used to treat ship ballast water, completely removing all zooplankton it also killed a large proportion of the microalgae [149]. The lethal temperature for particular zooplankton depends on the organism's physiological tolerances, its acclimatization capacity, and the rate of temperature change, and is usually less than that required for microalgal mortality. *Daphnia pulex* laboratory cultures acclimated at 20 °C had 95% mortality when exposed to temperatures of 35 °C for 4 h [150]. *Daphnia magna* had a LD_{50} (median lethal dose) of 34.8 °C after 24 h; 37.8 °C after 15 min; and 39.4 °C if the temperature was continuously increased by 0.2 °C/min [151]. Generally,

freshwater green microalgae can tolerate temperatures of ~35 °C [152], [153] and some species already used for microalgae mass culture, such as *Scenedesmus almeriensis,* can tolerate temperatures of ~44 °C [154]. The main constraint of zooplankton control by temperature is the need for a large source of waste heat; therefore, HRAPs would need to be constructed close to industry.

Cavitation

Cavitation occurs when a liquid is subjected to rapid changes of pressure, and the empty cavities generated at low pressures implode, releasing energy able to physically disrupt the zooplankton grazer's body. Cavitation can be generated by radiating sound waves (sonication) and generating hydrodynamic turbulences within a liquid media. Sonication has been utilized to treat ballast water [155], and was particularly effective to inactivate large zooplankton such as *Ceriodaphnia dubia*, *Brachionus plicatilis* and *B. calyciflorus*, with little effect on microalgae [156], [157], [158]. However, it is considered too costly to treat large volumes of water [159], [160], [161]. Moreover, the capital cost for a sonication unit with a capacity of 500 m^3/h (~US\$ 170k) [162] is twice the capital cost required to construct a one hectare HRAP [3], and it is, therefore, unlikely to be appropriate for hectare-scale HRAPs. Hydrodynamic cavitation can be produced mechanically with the creation of turbulence and pressure drops in a liquid flow using pumps or impellers to force the liquid through small openings [155], [163]. Hydrodynamic cavitation is less expensive and easier to set-up, maintain and control than sonication [164], [165]. Zooplankton are killed by a combination of mechanical effects (including collision impact, turbulence and bubble explosion shock waves), and chemical effects (e.g., from unstable radicals, OH· and $H_2O_2^{2-}$) [166], [167]. Hydrodynamic cavitation has been successfully used for cell disruption and inactivation of bacteria in water disinfection [163], [168], and ballast water treatment systems have been tested with promising results. Cavitation attained by forcing the liquid through a 26 mm pipe with an orifice (75% open) killed ~80% of zooplankton larger than 50 µm after a single pass and >95% after two passes, with an energy consumption of 0.31 kW/m^3 [166]. A device

using 21.5 mm diameter perforated plates and operated with different configurations (number of holes, size, shape and disposition) killed 28%-81% of zooplankton and 6%-46% of microalgae after a single pass [169]. Cavitation can selectively kill particular zooplankton and has greatest effect on adult zooplankton than on eggs [170]. The use of a plate with openings (slits) inserted in a 50 mm pipe to generate shear stress and cavitation, killed between 65.1% and 99.9% of zooplankton, with higher effects on individuals larger than 100 µm. The estimated running cost for a 150 m^3/h unit is ~US\$ 0.01 /m^3 with a capital cost of ~US\$ 100,000 [171]. Large zooplankton grazers may be killed simply by the generation of hydrodynamic shear stress, without the need for cavitation. Shear stress could be achieved using an inexpensive water pump connected to a valve or to a curved pipe. This option would have a milder effect on microalgae, requires simpler equipment, and would drastically reduce capital costs and energy use.

Solid shear stress

Large zooplankton may be killed by applying constant, mild mechanical stress using a bead mill. The solid shear stress is generated by the grinding action of the sphere friction [172], [173]. Bead mills are commonly used in pharmaceutical and biotechnology industries to extract enzymes or other components from cells. The bead mill has a chamber filled with small glass beads 0.1-10 mm in diameter and an impeller which stirs the beads [174]. Bead mills could be applied to HRAPs using a rotating cylinder partially open at either end (e.g., perforated plate or metal mesh), containing large beads (ø >10 mm) positioned longitudinally relative to the water flow. The algae suspension would gently flow through the slowly rotating device, with zooplankton killed by the friction generated between the beads. Various bead sizes and rotating speeds could be used to selectively kill particular zooplankton species. This system is potentially applicable to HRAPs because it is simply constructed and operated. Furthermore, if operated at low rotating speed (e.g., <30 rpm) and using large beads (e.g., ø >10 mm), it is expected to have low operation costs, and do not damage microalgae.

Filtration

Filtration is a cheap and simple technique that uses different filter mesh sizes to separate and capture zooplankton according to their size. In California, filtration using a 150 µm mesh size was used to partially control large zooplankton grazers in 12 m^2 HRAPs [70]. Automatic 100 µm and 50 µm mesh backwashing screen filters used for ballast water treatment [148], removed between 79% and 89% of zooplankton respectively [175]. Rotating drum filters with a 60 µm mesh size have been used to filter smaller zooplankton species [176]. Unfortunately, mesh sizes smaller than 200 µm are not suitable for HRAPs as the ponds grow algal-bacterial flocs of 50-200 µm [67], which would also be removed by such filters. Moreover, small mesh size filters clog faster resulting in higher operation costs [148]. Filters based on rubber granules are cheaper than traditional filters (e.g., sand) because they can be easily backwashed and operated at high flow rates. A filter packed with a 600 mm layer of 0.5-1.2 mm diameter granules operated at a flow rate of 49 m^3/h/m^2 removed ~66% of zooplankton larger than 50 µm, and also 50.5% of total microalgae [177]. To reduce microalgae retention in granule filters, larger granules could be used, so that only large zooplankton (>200 µm) are removed. The use of granules filters for zooplankton removal in HRAPS requires the pumping of pond water into the filter placed above the water level, and gravity flow of the filtered liquid back into the pond. Total separation of zooplankton would require 20 µm mesh filters [178]. However, in aquaculture systems zooplankton are harvested according to size: 80 µm meshes for small rotifers; 160 µm for large rotifers and early life stage copepods; 300-500 µm for small cladocerans and cyclopoid copepods; and 700 µm for adult *Daphnia* and large cyclopoid and calanoid copepods [179], [18]. Total harvesting of *Daphnia carinata* and *Moina australiensis* from experimental tanks in Adelaide (Australia) required a 200 µm mesh filter [140]. Adult cladocerans that are carrying eggs can be 50% larger than non-reproductive adults and therefore removable with large mesh sizes (600-800 µm) without removing algal biomass. Constant removal of reproductive adults is expected to eventually control their population. Estimated total costs for ballast water filtration are between US$ 0.06-0.19/m^3 [148]. In HRAPs, the control of cladocerans using large mesh

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filters may be achieved using a passive system which relies on the flow generated by the paddlewheel (no pumping required), with consequent reduction in costs.

Hydrocyclone

Cladocerans can be separated and recovered using hydrocyclones with continuous high speed centrifugal water flow, which require less maintenance than filters [147], [148]. Hydrocyclones are less efficient for particles smaller than 400 μm [148] and therefore have potential to separate large, mature cladocerans, copepods and ostracods from smaller algal flocs and rotifers [180]. Mudsnails (*Potamopyrgus antipodarum*) of various sizes, including 150 μm neonates, have been successfully separated from aquaculture water using a hydrocyclone [181]. In a pilot-scale ballast water treatment system, the removal efficiency of particles larger than 800 μm was 70-86%, while for particles smaller than 300 μm it was only 16.5-57% [182]. In addition, the high water velocity and pressure within the hydrocyclone system increased mortality due to mechanical damage. For example, in a ballast pilot plant, 62.7% of zooplankton were removed, and 95.3% of the zooplankton remaining in the treated water were dead [183]. In another pilot experiment, no living copepods were found after the hydrocyclone process [184]. Unfortunately there have been no studies on the effects for large colonial microalgae and microalgal-bacterial flocs, although a certain level of separation and mechanical disruption is expected. Estimated total costs of hydrocyclone systems are US$ 0.05-0.26 /m^3 [148].

Chemical control methods

An ideal zooplankton chemical control method should kill zooplankton with minimal effect on microalgae and it should be selective and not kill useful zooplankton species. Chemical treatments may be particularly useful to reduce the abundance of smaller zooplankton, such as rotifers, which are generally more difficult to remove using mechanical methods. Chemical control agents are often easy and relatively inexpensive to apply. However, these chemicals may also be assimilated by the algal biomass [15], and persist in the pond water affecting further treatment ponds (i.e., maturation

ponds) and receiving waters. As such, only control agents with short half-lives must be used in HRAPs. If safe levels are reached rapidly (e.g. in <48 h) the outflow from the treated HRAPs could be temporarily stopped for this period. Moreover, chemicals may select for chemically resistant zooplankton species that have higher tolerances to given concentrations and exposure times of chemicals. Options for chemical control of zooplankton include: raising of pH and NH_3-N concentration; addition of chemical agents; injection of CO_2; and the use of enzymes, enzyme inhibitors and infochemicals (chemicals that carry information between two individuals, and induce a behavioural or physiological response in the receiver).

pH

Alteration of pond water pH can be used to kill zooplankton. Cladocerans can tolerate a wide pH range, but their mortality rate drastically increased at values greater than 10.5 [185], [186]. In a highly eutrophic pond the survival rate of *Ceriodaphnia reticulata* drastically decreased at pH 11.2 [187], while in laboratory experiments the LC_{50} of *Daphnia magna* at pH 4.4 and pH 10.7 was 48h [188]. Some rotifers, including *Brachionus* species, can tolerate pH between <3 and 10 [189], [190]. In laboratory cultures, *B. calyciflorus* had the lowest reproduction rate and highest resting egg production at pH 10.5, while at pH 11.5 total mortality occurred in less than 24 h [191], [192]. In another laboratory experiment showed the LC_{50} at both pH 5 and pH 10 of a *B. calyciflorus* population was ~4 days, while at pH <6 a 50% reduction in the viability of eggs was achieved [190]. *Brachionus rubens* did not survive above pH 9.5 and below pH 4.5, even in the absence of ammonia [17]. Immediate zooplankton mortality by adjusting pond water pH is not achievable in HRAPs due to the large volumes of concentrated acid or base required to shift the pH to extreme values (<3 and >11) [19]. Furthermore, acidic pH is likely to be more detrimental to microalgae as they naturally shift pond water toward alkaline pH during day-time photosynthesis [16], [193], [194]. For example, in four 5,000 m^3 HRAPs located in Christchurch, New Zealand, the maximum daytime pH reached throughout the year was almost always above 10 [3]. Zooplankton reduction in HRAPs can be

achieved by augmenting the natural daytime pH increase, by stopping CO_2 addition and adding alkalizing chemicals (e.g., lime, $Ca(OH)_2$ or $NaOH$). However, pH levels should be carefully controlled because pH >11 can be harmful to pond microalgae [195], and induce auto flocculation of microalgae [196], [197] with resulting sedimentation on the bottom of HRAPs.

NH$_3$-N

Free ammonia (NH_3-N) toxicity can be used to control zooplankton in HRAPs at pH >9 [198], [199]. At pH levels higher than 7.5 the ammonium (NH_4^+) occurring in WW dissociates into un-ionized ammonia, NH_3-N. The proportion of NH_3-N increases with increasing pH: 50% at pH 9.5 and >80% at pH 10. The concentration of NH_4^+ in WW HRAPs generally ranges between 2 mg/L and 14 mg/L, and at high pH levels can generate sufficient NH_3-N to potentially depress the zooplankton population. Test-tube experiments showed that 3 mg/L of NH_3-N did not affect the reproduction of *Brachionus rubens*, while in the range of 3-5 mg/L the population reversibly declined, and concentrations over 5 mg/L killed all the rotifers within two days [200]. In a 0.1-ha HRAP constructed in Florida, USA, NH_3-N concentrations of 17 to ~20 mg/L completely eliminated *B. rubens* and the cladoceran *Diaphanosoma brachyurum* after 24 h, without affecting the microalgae [23]. In laboratory experiments *Daphnia carinata* did not survive 3 mg/L of NH_3-N for 24 h while 50% of *Moina australiensis* survived 48 h with NH_3-N concentration of 8.8 mg/L [137]. Acute treatment can be achieved by increasing the pH to ~11 for a few hours. For example, in two 8 M^3 HRAPs located in Ruakura Research Centre (Hamilton, New Zealand), complete zooplankton eradication was achieved by adding lime up to pH 11,2 for 4 hours followed by neutralization using concentrate acid (unpublished observation). Unfortunately, the cost of quickly raising and then lowering the pond pH using chemicals in hectare-scale HRAPs make this option uneconomic. Another possibility is to increase the pH of the pond by temporarily stopping the artificial CO_2 injection and to perform the NH_3-N treatment over a few days, using the natural rise of pH (~10) and increasing the NH_4^+ concentration by adding more WW. For example, full-scale HRAPs

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for WW treatment located in Christchurch, New Zealand, and operated without CO_2 addition over an entire year, reached pH values >10 during spring and summer [3], and had only a few minor zooplankton blooms (D. Sutherland, personal communication).

Commercial chemical products

Chemical control agents typically have higher activity on cladocerans than rotifers. For example, in laboratory experiments using three pesticides the 24 h LC_{50} for the rotifer *Brachionus calyciflorus* and cladoceran *Daphnia pulex* were 318.5 mg/L and 0.0036 mg/L, respectively, using Trichlorphon; 263.5 mg/L and 0.0273 mg/L using Buprofezin; and 0.4 mg/L and 0.0598 mg/L using Tralocythrin [22]. Cypermethrin is a neurotoxic insecticide [201], which at a concentration of 6.1 µg/L completely killed the populations of *Daphnia* and calanoid copepods located in lake enclosures in just two days. After the treatment, the abundance of rotifers, protozoans, bacteria and the chlorophyll-*a* all increased, probably due to the lack of competition and grazing activity of copepods and cladocerans [202]. The half-life of cypermethrin is 25 h [203] and costs ~U\$15/kg [204].

Temephos is an organophosphorus insecticide, which has been applied to enclosures in the shallow, hypereutrophic Lake Suwa (Japan). Temephos at a concentration of 58.6 µg/L killed all cladocerans and drastically reduced rotifer numbers. When the rotifer community recovered, *Trichocerca* spp., *Keratella cochlearis*, *Filinia longiseta* and *Lecane* spp. were replaced by *Polyarthra trigla*, *Hexarthra mira* and *Brachionus calyciflorus,* probably due to their higher tolerance to the chemical. Temephos half-life is 28.7 days at pH 7 [205], is rapidly adsorbed in suspended particles and sediments [206], and is not detrimental to microalgae, which always increased after treatments [207].

Carbaryl is an agricultural insecticide belonging to the carbamate family that at a concentration of 1 mg/L in enclosures placed in small, shallow and eutrophic ponds, killed all the cladocerans, rotifers and *Chaoborus* larvae. The absence of *Chaoborus* larvae (that can prey on zooplankton) promoted the re-establishment of cladocerans soon after treatment, in turn

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cladocerans competed with rotifers and reduced their population. The treatment did not show obvious detrimental effects on microalgae and the rapid recovery of cladocerans was probably also related to the rapid dissipation of the chemical [208]. Carbaryl half-life strongly depends on pH: in distilled water it is 3.2 h at pH 9; 12.1 days at pH 7; and 1,600 days at pH 5 [209].

Quinine sulphate is an anti-protozoal chemical that was used to control predatory ciliates in outdoor cultures of the algae *Dunaliella salina*, with only minor damage to algae itself. Doses required to inactivate ciliates are expected to be similar to those required to inactivate rotifers. The 24 h LC_{100} (absolute lethal concentration) for the ciliate was 12–14 mg/L, while for the algae the 72 h EC_{50} (half maximal effective concentration) was 14.5 mg/L [32]. Its effects on larger zooplankton are unknown. The organophosphate insecticide Dursban reduced >99% of *Moina micrura* in shallow experimental ponds located in Bakersfield (California). Doses of 0.028 kg/ha and 0.28 kg/ha inhibited *Moina* reestablishment for 1-3 weeks and 3-6 weeks respectively [210]. Permethrin is an insecticide, which at a concentration of 10 µg/L in pond enclosures eradicated the population of *Daphnia rosea*, without reestablishment in the following month. Photosynthesis and microalgae were not significantly affected, permethrin accumulated in the sediments at a concentration of 6 µg/L (wet weight), and 24 h after the treatment the permethrin concentration in water showed a six fold decrease [211].

Commercial products commonly used for ballast water treatment such as PeracleanOcean™, SeaKleen™ and Acrolein™ typically have a short half-life and are broad spectrum. PeracleanOcean™ is a fast-acting oxidizing liquid biocide based on peracetic acid and is active on bacteria, spores, yeasts, moulds, protozoa, algae and viruses between pH 5 and 9. In laboratory experiments performed under optimum environmental conditions for the tested species, 400 ppm of PeracleanOcean™ completely inactivated the microcrustaceans *Daphnia* sp., *Bosmina* sp. and *Cyclops* sp. after just 1-2 hours, while inactivation of the algae *Chlorella* sp. required 48 h. In water, PeracleanOcean™ rapidly decomposes into oxygen and acetic acid, the half-life in fresh water is 2-24 h depending on pH and temperature,

and the costs are between US$ 0.20-0.30/m^3 of treated water. Acrolein™ is an organic biocide, containing an unsaturated aldehyde (acraldehyde) marketed as "MAGNACIDER B Microbiocide". In laboratory tests, the 48 h LC$_{50}$ of *Daphnia magna* was 0.022 ppm; the 5-day EC$_{50}$ of the green algae *Selenastrum capriconutum* and the diatom *Navicula pelliculosa* were respectively 0.050 ppm and 0.068 ppm. Acrolein should be carefully managed because it is a strong irritant of skin, eyes, and nasal passages, the half-life is 8-24 h and estimated costs are between US$ 0.16-0.19/m^3 of treated water [171]. Menadione, the synthetic derivative of vitamins K1 and K2, also has biocide activity. It is marketed as SeaKleen™ and it is more effective on adult cladocerans and rotifers than green microalgae. In laboratory tests, the 24 h LC$_{50}$ of *Daphnia magna* juveniles, the adult copepod *Eucyclops* spp. and the adult *Brachionus calyciflorus* were respectively 0.46 mg/L, 0.43 mg/L and 0.45 mg/L. The freshwater green algae *Selenastrum* spp. completely survived a SeaKleen™ concentration of 80 mg/L for 48 h [212]. The resting eggs of *Brachionus plicatilis,* a freshwater copepod, and *Daphnia mendotae* had 24 h LC$_{50}$ and LC$_{90}$ respectively at a concentration of 1.1-2.6 mg/L*,* 0.8-4.9 mg/L, and 6.7-8.7 mg/L*.* The cost of SeaKleen™ used in concentrations of 2 ppm is estimated at US$ 0.20/m^3 of treated water [148], [213].

The use of chemicals to inactivate zooplankton in HRAPs appears to be a simple and effective option. However, the application of several chemicals still needs to be tested in hectare-scale HRAPs. Cypermethrin, Permethrin and Carbaryl can be used in low amounts and have quick inactivation times. SeaKleen™ and quinine sulphate are very promising because of their negligible effect on microalgae, but quinine sulphate requires testing on rotifers. More information is needed on persistence in the effluent and sediment, and selection should be based on costs, availability and effluent discharge limits for the specific chemical.

O$_2$ and CO$_2$

Zooplankton grazers are aerobic organisms and use metalloprotein haemoglobin, or similar smaller multidomain molecules to transport O$_2$ [214]. Hence, they can theoretically be controlled by removing all O$_2$ from

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the water or by inhibiting the O_2 binding capacity of haemoglobin. However, zooplankton are able to tolerate very low DO conditions. For example *Brachionus rubens* grows at 1.15 mg/L O_2, can tolerate 0.72 mg/L for ~5 days and will survive 0.3 mg/L for several hours [17]. Cladocerans can modify their haemoglobin concentration and its affinity for O_2 according to the DO of the water [215]. *Daphnia magna* has a 48-h LC_{50} of 0.6 mg/L and it is unable to remove O_2 from water at a concentration lower than ~0.3 mg/L. *Daphnia pulex* has a 48-h LC_{50} of 0.5 mg/L O_2 [216], [217] and *Moina mongolica* can tolerate DO levels between 0.14 mg/L and 0.93 mg/L [218]. Generally, aquatic species have low internal $ppCO_2$ due to the high CO_2 solubility in water [219] and are sensitive to CO_2 partial pressure variations. CO_2 partial pressure affects the capacity of haemoglobin to bind O_2 in two ways: directly by reversible CO_2 binding, and indirectly by pH changes that modify the binding affinity. Laboratory experiments have shown that when gaseous CO_2 was removed from the water, respiration was unaffected even at low pH indicating that gaseous CO_2 affects the respiration of *Daphnia magna* more than pH [220]. CO_2 addition in the form of dry ice was used to kill zooplankton in experimental enclosures [59]. In 1.5 m^3 microalgae cultures treated with pure CO_2, the zooplankton density was much lower than in control cultures without CO_2 and with the same pH (5 and 6) [221]. A combination of CO_2 and low O_2 using a gas mixture (2% O_2; 12% CO_2; 84% N_2) was proposed to kill zooplankton in ballast water treatment [222]. These results suggest the possibility of using CO_2 injection to control zooplankton in HRAPs. For hectare-scale HRAPs, very high $ppCO_2$ will be difficult to achieve due to gas exchange with the atmosphere. However, lower CO_2 concentrations maintained for long time periods (hours/days) could still control zooplankton grazers. Any CO_2 treatment should be performed at night, when algal/bacterial respiration reduces the DO, and produces CO_2 [11]. CO_2 can be derived from exhaust or flue gases, or as a by-product from processes such as fermentation [9]. Furthermore, CO_2 from flue gasses could contain CO (a known toxicant able to reduce the O_2 carrying capacity of hemoglobin), further increasing zooplankton mortality.

Enzymes

Chitin is a structural polysaccharide which largely makes up cladoceran exoskeletons [112], and it is periodically degraded and synthesised during moulting. The inhibition of chitin formation and degradation could be used to negatively affect cladoceran, copepod and ostracod growth. Chitin is hydrolysed by chitinases, a group of enzymes produced by bacteria, fungi, plants and insects during moulting [223], [224]. Chitinases have been proposed as a biopesticide to degrade fungal cell walls [225], and natural chitinolytic enzymes are commercially available. However, their costs are high and their use is limited [226]. A cheaper alternative is provided by substances that interfere with chitinase production and promote the mortality of crustacean zooplankton including diflubenzuron, chitosan and allosamidin [227], [228]. In laboratory tests, diflubenzuron at a concentration of 2.0 µg/L was lethal to *Daphnia magna* and had very low vertebrate toxicity. *Daphnia* were successfully eliminated from a 0.1 ha HRAP in one week using a 20 µg/L concentration of Dimilin™, a commercial formulation of diflubenzuron [229]. Chitosan and its derivatives repress chitinase activity through competitive inhibition that prevents larval moulting from occurring [230]. Laboratory toxicity tests have shown an artificial chitosan derivative added to the diet of the larva of the moth *Spodoptera littoralis* (Lepidoptera), at a concentration of 0.625 g/kg, resulted in 100% mortality [231]. The cost of food grade chitosan (95%) is ~ US$15 /Kg [232]. Allosamidin are pseudotrisaccharides that exert inhibitory activity on chitinase at very low concentrations. In laboratory experiments, 30 µg and 50 µg of allosamidin per ml of enzyme solution (200 units of chitinase/ml) respectively inhibited 50% and 70% of chitinase activity [233]. Unfortunately, data on the concentration of allosamidin needed for zooplankton inactivation and the half-life in water is not available, and the use on a large scale (e.g. HRAPs) is not possible due to production difficulties and high costs (~3,000 US$/mg) [228], [234]. In conclusion, chitinase inhibitors have potential for zooplankton control. However, their use in WW HRAPs requires further study, including their persistence in aquatic ecosystems.

Infochemicals

Infochemicals are substances excreted by organisms that may modify the behaviour, physiology and structure of individuals of another species. Kairomone infochemicals can provide selective advantages for organisms that detect them. For example, kairomones can induce defence mechanisms in microalgae against zooplankton grazing by promoting colony formation, bio-flocculation, or generation of spines [134], [235], [236], [237]. Formation of colonies and spines reduces the grazing effects of *B. calyciflorus*, *B. patulus*, *Ceriodaphnia dubia* and *Moina macrocopa* on algae [238], [239], with effects directly proportional to the chemical concentration [240], [241]. Some types of kairomones have been isolated and identified. For example, aliphatic sulfates and sulfamates [242], [243], [244] as well as an artificial substitute (octyl sodium sulfate) have been found to induce colony formation in *Scenedesmus* sp. and *Desmodesmus* sp. [245]. Moreover, lipophilic exudates of *Daphnia* [246] and man-made lipophilic surfactants derived from detergents both cause a defensive response in *Scenedesmus*. In laboratory experiments the commercially available FFD-6 linear alkyl benzene sulfonate, in a concentration between 0.001 and 0.01 g/L, induced the unicellular *S. obliquus* to form 4 and 8 cell colonies [247]. The FFD-6 drastically depressed the feeding and survival rates of *Daphnia magna*. The LC_{50}-24h and LC_{50}-48h were respectively 148 µl/L and 26 µl/L [248].

In hectare-scale HRAPs, infochemicals could potentially reduce zooplankton grazer activity by inducing defensive modifications in microalgae, and the addition of artificial substitutes (e.g., lipophilic surfactants) provides a low-cost option. Nevertheless, natural sources are also potentially viable. Infochemicals are expected to be abundant in zooplankton rich maturation ponds, and the outflow coming from these ponds could be filtered to remove zooplankton grazers and recirculated to the HRAPs to promote colony formation and bio-flocculation.

Biological control methods

The introduction of zooplanktivorous organisms to HRAPs could provide a natural zooplankton biocontrol method. A predator should be able to

permanently live in the WW HRAP but not be transferred to downstream ponds. For example, natural parasites of cladocerans such as bacteria and fungi [112] are not suitable because of the unavoidable contamination of maturation ponds. Fish, amphibians, and crustaceans are potentially effective although the physicochemical parameters of HRAPs are likely to limit the number of species that can be introduced, and their interactions with zooplankton have only been studied in natural and aquaculture systems. Moreover, selective predation on some zooplankton species can allow for the survival or high abundances of other zooplankton species. For example, water bodies containing zooplanktivorous fish are dominated by small zooplankton species, as the larger species are eliminated by fish predation. Water bodies without zooplanktivorous fish, on the other hand, are dominated by large invertebrates that either compete with or prey on small species [249].

The following section will review the potential for biological control of zooplankton by natural predators including Notonectidae, *Chaoborus* spp., predatory cladocerans, predatory rotifers, competing herbivorous cladocerans, ostracods and fish.

Notonectidae

Notonectidae (Order: Hemiptera) are aquatic insects commonly called backswimmers because they swim backwards. Notonectidae are predators of cladocerans and are distributed worldwide, typically inhabiting still and gently flowing freshwater lakes, ponds and marshes [250], [251], [252]. *Anisops* backswimmers live for one year and only reproduce once or twice in that time, with at least three months required to reach maturity [253]. *Anisops* have been found in polluted waters [254] as they are able to assimilate O_2 from the atmosphere [253]. However, despite being able to survive low DO waters, they also fly to new habitats when food and O_2 conditions are unfavourable [255]. *Anisops* prey on large cladocerans such as *Daphnia* spp. and *Moina* spp. [256], even when light is limited in highly turbid waters [257]. Laboratory experiments showed that *Anisops* are size-selective predators; a small *Anisops* (~3 mm long) can consume ~50 small (<0.5 mm) and ~30 large (0.5–0.9 mm) *Ceriodaphnia dubia* in 24 h, while

one large *Anisops* (~7 mm) can consume twice as much. In contrast, small *Anisops* were only able to eat small rotifers such as *Synchaeta pectinata* and *Polyarthra dolichoptera* but not the larger *Anuraeopsis fissa*, *Brachionus angularis*, *Keratella cochlearis* and *K. slacki* [258]. *Anisops* are common in WW treatment maturation ponds, but are less common in HRAPs, probably due to the flowing water, lack of a stable substrate for egg attachment, and extreme diurnal DO fluctuation. Without the implementation of specific systems to retain the organisms and a substrate on which to lay eggs, the use of Notonectidae to control zooplankton in HRAPs is unlikely due to their limited natural occurrence.

Chaoborus spp.

Chaoborus spp. are a type of a midge whose larvae (~6-23 mm) can live up to several months in anoxic sediments of small ponds. They are found on all continents excluding Antarctica, particularly in temperate and tropical climates and under eutrophic conditions [259], [260], [261], [262] and prefer environments with standing water [263]. They prey on cladocerans, copepods and rotifers, preferring the smallest available prey [264]. In experimental enclosures placed in Lake Ontario, *Chaoborus* resulted in the elimination of *Daphnia galeata,* and population reductions of the cladocerans *Bosmina*, *Diaphanosoma*, calanoid copepod species and the rotifer *Conochilus* [249]. In *in situ* predation experiments using 0.95 L enclosures placed in a fishless lake (northern Michigan), *Chaoborus* completely eliminated *Bosmina* [265]. In laboratory experiments, *Chaoborus punctipennis* effectively consumed rotifers such as *Synchaeta* spp., *Brachionus* and *Polyarthra* [266]. However, *Chaoborus* predation pressure is not expected to greatly reduce the rotifer population in lakes due to their high reproductive output [267]. *Chaoborus* sp. could therefore be established to moderate zooplankton population in HRAPs, as they do not feed on microalgae, and they are unable to disperse as larvae. However, more research is required to investigate their resistance to WW environments and ability to establish in the flowing waters of HRAPs.

Predatory cladocerans

Cladocerans such as *Cercopagis pengoi*, *Leptodora kindtii*, *Polyphemus pediculus*, and *Bythotrephes longimanus* are raptorial predators that feed on smaller zooplankton species [268], but not on microalgae. *Cercopagis pengoi* has been mainly found in brackish and fresh waters of Eastern and Central Europe, and has non-indigenous populations in and around the Great Lakes, USA [269], [270]. In Lake Ontario, *C. pengoi* reduced the populations of rotifers and crustaceans, such as *Bosmina longirostris*, *Daphnia retrocurva* and *Diacyclops thomasi*. In laboratory experiments, *C. pengoi* preyed on the rotifer *Asplanchna priodonta* and the cladocerans *B. longirostris, D. retrocurva, Ceriodaphnia lacustris, Scapheloberis kingi* and *Moina micrura* [271]. *Leptodora kindtii* is widespread throughout Europe, north of the Himalayas, northern Arabia, northern Africa, and North America [272], [273]. In laboratory experiments, *L. kindtii* fed preferentially on *Daphnia, Bosmina* and *Diaphanosoma* over *Chydorus, Keratella, Acanthocyclops, Leptodiaptomus* and copepod nauplii [274]. *Polyphemus pediculus* is mainly found in ponds and lakes of northern temperate zones such as the Great Lakes and the Caspian area [275], [276], [277]. *In situ* studies using zooplankton chambers showed that *P. pediculus* selected small prey such as the protozoan *Vorticella* and the rotifer *Keratella* over larger prey such as copepod nauplii. Moreover, prey without protective structures (e.g., spines and or hardened lorica) such as the rotifers *Conochilus* and *Polyarthra* were preferred over prey with protective structures such as *Kellicottia* [276]. *Bythotrephes longimanus* is native to central and northern Europe and Asia, and has spread into the Great Lakes [278], [279]. In Lake Ontario, the invasion of *B. longimanus* promoted a quick and long-lasting reduction in the average species richness of zooplankton [280], especially small cladocerans species such as the *Bosmina longirostris* and *Diaphanosoma birgei*, and the copepod *Tropocyclops extensus* [281]. Predatory cladocerans can potentially be used as a natural and cheap biocontrol of zooplankton grazers. However, they are not reported to inhabit hypereutrophic environments, and their establishment in HRAPs can be difficult because they have a generation time longer than herbivorous zooplankton [282]. Depending on the

geographical location of HRAPs, cladoceran species must be selected according to local availability or, if non-native species can be introduced, their ability to survive local climatic conditions. More research will be required to assess their survival and potential acclimatization to HRAP environments.

Predatory rotifers

Some species of large rotifers, such as Asplanchna species, are omnivorous and can grasp prey using their trophi [43]. For example, the number of food items found in the stomach of A. herricki collected in two natural lakes (Minnesota) included 69% rotifers such as Keratella, Brachionus and Ploesoma species, and just 25% microalgae colonies/cells; colonial Pediastrum and diatom species were tenfold less than Keratella bodies [283]. Asplanchna girodi fed mainly on smaller rotifers [284] and in a shallow hypertrophic lake, A. brightwelli suppressed the herbivorous rotifers Keratella cochlearis, feeding on reproductive females [285]. At higher prey densities A. brightwelli increased their ingestion rate; at 25 °C a single Brachionus calyciflorus was ingested in ~9-23 s, while the smaller Anuraeopsis fissa was ingested in just ~3-4 s [286]. Asplanchna brightwelli has been reported in sewage ponds [287], and other species such as Asplanchna sieboldi had optimal growth in raw WW [73]; hence, they are potentially suited to the HRAP environment. If established in HRAPs, Asplanchna spp. could reduce the rotifer population with minimal consumption of microalgae. However, it is crucial to select an appropriate species because some species such as A. girodi are mainly predacious, while other species such as A. priodonta can also feed on large algae (diameter up to 100 μm) [288], [284]. The main challenge for the use of predatory rotifers in HRAPs is to maintain their population when rotifer prey are absent.

Competing herbivorous cladocerans

Herbivorous cladocerans such as *Daphnia* spp. exert an inhibitory effect on smaller species such as rotifers, both by mechanical damage and food competition [289]. In waste stabilization ponds located in Luxemburg,

competition with *D. magna* and predation by *Cyclops strenuus* inhibited rotifer establishment [82]. In laboratory experiments the rotifer *B. calyciflorus* was largely suppressed by the cladoceran *Moina macrocopa*, with larger population reductions at lower food levels [290]. Low population densities (1-5 organism/L) of large *Daphnia* (>1.2 mm) were sufficient to cause high mortality rates of susceptible rotifer species such as *Keratella* spp. [291]. Hence, the establishment of a low density population of cladocerans is expected to depress rotifer abundance and maintain high microalgal settling by the preferential consumption of smaller particles [292], [38]. Cladocerans are normally undesirable in HRAPs. However, when able to reduce the abundance of less easily-managed rotifers they may be beneficial, particularly when the dominant microalgae are ingestible by both cladocerans and rotifers. Populations of cladocerans can be easily controlled to achievable densities by filtration, and they are expected to consume less microalgae than a large population of rotifers. Unlike zooplankton predators that generally require specific prey items to survive, competing herbivorous cladocerans such as *Moina* spp. can be easily established in HRAPs because they feed on microalgae and bacteria. The constant removal of cladocerans by means of filtration would also theoretically enhance the WW performance by removing nutrients from the system.

Ostracods

Ostracods are generally bottom dwellers that eat detritus. However, some species, such as *Heterocypris incongruens*, can prey on *Daphnia* and rotifers (up to 40 *Keratella quadrata/*day) [293]. In laboratory experiments, large adults (~2 mm) of *Cypris pubera* significantly reduced the growth of a *Keratella tropica* population by direct predation and mechanical damage [97]. In microcosm experiments the ostracod *Cypridopsis vidua,* at a concentration of $>10^3$ organisms/L, completely eliminated the rotifer *Lepadella* sp. from an initial concentration of $>10^3$ organisms/L, although it was not clear if the effect was caused by predation or competition for a common resource such as algal detritus. Furthermore, microalgal dominance shifted from *Scenedesmus* sp. to *Ankistrodesmus* sp. The

higher settleability of *Scenedesmus* sp. compared to *Ankistrodesmus* sp. may have promoted consumption of *Scenedesmus* sp. by the ostracod [294]. However, the increase of *Ankistrodesmus* sp. abundance was likely promoted also by the elimination of *Lepadella* sp. (a small rotifer expected to preferentially feed on the thin, needle-like *Ankistrodesmus* sp.).

Ostracods have the potential to be established in HRAPs to maintain low levels of rotifers, because ostracods are heavy and not easily suspended, and are not expected to have major effects on suspended microalgae. In addition, the shallow water level of HRAPs may facilitate the predatory activity of ostracods on rotifers through the water column [97].

Fish

Both larval and adult fish can prey on zooplankton, affecting community composition and abundance in natural lakes [295]. Fish have been suggested as zooplankton predators in algae production ponds [296], and could be potentially used to consume zooplankton species in HRAPs. Globally, various fish species tolerate a wide range of pH and DO and have been used in aquaculture ponds fed with human waste [62]. Species such as silver carp (*Hypophthalmichthys molitrix*) and Nile tilapia (*Oreochromis niloticus*), have proven to survive physicochemical conditions similar to those in HRAPs (297). The planktivorous common carp (*Cyprinus carpio*) can live in shallow, eutrophic, and turbid environments with pH ranging between 6.5-9.0 and low (0.3-0.5 mg/L) or very high DO [298]. Experiments conducted in 5 m^2 cement tanks for 90 days showed that Nile tilapia is particularly suitable for WW environments [299] and Pangasius (*Pangasius sutchi*) can survive the complete lack of O_2 by breathing at the surface [300]. In shock tests performed at 25 °C on three American freshwater fish species, the O_2 concentration was instantly decreased from optimal to critical levels, in which fish consistently survived for 24 h. The critical O_2 level was 0.75 mg/L for bluegill (*Lepomis macrochirus*), 0.92 mg/L for the largemouth bass (*Micropterus salmoides*), and 0.95 mg/L for the channel catfish (*Ictalurus punctatu*) [301]. The New Zealand native fish inanga (*Galaxias maculatus*) and common bully (*Gobiomorphus cotidianus*) placed in plastic tanks with low O_2 concentration (1 mg/L), required 48 h to exhibit

mortality rates between 27% and 80% [302], [303]. Eels are widely cultured throughout the world [304], they withstand polluted and shallow waters (<0.5 m deep), and juveniles can feed on small crustaceans [305], [306]. For example, *Anguilla dieffenbachia*, *A. australis* and *A. reinhardtii* can withstand a pH between 4.6 and 9.1 and water temperatures between 10 °C and 25 °C, and survive for 48 h at 1 mg/L of O_2 [303], [307]. Zooplankton control using fish requires the selection of species able to withstand local climatic conditions, and their enclosure in meshed cages to simplify their recovery and avoid damage by the paddlewheel. Enclosure of fish in confined areas of the pond has been implemented to harvest phytoplankton in partitioned aquaculture systems [308], and could potentially be used to harvest zooplankton in HRAPs. If fish are constantly kept in HRAPs, food can become scarce, hence HRAPs should be in the proximity of aquaculture ponds, where fish can be collected and transferred back as needed.

Concentration methods

Any control method applied to HRAPs at hectare-scale would require a large amount of water to be processed. The development of a method to concentrate the zooplankton in specific areas within the HRAP prior to the control method being applied could reduce costs. Control methods such as filtration, cavitation, hydrocyclone and bead mills could be applied only to the portion of the water column with a high density of zooplankton. For example, filtration of the upper layer of the water column (e.g., 50 mm), taking advantage of cladocerans natural migration toward the pond surface during the morning and evening, would reduce both the capital (smaller filtering surface) and operation (lower head loss) costs of filtration. The phototactic response of cladocerans could also be used for their night time concentration in areas of the HRAP with low velocity flow such as eddies, by using an artificial light source. Furthermore, cladocerans can be moved to the pond water surface by changing the concentration of respiratory gasses. In laboratory experiments, *Moina micrura* increased their swimming time when O_2 concentration was reduced from 10 mg/L to 1 mg/L [309]. However, at lower concentrations the swimming time drastically decreased. Similar behaviour was also observed in a laboratory experiment, with

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Daphnia carinata which moved to the surface of a 2 L chemostat when CO_2 concentration was between 100 and 150 mg/L. The response was not related to the O_2 concentration since that was always >1 mg/L (V. Montemezzani unpublished data). Both low O_2 and high CO_2 are likely to induce cladocerans to move toward the upper water layers (richer in O_2).

CONCLUSIONS

This review has provided an overview of the many potential options for zooplankton control in HRAPs. However, only a few of these are likely to be effective in controlling zooplankton on a large scale, and further research is required to demonstrate their effectiveness in full-scale HRAPs. The most promising options include:

- Increasing pond night-time CO_2 concentration by gas injection, for rapid control of a zooplankton bloom.
    - Selectivity, low capital cost and easy automation are promising.
    - Further research is needed to assess the lethal concentration of CO_2 for different zooplankton grazers.
- Promoting the temporary lethal un-ionized ammonia toxicity during daytime high pH periods, by raising the pond ammoniacal-N concentrations.
    - When naturally achievable this treatment is without cost.
    - Further research is required to quantify the adverse effects on phytoplankton, and to detect the optimal combination of pH, temperature, NH_3-N and treatment time for zooplankton species occurring in HRAPs.
- Continuous filtration of the upper 50-80 mm of the water column to remove mature cladocerans.
    - Filtration has low capital cost, simple operational and implementation requirements, and can be combined with CO_2 or phototactic induced migration to reduce the amount of water processed.
    - The harvested cladocerans are a potentially valuable by-product.
- Mechanical hydrodynamic cavitation and shear stress for a rapid mechanical disruption of zooplankton grazers, or the constant zooplankton reduction by regular disruption using a bead mill.

- o These technologies are effective although research is needed to optimize the energy input required to disrupt targeted species.
- Application of chemicals such as cypermethrin, permethrin, carbaryl, and commercial products such as PeracleanOcean™, SeaKleen™, and the chitinase inhibitor chitosan to quickly and easily reduce or eradicate the zooplankton population.
  - o Chemicals provide an interesting option to kill small zooplankton species that cannot be removed with mechanical methods but are applicable only when the outflow of HRAPs can be stopped for 24-48 h or temporary stored.
  - o Further research is needed to assess the degradation of these chemical in the HRAP environment, and their residual activity once in the maturation ponds.
- Application of infochemicals naturally occurring in zooplankton laden maturation ponds to promote algal colony and spine formation, and inhibit the grazing activity.
  - o Further research is needed to assess the type and amount of infochemicals present in maturation ponds and the required doses to induce the formation of spines, clumps and colonies.
- Biocontrol of zooplankton grazers by using competitor or predatory organisms.
  - o Biocontrol is potentially cost-effective, although it requires the screening of organisms able to establish in HRAPs and availability of taxa in specific geographical locations. The use of competing herbivores such as the cladoceran *Moina* spp. to control rotifers has the greatest potential because they can easily survive long time periods without prey.
  - o Further research on the densities of cladocerans required to depress specific rotifer species and their impact on microalgae is needed.

Although microalgae cultures may recover relatively quickly from "crashes" with re-growth of species that are not grazed by the zooplankton present, random changes of microalgal dominance are not desired and zooplankton

control is necessary to maintain stable and predictable HRAPs performance. Furthermore, to prevent the ecological imbalance caused by complete zooplankton removal, which could favour the establishment of other zooplankton that may be less easy to control, it may be most beneficial to control zooplankton grazers at low levels as part of a stable community. The selection of the most appropriate control method requires knowledge of the specific HRAP ecosystem, operational parameters, and local climate conditions. In addition, it should be clear which algal species are preferred, and the occurring zooplankton species should be classified accordingly to their beneficial or detrimental impact on the HRAPs main function (WW treatment performance, biomass production, culture of a specific alga).

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CHAPTER 3

ZOOPLANKTON COMMUNITY INFLUENCE ON SEASONAL PERFORMANCE AND MICROALGAL DOMINANCE IN WASTEWATER TREATMENT HIGH RATE ALGAL PONDS

ABSTRACT

High Rate Algal Ponds (HRAPs) with artificial addition of CO_2 provide improved tertiary-level wastewater treatment over conventional HRAPs. One of the greatest challenges for performance and management of HRAPs with CO_2 addition is the establishment of zooplankton grazers that can consume much of the algal biomass within a few days. High food availability and a near neutral pH provide optimal conditions for the establishment of zooplankton taxa and control strategies require an understanding of their population dynamics in HRAPs. Unfortunately, available literature lacks long-term assessment of such dynamics in HRAPs with CO_2 addition. Here, we assess the environmental and biological parameters of two wastewater HRAPs with CO_2 addition over a period of 14 months, in relation to HRAPs performance and operation. Eight species of zooplankton established in the HRAPs, with higher water temperatures and longer detention times promoting larger populations. Grazing pressure was associated with changes in: 1) the dominance of microalgal species; 2) large and rapid reductions of productivity; 3) reduced nutrient removal; 4) increased colony size, number of cells in colonies and formation of protective spines in microalgae; and 5) higher biomass settleability. Maintaining a dominance of colonial microalgae, operating with short retention times, and facilitating competition among zooplankton species showed greatest potential for reducing and controlling grazer populations.

Keywords: Wastewater treatment High Rate Algal Pond, Zooplankton, Grazers, Microalgae, HRAPs performance

INTRODUCTION

HRAPs for wastewater treatment

High Rate Algal Ponds (HRAPs) are used to reclaim water, nutrients and energy from organic wastes and can provide economical and efficient near tertiary-level wastewater (WW) treatment [1], [2]. HRAPs are 200-500 mm deep closed-loop, paddlewheel-mixed ponds (Figure 1), of up to a few hectares in size [3], which have a higher biomass yield when compared to conventional ponds, especially when operated with CO_2 injection as an additional carbon source and for pH control [4]. Algal biomass can be recovered by gravity settling of mainly colonial algae associated with bacterial flocs in harvest ponds, which can be used for biofuel production, fertilizer and animal feed [5], [6]. Before being discharged into the environment, the algal harvest pond effluent may be further treated in a series of maturation ponds where zooplankton graze on the remaining microalgae suspended in the water.

Figure 1 Pilot HRAPs at the Ruakura Research Centre, Hamilton, New Zealand

HRAP performance and management depend on environmental (light, temperature), operational (pH, CO_2 concentration, water depth, dissolved oxygen (DO), nutrient concentrations, hydraulic retention time (HRT)) and biological variables (parasites, fungi, grazers) [2], [7], [8], [9]. A major challenge with HRAPs is the establishment of herbivorous grazers, especially cladocerans (subphylum Crustacea; order Cladocera) and

rotifers (phylum Rotifera), which enter the ponds from the surrounding environment [10], [11]. When present at high concentrations, these taxa can severely reduce the algal biomass [12], [13], [14], and contribute to variable HRAP performance [15]. In addition, zooplankton grazing pressure can promote changes in microalgal dominance, modify size and structure of microalgal cells and colonies [16], [17], and induce structural modifications such as protective spines [18] that affect microalgal settleability [19] and the capacity of grazers to ingest them [20], [21], [22].

The HRAP environment provides selective pressures that reduce the diversity of zooplankton species able to survive [23], [24]. High nutrient concentrations, low nocturnal DO, high daily variation of temperature, free ammonia toxicity (when pH >10), short HRT, fast flowing water and large diameter (50-200 µm) colonial algae typically found in HRAPs, inhibit the development and feeding activity of most zooplankton species [25], [18], [26], [7], [1], [2], [27], [28], [29], [30]. In contrast, high food concentrations and the lack of predators allow for the development of large populations of those zooplankton species able to withstand the HRAP environment, especially when pH is controlled with CO_2 addition to pH ~8, providing favourable growth conditions [26], [31], [32]. The management of zooplankton in HRAPs is required both for consistent WW performance and algal productivity [33]. However, to develop appropriate control strategies it is necessary to understand the seasonal dynamics of zooplankton populations in HRAPs and the mutual interaction between grazers and phytoplankton, including the effect of grazing events on microalgal dominance and structural modifications. Published literature lacks a long term assessment of zooplankton in WW HRAPs, particularly those using CO_2 addition. In this study, we examined the physicochemical and biological parameters of two replicate WW HRAPs with CO_2 addition over 14 months. In particular, we focused on the response of the zooplankton community, and the biotic interactions between grazers and microalgae, in terms of HRAP performance (primarily concentration of total organic biomass (as volatile suspended solids, VSS) and of microalgae (as chlorophyll-*a*, Chl-*a*). We conclude with recommendations and provide a general strategy for zooplankton management in HRAP environments.

MATERIAL AND METHODS

Operation of pilot-scale high rate algal pond

Two identical pilot-scale WW HRAPs (West and East) were located at the Ruakura Research Centre, Hamilton, New Zealand (37°46'29.5"S - 175°18'45.4"E). Each HRAP consisted of a single-loop raceway separated by a central baffle, lined with black high-density polyethylene (HDPE) plastic, with semi-circular ends, a depth of 300 mm and volume of 8 m^3. Each pond was circulated at an average surface velocity of 0.15 m/s using a 1 m wide, steel paddlewheel with 8 blades. The HRAPs received 1 m^3/d of settled domestic WW collected from the main WW pump station at the Ruakura Research Centre, which was added at hourly intervals. In winter 1 m^3 of settled WW was added to each HRAP daily to give a HRT of 8 days. In spring/autumn and summer the HRT was reduced to 5 and 4 days, respectively, due to higher algal growth, by dilution of the influent with de-chlorinated tap water to simulate recirculation of treated effluent from which the algae had been harvested [34]. CO_2 was automatically added to the HRAPs as an additional carbon source, and to control the pH to a maximum of 8. The CO_2 was stored in CO_2 gas cylinders (BOC Gas Ltd, New Zealand), equipped with gas regulators and flow meters (0-12 L/min range). The pond water pH was measured every five seconds with a pH probe and when the pH exceeded 8, CO_2 was bubbled into the ponds (2 L/min), using gas diffusers placed on the bottom of HRAP downstream of the paddlewheel, until the pH was reduced to 7.8. The pH probes were calibrated monthly with pH standard solutions. The effluent flowed by gravity from a drainage pipe located on the bottom of the HRAPs into 250 L settling tanks where the biomass suspended in the culture was settled and removed from the tank bottom daily using a peristaltic pump (Masterflex, Cole-Parmer, HV-07523-60, Chicago, USA). The supernatant flowed from the settling tank into a cascade of four maturation ponds where the resident zooplankton community consumed the remaining microalgae. At the beginning of the monitoring period, the two HRAPs were emptied, carefully cleaned, their sediments removed including zooplankton resting eggs, and

inoculated with the same assemblage of naturally occurring algae that had established prior to cleaning.

Sampling protocol and environmental and physicochemical analyses

The HRAPs were sampled weekly in the morning (09:00h) over 14 months. The suspended biota (primarily rotifers, cladocerans, and microalgae) were sampled from the pond water from three areas with high mixing (Figure 2), using a PVC cylinder (diameter of 110 mm, length of 600 mm, volume 2.5 L). The cylinder was placed vertically into the water column, capped (firstly the top-end, and then the bottom-end), and the three samples were then combined into a single sample (~4 L) that was analysed on the same day. Active and diapausing benthic biota (resting eggs, copepods, and ostracods) were collected from three areas with water eddies, low mixing (<0.1 m/s water velocity) and high sedimentation [35], that were identified by visual inspection. Sediment was accumulated over 1 week in 100 ml plastic cylindrical beakers with open tops ($\varnothing$ 60 mm) that were held in position on the bottom of the HRAP using laboratory stands and clamps (Figure 2). Daily solar radiation, evaporation and rainfall were downloaded from the NIWA National Climate Database (http://cliflo.niwa.co.nz/). Pond environmental conditions (pH, and temperature) were continually measured using a Datasonde 4a (Hydrolab, HACH Environment, CO, USA). The data were logged at 15 min intervals using a data logger (CR10X, Campbell Scientific Inc., UT, USA). Samples for dissolved nutrients were filtered through Whatman GF/F filters (nominal pore size 0.7 µm) and concentrations of ammonium ($N\text{-}NH_4^+$), nitrate and dissolved reactive phosphorus (DRP) were determined using standard methods [36]. The tap water used to achieve a HRT <8 days was tested for nitrates to ensure that it was not a significant source of nitrates for the HRAPs.
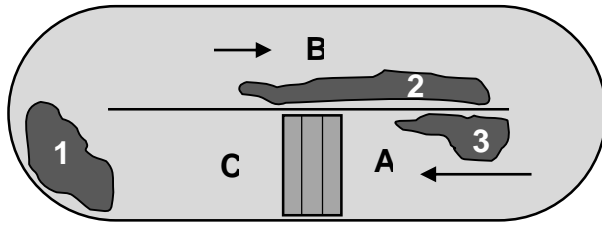
Figure 2 Suspended biota (high mixing) sampling areas (A,B,C) and sediment accumulation (low mixing) sampling areas (1,2,3)

Biomass measurements and settleability

To determine the weight of total suspended solids (TSS), a known volume of HRAP water was filtered through pre-rinsed, pre-combusted (450 °C for 4 h), and pre-weighed 47 mm Whatman GF/F filters, oven dried (85 °C) overnight using a drying chamber (270M Digital Series, Contherm), and weighed on an analytical scale (SI-234, Denver Instrument). The filters were then combusted at 450 °C for 1 h using a muffle furnace (F.E.Kiln, RTC1000, Bartlett Instrument Company, IA, USA), and weighed again to assess the ash weight. Total organic matter (or volatile suspended solids: VSS) was calculated as the difference between TSS and ash concentration [37]. Biomass (mainly microalgae and bacteria) productivity was calculated based on the VSS concentration, taking into account rainfall and evaporation [38].

Samples (10 ml) for chlorophyll-*a* analysis were filtered onto 25 mm Whatman GF/F filters and the chlorophyll-*a* extracted in 100 % methanol at 65 °C for 5 min, followed by 12 h at 4 °C in the dark. Samples were then centrifuged using a Sorvall/Dupont General Centrifuge GLC-2B at 3,000 rpm (RCF: ~1720 g) for 15 min and the absorbance of the supernatant was measured using a UV-Visible "Shimadzu UV 1601" spectrophotometer. Chl-*a* concentrations were calculated using the modified trichromatic equations for methanol [39]. Biomass settleability was estimated by settling 1 L of HRAP liquid in an Imhoff cone over a 1 h period [36], and the ratio TSS (g)/settled material (ml) was calculated.

Algal identification, relative abundance and average Maximum Cross-sectional Area

Algal species composition was determined weekly. Subsamples of HRAP water (1 ml) were settled in a 25 mm diameter Utermöhl chamber, and nine photographs of the HRAP algae were randomly taken using a Leica DM 2500 microscope (100x - field of view 1 mm), equipped with a digital Leica DFC 420 camera (Leica Microsystem, Switzerland), and the Leica Application Suite software (LAS version 4.1.0). Microalgae were identified to species level, where possible, according to taxonomic descriptions [40] and for each photograph the number of microalgal cells or colonies and the number of cells in each colony were counted. This methodology identified all the microalgae species with surface area ≥5 μm^2. The smallest species encountered (round unicellular species, and *Monoraphidium* sp.) have an average surface area >15 μm^2.

The relative abundances of microalgae were calculated by multiplying the average biovolume of each microalgal species by the total number of cell/colonies counted in all the pictures. The average biovolume for each microalgal species was calculated using 150 cells or colonies from samples collected throughout the entire monitoring period (Table 1) using the equations proposed by Vadrucci et al. [41]. The average number of colony cells (8) and the average thickness of a single cell (3 µm) of *Actinastrum* sp. were calculated by measuring 20 cells from samples collected throughout the entire monitoring period. The thickness of *Pediastrum* sp. colonies was assumed to be equal to the diameter of a single cell of the colony [42], and was calculated by dividing the diameter of the colonies by the number of cells aligned across the diameter (4-5-7-10 cells for colonies composed of 8-16-32-64 cells, respectively). As *Desmodesmus* sp. and *Scenedesmus* sp. cells are mostly circular [43], the thicknesses of colonies were assumed to be equal to the thickness of a single cell and were calculated by dividing the maximum length of a colony by the number of cells composing the colony (2-4-8).

Changes in microalgal species composition as well as changes in the shape and surface area of the cells/colonies of particular microalgal species occurred throughout the entire experimental period both affecting and

resulting from zooplankton grazing and species changes. To assess this variability, the average Maximum Cross-Sectional Area (MCSA) (μm^2) of the suspended microalgal particles included in the pictures taken from the samples of each monitoring was assessed weekly (excluding all particles $<5\ \mu m^2$ and any non-algal particles), using the freeware software "ImageJ" V 1.43u. MCSA is a term introduced to describe the average surface area of particles measured in their largest cross section, which is used to quantify the size of colonies and flocs, and thus to examine their growth or formation over time. Use of the MCSA measurement was based on our observation that as microalgae settled in the Utermöhl chamber they generally presented their largest sectional area downwards. Seasonal changes in the average MCSA of a microalgae species might indicate that its colony or cell size changes in response to environmental stimuli.

Table 1 Average (n: 150) colony or cell biovolume, major length (L) and minor length (l) of the microalgal species identified and measured over the entire monitoring period. The numbers after the microalgal species indicate the number of cells per colony. Large values of standard deviation indicate that microalgal species such as *Pediastrum* sp., *Micractinium* sp. and *Mucidosphaerium* sp., exhibited high size variability during the monitoring period.

	PEDIASTRUM SP. 8	*PEDIASTRUM SP. 16*	*PEDIASTRUM SP. 32*	*PEDIASTRUM SP. 64*	*SCENEDESMUS SP. 2*	*SCENEDESMUS SP. 4*	*SCENEDESMUS SP. 8*	*DESMODESMUS SP. 2*	*DESMODESMUS SP. 4*	*DESMODESMUS SP. 8*	*ANKISTRODESMUS SP.*	*MICRACTINIUM SP.*	*MUCIDOSPHAERIUM SP.*	*ROUND UNICELLULAR*	*ACTINASTRUM SP.*	*COELASTRUM SP.*	*STAURONEIS SP.*	*STAUROSIRA SP.*	*MONORAPHIDIUM SP.*	*CLOSTERIUM SP.*
L (µM)	25	36	41	89	8	13	36	21	22	39	37	50	52	5	13	13	26	85	12	102
L (µM)	21	32	35	73	7	8	10	14	17	13	5	32	44	4	10	12	5	11.0	2.4	9
BIOVOLUME (µM³)	2698	9550	9642	54138	223	382	1749	1659	2376	2801	302	82026	72330	59	110	1257	195	10067	16	2000
STDV	1785	12694	16378	63012	205	294	972	801	1706	2132	363	174011	60001	57	27	991	140	11954	6	562

Zooplankton and resting egg identification and enumeration

Subsamples (100 ml) of HRAP water were bubbled with pure CO_2 to asphyxiate all zooplankton and species were enumerated from triplicate 5 ml aliquots in a gridded counting chamber using a Leica M50 stereo microscope. Zooplankton were identified using available taxonomic guides [44], [45]. The volume of HRAP sediment that accumulated in the three 100

ml sediment collection beakers was quantified using graduated cylinders after 12 h settling at ~4 °C, and averaged. Sediments from each HRAP were then combined and the cladoceran diapausing eggs, copepods and ostracods were separated from the microalgae and detritus using a 125 µm mesh filter. The filtered particles were re-suspended in 300 ml of water and triplicate 5 ml aliquots were collected after mixing, and counted using the same methodology used for suspended zooplankton.

Spearman's rank correlations were used to statistically analyse the relationships between environmental variables, zooplankton density, and HRAP performance. Comparisons were made both using data from the whole experimental period (n: 63) and only for seasonal averages (n: 6). The p-values used for significance were p=0.01 (high statistical significance) and p=0.1 (moderate statistical significance).

RESULTS

Zooplankton occurrence

Zooplankton consisted of eight taxa which occurred in similar densities for both HRAPs averaged over the study period (Table 2). *Moina tenuicornis* was the only cladoceran recorded in the HRAPs, while rotifer species establishing large populations included *Brachionus calyciflorus*, *Cephalodella catellina* and *Filinia longiseta*. Bdelloid rotifers (rotifers of the subclass Bdelloidea) occurred at very low abundances during warmer months (November-February), and the colonial rotifer *Conochilus unicornis* occurred sporadically in winter (July) following sharp decreases (<60 mg/L of VSS) of biomass concentration in the HRAPs. Likewise, ciliates such as *Vorticella* sp. and *Paramecium* sp. occurred only after decreases in HRAP biomass concentration, were never dominant, and remained only a few days. Copepod and ostracod populations were dominated by the cyclopoid copepod *Paracyclops fimbriatus*, and the ostracod *Heterocypris incongruens*.

Table 2 Seasonal zooplankton abundance and *M. tenuicornis* resting eggs in West (W) and East (E) HRAPs. Values are averages of individuals per Litre, and include standard deviations in brackets. Winter 2013 (n: 6), spring 2013 (n: 13), summer 2013-14 (n: 13), autumn 2014 (n: 13), winter 2014 (n: 13), spring 2014 (n: 4). Concentrations of *M. tenuicornis*, *B. calyciflorus*, *C. catellina*, *F. longiseta* and bdelloid rotifers are expressed as organisms/L of water. Concentrations of *Moina* resting eggs, *P. fimbriatus*, and *H. incongruens* are given as organisms/L of sediment. A lack of a species during a season is indicated with "-"

		Winter 2013	Spring 2013	Summer 2013-14	Autumn 2014	Winter 2014	Spring 2014	Experiment Average (14 months)
Period		*30/07 - 03/09*	*03/09 - 03/12*	*03/12 - 04/03*	*04/03 - 03/06*	*03/06 - 02/09*	*02/09 - 30/09*	
Cladocera, *M.tenuicornis* (ind./L)	W	1017 (1135)	284 (480)	10 (36)	36 (74)	359 (391)	2167 (2027)	392 (884)
	E	998 (1068)	221 (435)	700 (1022)	615 (873)	331 (383)	1220 (331)	501 (712)
Rotifer, *B. calyciflorus* (ind./L)	W	1267 (1241)	15891 (10407)	1343 (2544)	51 (87)	21 (52)	2167 (2027)	3954 (8040)
	E	489 (465)	12636 (9241)	995 (1238)	351 (1104)	1941 (687)	12293 (16064)	4088 (8087)
Rotifer, *C. catellina* (ind./L)	W	-	36924 (61844)	44581 (93840)	100 (147)	-	-	17850 (55021)
	E	-	75429 (132953)	13738 (14704)	385 (556)	-	-	19583 (67889)
Rotifer, *F. Longiseta* (ind./L)	W	-	9463 (15194)	10648 (11867)	2656 (2904)	3 (9)	1007 (1627)	5018 (9976)
	E	-	4115 (11848)	8081 (8059)	4059 (5254)	-	-	3492 (7544)
Rotifer, Bdelloid sp. (ind./L)	W	-	1 (4)	491 (759)	44 (99)	26 (93)	-	122 (402)
	E	-	87 (170)	700 (1022)	31 (66)	15 (38)	-	182 (548)
Ostracod, *H. incongruens* (ind./L sediment)	W	744 (457)	952 (1479)	27952 (46356)	175646 (108852)	97749 (90730)	179453 (204021)	74154 (110383)
	E	1344 (1170)	1767 (1985)	16129 (15425)	108308 (83633)	30036 (20617)	40973 (35643)	34469 (55552)
Copepod, *P. fimbriatus* (ind./L sediment)	W	700 (809)	4214 (6935)	1114 (1214)	67 (109)	-	-	1245 (3603)
	E	144 (236)	4214 (6935)	1057 (860)	5 (19)	-	-	862 (1850)
Eggs *Moina* (ind./L sediment)	W	78 (129)	124 (58)	286 (257)	1103 (853)	3528 (5753)	5347 (1861)	1356 (3047)
	E	611 (394)	1152 (595)	23238 (24722)	35697 (14131)	41702 (26122)	31373 (28422)	23434 (24226)

In both HRAPs, rotifers had large populations during spring and summer (September-December) and *M. tenuicornis* had large populations throughout the year excepting November-March (Figure 3). Generally, at lower water temperatures zooplankton required a higher HRT (8 days) to establish populations. For example, *M. tenuicornis* established long lasting (two months) populations with high densities (up to 5,600 individuals/L) when HRT was 8 days and the average water temperature was ~13 °C (September 2013-14). Conversely, when the average water temperature was >18 °C, *M. tenuicornis* established short lasting (one month) large

populations (up to 3,400 individuals/L) when HRT was only 5 days (late December, April). Following *M. tenuicornis* establishment in the HRAPs, their resting eggs were constantly present in the sediment, reaching densities up to 100,000 individuals/L of sediment (Figure 3). The abundance of resting eggs was higher in the East HRAP than in the West HRAP, and generally increased following *M. tenuicornis* blooms. The large (200-300 μm in length) *B. calyciflorus* established long lasting (one to four months) populations with high densities (up to ~35,000 individuals/L) when HRT was 8 days and the average water temperature was >15 °C (September-November 2013, and September 2014). When HRT was 5 days, *B. calyciflorus* maintained moderate (<9,000 individuals/L) densities only when the average water temperature was >20 °C (late December-January, West and East, and mid-April, East) (Figure 3). Sudden but relatively short, one to two month, blooms of the small (50-90 μm length) *C. catellina* reached very high densities (between 160,000 and 350,000 individuals/L) and occurred in November-December, when the average water temperature was >20 °C, and HRT was 5 days (Figure 3). *F. longiseta* (~100 μm in length) bloomed during summer-early autumn (December-April), and densities up to 45,000 individuals/L occurred in December when the average water temperature was >20 °C, and HRT was 5 days. In the period from 22 November to 10 December, the HRT was reduced from 5 to 4 days, at which time the densities of all rotifer species rapidly decreased. The populations partially recovered when the HRT was increased to 5 days, although in much lower densities compared to the period with HRT of 8 days. When the average water temperature was <20 °C and HRT still 5 days, all the rotifer species were absent, excluding *F. longiseta* which persisted into May, when the average water temperature was >15 °C (Figure 3).
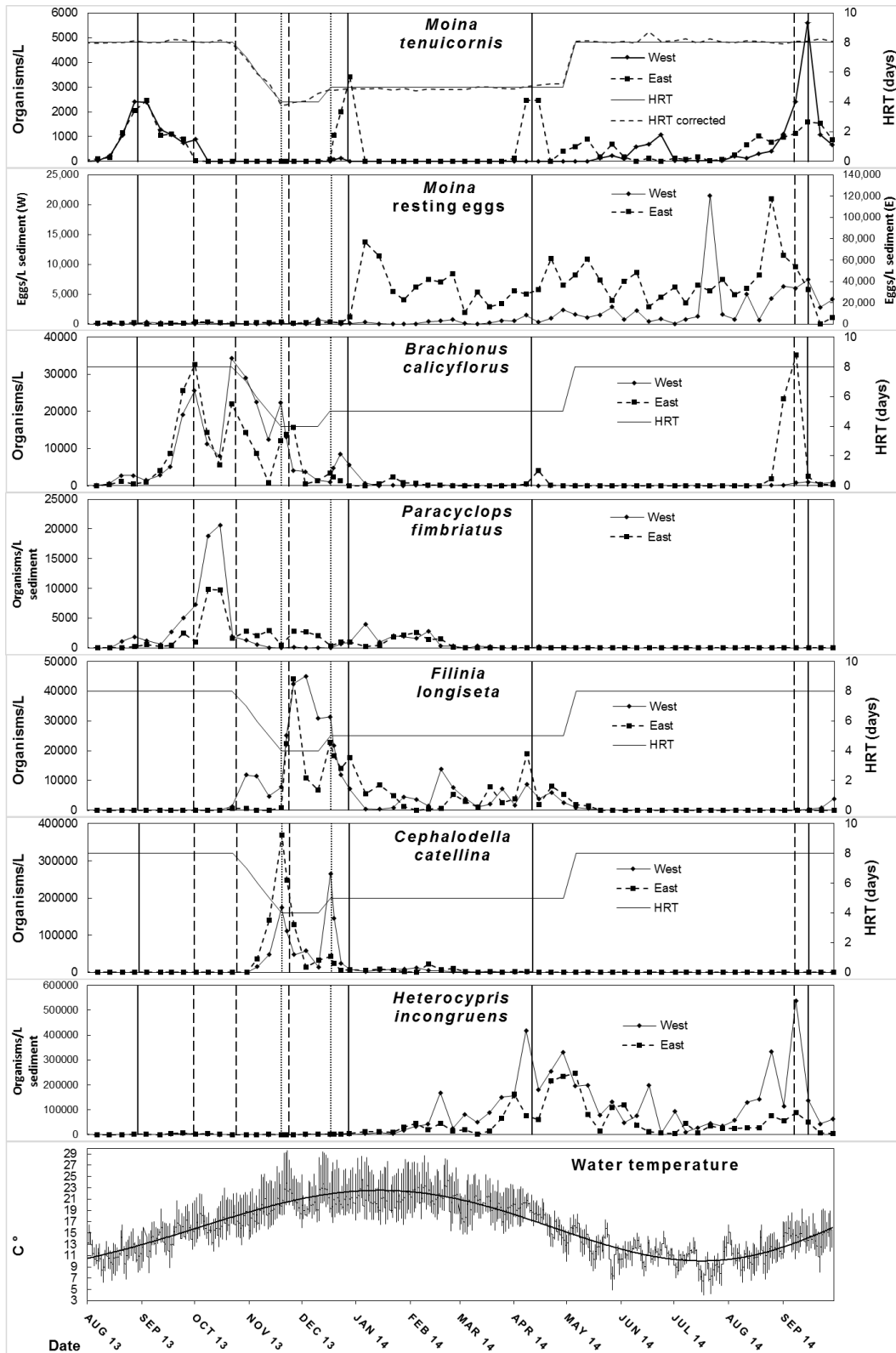
Figure 3 West and East HRAP zooplankton species density, *M. tenuicornis* resting egg density in the sediments, and pond water temperature. The HRT is plotted together with *M. tenuicornis, B. calyciflorus, C. catellina* and *F. longiseta* abundances. Resting eggs of West and East HRAPs are plotted on vertical axes with different scales due to the large variance between concentrations in the two HRAPs. Concentrations of *M. tenuicornis, B. calyciflorus, C. catellina* and *F. longiseta* are in organisms/L of water. Concentrations of *Moina* resting

During spring and summer (September 2013 to February) the copepod *P. fimbriatus* established in the sediments of both HRAPs (up to 20,000 individuals/L of sediment) with highest densities around mid-October and none were recorded in March (Figure 3). After six months of operation (late January), the ostracod *H. incongruens* established in the HRAP sediment, reaching densities of up to 500,000 individuals/L of sediment and persisting until the end of the monitoring period. Ostracod densities were higher during autumn and spring than in winter (July 2014; Figure 3).

Table 3 Dimension (length), preferred food, feeding mode and ingestible food particle size of four zooplankton groups and two species found in HRAPs. [26][1], [46][2], [47][3], [48][4], [49][5], [45][6], [50][7], [51][8], [52][9], [53][10], [54][11].

Taxon	Taxon size range (mm)	Preferred food	Mode of feeding	Ingestible food particle size range (µm)
Rotifera [5,7,8]	L (0.2-0.6)	Bacteria, algae and protozoa	Filter feeding via coronal cilia	1-20
Cladocera [3,8]	L (0.3-3)	Bacteria, algae, protozoa, ciliates, rotifers and tiny crustaceans	Filter feeding via thoracic appendages	1-50
Copepoda [1,5,9,8,9,10,11]	L (0.5-5)	Bacteria. algae, ciliates, small rotifers	Filter feeding/raptorial	<60
Ostracoda [6,8]	L (3-5)	Organic detritus, bacteria, protozoa, algae and filamentous algae, plant material and dead animals	Filter feeding via thoracic appendages	5-100
Brachionus spp. (rotifer) [2,3,5,7]	L (0.10-0.34)	Bacteria, algae and protozoa	Filter feeding via coronal cilia	1<10
Moina spp. (cladoceran) [1,3,4,5]	L (0.6-1.5)	Bacteria, algae, ciliates, protozoans, rotifers	Filter feeding via thoracic appendages	<60

Grazer inhibition via direct and indirect competition

Some grazer species that established in HRAPs were associated with a reduction in densities or complete absence of other grazer species. The presence of *M. tenuicornis* (>1,500 individuals/L) was always associated with an absence or declining densities (if already established) of other planktonic grazers (the rotifers *B. calyciflorus, C. catellina,* and *F. longiseta*) (Figure 3). Large populations of the rotifer *F. longiseta* established only

when other rotifer species were absent or had very low densities (Figure 3). Large population densities (>100,000 individuals/L of sediment) of the ostracod *H. incongruens* were always associated with an absence or very low densities of rotifers, and the establishment of the copepod *P. fimbriatus* in the sediments was associated with an absence or decline of all other grazers. In particular, maximum densities of *P. fimbriatus* (mid-October) (Figure 3) corresponded with sharp declines in *B. calyciflorus* populations in both HRAPs. Once the ostracod *H. incongruens* established in the HRAP sediment, *P. fimbriatus* was absent in both HRAPs (February), and *B. calyciflorus* was absent from the West HRAP, which had a higher (>100,000 individuals/L of sediment) population of the ostracod relative to the East HRAP (September, 2014) (Figure 3).

HRAP performance

During the 14 months of monitoring, the West and East HRAPs had similar average performances (Table 5). During warmer months (October-March), HRAP productivity, VSS and Chl-*a* were higher and fluctuated widely. During colder months (August-September 2013, and April-September 2014), HRAP productivity, VSS and Chl-*a* were lower, and changes occurred gradually and with moderate fluctuations (Figure 4).
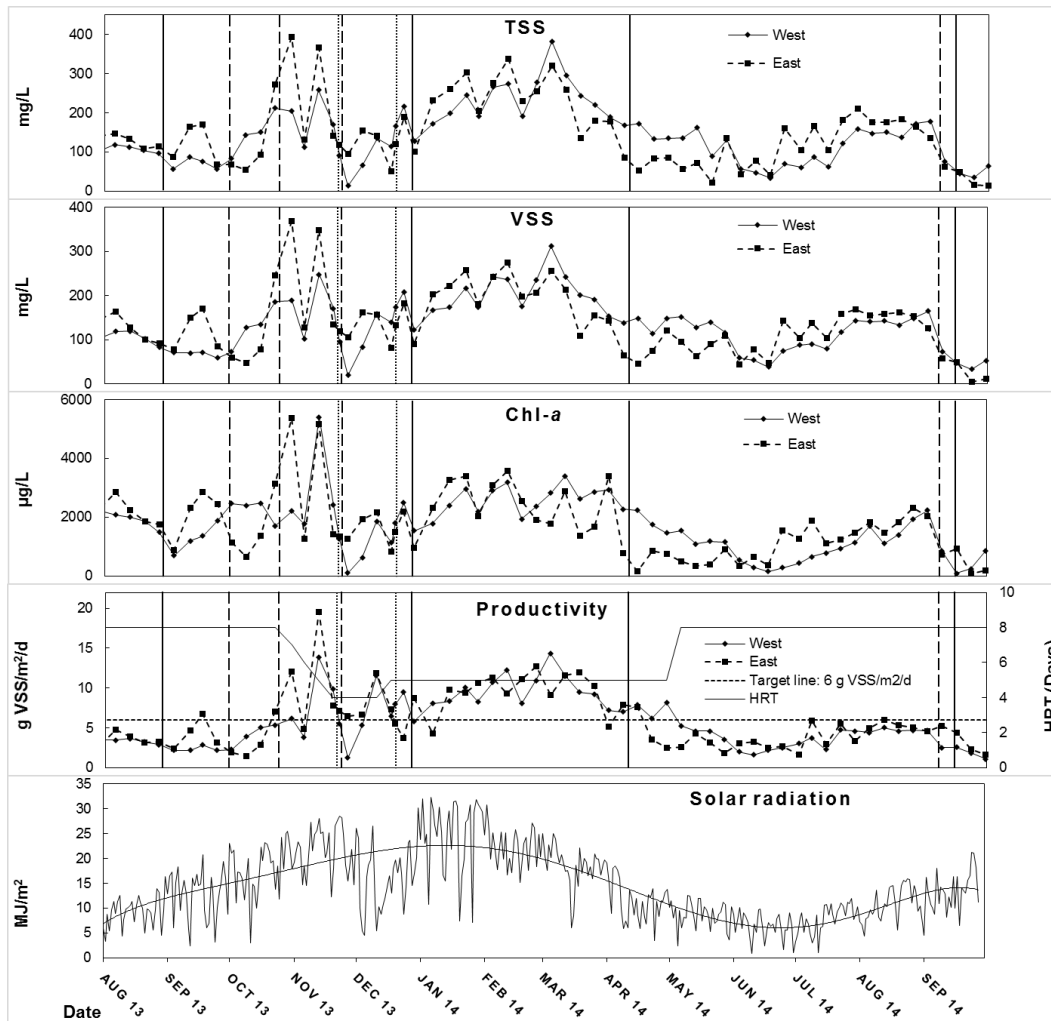
Figure 4 Variation of total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll-*a* (Chl-*a*), and productivity of West and East HRAPs. The target line in the productivity chart indicates a productivity of 6 g VSS/m²/d. Vertical lines show the main population peaks of zooplankton species associated with productivity reduction and changes of microalgal relative abundance. Full line, *M. tenuicornis***, large dotted line,** *B. calyciflorus***, small dotted line,** *C. catellina***. To identify the HRAP (West or East) where the bloom occurred, refer to Figure 3.**

The average productivities of the West and East HRAPs were 5.69 g VSS/m²/d, and 5.99 g VSS/m²/d, respectively, and productivity correlated positively with daily solar radiation (Table 4 and Table 5). The density of planktonic grazers showed a weak positive correlation with the productivity of HRAPs, although was not significantly correlated with VSS or Chl-*a* (Table 4) However, peaks in zooplankton density were generally followed (one week) by reductions in TSS, VSS, Chl-*a*, and productivity (Figure 4). One exception was the very low biomass and productivity measured during

the period 27 May-17 June, which was a consequence of low concentrations of $N-NH_4^+$ and $P-PO_4^{3-}$ in the inflow due to excessive dilution of WW with storm water. When densities of *M. tenuicornis*, *B. calyciflorus* and *C. catellina* were greater than ~1,500 individuals/L, ~15,000 individuals/L and ~50,000 individuals/L respectively, HRAP productivity showed large reductions (between ~35% and ~90%) that generally lasted between two and five weeks (Figure 3 and Figure 4). When microalgal biomass (Chl-*a*) reached its lowest concentrations, recovery to pre-bloom levels required one to three weeks, and was faster during warmer periods (October-April) (Chl-*a* concentrations, Figure 4). Productivity reductions in the West HRAP coincided with high densities of *M. tenuicornis* (September 2013, ~50% reduction), *B. calyciflorus* (October, ~35% reduction), *C. catellina* (November, ~93% reduction, and December, ~50% reduction), and *M. tenuicornis* (September 2014, ~80% reduction). Productivity reductions in the East HRAP coincided with high densities of *M. tenuicornis* (September 2013, ~50% reduction), *B. calyciflorus* (October, ~85% reduction, and November, ~60% reduction), *C. catellina* (November, ~70% reduction), *M. tenuicornis* (December, ~55% reduction, and April, ~50% reduction), and *B. calyciflorus* (September 2014, ~60% reduction). Generally, minimum productivity periods were followed by rapid (one to two weeks) reductions in grazer densities.

Table 4 Spearman's rank correlation coefficients (r_s) between variables in the West (W) and East (E) HRAPs. Correlations have been calculated using weekly data (n: 63), excluding those between VSS and nutrient removal, which have been calculated using seasonal averages (n: 6) to eliminate the very high or low peaks promoted by rapid changes of nutrients in WW inflow. Positive r_s values indicate a positive correlation between variables, negative r_s values indicate a negative correlation between variables, * indicates a p<0.01 with high statistical significance, ** indicates a p<0.1 with moderate statistical significance, * indicates a p>0.1 without statistical significance.**

		Productivity	VSS	Chl-a	MCSA	N-NH$_4^+$ removal	P-PO$_4^{3-}$ removal	Pediastrum cells per colony	Scenedesmus cells per colony	Desmodesmus cells per colony
VSS	W	0.9*	-	-	-	0.9**	0.9**	-	-	-
	E	0.6*	-	-	-	0.7**	0.7**	-	-	-
Planktonic grazers	W	0.4*	0.3**	0.3**	0.5*	-	-	0.7*	-0.1***	0.4*
	E	0.4*	0.1***	0.2***	0.5*	-	-	0.6*	0.0***	0.6*
Chl-a	W	0.7*	0.8*	-	-	-	-	-	-	-
	E	0.5*	0.9*	-	-	-	-	-	-	-
Solar radiation	W	0.6*	06*	0.6*	-	-	-	-	-	-
	E	0.6*	0.5*	0.5*	-	-	-	-	-	-
B. calyciflorus	W	-	-	-	0.7*	-	-	-	-	-
	E	-	-	-	0.6*	-	-	-	-	-

Settling of microalgae and the average amount of sediment accumulated on the pond bottom were highest during the warm period (October-March) when VSS was the highest (Table 5), rotifers were abundant (Table 2), and large and highly settling microalgae such as *Micractinium* sp. and especially *Pediastrum* sp. dominated the HRAPs (Table 5 and Figure 6).

Table 5 Seasonal biomass (TSS and VSS), chlorophyll-*a*, productivity, MCSA, settleability, and average sediment accumulated over 7 days in three beakers of West (W), East (E) HRAPs, and wastewater (WW) inflow. Values are averages and include standard deviations in brackets. Winter 2013 (n: 6), spring 1013 (n: 13), summer 2013-14 (n: 13), autumn 2014 (n: 13), winter 2014 (n: 13), spring 2014 (n: 4).

		Winter 2013	Spring 2013	Summer 2013-14	Autumn 2014	Winter 2014	Spring 2014	Experiment Average (14 months)
Period		*30/07 - 3/09*	*03/09 - 03/12*	*03/12- 04/03*	*04/03 - 03/06*	*03/06 - 02/09*	*02/09 - 30/09*	
TSS (mg/L)	WW	39 (18)	28 (13)	53 (47)	60 (30)	85 (38)	57 (15)	54 (37)
VSS (mg/L)	WW	40 (19)	28 (13)	57 (35)	67 (37)	87 (32)	82 (29)	59 (36)
TSS (mg/L)	W	99 (22)	129 (70)	208 (76)	164 (64)	110 (51)	56 (18)	128 (53)
	E	122 (22)	166 (107)	211 (94)	107 (68)	145 (49)	36 (24)	131 (59)
VSS (mg/L)	W	99 (20)	122 (64)	193 (52)	149 (45)	109 (40)	52 (17)	121 (48)
	E	118 (34)	158 (99)	188 (65)	102 (48)	131 (37)	31 (26)	122 (54)
Chlorophyll *a* (µg/L)	W	1720 (562)	2032 (1216)	2329 (658)	1928 (862)	1003 (664)	860 (842)	1713 (965)
	E	1993 (670)	2274 (1454)	2207 (925)	1106 (1004)	1467 (546)	796 (780)	1720 (1105)
Productivity (g/m²/d)	W	3.1 (0.6)	4.9 (3.5)	9.3 (2.6)	7.2 (3.3)	3.6 (1.2)	2.6 (1.3)	5.7 (3.4)
	E	3.5 (0.9)	6.3 (4.8)	9.0 (2.7)	6.1 (3.6)	4.1 (1.4)	3.6 (1.6)	6.0 (3.7)
MCSA (µm²)	W	240.7 (78.1)	298.0 (135.7)	208.2 (113.0)	77.6 (11.9)	109.7 (17.5)	126.1 (46.4)	179.2 (118.8)
	E	263.9 (60.3)	335.6 (238.8)	189.5 (44.1)	144.0 (43.0)	53.3 (24.6)	137.9 (74.5)	191.1 (153.0)
Settleability (ml/g TSS)	W	0.002 (0.004)	0.02 (0.03)	0.04 (0.02)	0.01 (0.01)	0.02 (0.02)	0.01 (0.01)	0.016 (0.02)
	E	0.004 (0.06)	0.02 (0.03)	0.06 (0.03)	0.02 (0.02)	0.02 (0.01)	0.07 (0.06)	0.032 (0.03)
Settled sediment (ml)	W	45 (17)	82 (22)	85 (20)	29 (10)	61 (24)	51 (16)	62 (29)
	E	50 (12)	91 (22)	96 (16)	84 (16)	81 (23)	87 (18)	84 (22)

The removal efficiency of nutrients correlated positively with the biomass concentration (as VSS) (Table 4). Average net reduction efficiencies of $N-NH_4^+$ and $P-PO_4^{3-}$ over 14 months ranged from 66-70% and 40-47%, respectively, and higher during the second half of spring, summer and autumn, when HRAP productivity was highest (Table 6). The nutrient content of the WW inflow showed high variability with $N-NH_4^+$, $P-PO_4^{3-}$ and NO_3-N varying in the range 0.7-54.0 mg/L, 0.3-6.2 mg/L, and 0-6.8 mg/L, respectively, while the average concentrations of $N-NH_4^+$ and $P-PO_4^{3-}$ over 14 months were 26.7 mg/L and 3.6 mg/L.

Table 6 Seasonal environmental and physicochemical parameters of West (W), East (E) HRAPs, and wastewater inflow (WW). Values are averages and include standard deviations in brackets. Winter 2013 (n: 6), spring 1013 (n: 13), summer 2013-14 (n: 13), autumn 2014 (n: 13), winter 2014 (n: 13), spring 2014 (n: 4).

		Winter 2013	Spring 2013	Summer 2013-14	Autumn 2014	Winter 2014	Spring 2014	Experiment Average (14 months)
Period		*30/07 - 3/09*	*03/09 - 03/12*	*03/12 - 04/03*	*04/03 - 03/06*	*03/06 - 02/09*	*02/09 - 30/09*	
N-NH$_4^+$ (mg/L)	WW	22.1 (7.4)	16.5 (29.8)	25.3 (13.3)	26.6 (16.4)	26.1 (10.6)	24.0 (5.9)	26.7 (17.5)
NO$_3$-N (mg/L)	WW	1.5 (1.3)	0.7 (0.7)	2.5 (1.7)	1.9 (1.7)	1.3 (0.9)	2.5 (1.1)	1.6 (1.4)
P-PO$_4^{3-}$ (mg/L)	WW	3.1 (0.9)	4.0 (2.3)	4.1 (1.7)	3.1 (2.0)	3.6 (1.4)	3.2 (0.9)	3.6 (1.8)
Solar radiation (MJ/m^2)	-	9.9 (3.4)	17.6 (6.2)	21.0 (7.1)	12.5 (5.4)	8.5 (3.5)	13.6 (3.8)	14.4 (7.2)
Pond temperature (°C)	W	11.8 (1.8)	17.7 (3.5)	21.6 (2.8)	16.4 (3.9)	10.9 (2.1)	14.5 (2.0)	16.7 (5.0)
	E	11.6 (1.8)	17.7 (3.6)	21.6 (7.1)	16.7 (3.8)	11.4 (2.1)	14.2 (2.0)	16.9 (4.9)
pH	W	7.6 (0.4)	7.2 (0.5)	7.2 (0.6)	7.0 (0.6)	7.3 (0.5)	6.9 (0.5)	7.17 (0.6)
	E	7.4 (0.7)	7.2 (0.5)	7.2 (0.5)	7.2 (0.5)	7.4 (0.4)	6.8 (0.4)	7.24 (0.5)
N-NH$_4^+$ effluent (mg/L) + [% net reduction]	W	12 (4) [43%]	7 (8) [74%]	3 (3) [80%]	6 (5) [76%]	13 (7) [60%]	10 (5) [59%]	8 (7) [66%]
	E	10 (3) [52%]	5 (4) [78%]	2 (2) [80%]	8 (7) [67%]	13 (7) [58%]	5 (2) [54%]	7 (6) [70%]
P-PO$_4^{3-}$ effluent (mg/L) + [% net reduction]	W	2 (1) [28%]	2 (1) [30%]	1 (1) [57%]	1 (1) [50%]	3 (1) [31%]	3 (1) [17%]	3 (2) [40%]
	E	2 (1) [37%]	2 (1) [41%]	1 (1) [64%]	2 (1) [53%]	2 (1) [39%]	3 (1) [22%]	2 (4) [47%]
NO$_3$-N effluent (mg/L) + [% increase]	W	2 (2) [93%]	2 (1) [623%]	4 (3) [196%]	4 (2) [363%]	4 (3) [206%]	5 (2) [155%]	4 (2) [189%]
	E	2 (1) [63%]	3 (1) [424%]	5 (3) [205%]	3 (2) [247%]	3 (3) [159%]	6 (2) [163%]	2 (1) [215%]

When biomass was >~120 mg/L, the concentrations of N-NH$_4^+$ and P-PO$_4^{3-}$ in the HRAPs were always <~10 mg/L, and <~2 mg/L, respectively, and only moderately affected by increases of nutrient concentrations (up to ~50 mg/L of N-NH$_4^+$, and ~6 mg/L of P-PO$_4^{3-}$) in WW inflows (Figure 5). Conversely, when VSS was <~120 mg/L, the concentrations of N-NH$_4^+$ and P-PO$_4^{3-}$ in HRAPs had larger fluctuations, with peaks up to ~20 mg/L, and ~4 mg/L, respectively, and were more likely to increase when the nutrients concentration of WW inflow increased.
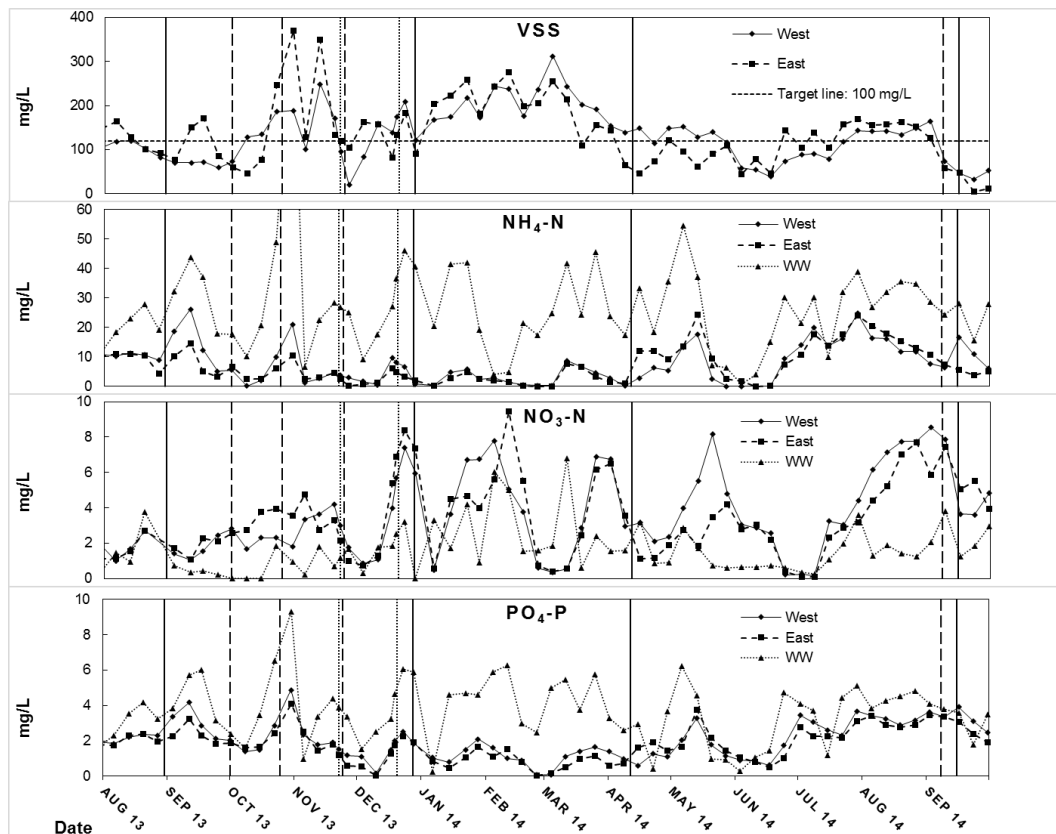
Figure 5 Comparative variation of nutrients (N-NH₄⁺, NO₃-N, and P-PO₄³⁻) in West and East HRAPs, and WW inflow over 14 months. The target line in the VSS chart indicates a VSS of 120 mg/L of VSS. This was the threshold above which the HRAPs had a good capacity to remove WW nutrients. Vertical lines show the main population peaks of zooplankton species associated with productivity reduction and changes of microalgal relative abundance. Full line, *M. tenuicornis*, large dotted line, *B. calyciflorus*, small dotted line, *C. catellina*. To identify the HRAP (West or East) where the bloom occurred, refer to Figure 3. The out of scale N-NH₄⁺ peak (late October, WW inflow) reached 126 mg/L.

Microalgal dynamics

Microalgal communities were similar in both HRAPs, and included mainly 13 taxa (Table 1) (Figure 6). In August 2013, both HRAPs were dominated by the unicellular, needle-shaped *Ankistrodesmus* sp. that was absent by the end of September 2013. The HRAPs were then dominated by colonial species such as *Micractinium* sp., *Pediastrum* sp. and *Mucidosphaerium* sp. East HRAP was temporarily dominated by smaller species such as the colonial *Desmodesmus* sp. (June, 2014) and the unicellular needle shape *Monoraphidium* sp. (July, 2014), although by September 2014, the colonial *Micractinium* sp. was dominant again.

Dominant microalgal species that showed sudden (<2 week) declines were the unicellular *Ankistrodesmus* sp. and *Monoraphidium* sp., and the colonial *Mucidosphaerium* sp. Declines of unicellular *Ankistrodesmus* sp. and *Monoraphidium* sp. were followed by reestablishment of colonial *Mucidosphaerium* sp., and *Micractinium* sp. (Figure 6). Declines of colonial *Mucidosphaerium* sp. and non-spined *Micractinium* sp. were followed by establishment of *Pediastrum* sp. The abundance of round unicellular microalgae was higher when filter feeders grazers did not occur in the pond water (February-July).
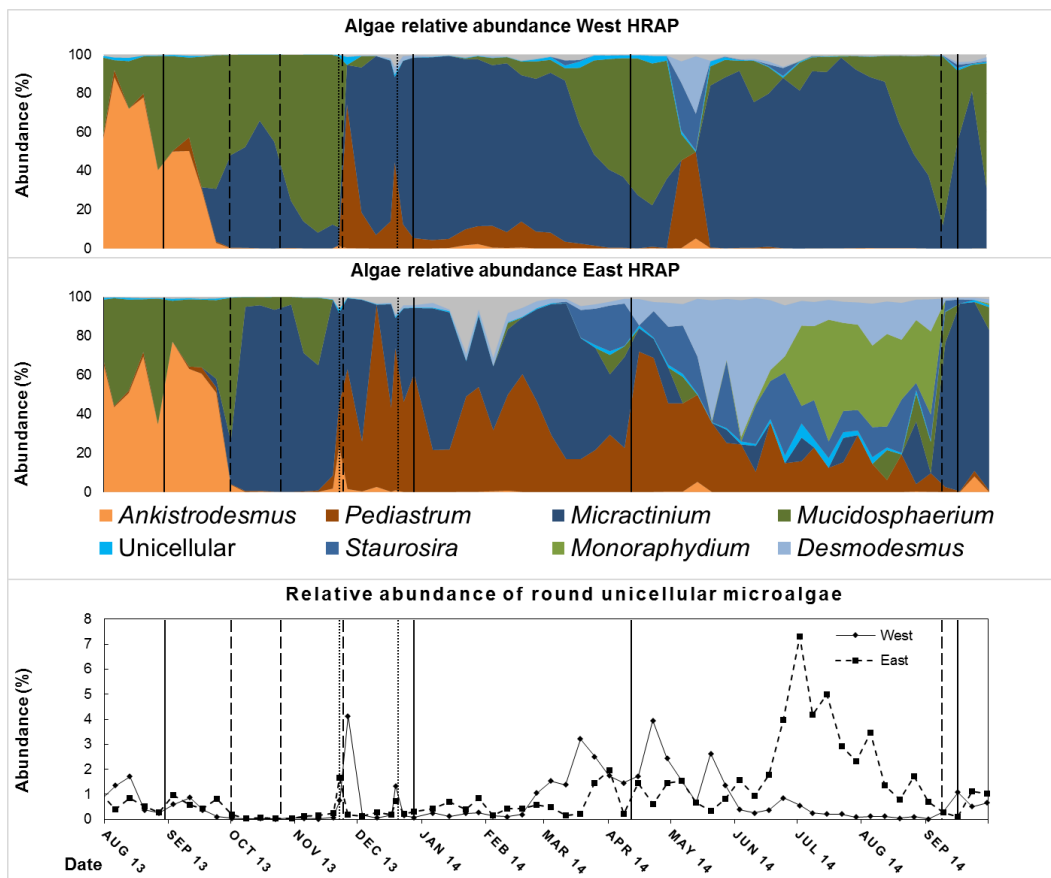
Figure 6 Comparative variation of relative abundance of the main microalgal species in West and East HRAPs over 14 months. The grey colour refers to the cumulative abundance of *Actinastrum* sp., *Coelastrum* sp., *Stauroneis* sp., and *Closterium* sp. The bottom plot includes only the relative abundance of all the small unicellular microalgae species with spherical shape. Vertical lines show the main population peaks of zooplankton species associated with productivity reduction and changes of microalgal relative abundance. Full line, *M. tenuicornis*, large dotted line, *B. calyciflorus*, small dotted line, *C. catellina*. To identify the HRAP (West or East) where the bloom occurred, refer to Figure 3.

Microalgae maximal cross sectional area (MCSA) and structural modifications induced by grazers

Microalgae MCSA correlated positively with the density of planktonic grazers (Table 4), ranging from ~50 -150 μm^2 in periods when grazers were absent up to ~200 - 600 μm^2 in periods of grazer high density (Table 5 and Figure 7). In both HRAPs, high densities of the rotifer *B. calyciflorus* (>15,000 individuals/L) were associated with a large and rapid (1-2 weeks) increase in average microalgae MCSA. Microscopic analyses showed that *Micractinium* sp. clumped into larger colonies and *Mucidosphaerium* sp. increased its colony size and density by packing individual cells more tightly (Figure 8). When *Micractinium* sp. or *Mucidosphaerium* sp. were dominant and the abundance of *B. calyciflorus* was low, the average MCSA was in the range of ~100-200 μm^2 for both microalgae species. In contrast, with high densities of *B. calyciflorus* the MCSA increased to ~200-600 μm^2 and ~200-400 μm^2, respectively (Figure 7). Conversely, following the decline in *B. calyciflorus* densities, high densities of the small rotifer *C. catellina* were associated with a rapid and short lasting reduction of the average MCSA of microalgae in both HRAPs. The West pond was dominated by *Mucidosphaerium* sp., and the average MCSA decreased from 573 μm^2 to 178 μm^2 in two weeks. The East pond was dominated by *Micractinium* sp., and the average MCSA decreased from 608 μm^2 to 243 μm^2 in one week.
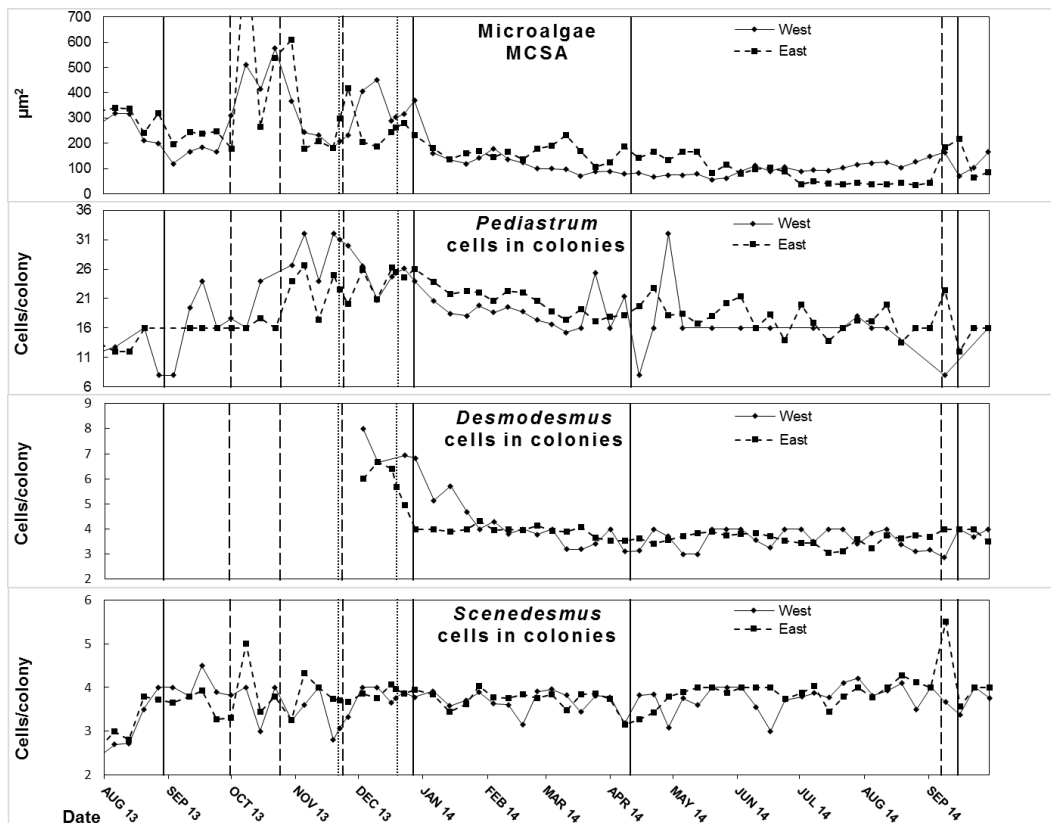
Figure 7 Microalgal maximal cross sectional area, and *Pediastrum* sp., *Desmodesmus* sp. and *Scenedesmus* sp. average colony cells. Vertical lines show the main population peaks of zooplankton species associated with productivity reduction and changes of microalgal relative abundance. Full line, *M. tenuicornis*, large dotted line, *B. calyciflorus*, small dotted line, *C. catellina*. To identify the HRAP (West or East) where the bloom occurred, refer to Figure 3. The out of scale MCSA peak (October, East HRAP) reached 1,012 µm².

Under intense grazing pressure by the rotifers *B. calyciflorus* (>15,000 individuals/L) and *C. catellina* (>300,000 individuals/L), *Micractinium* sp. generated long protective spines, and the colonial microalgae *Desmodesmus* sp. (that has small spines in absence of grazers) grew longer spines. High densities of rotifers and the cladoceran *M. tenuicornis* were also associated with the formation of long protective spines in *Pediastrum* sp. (Figure 8). Densities of planktonic grazers correlated positively with the number of cells in colonies of *Pediastrum* sp. and *Desmodesmus* sp., but not of *Scenedesmus* sp. (Table 4). Under high densities of *B. calyciflorus* (October-December) the average number of cells in *Pediastrum* sp. colonies increased to ~25-32 (West) and ~20-27 (East) (Figure 7), compared to periods of low densities of *B. calyciflorus* when it was ~17-20 (West) and ~18-22 (East). In both HRAPs, the average number

of cells per colony of *Desmodesmus* sp. increased from ~4 when grazers were absent to ~7 during periods of high grazing pressure (December) (Figure 7). In contrast, *Scenedesmus* sp., which has a similar structure but is smaller than *Desmodesmus* sp., had only minor variations in the number of colony cells. Before the establishment of zooplankton in the HRAPs, the average number of cells per colony of *Scenedesmus* sp. was ~3 (August 2013) (Figure 7), and increased to 4.5-5.5 when *B. calyciflorus* reached densities >30,000 individuals/L (September 2013-2014, October, November).
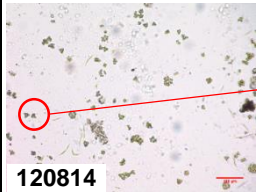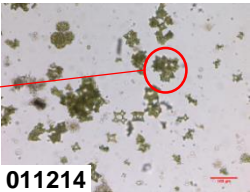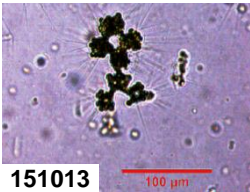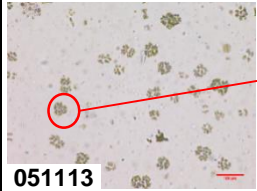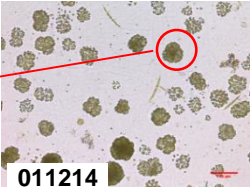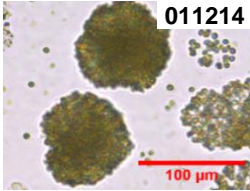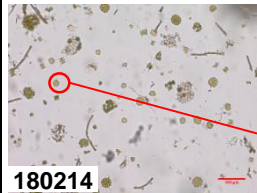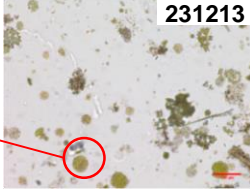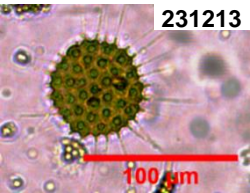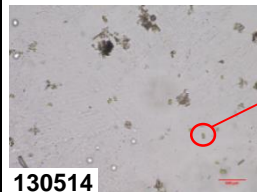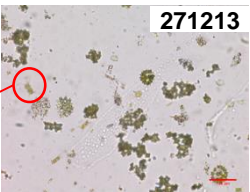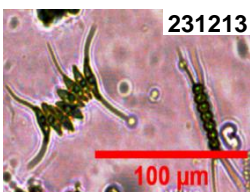
Microalgae	No grazing pressure	Grazing pressure effect on colonies	Grazing pressure effect on structure
Micractinium sp. Colonies clumping and spine generation	120814	011214	151013
Mucidosphaerium sp. Increase of colony size and density	051113	011214	011214
Pediastrum sp. Increase of colony cells and generation of spines	180214	231213	231213
Desmodesmus sp. Increase of colony cells and generation of spines	130514	271213	231213

Figure 8 Structural modifications (colony size and density, number of colony cells, and generation of spines) induced by grazing pressure on microalgae. The labels indicate the date and the HRAP from where the sample was collected (DDMMYY). Red marks show the changes in the colony of microalgae species using the same scale. Scale bar: 100 µm.

DISCUSSION

Zooplankton occurrence

Compared with natural environments, HRAP zooplankton taxa have a lower species diversity but up to three orders of magnitude higher population densities. The eight species found in the HRAPs compares with up to 100 rotifer species and 30 micro-crustacean species found in natural freshwater environments [44], [55], [56]. Typical average densities of rotifers, cladocerans and copepods in natural lakes and rivers vary between ~15-820 individuals/L, ~1-30 individuals/L, and ~7-11 individuals/L, respectively [57], [58], [59]. This contrasts with 5,000 *M. tenuicornis*/L and 380,000 rotifers/L found in the HRAP environment. Zooplankton densities in natural aquatic environments that do not have fish can be four-fold higher than those that do [60]. However, the population densities in such environments are still 2-3 orders of magnitude lower than those found in this study. The low diversity of zooplankton species established in HRAPs was likely a consequence of the selective pressures of the fast flowing water and WW environment on biota [25], [18], [7], [2]. Conversely, the very high maximum population densities of species able to survive conditions in HRAPs was likely promoted by an abundance of food, the exclusion of many predators and competitors in the WW environment, and the neutral pH optimal for grazer growth [26] that also prevented free ammonia (N-NH$_4^+$) toxicity.

The establishment of zooplankton able to survive the physical and chemical conditions of a treatment pond are influenced by the availability of food, water temperature and HRT [61], [62], [11]. Moreover, HRAPs are mixed systems and the water flow rate (~0.3 M/s) could also affect zooplankton establishment. In natural flowing waters (e.g. rivers) high flow rates generally limit the retention of zooplankton [63], [64]. However, HRAPs are closed loops, and turbulence, caused by high water flow rates, will generally favour rotifers over microcrustaceans [65]. The comparatively high algal (Chl-*a*) and bacterial biomass in the HRAPs throughout the monitoring period should provide sufficient food [66], [67], [68] for zooplankton to establish and reproduce. Hence, zooplankton establishment in HRAPs was

likely to be mainly related to the combination of water temperature and HRT, with longer HRTs increasing the number of individuals reaching reproductive maturity (at a given temperature) before being washed out of the HRAP. Canovas et al. [11] reports similar results and related the dominance of zooplankton species to different generation times and changes in water temperature in a pilot-scale HRAP (16.8 m^3, depth of 350 mm, HRT of 8 days, surface velocity of 0.15m/s, and without CO_2 addition) located in Mèze, southern France. In cooler seasons, the HRAP was dominated by protozoa with their generation being much shorter than those of metazoans (such as rotifers and cladocerans). Conversely, metazoans that have a competitive advantage over protozoans due to competition for shared food and direct predation [69], [70], tended to dominate the HRAPs during warmer periods when their generation times were sufficiently lower than the pond HRT for them to reproduce and establish populations. Accordingly, the HRT of HRAPs must be long enough for grazers to complete their reproductive cycle at a given temperature (Table 7). Species that do not reach reproductive maturity under the combination of water temperature and HRT of the HRAP will be unable to establish unless they can actively avoid the pond outflow. In very warm regions, short HRTs (2-4 days) can be used to reduce grazer abundance [25] and limit grazing of HRAP microalgae. For 12 m^2 pilot-scale HRAPs in western U.S.A., when the HRT was reduced from >4 to 2-3 days, zooplankton blooms did not occur [61].

The large cladoceran *M. tenuicornis* was probably able to establish in our HRAPs due the capacity of *Moina* species to tolerate WW conditions and low DOs [71], [23], [72]. Maximum *M. tenuicornis* densities in our HRAPs were similar to those observed in both laboratory and mass cultures [48], [68], [73], and were likely limited by overcrowding and consequent intraspecific competition [74]. *M. tenuicornis* has a longer embryonic development [33], and reproductive age relative to rotifers at all pond water temperatures (Table 7). However, it was still able to establish populations in HRAPs even when the HRT was shorter than its generation time for the pond temperature. For example they established when HRT was 8 days, even though *Moina* spp. are expected to become reproductive after >11

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days (13 °C), and when HRT was 5 days and *Moina* spp. are expected to become reproductive after ~8 days (18 °C). The densities of *M. tenuicornis* diapausing eggs in the pond sediment increased with high densities of active *M. tenuicornis* due to the production of new eggs, and decreased when *Moina* were absent, either from natural degradation [75] or being washed from the system. The rotifers *B. calyciflorus*, *C. catellina* and *F. longiseta* are all common inhabitants of WW environments [76], [62], [77]. Maximum HRAP densities of *B. calyciflorus* were lower than those achieved in mass cultures [78], [79], [80], likely a consequence of the relatively short HRT of the HRAPs and competition with other organisms such as *M. tenuicornis*, ostracods and copepods. In contrast to the cladoceran *M. tenuicornis*, rotifers required HRTs that were (~2 x) longer than their generation time to establish high densities in the HRAPs. For example, they established when HRT was 8 days and rotifers are expected to become reproductive after 3-4 days (17 °C), and when HRT was 5 days and rotifers are expected to become reproductive after ~2-3 days (20 °C).

The greater capacity of cladocerans relative to rotifers to establish large populations in HRAPs, despite having a life cycle longer than the pond HRT, likely was caused by cladocerans migrating to near the pond surface at night [81], and resulting in lower densities in the deep water column where the outflow was located. Consequently, cladocerans were retained longer within the HRAP compared with the smaller, slower swimming rotifers that were more evenly distributed throughout the water column. The maximum temperatures reached in the HRAPs were unlikely to limit the establishment of grazers since both rotifers and cladocerans can withstand temperatures exceeding 25-30 °C [78], [82].

Bdelloid rotifers, that have previously been shown to withstand WW environments [83], were only present in the HRAPs at very low abundances during the warmer months (November-February) when reproduction was faster. The obligate asexual reproduction and consequent inability of bdelloid rotifers to generate resting eggs [84] probably reduced their chances of establishing for long periods in the HRAPs. Zooplankton that preferentially feed on bacteria, including the colonial rotifer *Conochilus* sp. [85], [86], ciliates such as *Vorticella* sp., and *Paramecium* sp. [87], occurred

sporadically after HRAP algal concentrations declined, when the bacteria concentration was probably higher [88], and the numbers of larger competing grazers were reduced due to the decreased availability of algal food.

The cyclopoid copepod *P. fimbriatus* and the ostracod *H. incongruens* have been both found to tolerate WW conditions [53], [89]. Copepods typically have a reproductive age that is longer (7-30 days) than the HRAP HRT. However, *P. fimbriatus* is a benthic species [45], and is likely to be more easily retained in HRAPs. Furthermore, food was available during the entire monitoring period, since copepods feed on microalgae, bacteria, ciliates, juvenile rotifers, cladocerans, and chironomid larvae (blood worms that are commonly abundant in HRAP sediment) [90], [52], [53]. Ostracods such as *H. incongruens* have much longer generation times (up to three months) than other zooplankton. However, since they are also mainly bottom dwellers, they were still able to establish in the sediments of HRAPs [91], [92], which would have provided abundant food including bacteria, algae, protozoa, zooplankton, and organic detritus [45]. During the coldest months (June and July) *H. incongruens* were at their lowest densities, likely due to the very long generation time that ostracods have at temperatures ~10 °C (Table 7).

Table 7 Comparison of life-span, reproductive age and life-time asexual egg production of different zooplankton groups at different temperatures [51][1], [93][2], [45][3], [50][4], [94][5], [26][6], [95][7], [96][8], [97][9], [98][10]. Reproductive age of cladocerans is based mainly on data for *Moina* spp.

Zooplankton Type	Rotifers			Cladocerans				Ostracods				
Temperature (°C)	*10*	*20*	*25*	*10*	*15*	*20*	*25*	*10*	*17*	*20*	*23*	*25*
Life-span (d) [1,2,5]	20	12	5	85		50	40	85	79-88	50	38-40	40
Reproductive age (d) [3,4,1,5,6,7,8, 9,10]	5-7	2-3	1.25-1.75	19-24	8-11	3-8	3.5-6.5	28-32	48-54	13-15	22-27	7-11
Life-time egg production [1]	15-25	15-25	15-25	400-600		400-600	500-700	250-500		250-500	500-750	

Grazer inhibition via competition and predation

Zooplankton species can compete directly via mechanical interference [99], [100], [101], and indirectly via exploitative competition over a common food resource [102]. Over the 14-month study, mutual exclusion among

the grazer species that established in the HRAPs was observed. High densities of the cladoceran *M. tenuicornis* (>1,500 individuals/L) were associated with lower densities of rotifers *B. calyciflorus, C. catellina* and *F. longiseta*, likely due to mechanical damage to the rotifers caused by the fast movement of *M. tenuicornis'* appendages, and competition over the shared food [103], [104], [105]. Cladocerans have a preferential feeding on smaller food particles and likely reduced the availability of smaller food particle that can be ingested by rotifers.

F. longiseta established in spring-summer during periods when other rotifer and microcrustacean species were absent or had very low densities, resulting in lower competition for its preferred food including smaller algal particles (~6 μm) [106], and bacteria [107]. Large densities of the cyclopoid copepod *P. fimbriatus* were associated with a significant decrease in *B. calyciflorus* density (October), probably due to direct predation by *P. fimbriatus* [108], [109]. The ostracod *H. incongruens* also appeared to limit rotifer densities although it is not clear if this was a causal relationship. However, direct predation by the ostracod or mechanical damage of rotifers captured and then rejected during ostracod feeding, are possible explanations [110], [92]. *H. incongruens* presence was also associated with an absence of the copepod *P. fimbriatus* from both HRAPs and *H. incongruens* is known to prey on cyclopoid copepods [111].

HRAP performance and microalgal dynamics

Variations of biomass, productivity, microalgae relative abundances and nutrient removal in HRAPs depended on factors including season (temperature and solar radiation), WW nutrients, and grazer densities. An increase in zooplankton density occurred during warmer seasons and often initially coincided with periods of high microalgal productivity (>6 g VSS/m^2/d) (October-December). However, the high population density of rotifers and cladocerans resulted in reductions in productivity, TSS, VSS and Chl-*a* that lasted between two and five weeks and decreased the capacity of the HRAPs to remove N-NH$_4^+$ and P-PO$_4^{3-}$ from the water. During winter (May-September) the productivity in the HRAP was low (<~6 g VSS/m2/d), even without high zooplankton densities. *B. calyciflorus*

efficiently consumed the small and needle shaped *Ankistrodesmus* sp. and *Monoraphidium* sp. as previously reported [112], [113], [114]. However, the colonial *Mucidosphaerium* sp., and *Micractinium* sp. were likely too large to be ingested. When HRAPs were dominated by colonial microalgal species the presence of *B. calyciflorus* did not result in changes of microalgae relative abundances likely due to preferential feeding on bacteria [115] and smaller unicellular microalgal species present (albeit in low concentrations). *C. catellina* could feed on colonies of microalgal species with loosely combined cells such as *Mucidosphaerium* sp. and *Micractinium* sp. In contrast, they would be unable to feed on the colonial *Pediastrum* sp. that has cells fused together within the colony, and a silica skeleton within the cell wall. Microscope observation of the feeding mechanism of *C. catellina* showed that the small rotifers moved around the microalgae colonies and grazed on single cells using their protrusible grasping mastax (grazing pharynx) [116]. *M. tenuicornis* efficiently consumed unicellular *Ankistrodesmus* sp., unicellular round microalgae, and small and non-spined *Micractinium* sp., although were not able to consume colonial *Mucidosphaerium* sp., *Pediastrum* sp., and large colonies of *Micractinium* sp. *Moina* spp. can graze on both small and large particles, although preferentially feeds on smaller particles such as unicellular microalgae and bacteria [22], [113]. Populations of *F. longiseta* did not alter the HRAPs productivity and the relative abundance of microalgal species, likely due to their inability to ingest large particles [106], [85], and their preferential feeding on bacteria [107].

Generally, the reduction of ingestible microalgae species was followed by the decline in grazer densities, probably due to a lack of ingestible food. After zooplankton densities declined, less ingestible microalgal species (large or colonial) increased to previous levels in two to four weeks during colder periods (May-September), and one to two weeks during warmer periods (October-April).

The 13 microalgae species that established in the HRAPs were typical of those in New Zealand WW HRAPs operating without species control (microalgae inoculation or recirculation to promote specific species) [3], [38]. The species succession during the warm season (October-April) was

similar to that reported by Park et al. [29], with dominance of colonial species such as *Micractinium* sp., *Pediastrum* sp., and *Mucidosphaerium* sp. However, in their study, the cold season (April-September) was dominated by *Mucidosphaerium* sp. followed by *Pediastrum* sp. In contrast, we found *Micractinium* sp. followed by *Mucidosphaerium* sp. in West HRAP, and *Pediastrum* sp., and *Desmodesmus* sp., followed by *Monoraphidium* sp., and then *Micractinium* sp. in East HRAP.

When VSS was <120 mg/L, the capacity of HRAPs to remove $N-NH_4^+$ and $P-PO_4^{3-}$ from the pond water was reduced. Conversely, when VSS was >~120 mg/L, even large increases in the concentrations of $N-NH_4^+$ and $P-PO_4^{3-}$ in WW inflow caused only minor, or no increase in the concentration of $N-NH_4^+$ and $P-PO_4^{3-}$ in the HRAPs.

Microalgae maximal cross sectional area (MCSA), structural modifications induced by grazers and biomass settleability

Higher average MCSAs, larger and denser colonies, higher numbers of cells in colonies, and the formation of protective spines in microalgae occurred during periods of high densities of grazers. MCSA changed according to the dominant microalgal species present, grazing mechanisms of zooplankton species present, as well as the size of microalgal cells and colonies. Low average MCSAs (<100 μm^2) were indicative of a high abundance of small unicellular microalgal species (e.g., *Monoraphidium* sp. and round unicellular algae), a low abundance of colonial species (e.g., *Micractinium* sp*., Pediastrum* sp. and *Mucidosphaerium* sp.), or a high abundance of small colonial species (e.g., *Coelastrum* sp.). Conversely, high average MCSAs (>200 μm^2) indicated a high abundance of colonial species, a low abundance of unicellular species, or a high abundance of large unicellular species (e.g., *Ankistrodesmus* sp.). Higher average MCSAs occurred in periods of high densities of the filter feeder *B. calyciflorus*, possibly because they are unable to ingest larger (>20 μm) food particles and instead consumed the smaller microalgal species. Conversely, *C. catellina* with its grasping mastax could consume cells of large colonial microalgae such as *Micractinium* sp. and *Mucidosphaerium* sp., causing severe reductions in the average MCSA.

The generation of long protective spines and the increase in the number of colony cells also occurred when *B. calyciflorus, C. catellina,* and *M. tenuicornis* had high densities. Spines and larger colonies are defensive responses that reduce the chances of microalgae being grazed by zooplankton [117], [17]. Those formations are generally induced by infochemicals [16], [118] released by grazers. High densities of planktonic grazers were associated with clumping of cells into larger and denser colonies for *Micractinium* sp. and *Mucidosphaerium* sp., and with an increase in the average number of cells in colonies of *Pediastrum* sp. and *Desmodesmus* sp., and to a lesser extent *Scenedesmus* sp. Increases in the number of cells within a colony have been reported for *Scenedesmus* sp. and *Desmodesmus* sp. [119], [120]. However, the structural modifications induced by grazing pressure on *Pediastrum* sp. colonies have not been examined. Rojo et al. (2009) [121] reported that the infochemicals of the large cladoceran *Daphnia magna* increased the density of the largest colonies of *Pediastrum tetras*, although the incubation of the small *P. tetras* together with the cladoceran led to a decrease in the density of the largest colonies due to selective size predation. Therefore, it is expected that the presence of rotifers and their infochemicals can promote the increase of large (32 and 64 cells) *Pediastrum* sp. colonies. While the large *D. magna* can consume and reduce the abundance of particles up to 80 µm [20], including larger *Pediastrum* sp. colonies, *M. tenuicornis* and rotifers cannot consume such large particles. *Scenedesmus* sp. had only minor variations in the number of colony cells compared to previous studies that reported the colony formation of unicellular *Scenedesmus* sp. when incubated with *B. calyciflorus* infochemicals [122], and *B. calyciflorus* individuals [17]. This was likely due to the lack of unicellular *Scenedesmus* sp. in our HRAPs, as filter-feeding rotifers likely grazed mainly on the smaller one and two celled colonies.

The settling of biomass in HRAPs was mainly related to the dominant microalgal species present and was higher during periods when the colonial algae *Pediastrum* sp. and *Micractinium* sp. were dominant and when large populations of zooplankton grazers were established (October-March). Moreover, the sediment accumulated on the pond bottom beneath eddies

was higher when heavier microalgae dominated, especially *Pediastrum* sp. Higher settleability during periods of high densities of grazers can be explained by the selective consumption of undesirable small diameter (1-10 µm) unicellular microalgae (*B. calyciflorus* and *M. tenuicornis*) [61] that promoted the dominance of colonial microalgae; the production of settleable faecal pellets composed of smaller algae clumped together (likely all species) [19]; the induction of colony formation (likely all species) [120]; and the promotion of large and stable algal/bacterial flocs by aggregation of suspended particles with substances that rotifers excrete [83]. However, large suspended particles (high MCSA) were not always associated with high settling because some large colonial microalgae such as *Mucidosphaerium* sp. have similar densities to that of water.

A previous study using the same HRAP system [38], but with partial recycling of biomass back into the pond water to promote the dominance of large colonial and highly settling microalgal species, had average productivities higher than in this study. HRAPs were also operated with shorter HRTs (4 days instead of 5 days in the period December-March, and 6 days instead of 8 days during September-November and April-May). The higher productivity was probably a result of improved light availability in the more dilute pond culture resulting in higher microalgae growth rates, and a reduced grazer population in the HRAP during periods of shorter HRT. Furthermore, the dominance of the grazer-resistant large colonial alga *Pediastrum boryanum* in the HRAP was also likely to have contributed to the higher productivity, while the biomass recycling provided a constant microalgae inoculum that facilitated recovery of HRAPs after unfavourable events. Higher productivities were also achieved in four full-scale HRAPs (1.25 ha) located in Christchurch (New Zealand) [3], despite the ponds being operated without CO_2 addition and in a colder location. The higher productivity of the Christchurch HRAPs may also have been due to the lower densities of grazers in these ponds (personal communication, D. Sutherland, NIWA, Christchurch). The low population of grazers may have resulted from two factors: the dominance of the large colonial microalgae *Micractinium* sp., which is generally difficult for zooplankton grazers to ingest, and the average pH was >9 with peaks >10, during spring and

summer (when increases in grazer densities tend to occur). With such high pH, free ammonia (NH_3-N) toxicity could have inhibited both rotifer and microcrustacean populations [33].

Implications for HRAP operation

Zooplankton grazer establishment can be either detrimental or beneficial to HRAP performance. When grazers are able to rapidly consume the dominant microalgal species, productivity and WW nutrient removal can be dramatically reduced. However, a low-moderate grazing activity that leads to structural modification and/or aggregation of the microalgae, can promote a better harvest of the biomass.

Filter-feeding grazers can quickly consume easily ingestible (e.g. small size, unicellular) microalgae species when they are dominant. This may be beneficial since these small microalgae provide a poor harvest. Hence, it may be beneficial to promote a permanent population of filter feeding rotifers (e.g., *B. calyciflorus*) sufficient to consume the poorly settling microalgae and induce structural modifications of colonial microalgae that would increase their settling capacity, provided that there is only a minor loss of overall productivity. In particular, the dominance of colonial microalgae such as *Pediastrum* sp. and *Micractinium* sp. should be promoted because they are difficult to ingest and are highly settleable.

Rotifer species able to consume colonial microalgae (e.g., *C. catellina*) may be beneficial in removing poorly settling species such as *Mucidosphaerium* sp., although they should be controlled when highly settling colonial microalgae such as *Micractinium* sp. are dominant. A moderate population of *M. tenuicornis* may be beneficial to control numbers of rotifers such as *B. calyciflorus*, again provided that there is only a minor loss of overall productivity. Copepods and ostracods do not appear to be detrimental to HRAP productivity and nutrient removal, and have the potential to reduce the abundance of rotifers.

Short HRTs decreased the capacity of grazers to establish in HRAPs and when possible, HRT could be temporary reduced in order to depress or even deplete undesired grazer species. Furthermore, the establishment of *M. tenuicornis* that likely can avoid the HRAP outflow due to daily migration,

could be reduced by placing the outflow higher in the water column where individuals have higher concentrations, or just after the paddle wheel, where individuals are evenly mixed.

CONCLUSIONS

The selective pressure of WW physicochemical conditions limited the establishment of zooplankton in HRAPs to only eight species. However, these species reached very high population densities, likely due to high food availability, the control of pH using CO_2, and reduced competition from other species. The establishment and growth of zooplankton populations were favoured when higher average water temperatures promoted faster reproduction rates, and longer HRTs increased the retention of individuals in the HRAPs. During the monitoring period, 13 microalgal species typical of New Zealand WW HRAPs established in our HRAPs. The establishment of large populations of grazer species, which were able to efficiently consume the dominant microalgae, was associated with changes in microalgal dominance and large, rapid reductions in productivity, TSS, VSS and Chl-*a*, and decreased the capacity of HRAPs to remove nutrients from the pond water. Following declines in zooplankton densities, microalgae returned to pre-bloom levels although they were usually replaced with species more resistant to grazing pressure (e.g., colonial).

Episodes of inhibition between the grazer species that established in HRAPs were observed, and large populations of cladocerans, copepods and an ostracod appeared to reduce the populations of rotifers. Grazing pressure and possibly infochemicals released from the grazers into the pond water were associated with structural modifications of microalgae cells and colonies that increased the average MCSA, the size and density of colonies, the number of cells in colonies, and promoted the formation of protective spines in microalgae. The settling of biomass in HRAPs, and the accumulation of sediments on the pond bottom, were higher when colonial algae were dominant, and when microalgae had structural modifications induced by grazing pressure.

Future research should focus on the development of cost effective and environmentally friendly technologies to reduce detrimental taxa and select for beneficial grazer populations in HRAPs.

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CHAPTER 4

SCREENING OF POTENTIAL ZOOPLANKTON CONTROL TECHNOLOGIES FOR WASTEWATER TREATMENT HIGH RATE ALGAL PONDS

ABSTRACT

Zooplankton taxa including cladocerans and rotifers are one of the greatest challenges for effective management of High Rate Algal Ponds (HRAPs) for wastewater treatment as they can establish and rapidly consume beneficial microalgae. Harmful zooplankton need to be controlled using cost effective treatments, and here we tested under controlled laboratory conditions the efficacy of chemical CO_2 asphyxiation, biological control of rotifers using competitor species expected to graze only moderately on colonial microalgae, and mechanical disruption using hydrodynamic shear stress. CO_2 asphyxiation caused acute mortality of all zooplankton species (t<10 min). Increasing the cladoceran *Moina tenuicornis* to densities >2,500 individuals/L was associated with a decrease in rotifer populations (~23% of the population in the control). The ostracod *Heterocypris incongruens* at densities >1,000 individuals/L were also associated with a decrease in rotifer densities that were ~27% of the population in the control. Hydrodynamic shear stress killed 100% of *M. tenuicornis* and ~80% of the rotifer *Brachionus calyciflorus* after a single pass. Furthermore, *M. tenuicornis* was concentrated in the upper 50 mm of a 300 mm deep water column using vertical migration induced by CO_2 concentrations of between 25-180 mg/L. All of these treatments have potential for use to control zooplankton blooms in WW HRAPs.

Keywords: Zooplankton control, High Rate Algae Ponds, CO_2 asphyxiation, CO_2 induced migration
Hydrodynamic stress, grazers' biocontrol.

INTRODUCTION

HRAP for wastewater treatment

High Rate Algal Ponds (HRAPs) are open, shallow, paddlewheel-mixed ponds with dimensions up to a few hectares [1]. HRAPs are globally known as effective and technically simple reactors to reclaim water, nutrients and energy from wastewater (WW) as algal biomass [2], [3], [4], [5], [6], [7], particularly when operated with pH regulation and CO_2 addition as an extra carbon source [8]. The algal biomass is settled and recovered by gravity in settling tanks, and can be used for biofuel production, fertilizer and animal feed [9], [10]. Before being discharged into the environment, the algal settling pond effluent is further treated in a series of maturation ponds where zooplankton graze on any remaining microalgae. One of the main challenges that can severely reduce the performance of HRAPs is the contamination and establishment of zooplankton from the surrounding environment. Species of cladocerans and rotifers that can survive WW conditions can attain high population densities because HRAPs have high levels of food (mainly bacteria and microalgae), and a lack of predatory fish. Large populations of zooplankton can rapidly consume the microalgal biomass [11], [12], and reduce the nutrient recovery from WW [13]. The need for treatments to control zooplankton in large and open algal cultures is well recognized [14], [15], [16]. However, to date low-cost and effective treatments applicable to full scale HRAPs have not been assessed.

Montemezzani et al. [16] proposed physical, chemical, and biological options for zooplankton control in HRAPs. Physical treatments included filtration, hydrodynamic cavitation, shear stress and bead mills and chemical treatments included a night-time increase in CO_2 concentrations, promotion of lethal un-ionized ammonia toxicity, use of biocides, and the chitinase inhibitor chitosan. Biological controls included using competitor and predatory organisms that exert a low grazing pressure on colonial microalgae, and which can be easily controlled themselves, such as the cladoceran *Moina tenuicornis*, the ostracod *Heterocypris incongruens*, and carnivorous rotifers such as *Asplanchna* species. These treatments were derived from strategies to control zooplankton in ballast waters [17], [18],

[19], and to manage zooplankton in aquaculture ponds [20], and their efficacy in WW environments requires testing.

Here, using controlled laboratory experiments we tested the efficacy of CO_2 asphyxiation, biocontrol using the cladoceran *M. tenuicornis*, biocontrol using the ostracod *H. incongruens*, and hydrodynamic stress. Chemical approaches were excluded owing to the extensive existing literature [21], [22], [23], [24], [25], which indicates that chemicals would likely affect the zooplankton community within the subsequent maturation ponds and contaminate the final treated effluent. For example, the low-cost pesticide rotenone and the disinfectant hypochlorite were both used to control the rotifer *Brachionus calyciflorus* in laboratory cultures of the microalga *Chlorella kessleri* [26], [27]. However, rotenone can persist in water for >2 weeks, and doses of hypochlorite sufficient to control rotifers without affecting the microalgae, require dosing for at least 3 days. This is not very practical for WW HRAP operation, particularly during summer when the HRT is only 4 days. Moderate heating was also excluded because although temperatures of 35–40 °C for a few hours can kill all zooplankton species [28], [29], [30], a large portion of the microalgae may also die. Moreover, the energy cost necessary to increase the temperature of large amounts of water (3,000-5,000 m^3) of full scale HRAPs, and to rapidly reduce it after zooplankton death to reduce microalgal mortality, make moderate heating hardly applicable in full scale HRAPs.

Acute zooplankton CO_2 asphyxiation was selected because it is a novel option that can be applied using the hardware to inject CO_2 for pH control that may already be installed in HRAPs [31] and was based on the assumption that higher $ppCO_2$ (partial pressure of CO_2) can inhibit the aerobic metabolism of cladocerans and rotifers. Acute application of CO_2 has previously been used to inactivate zooplankton in experimental enclosures using dry ice [32], and to reduce the zooplankton density in 1.5 m^3 microalgae cultures using pure CO_2 [33]. Biocontrol of rotifers using the competitive action of *M. tenuicornis* [34] was selected because *Moina* is a cladoceran sufficiently large to be controlled to desired densities using filtration, and that can establish and thrive in WW HRAPs [13]. *Moina* also have a competitive advantage in terms of shared food, size, and mechanical

strength over smaller species such as rotifers [35], [36], [37], [38]. *Moina* preferentially feed on smaller microalgae and bacteria [39], [40] reducing or depleting the only food particles (1-20 μm) that can be ingested by the smaller rotifers [41]. Furthermore, the fast movement of *M. tenuicornis'* appendages can cause mechanical damage to rotifers [42]. Biocontrol of rotifers using the competitive action of ostracods was selected because high population densities of the detrital feeding ostracod *H. incongruens* in pilot HRAPs in New Zealand have been found to be associated with low densities of microalgal eating rotifers [13]. Ostracods can directly prey on rotifers [43], [44], and can indirectly exert mechanical damage on rotifers with the rapid movement of their appendages during feeding [45]. Control of zooplankton using hydrodynamic shear stress was chosen because it is a simple, reliable, and easy to automate treatment that can selectively kill or damage zooplankton species [46], [47] more than microalgae cells [48]. Hydrodynamic cavitation can be generated by a pressure drop in a liquid flow using pumps to force the liquid through small openings [49], [50], and has been successfully used to kill zooplankton in ballast water tanks [48], with higher death rates achieved with larger (>100 μm) organisms [51]. However, hydrodynamic cavitation is too costly to treat large volumes of water [52], so here we test a reduced treatment intensity that produced milder hydrodynamic shear stress without achieving cavitation. Finally, we used raised concentrations of CO_2 to induce migration of *M. tenuicornis* to the upper layers of the water column (a behavior observed during the CO_2 asphyxiation experiments) with the aim of concentrating *Moina* and reducing the amount of water required to process with filtration or hydrodynamic stress.

The efficacies of CO_2 asphyxiation, biocontrol using the cladoceran *M. tenuicornis*, biocontrol using the ostracod *H. incongruens*, and hydrodynamic stress were tested using controlled laboratory experiments conducted using batch microalgae and zooplankton cultures. We exposed cladoceran and rotifer populations to increasing CO_2 concentrations and hydrodynamic stress intensities with the aim of enhancing death rates, and incubated given densities of *M. tenuicornis* and *H. incongruens* with rotifer populations to assess the rotifer growth dynamics and the reductions in

population densities. Treatments were selected based on minimal environmental impact, selectivity for specific zooplankton species, and low cost.

MATERIAL AND METHODS

General analyses, experimental set up, and sampling protocol

Microalgal biomass, zooplankton identification and counts

Phytoplankton and zooplankton used in the experiments were sourced from two 8 m^3 pilot-scale WW HRAPs (West and East) located at the Ruakura Research Centre, Hamilton, New Zealand (37°46'29.5"S - 175°18'45.4"E). Total suspended solids (TSS) of microalgae cultures was measured by filtering a known volume of culture through pre-rinsed, pre-combusted and pre-weighed 47 mm Whatman GF/F filters (nominal pore size 0.7 μm), oven dried (85°C) overnight using a drying oven (270M Digital Series, Contherm), and weighed on an analytical scale (SI-234, Denver Instruments). The filters were then combusted at 450°C for 1 h using a muffle furnace (F.E.KILN, RTC1000, Bartlett Instrument Company, UK), and weighed again to determine the ash weight. Total organic matter (volatile suspended solids: VSS) was calculated as the difference between TSS and the ash concentration [53]. Samples (10 ml) for chlorophyll-*a* analysis were filtered onto 25 mm Whatman GF/F filters (nominal pore size 0.7 μm) and the chlorophyll-*a* extracted in 100% methanol at 65 °C for 5 min, followed by 12 h at 4 °C in the dark. Samples were then centrifuged using a Sorvall/Dupont General Centrifuge GLC-2B at 3,000 rpm (RCF: ~1720 g) for 15 min and the absorbance of the supernatant was measured using a UV-Visible Shimadzu UV 1601 spectrophotometer. Chl-*a* concentrations were calculated using the modified trichromatic equations for methanol [54]. Zooplankton were identified according to taxonomic descriptions [55] from triplicate 5 ml samples of experimental cultures that were placed in a gridded counting chamber, mixed with 3 drops of Lugol's Iodine solution (10% v/v) to inactivate zooplankton, and counted using a Leica M50 stereo microscope [56].

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Microalgal identification and relative abundance

Microalgal species composition was determined from 1 ml samples of experimental culture that were settled in a 25 mm ø Utermöhl chamber. Three images per sample were taken in random fields of view using a Leica DM 2500 microscope (100x - field of view (ø) 1 mm), equipped with digital Leica DFC 420 camera (Leica Microsystem, Switzerland), and the software Leica Application Suite (LAS version 4.1.0). Microalgae were identified to species level, where possible, according to taxonomic descriptions [57]. The relative abundance of microalgae was calculated by multiplying the average biovolume of each microalgal species by the total number of cells or colonies counted (depending on the species) in all the pictures. The average biovolume for each microalgal species was calculated using the equations proposed by Vadrucci et al. [58] and the methodology and assumptions described in [13].

Experimental set up

The experiments were conducted using triplicate 500 mL batch cultures in conical flasks. To avoid mechanical damage of zooplankton the cultures were mixed and aerated using an air pump (Aqua One Infinity AP-950 Twin Outlet) with one 25x10 mm stone air spargers placed at the bottom of each flask. The two outlets with a flow rate of 280 l/h were connected to air splitters and valves to obtain a constant air flow rate of ~300 ml/min/flask. The bubbles were sufficiently large to avoid entrapment under the carapace of cladocerans which can result in their flotation. The flasks were incubated in a growth chamber (Biosyn 6000 CP, Contherm, Australia) under standard conditions (20°C x 12 h - 19°C x 12 h; light intensity 250 µmol/m^2/s), with the pH kept between 7 and 8 by constant injection of ~0.2% v/v CO_2 to avoid ammonia toxicity. Samples (5 mL) were collected from the centre of each flask after mixing by inverting 5 times. To minimize the removal of ostracods in experiments where these needed to be retained in the culture, sampling was performed 30 s after the mixing ceased to enable ostracod settling. Densities of rotifers and ostracods used in the experiments were in the same order of magnitude of maximal densities observed in pilot HRAPs during 14 months monitoring period [13]. The concentration of zooplankton

was measured every one to three days, depending on the experiment, and microalgal relative abundance, VSS, and Chl-*a* concentrations were measured at the beginning and at the end of the experiments. Pearson's correlations were used to statistically analyse the relationships between zooplankton mortality and CO_2 concentration (%), *M. tenuicornis* density (individuals/L), and water pressure before the plate (bar), and the relationships between the density of *M. tenuicornis* in the upper 50 mm of the water column and the CO_2 concentration (%). Comparisons were made using the averages of triplicate repetitions. We used p-values of <0.05 and 0.1 to assess levels of statistical significance. Error bars in the graphs are ± standard deviation calculated for data from the triplicate flasks.

Specific treatment conditions

CO_2 asphyxiation of M. tenuicornis, Brachionus spp., and Paramecium sp.

The acute (up to 60 minute) influence of CO_2 concentration on the mortality of the cladoceran *M. tenuicornis*, the rotifers *B. calyciflorus* and *B. rubens*, and the ciliate *Paramecium* sp. was assessed on five single individuals. Eleven different CO_2 concentrations in N_2 were tested, ranging from saturation (~2,000 mg/L at 17 °C) down to 330 mg/L. The lowest CO_2 concentration was determined in preliminary experiments as that at which zooplankton mortality still reliably occurred within 60 minutes. CO_2 asphyxiation was assessed using a 1 L transparent measuring cylinder containing a 300 mm depth (900 ml volume) of tap water that was bubbled until saturation (constant pH) for each CO_2/N_2 gas mix, using an air stone sparger (25 mm x 10 mm) placed at the bottom of the cylinder. The concentration of CO_2 in the water bubbled with the gas mix was measured in three 100 ml samples by titration of carbonic acid against NaOH standards, with phenolphthalein (0.5%) indicator until colour change at pH 8.3 [59], [60]. Another 300 ml of tap water was added to the cylinder to replace the water used for the CO_2 concentration analyses, and it was bubbled again until a constant pH was achieved. The gas injection was stopped just before the zooplankton were introduced to the water to prevent them from being harmed by shear stress, five individuals of *M. tenuicornis*

(body length comprised between 0.5 and 1.5 mm and easily visible in the transparent water column) were added into the cylinder, and the cylinder was immediately capped. Immobility of *M. tenuicornis* was determined as the time required for individuals to cease any visible movement. Mortality of *M. tenuicornis* (complete and permanent failure of the heart), rotifers and ciliates (no body movements, including cilia and mastax) were assessed by placing single individuals of *Moina*, or 0.5 ml of rotifer/ciliate culture into a gridded counting chamber that was then completely filled with 10 ml of tap water that had previously been bubbled with the given gas mix. The chamber was immediately capped using a petri dish lid placed over the water surface to prevent CO_2 outgassing. The counting chamber and the microscope were used because rotifers, ciliates, and the heart activity of *Moina* are not visible with bare eyes. When zooplankton individuals were placed in the cylinder or in the counting chamber, a stopwatch was activated. Individuals were observed constantly and the immobility or mortality times were assessed. To assess if low O_2 concentration (likely to be partially displaced by injection of the gas) contributed to zooplankton mortality, individuals were introduced into water bubbled (1 L/min) with pure N_2 for 60 min, to severely reduce the O_2 concentration (~0.2–0.4 ppm) [61]. All zooplankton species used in the experiments were cultured and kept under the same room conditions (~18°C) throughout the study. The tap water used in the experiments had been placed in an open container (5 L) for 24 h to ensure off-gassing of chlorine before use.

Rotifer control using the cladoceran M. tenuicornis
The inhibitory effect of the cladoceran *M. tenuicornis* (initial density ~1,600 individuals/L) on rotifers was tested on a rotifer community containing *B. calyciflorus*, *B. rubens* and *F. longiseta* (initial combined density of ~5,000 individual/L). Rotifers were incubated with *M. tenuicornis* in a microalgae culture dominated by *Actinastrum* sp. (initial VSS concentration ~100 mg/L, and Chl-*a* concentration ~1,600 µg/L). The experiment was conducted over 21 days, and the densities of *M. tenuicornis* and rotifers were assessed daily. To prevent food depletion, VSS and Chl-*a* were maintained at concentrations of >~100 mg/L, and >~1,500 µg/L, respectively, by adding

15 ml of a concentrated *Actinastrum* sp. culture to each flask on days 4 and 8.

Rotifer control using the ostracod H. incongruens

A preliminary experiment was performed to test the capacity of ostracods (*H. incongruens*) to reduce the density of the rotifer (*B. calyciflorus*). Microalgae and rotifers were grown separately and mixed before the experiment. The microalgae TSS concentration in the rotifer culture was maintained at >250 mg/L, and was composed of *Pediastrum* sp. and *Ankistrodesmus* sp. with initial relative abundances of ~60% and ~40%. The initial density of *B. calyciflorus* was ~20,000 individuals/L. *H. incongruens* were collected from HRAP sediment using a 500 μm filter, counted, and added to the microalgae-rotifer culture to give a density of 1,518±59 *H. incongruens*/L. Ostracod concentrations, microalgae relative abundances, and TSS concentrations were assessed at the beginning and end of the experiment, and the concentrations of rotifers were assessed daily. The experiment was concluded when the density of rotifers was reduced to <1,000 individuals/L.

Two successive experiments were performed to assess the rotifer reduction under different mixing intensities and exposures (i.e., direct versus indirect contact between rotifers and ostracods). The first experiment was conducted over 10 days with low mixing conditions using an air flow rate of ~50 ml/min/flask. The microalgae culture was dominated by *Actinastrum* sp. (90%), TSS was constantly >100 mg/L, and the initial rotifer population was composed of 6,000 *B. calyciflorus*/L, and 6,600 *B. rubens*/L. Initial and final average densities of *H. incongruens* were 1,080±190 individuals/L, and 817±189 individuals/L, respectively. The experiment was performed in triplicate and included a control without *H. incongruens*; a treatment with *H. incongruens* loose in the flasks to allow direct contact with the rotifers; and a treatment with *H. incongruens* enclosed in 60 μm mesh filter bags placed into the flasks to avoid direct contact with the rotifers. Settled organic matter was collected from the bottom of WW HRAPs and added (10 ml) to flasks and filter bags as a food source for the ostracods. The second experiment was conducted over 5 days with high mixing

conditions using an air flow rate of ~300 ml/min/flask. High mixing conditions enhanced the interaction between ostracods and rotifers (when *H. incongruens* were loose in the flasks) and the distribution of chemicals in the culture (when *H. incongruens* were enclosed in 60 μm mesh filter bags). The microalgae culture was dominated by *Actinastrum* sp. (90%), TSS was constantly >100 mg/L, and the initial rotifer population was composed of ~14,000 *B. calyciflorus*/L. The experiment was performed in triplicate and included a control without *H. incongruens*, and four treatments. The treatments were identical to those of the previous experiment except that two different concentrations of *H. incongruens* (low: 946±197 individuals/L, and high: 1,893±394 individuals/L) were used. The final average densities of *H. incongruens* were 1,013±317 individuals/L, (low), and 1,680±711 individuals/L (high).

Zooplankton control using hydrodynamic shear stress

The mortality of zooplankton caused by hydrodynamic shear stress was assessed by pumping 10 L of *M. tenuicornis* and *B. calyciflorus* cultures through aluminium perforated plates with different numbers and diameters (mm) of orifices, and total open surface area of orifices (mm^2). The different configurations of plates were: 1x9 mm (64 mm^2), 1x6 mm (28 mm^2), 1x4 mm (13 mm^2), 25x1 mm (20 mm^2), 9x2 mm (28 mm^2), 4x3 mm (28 mm^2), and 5x2 mm (16 mm^2). A 23 L tank supplied the liquid culture to a pump Lowara PSAM 70/A, with maximum flow rate of 16 L/min, and power of 0.37 kW. The perforated plate was located inside a 20 mm pipe connected to the pump outflow, and two pressure gauges were used to detect the pressure (Bar) before, and after the perforated plate. The discharge rate (m^3/s) was calculated by dividing the pumped volume by the time required to pump it, and the velocity (m/s) was calculated by dividing the discharge rate by the open surface (m^2). Triplicate samples of 100 ml were collected before and after a single pass through the treatment, total zooplankton were counted, and death rate was assessed. Species death rate was calculated including both dead and disrupted (fragmented into small pieces and not visible) individuals using equation (2). Where *a* was total number of zooplankton before the treatment, *b* was the total number of visible dead zooplankton

after the treatment, c was the total number of visible zooplankton after the treatment (visible dead and alive individuals, after inactivation of living individuals with two drops of Lugol solution 10% v/v).

$$Death\ rate=100 - \left(\frac{c-b}{a}*100 \right) \tag{2}$$

The number of zooplankton remaining alive after the treatment was calculated as $c\text{-}b$. The number of zooplankton disrupted (and not visible) during the treatment was calculated as $a\text{-}c$. Counting was performed 60 min after the treatment to allow for death, or recovery of partially damaged zooplankton.

M. tenuicornis CO_2 induced surface migration

The migration of *M. tenuicornis* to the upper 50 mm of the water column under increasing concentration of CO_2 was assessed with the same method described in Section 2.2.1. The cylinder was placed in a room with diffuse neon light to minimize phototactic induced migration, the water bubbled with CO_2, and single individuals where introduced into the cylinder. When individuals were able to reach the surface, the time spent in the upper 50 mm of the water column was measured with a stopwatch. When they could not reach the surface and irreversibly sank below the 50 mm mark, the time was considered to be zero. Measurements were performed on five individuals for each concentration of CO_2 over 180 minutes, and the time spent in the upper 50 mm was averaged.

RESULTS

CO_2 asphyxiation of *M. tenuicornis*, *Brachionus* spp., and *Paramecium* sp.

Gas mixes (N_2+CO_2) with increasing percentages of CO_2 injected into the water promoted higher concentrations of dissolved CO_2, and lower pH values (Figure 1). Higher concentrations of dissolved CO_2 caused a shorter mortality time for all zooplankton tested (Figure 2; Table 1). When the water

CO_2 concentration increased from ~5 mg/L to ~50 mg/L, the pH decreased from 7.2 to 5.8.
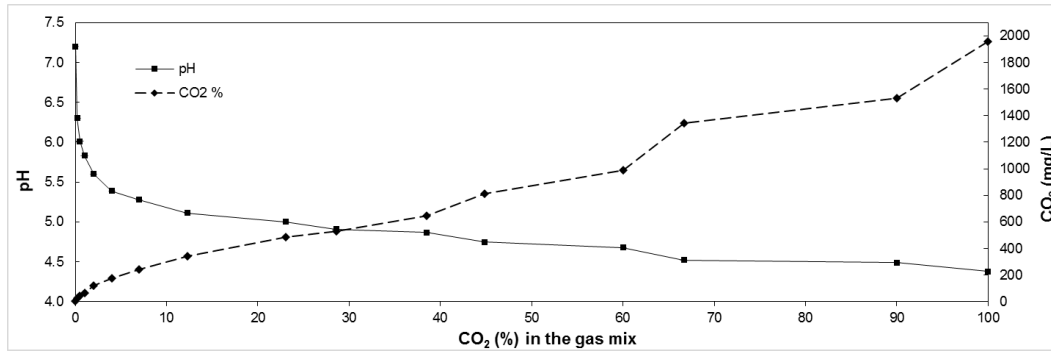
Figure 1 Amount (%) of CO_2 in the gas mix (N_2/CO_2) injected, and corresponding water CO_2 concentration (mg/L), and pH.

Injecting pure CO_2 raised the dissolved CO_2 concentration to ~2,000 mg/L and lowered the water pH to less than 4.5, which caused complete (100%) mortality of *M. tenuicornis* after < 2 minutes, and of rotifers after < 16 minutes, depending on species (Figure 2 - top, Figure 3). Mortality of *B. calyciflorus* occurred in 50-75% of the time required for *B. rubens* mortality.

Table 1 Pearson`s correlation coefficients (r) between treatment intensity and zooplankton density. Positive values denote positive linear correlation, negative values denote negative linear correlation, *p<0.05 (statistical significance), and **p<0.1 (moderate statistical significance). The number of different intensities of treatment is indicated with "n". Increasing CO_2 concentrations are correlated with the corresponding zooplankton mortality times (s). *M. tenuicornis* densities are correlated with corresponding rotifer densities (individuals/L). Increasing water pressure before the plate (bar) are correlated with zooplankton death rates (%) after the treatments. Correlations not related to the discussion are indicated with "-".

	M. tenuicornis immobility time	*M. tenuicornis* mortality time	*B. calyciflorus* mortality time	*B. rubens* mortality time	*Paramecium* spp. mortality time	*F. longiseta* mortality time	*Moina* in 50 mm (%)
CO_2 concentration	-0.67* (n=15)	-0.90** (n=5)	-0.87* (n=9)	-0.78* (n=9)	-0.79* (n=9)	-	0.56* (n=15)
***M. tenuicornis* density**	-	-	-0.53* (n=22)	-0.51* (n=22)	-	-0.62* (n=22)	-
Water pressure before the plate	-	0.60** (n=8)	0.87* (n=8)	-	-	-	-
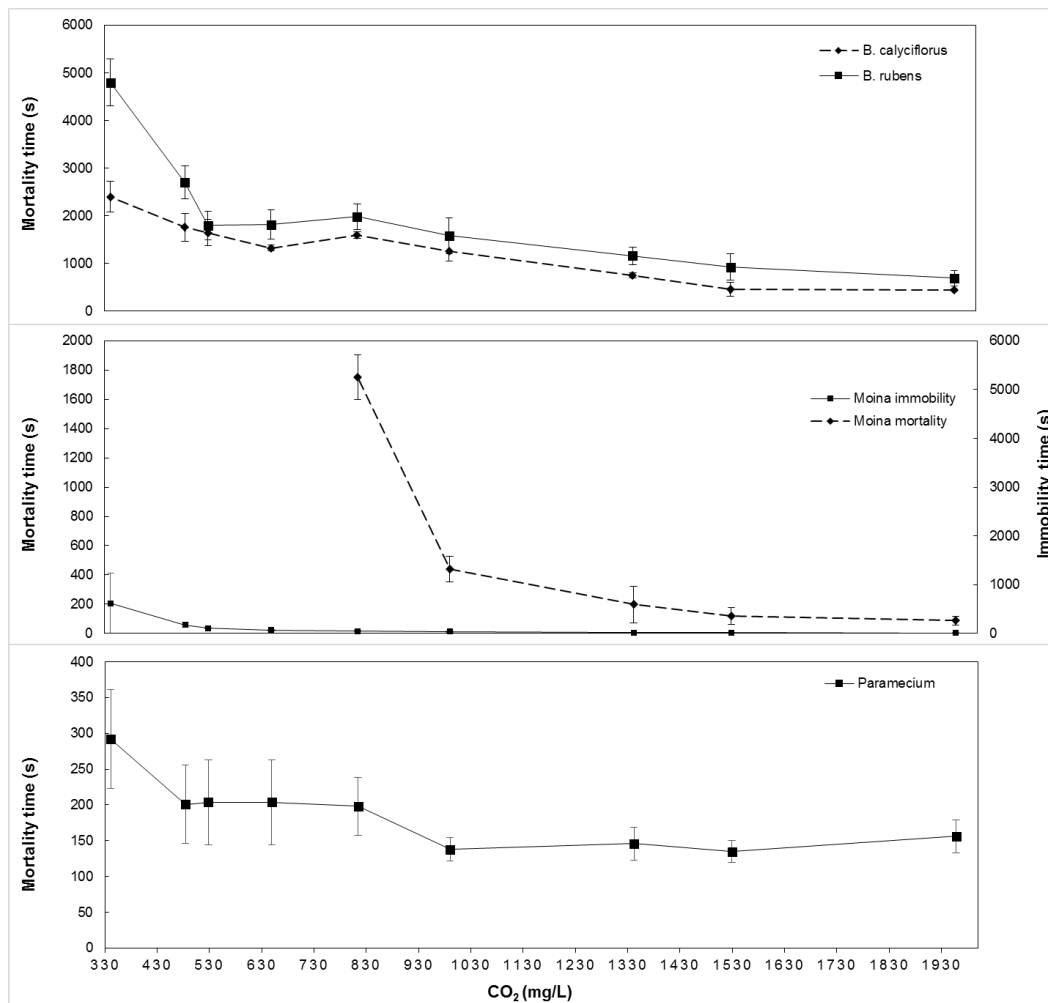
Figure 2 Average mortality and immobility times (n: 5) of different zooplankton exposed to dissolved CO_2 concentrations of between ~2,000 mg/L and ~330 mg/L. Top: Rotifers *B. calyciflorus* and *B. rubens*; Centre: the cladoceran *M. tenuicornis*; Bottom: the ciliate *Paramecium* spp. Error bars are ± standard deviations.

M. tenuicornis acute mortality did not occur at CO_2 concentrations below 830 mg/L and at all CO_2 concentrations at which it occurred, mortality times were ~4 times shorter than for the rotifers (Figure 2, top and centre). Moreover, *Moina* immobilisation occurred before mortality of both *Moina* (at ~2,000 mg/L of CO_2 individuals were motionless within 10 s and died within 90 s), and rotifers (Figure 3). At all CO_2 concentrations younger pale (low hemoglobin) individuals died faster and showed earlier immobility than larger red (high hemoglobin) adults (visual observation). Mortality of the ciliate *Paramecium* sp. (Figure 2, bottom) occurred more rapidly than for *Moina* and the *Brachionus* species, and was similar (between 130 s and 300 s) at all CO_2 concentrations (330-2,000 mg/L). *M. tenuicornis*, the

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Brachionus species, and *Paramecium* sp. introduced into water bubbled with pure N_2 showed zero mortality and normal swimming behaviour after 4 h.
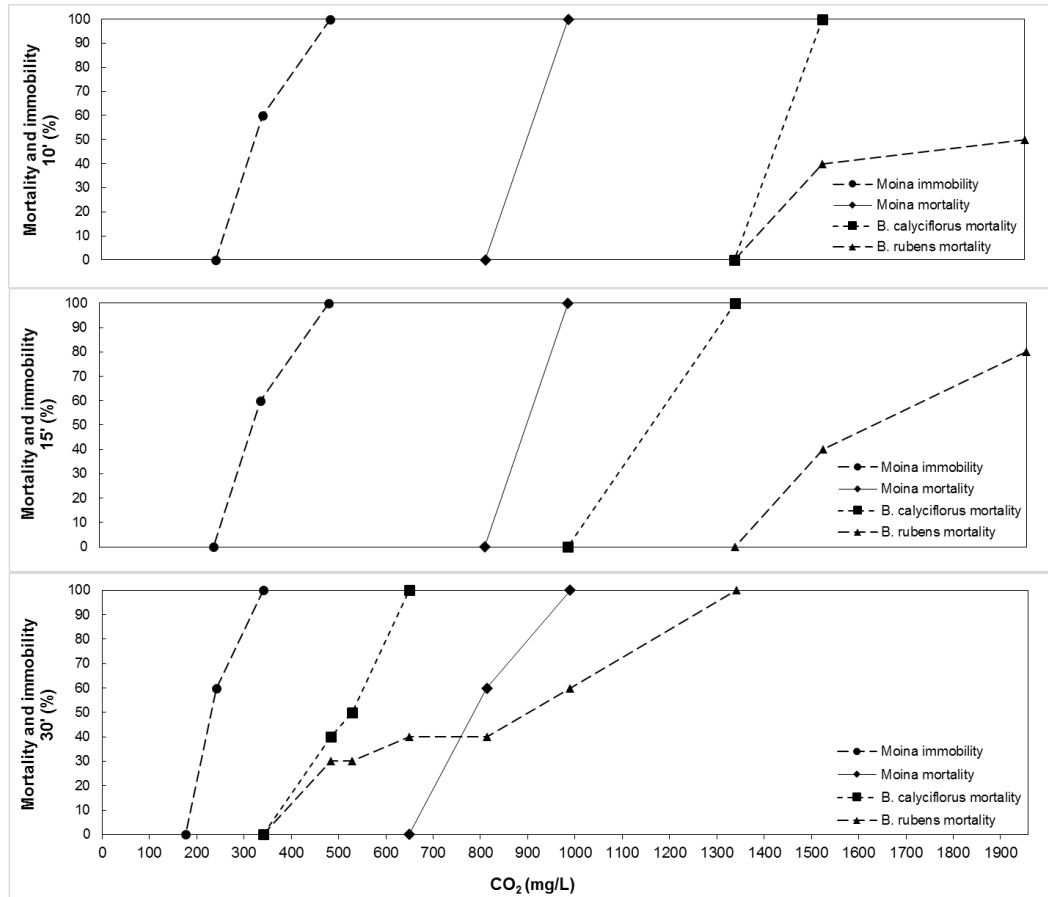
Figure 3 Zooplankton mortality and immobility (%) after 10, 15 and 30 minutes of exposure to increasing concentrations of CO_2.

Rotifer control using the cladoceran *M. tenuicornis*

High densities of *M. tenuicornis* (particularly >2,000 individuals/L) reduced the growth of all rotifers compared with the control, although the effect varied depending on the rotifer species (Figure 4). *F. longiseta* and particularly *B. rubens*, (the smallest of the rotifer species) were most influenced by *M. tenuicornis* (Figure 4), while the growth of *B. calyciflorus* appeared to be delayed by the presence of *M. tenuicornis.* Over the 21 days of the experiment the average density of *B. rubens*, *F. longiseta*, and *B. calyciflorus* in the treatment were 22.6%, 59.2%, and 89.0% of the control, respectively.
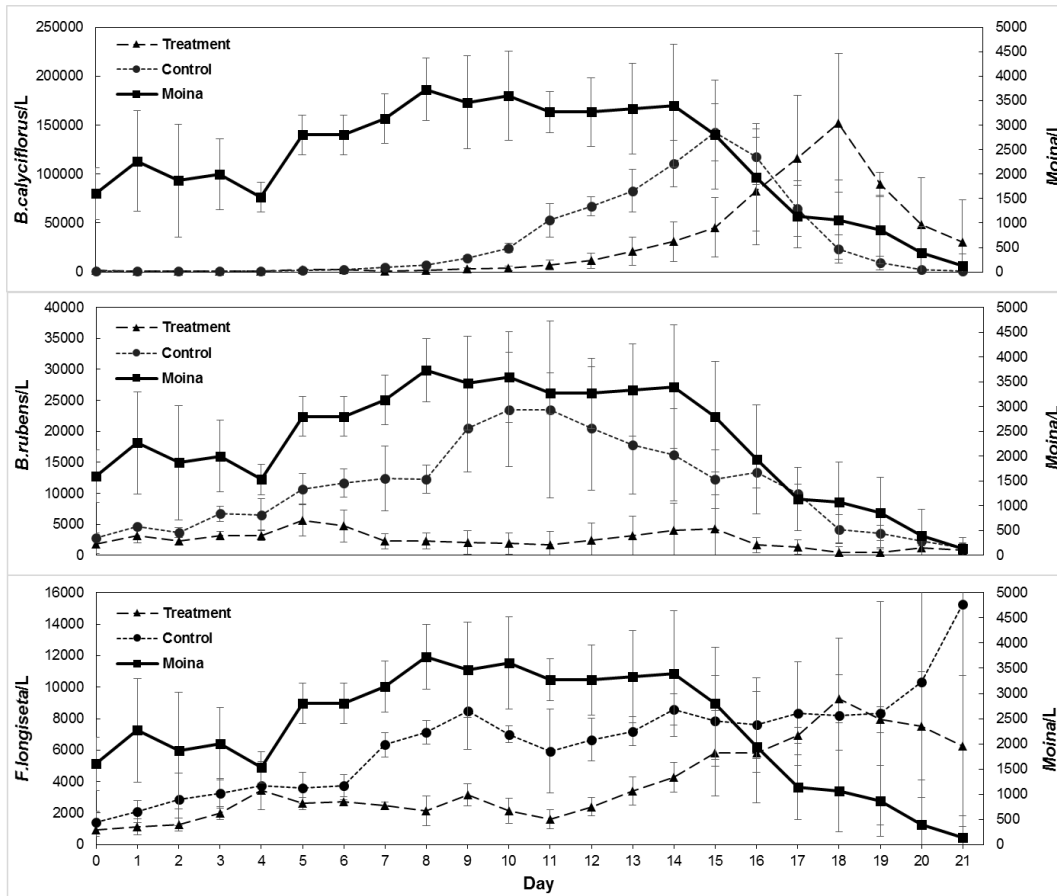
Figure 4 The inhibitory effect of the cladoceran *M. tenuicornis* (thick line) on a rotifer community containing *B. calyciflorus, B. rubens* and *F. longiseta*. Top: average population densities of *B. calyciflorus* in control and treatment. Centre: Average population densities of *B. rubens* in control and treatment. Bottom: average population densities of *F. longiseta* in control and treatment. After day 14 zooplankton dynamics were likely affected by other factors such as scarce food availability and self-inhibition from their own metabolites. Values are averages of triplicate batch cultures, error bars are ± standard deviations.

Rotifer control using the ostracod *H. incongruens*

Direct incubation of ~1,500 *H. incongruens*/L with a dense population of *B. calyciflorus* reduced the average density of the rotifer by half compared with the control over 11 days (Figure 5). The lowest concentration of *H. incongruens* observed to clearly reduce the density of *B. calyciflorus* was ~700 individuals/L (data not shown).
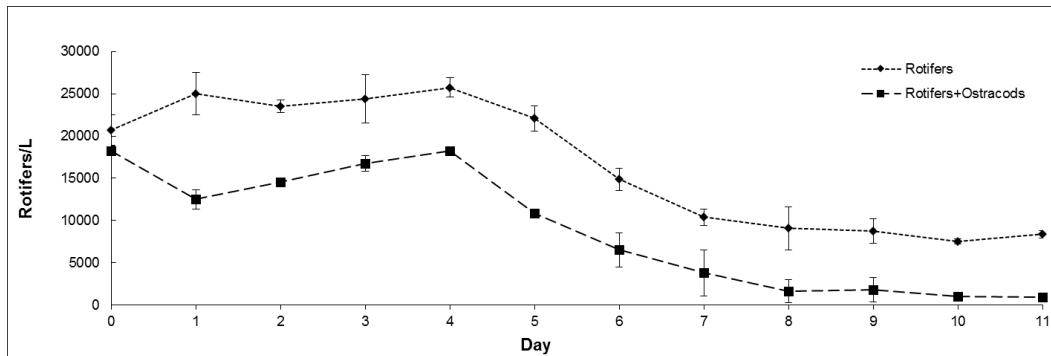
Figure 5 Changes in the density *B. calyciflorus* populations incubated with and without the ostracod *H. incongruens* (~1,500 individuals/L). Values are the averages of three replicates ± standard deviations.

Repeating the experiment with ~1,000 *H. incongruens*/L, low mixing and a rotifer culture of *B. calyciflorus* and *B. rubens* showed their average densities reduced to 69.4% and 46.8% of those in the control. Enclosing the ~1,000 *H. incongruens*/L in 60 µm mesh filter bags (indirect contact) gave average densities of *B. calyciflorus* and *B. rubens* of 62.9% and 64.9% of the control. The growth rate of both rotifers was slower during the first four days in both treatments, and increased thereafter. A further experiment with highly mixed cultures of 1,000 and 2,000 *H. incongruens*/L either loose in the flasks, or enclosed in 60 µm mesh filter bags gave lower average densities of *B. calyciflorus* compared with controls than under low mixing conditions. Average population densities of *B. calyciflorus* incubated with 1,000 and 2,000 *H. incongruens*/L were 29.9% and 27.3% (direct contact), and 34.3% and 29.2% (indirect contact) of the controls. At the end of the five-day experiment, the density of *B. calyciflorus* in all the treatments was only 10% of the control (Figure 6).
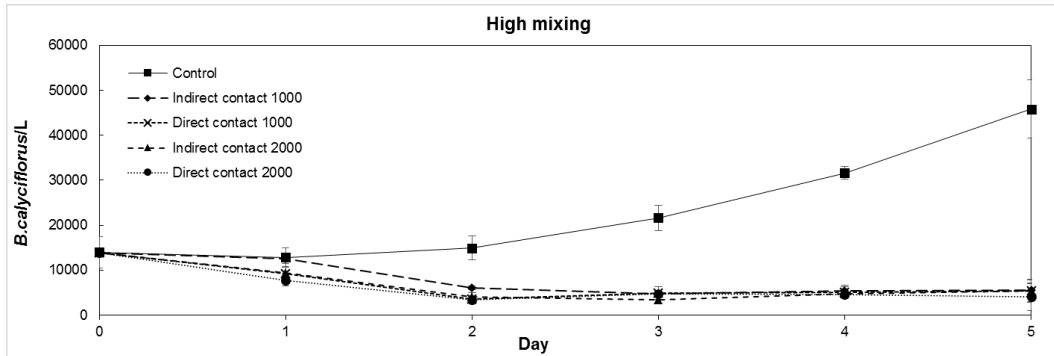
Figure 6 Changes in abundance of *B. calyciflorus* populations incubated directly with, indirectly with, and without (control) the ostracod *H. incongruens* (in concentrations of 1,000 and 2,000 individuals/L), in a highly mixed environment. Values are averages of three replicates ± standard deviations.

Under low mixing conditions, the percentage of rotifers carrying parthenogenetic eggs in the treatment and control were comparable and decreased similarly throughout the experiment (Figure 7). However, under highly mixed conditions, the percentages of rotifers carrying eggs increased from Day 3 in all the treatments and were 300% of the control by the end of the experiment (Figure 8).
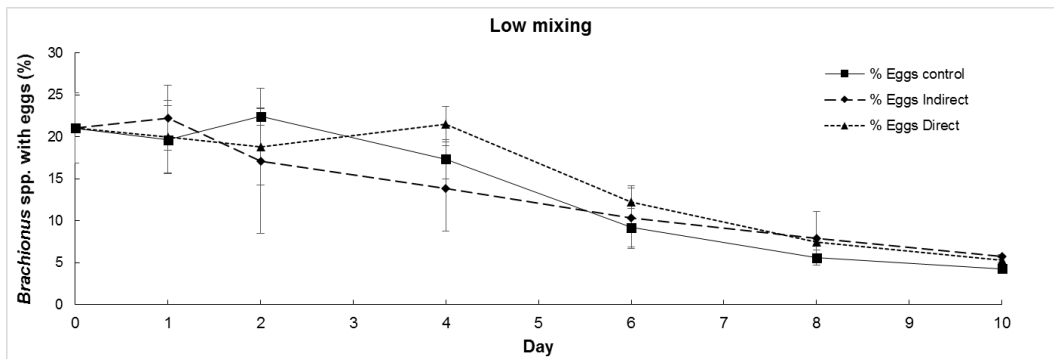
Figure 7 Percentage of individuals carrying eggs in rotifers populations (*B. calyciflorus* and *B. rubens*) incubated directly with, indirectly with, and without (control) the ostracod *H. incongruens*, under low mixing conditions. Values are averages ± standard deviations.

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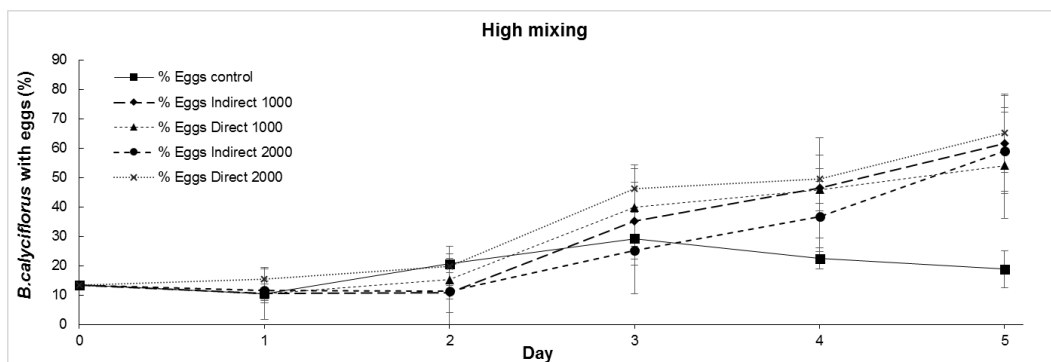
Figure 8 Percentage of individuals carrying eggs in *B. calyciflorus* populations incubated directly with, indirectly with, and without (control) the ostracod *H. incongruens* (in concentrations of 1,000 and 2,000 individuals/L), under high mixing conditions. Values are averages of three replicates ± standard deviation.

Cladoceran and rotifer control using hydrodynamic shear stress

Hydrodynamic shear stress reduced the density of zooplankton by mechanically damaging their body structure. The seven configurations of perforated plates promoted specific hydrodynamic parameters (Table 2), cavitation conditions were never achieved, and smaller total open surface areas of plates promoted higher pressure before the plate, higher fluid velocity, and likely harsher hydrodynamic shear stress.

Table 2 Different configurations of perforated plates with total open surface, flow rate, pressure before the plate, and fluid velocity through the perforated plate.

Orifice(s) (number and size)	Open Surface (mm²)	φ (m³/s)	Pressure before plate (Bar)	Velocity (m/s)
None	227.0	0.00034	0.0	1.5
1x9 mm	63.6	0.00032	0.0	5.1
9x2 mm	28.3	0.00031	0.6	10.8
4x3 mm	28.3	0.00030	0.8	10.7
1x6 mm	28.3	0.00029	1.0	10.2
5x2 mm	19.6	0.00026	1.5	13.5
25x1 mm	15.7	0.00025	1.9	15.9
1x4 mm	12.6	0.00022	2.6	17.3

The death rate of *M. tenuicornis* was always higher than that of *B. calyciflorus*, and the positive correlation between death rates of *B. calyciflorus* and the before-the-plate water pressure was stronger than that of *M. tenuicornis* (Table 1). The mildest treatment was achieved without

using the perforated plate and the orifice completely open (227 mm^2), and the hydrodynamic stress generated was sufficient to kill 65% of *M. tenuicornis* with a single passage. The use of any of the perforated plates caused death rates of *M. tenuicornis* of >95%. The death rate of *B. calyciflorus* was >50% only at higher levels of pressure before the plate, with the highest death rate (~80%) achieved using a perforated plate with a single orifice (ø 4 mm) (Figure 9).
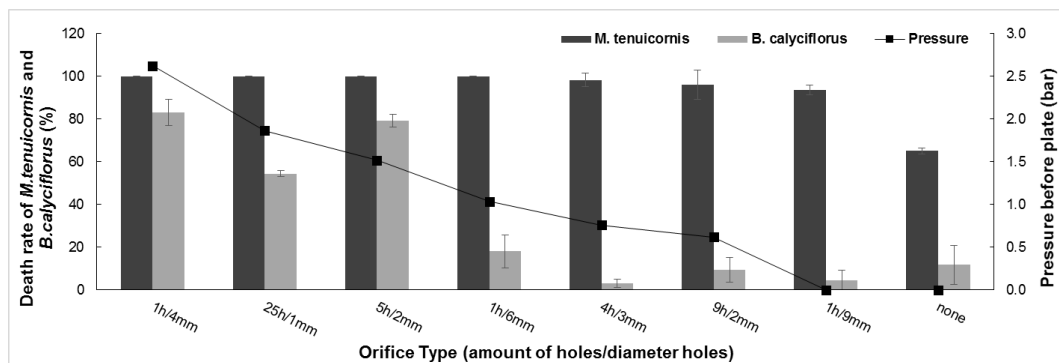
Figure 9 Death rates of *M. tenuicornis* and *B. calyciflorus* using different perforated plates (bars). The pressure of the liquid before the perforated plate is plotted on the second vertical axes (line). Values are the averages of three replicates ± standard deviations.

The hydrodynamic shear stress also caused the fragmentation of algae-bacteria flocs and the separation of cells from the colonies of *Micractinium* sp. and *Mucidosphaerium* sp., but not those of *Pediastrum* sp. (visual observation, Figure 10).
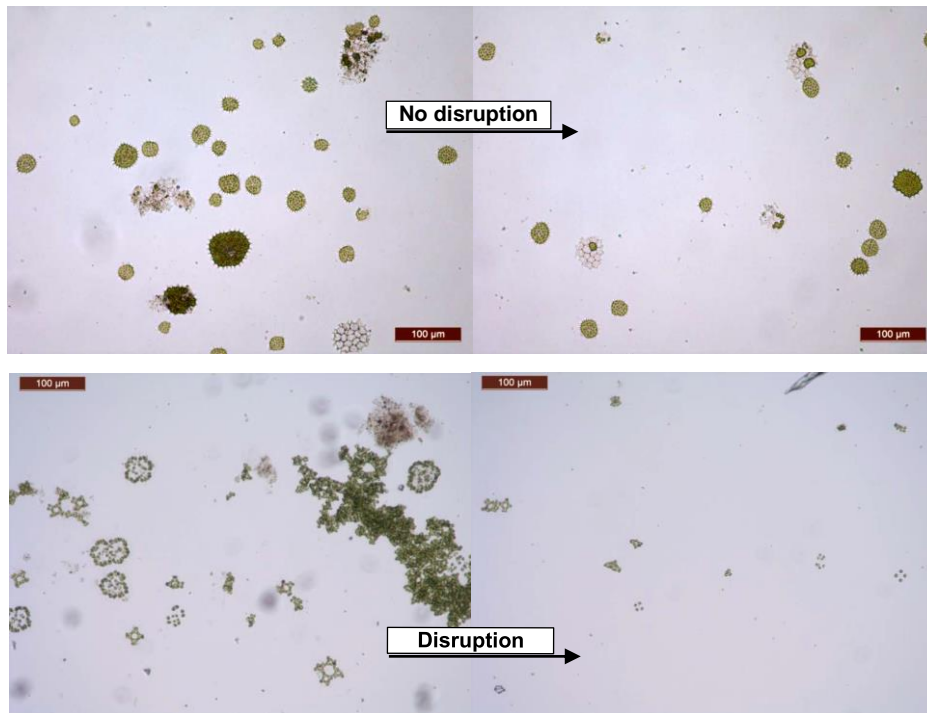
Figure 10 Effects of hydrodynamic shear stress on the colonial structure of *Pediastrum* sp. (top, no colony disruption), *Micractinium* sp. and *Mucidosphaerium* sp. (bottom, colony disruption).

M. tenuicornis CO_2 induced surface migration

Without CO_2 addition (~5.5 mg/L of CO_2) *M. tenuicornis* swam evenly throughout the entire 300 mm deep water column, spending minimal time at the surface (top 50 mm). However, a CO_2 concentration of just 25 mg/L caused the *M. tenuicornis* individuals to migrate to the surface of the water column where they spent on average 6,000s of the experimental period (Figure 11). CO_2 concentrations between 5.5 mg/L and 25 mg/L could not be assessed due to the limits of the valves used to control the flow rate of CO_2 and N_2. As CO_2 concentration was increased above 25 mg/L up to 2,000 mg/L, the duration of time individuals spent at the surface progressively declined (Table 1). Higher CO_2 concentrations caused individuals to become immobilised in less time and subsequently sink to the bottom of the water column and die. When individuals descended only a few millimetres (10-40 mm) from the water surface, they were usually able to swim to the surface again for 3 or 4 times. However, when they descended deeper than 50 mm due to decreased swimming frequency (strokes), sinking was almost always irreversible. When the water was bubbled with

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pure N_2 to reduce the O_2 dissolved into the water, individuals of *M. tenuicornis* did not show preferential surface migration and swam throughout the entire water column.
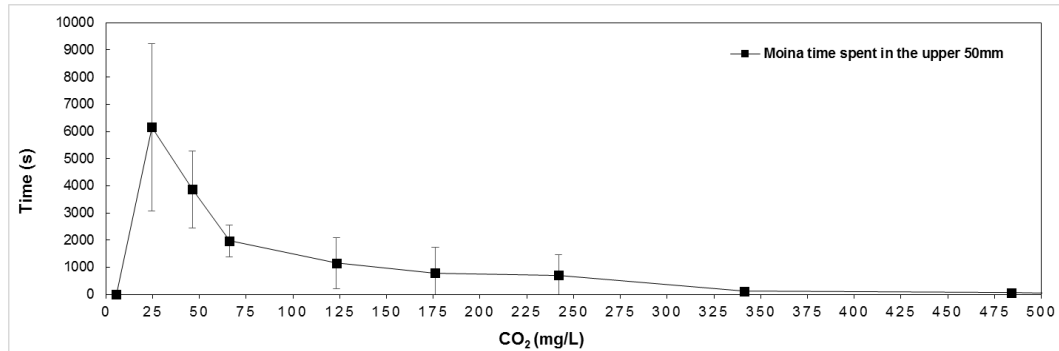
Figure 11 Average time that *M. tenuicornis* spent in the upper 50 mm of a 300 mm water column, at different concentrations of CO_2. Each data point is the average of five individuals assessed separately ± standard deviation.

DISCUSSION

Zooplankton control using CO_2 asphyxiation

CO_2 asphyxiation of *M. tenuicornis* occurred in less time than for rotifers despite cladocerans (particularly *Moina* species) typically being reported as having similar or greater tolerance to low O_2 levels than rotifers [62], [63], [64], [65]. Cladocerans transport O_2 using molecules similar to haemoglobin [66], and CO_2 can both directly and indirectly (by pH reduction) lower the O_2 binding capacity of these molecules [67]. Rotifers exchange gasses via diffusion [68] and through ingested water. The higher environmental $ppCO_2$ probably limited the capacity to release the CO_2 produced during respiration back into the water. The more complex mechanisms based on haemoglobin of cladocerans was likely more greatly affected than the simpler mechanisms based on gas diffusion of rotifers.

Mortality of *M. tenuicornis* required longer periods of asphyxiation than immobility. Immobilized *Moina* which still had a heart beat were able to survive after short periods of asphyxiation if rapidly transferred to water with low levels of CO_2 (e.g., pure tap water). However, the surviving individuals were often crippled and had impaired mobility, with greater damage associated with longer exposure to high $ppCO_2$. The high variability (e.g.,

standard deviation) of *Moina* mortality time at a CO_2 concentration of 330 mg/L suggests that individuals have a diverse tolerance to asphyxiation at lower concentrations of CO_2. The longer survival time of red adults and their ability to swim for longer periods of time compared to pale juveniles can be explained by the capacity of adult cladocerans to modify their haemoglobin concentration and hence affinity for O_2 according to the DO concentration of the surrounding water [69]. Red adults with higher concentrations of haemoglobin were likely more resistant to higher CO_2 concentrations, conversely, pale juveniles were unable to adapt as this may require up to 10 days [70], making them more vulnerable to higher concentrations of CO_2. In conditions of low O_2, red *Daphnia* that are adapted to live in a low O_2 environment have been reported to swim for longer periods of time and distances (>10 fold) relative to pale individuals adapted to normoxic environments [71]. It is likely that zooplankton mortality was a result of the high CO_2 concentrations rather than the low pH obtained with the treatments. This assumption is firstly based on previous studies on the cladoceran *Daphnia magna,* where high concentrations of CO_2 reduced the diffusion of CO_2 from the gills of *Daphnia* to the water, increasing blood CO_2 tension, and reducing the affinity of the blood for O_2 and the respiration rate of *Daphnia*. The diffusion of CO_2 from the gills of *Daphnia* into the water was inhibited by the high water CO_2 concentration and not by the low pH associated with the high CO_2 concentrations [72]. Secondly, zooplankton (especially rotifers) are resistant to pH as low as 4 [62], [73], [74], [75], and the minimum pH reached during this experiment was ~4.4. Lastly, low O_2 concentration (likely to be partially caused via stripping or displacement during CO_2 addition) was not expected to contribute to zooplankton mortality during this experiment, as previous work has shown that water bubbled with pure N_2 to reduce the O_2 concentration did not cause zooplankton mortality [61].

Injection of CO_2 into HRAPs has the potential to selectively kill different zooplankton species (e.g., cladocerans, followed by rotifers) by increasing CO_2 concentration and exposure time. Acute CO_2 treatment in full scale HRAPs could be implemented using the same hardware used for daytime CO_2 addition to overcome carbon limitation of photosynthesis. If used after

midnight, when microalgal/bacterial respiration has already increased pond water CO_2, acute CO_2 treatment may be cost effective if the CO_2 is available from exhaust or flue gases or as a byproduct of near-by industrial processes such as fermentation [8]. It is expected that in a full-scale HRAPs immobilised *Moina* would settle to the pond bottom and eventually die. Hence, assuming a 100% efficient sparging system (e.g., all the CO_2 bubbled is dissolved into the pond water) and a moderate degree of CO_2 outgassing (~10%/h), the eradication of *M. tenuicornis* in a 3,000 m^3 HRAP might be achieved overnight by a pond water CO_2 concentration of ~340 mg/L which would require ~1,000 kg of CO_2. Similarly, eradication of *B. calyciflorus* might be achieved overnight with a CO_2 concentration of ~530 mg/L and ~1,500 kg of CO_2. Further, the rapid mortality of the ciliate *Paramecium* sp. (less than 300 s) at low CO_2 concentrations (~150 mg/L) suggests that CO_2 treatment could be effectively used in systems where ciliates can promote severe biomass reductions, such as those that grow unicellular microalgae (e.g., *Dunaliella salina*) [23]. An alternative would be the use of chronic CO_2 treatments, with lower CO_2 concentrations over longer (several days) exposure times. However, these levels would need to be verified by pilot-scale trials since zooplankton sourced from different environments are adapted to different CO_2 concentrations and will have different mortality and immobility times at given concentrations of CO_2. Moreover, the assessment of the effect of high concentration of CO_2 on microalgae is essential to decide if the treatment can be used, and requires further investigation.

Rotifer control using the cladoceran *M. tenuicornis*

M. tenuicornis densities of ~1,500 individuals/L reduced the population of the smallest rotifer *B. rubens* (~100 µm in diameter), whereas densities of >2,500 individuals/L were required to reduce the population of the larger rotifer *B. calyciflorus* (~300 µm in length). These results are in agreement with the higher inhibition that large cladocerans exert on smaller species such as rotifers, than on larger species such as small cladocerans [76], [77]. The delayed bloom of *B. calyciflorus* was likely a consequence of the reduction of *M. tenuicornis* density (Day 15). The lower density of *B.*

calyciflorus in the treatment compared to the control between Day 7 (beginning of the exponential growth of rotifers) and Day 15, suggests that densities of *B. calyciflorus* are expected to be low in HRAPs with HRTs ≤8 days and a comparable biocontrol in place. The reduction of rotifers is expected to be enhanced in continuous cultures where the liquid (and individuals) is continuously removed from the culture. The establishment of *Moina* populations in microalgal cultures has the potential to provide an effective biocontrol of rotifers, especially smaller species, which are more difficult to control by mechanical treatment (see 3.4). Being a large organism, *Moina* density may be easily controlled using filtration. However, the permanent establishment of Moina in HRAPs may be difficult to achieve, and Moina may need to be inoculated and maintained at moderate densities during periods when rotifers have the potential to reach harmful densities for HRAP performance such as spring and early summer [13]. Crashes of a *Moina* population could be avoided by preventing overcrowded cultures with resulting accumulation of metabolites, toxins, reduced food quality, and production of resting eggs [78], [79]. This could be achieved by removing individuals (e.g., using filtration) to maintain a moderate population density. Although *Moina* is expected to exert only a moderate grazing pressure on desirable colonial microalgae because they preferentially feed on smaller particles, the rate of consumption of colonial microalgae still needs to be quantified.

Rotifer control using the ostracod *H. incongruens*

Incubation of a population of ~1,000 *H. incongruens*/L either loose in the flasks (direct contact) or enclosed in 60 µm mesh filter bags (indirect contact) with rotifers reduced the average density of the rotifer populations compared with the controls. Rotifer inhibition via competition for shared food can be excluded as a potential cause because the concentration of microalgal species ingested by rotifers in the treatment was always higher than in the control. The similar reductions of *B. calyciflorus* found with both direct and indirect contact with *H. incongruens* suggests that inhibition of large rotifers was probably mainly due to chemicals (e.g., infochemicals or metabolites) released into the water by the ostracods, and to a lesser extent

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by direct predation or mechanical disruption. It is likely that metabolic products released into the water reduced the hatching rate of the rotifers' eggs [80], [81], [82], since in the high mixing experiments with both direct and indirect contact with *H. incongruens*, rotifer populations had more individuals carrying eggs (>3 fold) than those in the controls.

The greater reduction in the density of the small rotifer *B. rubens* than the larger *B. calyciflorus* under direct contact with ostracods was likely due to a higher contribution that direct predation and mechanical disruption had on the reduction of the smaller, more ingestible rotifer. The higher reduction of rotifers and higher percentage of individuals carrying eggs observed with more mixing were probably caused by the greater contact between rotifers and ostracods (which being heavy tend to stay on the bottom), and better diffusion of chemicals into the liquid. Higher concentrations of ostracods (~2,000 individuals/L) promoted only slightly lower densities of rotifers compared to the lower concentrations of ostracods (~1,000 individuals/L), indicating that ~1,000 ostracods/L were nearly sufficient to exert the maximum effect.

The establishment of an ostracod population in microalgal cultures proved to be very effective in reducing the growth of rotifers and offers a potential option for biocontrol of rotifers in full scale HRAPs. Ostracods are bottom dwelling organisms that can be easily retained in the HRAPs [13], and are not expected to consume suspended microalgae because they preferentially feed on more digestible detrital solids [83] (that are abundant in HRAP sediment). Moreover, HRAPs are mixed systems that would promote the diffusion of chemicals and the contact between rotifers and ostracods, favouring the treatment. In an 8 m^3 pilot HRAP *H. incongruens* naturally occurred at densities associated with reduced rotifer populations (>100,000/L of sediment) for 2-3 months [13], and it is likely that inhibiting concentrations of ~1,000 ostracods/L can be achieved in hectare scale HRAPs.

Further research should assess the capacity of ostracods to inhibit rotifer populations for longer periods of time (seasons) with HRT and physicochemical conditions similar to those of operating HRAPs. Further, the effects of a permanent population of ostracods on the dominance and

productivity of suspended microalgae, and the mechanism(s) involved in rotifer inhibition all warrant further investigation.

Zooplankton control using hydrodynamic shear stress

Hydrodynamic stress was very effective in the elimination of cladocerans, and to a lesser extent rotifers. Zooplankton were likely killed by a combination of collision impact, turbulence and bubble explosion shock waves [46], [47]. Probably the larger, more rigid and more complex body structure of the microcrustaceans was more easily damaged than the more flexible body of rotifers. Perforated plates that promoted higher pressure before the plate and higher fluid velocity through the plate, likely generated harsher shear forces that caused higher mortality of zooplankton. The treatment also caused the disruption of microalgal-bacterial flocs and colonies of microalgal species with weakly bound cells such as *Micractinium* sp. and *Mucidosphaerium* sp., although colonies of *Pediastrum* sp. that have cells that are fused to each other were not disrupted. The treatment of a 1.25 ha HRAP (channel width of 12 m, depth of 0.3 m, horizontal flow of 0.2 m/s, [84]) in a single pass, would require a pumping capacity of 2,500 m^3/h, achievable with two commercial centrifugal pumps. Hydrodynamic stress is likely to be an ideal 'emergency treatment' for rapid control of cladocerans, and to a lesser extent larger rotifers. However, the eradication of larger zooplankton species (e.g., cladocerans) may decrease the competition in terms of shared food and mechanical interference, resulting in higher densities of smaller species such as rotifers that are potentially more difficult to control [13]. Further research should assess the effectiveness of using hydrodynamic stress over longer durations, under HRTs and physicochemical conditions of HRAPs, and the effects on microalgae growth, colony disruption, and biomass settleability. The treatment intensity and duration should also be determined for each zooplankton species typical of WW HRAPs.

M. tenuicornis CO_2 induced surface migration

The migration of *M. tenuicornis* to the water surface at low CO_2 concentrations (~10% of that required for mortality) probably resulted from

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the individuals trying to reach surface waters which naturally would have a lower ppCO$_2$ and a higher concentration of O$_2$. Previous research found that when *Moina micrura* was placed in experimental chambers under conditions of declining O$_2$ concentration (from 10 mg/L to 1 mg/L), their swimming activity initially increased, and then subsequently decreased at lower O$_2$ concentrations [85]. This was likely due to changing the allocation of energy from locomotion to feeding (e.g., decreasing swimming strength and frequency to invest more energy into feeding). Anoxia and CO$_2$ asphyxiation perhaps induce similar responses, and the low CO$_2$ increase in swimming activity could be a strategy to prevent sinking and reach the water surface. The lack of surface migration when the cylinder water was bubbled with pure N$_2$, suggests that surface migration was mainly promoted by the higher ppCO$_2$ rather than the low O$_2$ concentration. The decline in *Moina* swimming activity with greater depth in the water column was likely due to higher ppCO$_2$ and increased asphyxiation. Different levels of hemoglobin among individuals (pale and red) may explain the high variability of time spent at the surface at lower concentrations of CO$_2$ (~25 mg/L). CO$_2$ injection at a ~25 mg/L concentration could be used to concentrate cladocerans in the upper layer of the water column of HRAPs, so that filtration and cavitation treatments only need to be performed on the upper portion of the water column, reducing the amount of water processed and the overall treatment costs.

Selection of treatments

The treatments assessed in this study have different capital and operation costs, simplicity of application, and effectiveness at zooplankton control (Table 3). Selection of the most appropriate treatment method depends mainly on controlling the zooplankton species that most affect HRAP productivity and nutrient removal; the typical frequency of zooplankton contamination, and the available budget. CO$_2$ asphyxiation has the potential to be selective and effective on all zooplankton groups although it may be costly if unavailable locally as a waste product. Biocontrol has the potential to be effective for rotifer control, is virtually costless, and is the most environmentally friendly control method. However, establishing and

maintaining populations of cladocerans for long period of times in HRAPs may be difficult and the establishment of ostracods would be an easier option. Mechanical hydrodynamic shear stress is ideal for the control of cladocerans, although it may require large amounts of energy to control rotifers, particularly smaller species, and the capital cost for high capacity pumps may be high. However, it has the potential to be rapid, consistent, and easy to apply.

Table 3 Relative expected capital and operation cost, simplicity of application, and target organisms of different treatments. Costs are sourced from Chapter 2.

Treatment	Expected capital cost	Expected operation cost	Application	Target organisms
CO₂ asphyxiation	Medium-Low	High-Low	Easy	All species
M. tenuicornis biocontrol	Low	Low	Difficult	Rotifers
H. incongruens biocontrol	Low	Low	Medium	Rotifers
Hydrodynamic shear stress	High	Low	Easy	Mainly cladocerans

CONCLUSIONS

This study indicates that treatments such as acute injection of CO_2, hydrodynamic shear stress, and biocontrol using populations of ostracods or cladocerans are promising options to control typical contaminant zooplankton species of wastewater treatment HRAPs. In particular: 1) Acute injection of CO_2 was more effective in controlling cladocerans than rotifers and the treatment has the potential to be used to selectively kill specific zooplankton species according to their tolerance to high $ppCO_2$; 2) The cladoceran *M. tenuicornis* is a potentially effective option for biocontrol of rotifers, particularly smaller species, while the ostracod *H. incongruens* can reduce rotifer populations under high mixing conditions; 3) Hydrodynamic shear stress effectively killed cladocerans, and to a lesser degree rotifers, offering a treatment to selectively kill cladocerans; and 4) Exposure to moderate concentrations of CO_2 could be used to cause *M. tenuicornis* to migrate to the upper layer of the water column. Further research is required to assess the long term effectiveness of the treatments on zooplankton populations under physicochemical (nutrient concentration, pH, temperature, light radiation), and operational (HRT, mixing, CO_2 addition) conditions typical of WW HRAPs. This would include determining the effects on the microalgal community, productivity, settleability, and nutrient removal. Since the infrastructure to add CO_2 may already be used to promote pond treatment and productivity, CO_2 asphyxiation is particularly worth further investigation.

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CHAPTER 5

ASSESSMENT OF POTENTIAL ZOOPLANKTON CONTROL TREATMENTS FOR WASTEWATER TREATMENT HIGH RATE ALGAL PONDS

ABSTRACT

Cladocerans and rotifers rapidly consume beneficial microalgae and reduce the performance of High Rate Algal Ponds (HRAPs) for wastewater treatment and algal production. Potential zooplankton control treatments for HRAPs have been proposed and tested at a laboratory scale including CO_2 asphyxiation, biological control using competitor species, filtration, and mechanical disruption using hydrodynamic shear stress. This paper aims to validate these treatments using outdoor mesocosms with physicochemical and operational conditions similar to those of full scale HRAPs. A continuous CO_2 concentration of ~100 mg/L maintained low pond water zooplankton densities, while a continuous concentration of ~180 mg/L killed all microcrustaceans and rotifers present. As biocontrol agents, the cladoceran *Moina tenuicornis* at ~2,000 individuals/L reduced average rotifer densities by 90% while the ostracod *Heterocypris incongruens* at ~1,000 individuals/L removed all rotifers. Mechanical filtration using 300 μm and 500 μm filters eradicated *M. tenuicornis* after one and four filtration periods, respectively. Mechanical hydrodynamic stress killed up to 100% of microcrustaceans, and ~50% of larger rotifers. Furthermore, phototaxis-induced migration promoted higher densities of *Moina* in the upper layer of the water column in an 8 m^3 HRAP during periods of low solar radiation, suggesting that mechanical treatments should be performed at night and to the upper layer of the pond water. Overall, CO_2 asphyxiation appeared to be the most reliable, versatile, and effective zooplankton control treatment.

Keywords: Zooplankton control, High Rate Algae Ponds, CO_2 asphyxiation, Hydrodynamic stress, Filtration, Grazers' biocontrol.

INTRODUCTION

Zooplankton control in wastewater treatment HRAPs

High Rate Algal Ponds (HRAPs) with artificial CO_2 addition are simple reactors to reclaim nutrients and energy from wastewater (WW) as algal biomass, and provide higher productivity and nutrient removal compared to traditional pond systems [1], [2]. However, being open systems with near neutral pH and high food concentration, HRAPs are particularly susceptible to contamination with zooplankton species that can establish and survive at high densities in the wastewater environment. When high densities of the zooplankton species consume the dominant microalgal species, they can reduce the microalgal biomass and negatively affect HRAP performance [3], reducing both productivity and nutrient removal [4].

The necessity of treatments to control zooplankton populations in full scale HRAPs is widely recognized [5], [6], [7]. However, it may be more beneficial to control zooplankton to low densities rather than completely eradicate them [4]. In particular, the eradication of larger zooplankton species such as cladocerans may reduce competition for shared food resources and mechanical interference, which naturally limit densities of less desirable smaller species, such as rotifers, that generally are more difficult to control [4], [8]. Furthermore, moderate densities of filter feeding zooplankton species such as the rotifer *Brachionus* spp. and cladoceran *Moina* spp. can be beneficial by altering algal morphology to forms that enhance biomass harvestability [4].

Montemezzani et al. (2015) proposed potential options to control zooplankton in HRAPs [7]. Treatments included mechanical treatments such as filtration, hydrodynamic cavitation, shear stress and bead mills; chemical treatments such as CO_2 asphyxiation, promotion of the lethal un-ionized ammonia toxicity, use of biocides, and the chitinase inhibitor chitosan; and biocontrol treatments including competitor and predatory organisms such as the cladoceran *Moina tenuicornis*, the ostracod *Heterocypris incongruens*, and species of the carnivorous rotifer *Asplanchna*. Acute (<60 min) CO_2 asphyxiation, biocontrol of rotifers using the cladoceran *M. tenuicornis* and the ostracod *H. incongruens*, and

mechanical hydrodynamic disruption of zooplankton have been tested at a laboratory scale [8], and showed potential for zooplankton control in HRAPs. Acute injection of CO_2 into the wastewater resulted in more rapid asphyxiation of cladocerans than rotifers, showing potential for use to selectively control particular types of zooplankton. Biocontrol using *M. tenuicornis* was effective in reducing smaller species of rotifers while biocontrol using *H. incongruens* severely reduced the densities of all rotifer species, particularly in high mixing conditions to ensure they were brought into contact. Hydrodynamic shear stress was more effective in killing cladocerans (by disrupting their large, brittle exoskeleton) than smaller soft-bodied rotifers, showing the potential to select for larger zooplankton species. The implementation of these treatments into full-scale HRAPs requires further validation under physicochemical (nutrient concentration, pH, temperature, light radiation), and operational (hydraulic retention time (HRT), mixing, CO_2 addition) conditions typical of WW HRAPs. Here we assessed the chronic (1-2 month) CO_2 asphyxiation of zooplankton, the biocontrol of rotifers using the cladoceran *M. tenuicornis* and the ostracod *H. incongruens*, and the hydrodynamic disruption of zooplankton using outdoor mesocosms operated with typical WW HRAP conditions. The mechanical control of *M. tenuicornis* by filtration was also tested. Chronic CO_2 injection was tested instead of the acute injection used in previous laboratory experiments [8] as we expected this alternative treatment strategy to maintain more stable long-term HRAP performance and reduce the amount of CO_2 required for zooplankton control.

All the treatments tested were chosen based on their minimal negative environmental impact; potential selectivity for particular zooplankton taxonomic groups; cost effectiveness, and lack of effects on the beneficial zooplankton communities present in downstream maturation ponds of WW HRAP systems. We exposed cladoceran and rotifer populations to increasing chronic CO_2 concentrations and hydrodynamic stress intensities, incubated zooplankton populations with specific densities of *M. tenuicornis* and *H. incongruens*, and used different filter sizes to remove *M. tenuicornis*. Zooplankton control treatments were assessed in terms of the magnitude of zooplankton reduction, the changes in microalgal

biomass concentration, productivity, relative abundance, and settleability. Phototaxis-induced vertical migration of *M. tenuicornis* was demonstrated in the water column of an 8 m^3 HRAP with the aim of only applying mechanical treatments to the surface (zooplankton dense) portion of the pond, to reduce treatment time and costs.

MATERIAL AND METHODS

Experimental set up

Each experiment was conducted with triplicate treatments and controls using outdoor 20 L mesocosms. Mesocosms had a water depth of 300 mm, liquid volume of 16 L, and water surface area of 0.06 m^2. They were foil-wrapped to prevent light penetration through the sides, mixed and aerated with aquarium air stone spargers (100 x 15 mm), using a Hailea ACO 160W air pump, with maximum flow rate of 145 L/min and 160 W power. The flow rate was ~10 L/min per mesocosm and the air bubbles were sufficiently large to avoid their entrapment under the carapace of cladocerans and resulting flotation of individuals. The mesocosms were located at the Ruakura Research Centre, Hamilton, New Zealand (37°46'29.5"S - 175°18'45.4"E), adjacent to two 8 m^3 pilot-scale WW HRAPs (West and East) which were the source of microalgae and zooplankton used in the experiments. The pilot-scale WW HRAPs were single-loop raceways with semi-circular ends lined with black high-density polyethylene (HDPE) plastic, with a depth of 300 mm, volume of 8 m^3, mixed with an 8 blade steel paddlewheel (1 m wide), average surface velocity of 0.15 m/s, pH controlled between 7 and 8 by addition of CO_2, and a HRT of 8 days achieved by addition of 1 m^3/d of settled domestic WW. The pH of mesocosm cultures was maintained between 7 and 8 by continuous addition of ~0.2% CO_2 v/v in air. A four day HRT was used during January (Austral summer) (CO_2 summer experiment) and an eight day HRT was used during March-April and July-September (Austral autumn, winter, and spring) (all remaining experiments), which were achieved with daily (~9 am) replacement of 2 and 4 L of mesocosm culture with primary settled WW.

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Specific treatment conditions

Zooplankton control using CO$_2$ asphyxiation

Different intensities of zooplankton CO$_2$ asphyxiation were achieved by continuous (chronic) injection of CO$_2$/Air gas mixes with different percentages of CO$_2$ (0.5%, 2%, 5%, and 10%) into the mesocosm cultures. The control mesocosms were injected with air, and the experiment was performed twice: initially during summer (21 days, 10/01/2014 - 30/01/2014), and then during winter (62 days, 15/07/2014 - 15/09/2014). The starting microalgae and zooplankton cultures were collected on 09/01/2014 (summer) from the East HRAP, and on 15/07/2014 (winter) from the West HRAP. Different ponds were used to have a microalgae consortium composed of colonial species similar to that of WW HRAPs [9]. CO$_2$ concentration and pH were monitored and adjusted three times per day (09:00 am, 12:30 pm and 04:00 pm) using a gas analyser (Biogas 5000, Geotech), and pH meter (TPS WP-91, TPS Pty. Ltd., Springwood Australia). Ammonia concentration was determined twice per week in the first month, and once per week during the second month (winter experiment) using standard methods [10]. In the summer experiment, algal biomass, relative abundance, settleability, MCSA (Maximum Cross-sectional Area), and zooplankton abundance, were assessed every Monday and Thursday as described in the sampling protocol (below). In the winter experiment, the sampling frequency was identical until day 27. After this time, samples were collected weekly (Monday). The concentration of CO$_2$ in 100 mL samples of the mesocosm cultures was assessed by titration of the carbonic acid formed by CO$_2$ with NaOH standard solutions and phenolphthalein (0.5%) indicator until colour change at pH 8.3 [10], [11].

Rotifer control using the cladoceran M. tenuicornis

The inhibitory effect of *M. tenuicornis* on rotifers was assessed by inoculating *M. tenuicornis* into a microalgae culture sourced from East HRAP on 16/03/2015. This was dominated by *Mucidosphaerium* sp. and had a mixed rotifer population (*B. calyciflorus, C. catellina, F. longiseta,* and bdelloid rotifers). The experiment was conducted over 28 days (16/03/2015 - 13/04/2015). *M. tenuicornis* were sourced from the maturation ponds of

the HRAP using a 500 μm net. Mesocosm densities were maintained at >1,500 individuals/L by daily counting of 5 mL subsamples and addition of individuals when necessary. The average density of *M. tenuicornis* in the treatment mesocosms over the entire experiment was 2,166 ± 1,252 individuals/L. The control mesocosms did not contain *M. tenuicornis*. Algal biomass and settleability were measured, and zooplankton identified and counted, every Monday and Thursday as described in the sampling protocol (below). Algal identification, relative abundance and MCSA (Maximum Cross-sectional Area) were measured every Monday as described in the sampling protocol (below).

Rotifer control using the ostracod H. incongruens

The inhibitory effect of *H. incongruens* on rotifers was assessed over 28 days (16/03/2015 - 13/04/2015) by inoculating the same microalgae culture used in previous experiment with *H. incongruens*. The ostracods were sourced from the sediment of the West HRAP on 13/03/2015 and separated from the organic sludge using a 500 μm mesh filter and high water pressure from the sink head directed through the filter. Ostracods were recovered in the form of a wet paste, and at the beginning of the experiment each mesocosm was inoculated with 100 mL of wet paste (~30,933 individuals). Ostracods were enumerated by collecting a defined volume of wet paste, suspended in 500 mL of water, and subsampling (5 mL) the water after mixing. The density of *H. incongruens* in the mesocosms ranged between ~2,000 individuals/L (day 1) and ~1,200 individuals/L (day 28). To prevent the removal of ostracods during the daily replacement of water with primary settled WW, ostracods were separated using a 300 μm mesh filter and reintroduced into the mesocosms. The control cultures did not contain *H. incongruens*. Analyses and monitoring frequency were performed as described in the previous experiment and the sampling protocol.

M. tenuicornis control using filtration

The control of *M. tenuicornis* using filtration was assessed over a 35 day period (20/03/2014 - 24/04/2014) using a microalgae culture dominated by *Microactinium* sp. and *Pediastrum* sp. sourced from East HRAP on

20/03/2014. Zooplankton that were initially present in the treatment mesocosms were inactivated by sparging with pure CO_2 for 2 h. After 24 h the treatment mesocosms were inoculated with *M. tenuicornis* that had been collected from the HRAP system maturation ponds on 19/03/2014 using a 500 µm filter. *M. tenuicornis* were removed and counted from different triplicate treatment mesocosms daily after filtration using either 300, 500 or 800 µm filters. Two controls were set up in triplicate: 1) *Moina* inoculated but without control by filtration; 2) No *Moina* inoculated and without filtration. TSS, VSS, Chl-*a* concentration, and settleability were measured every three days. The relative abundances of rotifers and microalgae were assessed on days 1, 14 and 35. After day 14 the treatment mesocosms that were filtered with the 300 µm and 500 µm filters were discarded due to a lack of *M. tenuicornis*, the bloom of rotifers and depletion of microalgal biomass.

The effect of filtration on the retention of total suspended biomass on the filter was assessed on a microalgae culture collected from East HRAP and dominated by colonial *Micractinium* sp. (60%), *Mucidosphaerium* sp. (22%), and *Pediastrum* sp. (13%), with a laboratory test. Triplicate samples (1.0 L) of microalgae culture were filtered using 200, 300, 500, 800 and 1,000 µm filters. VSS, chlorophyll-*a*, MCSA (including bacterial flocs), and settleability of the filtrates were measured, and reductions were compared to the control (without filtering).

Zooplankton control using hydrodynamic stress
Zooplankton mortality from hydrodynamic stress was assessed by daily pumping of mesocosms filled with the same microalgae culture used in the "Rotifer control using the cladoceran *M. tenuicornis*" experiment through an aluminium plate (ø 20 mm) with one central orifice (ø 4 mm), using a pump (Lowara PSAM70/A) with 16 L/min maximum flow rate and 370 W power. The control cultures were not pumped. The perforated plate was located inside the 20 mm pipe connected to the pump outflow, and a pressure gauge was used to measure the pressure before the perforated plate (2.6 bar) for a discharge rate of 0.22 L/s and fluid velocity 17.3 m/s. The experiment was conducted over 28 days (16/03/2015 - 13/04/2015). Triplicate samples of 100 mL were collected before and after the treatment, total zooplankton

were counted, and death rate was assessed. Analyses and monitoring frequency were performed as described in the sampling protocol and in the "Rotifer control using the cladoceran *M. tenuicornis*" experiment. The percentage of surviving *B. calyciflorus* individuals that had detached eggs was also measured.

M. tenuicornis control in an 8 m³ HRAP using mild hydrodynamic stress

The effect of mild hydrodynamic stress was assessed on the East pilot-scale HRAP with an initial *M. tenuicornis* density of ~1,100 individuals/L, and dominant alga *Ankistrodesmus* sp. The HRAP water was pumped (Lowara Domo 15/B centrifugal pump, maximum flow rate: 600 L/min; power: 1.1 kW) through three 1 m lengths of 25 mm internal diameter alkathene pipe connected by two 90° elbows. The treatment was milder than that used in the previous mesocosm experiment as a perforated plate was not used. HRAP water was channeled into the pump inflow using a horizontal rectangular funnel (600 x 300 x 200 mm), and the outflow was discharged downstream. The discharge rate was ~105 L/min, meaning that a volume equivalent to the whole pond volume (8 m³) was treated in 75 minutes (one cycle). The HRAP water was sampled in front of the paddlewheel using a 2.5 L bucket that was dipped into the water down to 5 cm above the pond bottom and with the opening facing the paddlewheel (downstream). The death rate of *M. tenuicornis* was calculated including both dead and disrupted (fragmented into small pieces and not visible) individuals using equation (1). Where *a* was the total number of *Moina* before the treatment, *b* was the total number of visible dead *Moina* after the treatment, *c* was the total number of visible *Moina* after the treatment (visible dead and alive individuals, after inactivation of living individuals with two drops of Lugol solution 10% v/v).

$$\text{Death rate} = 100 - \left(\frac{c - b}{a} * 100 \right) \tag{1}$$

The number of *Moina* remaining alive after the treatment was calculated as *c-b*. The number of *Moina* disrupted (and not visible) during the treatment was calculated as *a-c*. Counting was performed 60 min after the treatment to allow for death, or recovery of partially damaged *Moina*.

Vertical distribution of M. tenuicornis in an 8 m³ HRAP

The vertical distribution of *M. tenuicornis* in an 8 m³ and 300 mm deep pilot-scale HRAP (West) constructed and operated as previously described by Montemezzani et al. (2016) [4], was assessed at 3 h intervals over 24 h of a sunny spring day (02/10/2013). Four filters were constructed from sections of plastic pipe (ø 50 mm, length 60 mm) with 500 µm filter material over the downstream end, and aligned vertically using laboratory stands and clamps at different depths (50, 135, 200, and 280 mm) measured from the pond surface to the centre of the filter. To recover sufficient *M. tenuicornis* biomass for the analyses without clogging the filter, the filters were introduced into the pond water for a period of only 20 s. The content of each filter was washed into pre-weighed aluminium trays and all floating and settled debris were removed using a pipette and tweezers with multiple washings with clean water. Samples were then oven dried at 85°C (270M Digital Series, Contherm) for a period of at least 24 h, cooled in a desiccator and weighed to measure the *Moina* biomass. The percentage of total *Moina* biomass occurring in the upper 50 mm of the water column was then calculated.

Sampling protocol and analyses

Microalgal biomass, settleability, zooplankton identification and counting

In all the experiments samples were collected from the centre of each mesocosm using triplicate 100 mL plastic beakers, after complete mixing of the liquid with circular and vertical movements using a 200 mL measuring cylinder with circular bottom (ø 80 mm) as a mixing tool. The total suspended solids (TSS) concentration was assessed using sub-samples from each 100 mL plastic beaker, by filtering a known volume of sample through a pre-rinsed, pre-combusted (450°C for 4 h), and pre-weighed 47 mm Whatman GF/F filter (nominal pore size 0.7 µm). Filters were then oven dried (85°C) overnight using a drying chamber (270M Digital Series, Contherm), and weighed on an analytical scale (SI-234, Denver Instruments). The ash weight was assessed by weighing the filters after combustion at 450°C for 1 h using a muffle furnace (F.E.KILN, RTC1000,

Bartlett Instrument Company, UK). Total organic matter (or volatile suspended solids: VSS) was calculated as the difference between the TSS and ash concentrations [12]. Biomass (mainly microalgae and bacteria) productivity was calculated based on the VSS concentration, taking into account rainfall and evaporation [13]. Evaporation, rainfall, and daily solar radiation were acquired from the NIWA National Climate Database (http://cliflo.niwa.co.nz/). The concentration of chlorophyll-*a* was determined using triplicate 10 mL subsamples which were filtered onto 25 mm Whatman GF/F filters (nominal pore size 0.7 μm). Chlorophyll-*a* was extracted in 100% methanol at 65°C for 5 min, followed by 12 h at 4°C in the dark. Samples were then centrifuged (Sorvall/Dupont General Centrifuge GLC-2B) at 3,000 rpm (RCF: ~1720 g) for 15 min and the absorbance of the supernatant was measured using a UV-Visible Shimadzu UV 1601 spectrophotometer. Chl-*a* concentrations were calculated using the modified trichromatic equations for methanol [14]. Biomass settleability was estimated by settling 1 L of the microalgae culture in an Imhoff cone over a 1 h period, and oven drying (85°C, overnight) the settled material in pre-weighed aluminium trays [10]. Settleability was calculated as the % of the total suspended biomass (TSS) that settled. Zooplankton were inactivated by bubbling pure CO_2 into 100 mL samples from each triplicate mesocosm, and counted in triplicate 5 mL aliquots using a gridded counting chamber and a Leica M50 stereo microscope. Zooplankton were identified according to [19] and [20]. The average maximum density of zooplankton in the treatment mesocosms at single sampling points was used to describe the variation of zooplankton density throughout the experiment. The capacity of treatments to reduce the density of zooplankton was described using the average density of zooplankton in the treatment mesocosms during the entire experimental period.

Statistical analyses were performed using Pearson's correlations between the different intensities of treatments: CO_2 concentration (%); filter size (μm); hydrodynamic stress events (n°), and mesocosm biological measurements: zooplankton density; biomass productivity; Chl-*a*; MCSA and settleability, using averages for the entire experimental period. The p-values used for significance were p=0.05 (statistical significance), and p=0.1

(moderate statistical significance). A p>0.1 indicated no statistical significance. Error bars in the figures are ± standard deviation calculated from the data of triplicate mesocosms.

Algal identification, relative abundance and average Maximal Cross-sectional Area

Algal species composition was determined from 1 mL subsamples that were settled in a 25 mm ø Utermöhl chamber. Three images per sample were taken in random fields of view using a Leica DM 2500 microscope (100x - field of view (ø) 1 mm), equipped with a digital Leica DFC 420 camera (Leica Microsystem, Switzerland), and the software Leica Application Suite (LAS version 4.1.0). Microalgae were identified to species level, where possible, according to taxonomic descriptions [15]. The relative abundance of microalgae was calculated by multiplying the average biovolume of each microalgal species by the total number of cells or colonies (depending on the species) counted in the three images. The average biovolume of each microalgal species was calculated using the equations proposed by Vadrucci et al. [16], and the methodology and assumptions of [4]. Changes in microalgal species composition, shape, and surface area of their cells/colonies were assessed by measuring the average surface area of suspended microalgal particles in their largest cross section (Maximum Cross-Sectional Area, MCSA (μm^2)) [4]. MCSA measurement was based on our observation that as microalgae settled in the Utermöhl chamber they generally presented their largest sectional area downwards. MCSA was used instead of biovolume because it can be rapidly calculated on a high number of samples using image analyses software. The average MCSA was calculated by measuring the surface area of all the particles of the three images previously used to assess the relative abundance of microalgae excluding particles <5 μm^2, and non-algal particles, using the freeware software 'ImageJ' V 1.43u. Generally, higher average MCSAs occur when colonial microalgae are dominant (e.g., *Micractinium* sp., *Pediastrum* sp., and *Mucidosphaerium* sp.) and cells and colonies are larger. Smaller average MCSAs occur when small unicellular microalgal species are dominant, or colonial species are small (e.g., *Coelastrum* sp.).

RESULTS

Zooplankton control using CO_2 asphyxiation

Higher percentages of CO_2 in the CO_2/Air gas mix bubbled into the mesocosms promoted higher dissolved CO_2 concentrations, lower pH (Table 1), and lower average densities of zooplankton (Table 2).

Table 1 HRT, pH, CO_2 concentration, VSS concentration, productivity, Chl-*a* concentration, MCSA, and settleability in mesocosms treated with different levels of CO_2 in a CO_2/Air gas mix (0.5%, 2%, 5%, and 10%) and control mesocosms (bubbled with air), in summer and winter experiments. Values are seasonal averages of triplicate repetitions and include standard deviations in brackets. The number of seasonal sampling sessions were: n=7 (summer), and n=14 (winter).

	CO_2 (%)	HRT (days)	pH range	CO_2 concentration (mg/L)	VSS (mg/L)	Productivity (g/m²/d)	Chl-*a* (µg/L)	MCSA (µm²)	Settleability (%)
Summer (10/01/2014- 30/01/2014)	Air	4	8.1 - 10.1	5.1 (1.3)	214 (27)	14.7 (2.3)	2840 (549)	209 (37)	30 (11)
	0.5	4	6. 8 -7.9	19.8 (4.4)	230 (40)	16.0 (3.1)	3035 (694)	190 (25)	49 (8)
	2	4	6.2 - 7.2	53.8 (8.6)	281 (56)	20.1 (3.9)	3773 (844)	201 (50)	42 (6)
	5	4	6.0 - 6.8	99.3 (2.7)	294 (54)	21.1 (3.5)	4696 (1287)	181 (52)	41 (15)
	10	4	5.9 - 6.3	180.9 (12.9)	315 (57)	22.7 (2.6)	5081 (1710)	118 (44)	23 (8)
Winter (15/07/2014- 15/09/2014)	Air	8	8.3 - 9.1	6.0 (0.9)	166 (52)	6.3 (1.8)	2133 (156)	137 (46)	32 (13)
	0.5	8	6. 8 -7.9	19.8 (4.4)	177 (58)	6.8 (2.0)	2470 (166)	122 (51)	38 (19)
	2	8	6.2 - 7.2	53.8 (8.6)	186 (65)	7.1 (2.2)	2420 (119)	97 (40)	38 (16)
	5	8	6.0 - 6.8	99.3 (2.7)	191 (81)	7.4 (3.0)	2255 (323)	77 (19)	35 (15)
	10	8	5.9 - 6.3	180.9 (12.9)	166 (67)	6.4 (2.5)	1548 (85)	81 (11)	37 (16)

Table 2 Average (Av) and maximum (Max) densities of zooplankton species in mesocosms treated with different levels of CO$_2$ in a CO$_2$/Air gas mix (0.5%, 2%, 5%, and 10%) and control mesocosms (bubbled with air), in summer and winter experiments. Values are seasonal averages of triplicate repetitions calculated including the initial zooplankton populations and include standard deviations in brackets. The number of seasonal sampling sessions were: n=7 (summer), and n=14 (winter). A lack of a species during a season is indicated with "-".

Season	CO$_2$ (%)	M. tenuicornis (ind./L) Av	Max	B. calyciflorus (ind./L) Av	Max	C. catellina (ind./L) Av	Max	F. longiseta (ind./L) Av	Max	Bdelloid rotifers (ind./L) Av	Max	Copepod (ind./L) Av	Max	Ostracod (ind./L) Av	Max	Rotifer (ind./L) Av	Max	Microcrustacean (ind./L) Av	Max
Summer (10/01/2014 - 30/01/2014)	Air	-	-	9095 (6566)	22000	3857 (6673)	19000	9543 (10310)	33000	29 (49)	0	371 (663)	533	-	-	25524 (14022)	25400	371 (663)	530
	0.5	-	-	18457 (16089)	46000	25562 (21166)	63000	22752 (12558)	40000	3286 (5637)	17000	429 (635)	533	-	-	70057 (41086)	136000	429 (635)	580
	2	-	-	11905 (7817)	25000	8762 (6727)	22000	15810 (3175)	21000	7352 (11298)	31000	391 (662)	600	10 (23)	70	43829 (17623)	68200	400 (657)	600
	5	-	-	10286 (9411)	12000	1457 (2634)	0	4876 (4717)	11800	2800 (4737)	14000	324 (664)	267	10 (23)	70	19419 (16630)	53500	333 (663)	330
	10	-	-	67 (118)	0	1105 (2706)	0	1695 (4153)	0	210 (487)	0	276 (677)	0	-	-	3076 (6902)	70	276 (677)	0
Winter (15/07/2014 - 15/09/2014)	Air	71 (71)	233	1274 (2800)	9300	0	0	-	-	26 (81)	300	-	-	31 (54)	133	1300 (2799)	9300	71 (71)	233
	0.5	164 (207)	733	3862 (8913)	26200	1624 (5663)	21300	-	-	5 (18)	70	-	-	29 (45)	133	5491 (13725)	48000	164 (207)	730
	2	76 (100)	400	714 (2328)	8700	2788 (10423)	39000	-	-	0	0	-	-	29 (37)	100	3502 (12734)	47800	76 (100)	400
	5	26 (40)	66	0	0	786 (2940)	11000	-	-	0	0	-	-	26 (37)	67	786 (2940)	11000	26 (40)	230
	10	7 (27)	0	0	0	0	0	-	-	0	0	-	-	26 (30)	67	0	0	7 (27)	130

In the summer experiment (average daily water temperature: ~21°C, Figure 1, F) the rotifers *B. calyciflorus*, *C. catellina, F. longiseta*, and bdelloid rotifers established in the mesocosms with % of CO$_2$ addition affecting individual species average densities (Table 2) and growth profiles (Figure 1). The maximum and average densities of zooplankton were measured in the treatment mesocosms with 0.5% of CO$_2$, and declined in the treatment mesocosms with higher % of CO$_2$. Treatments with 5% and 10% CO$_2$ were required to reduce zooplankton densities below those found with air (Table 2). Cladocerans did not establish, the cyclopoid copepod *Paracyclops*

fimbriatus and the ostracod *H. incongruens* were the only microcrustacean species present, although in very low densities (Table 2).
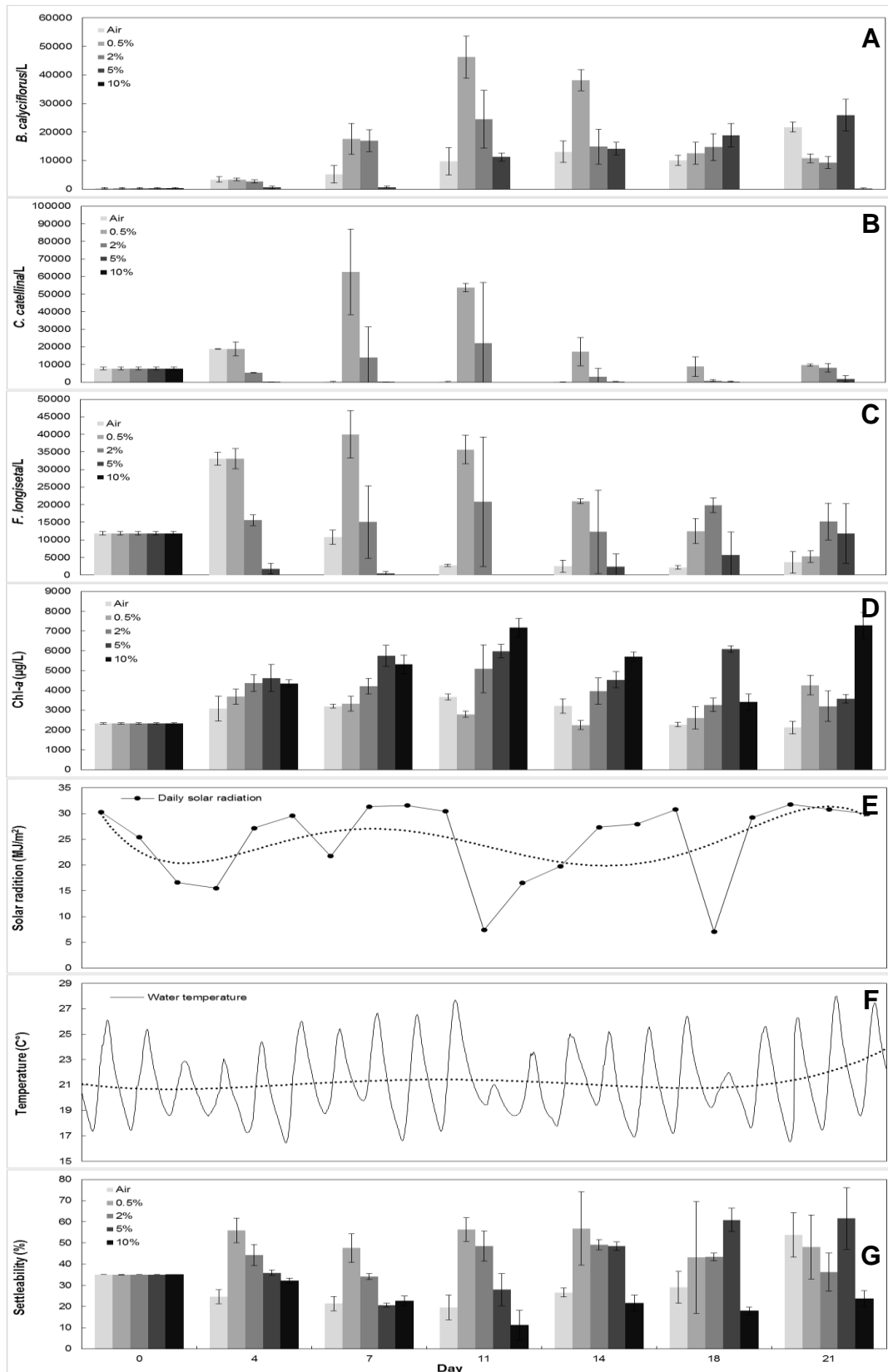
Figure 1 Density of the rotifers *B. calyciflorus* (A), *C. catellina* (B), and *F. longiseta* (C), Chl-*a* concentration (D), average daily solar radiation (E), water temperature (F), and biomass settleability (E), in triplicate mesocosms treated with different levels of CO_2 in a CO_2/Air gas mix (0.5%, 2%, 5%, and 10%) and control mesocosms (bubbled with air), in the summer experiment. Values are averages of triplicate mesocosms ± standard deviations.

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During the winter experiment the average daily water temperature ranged from 7°C to 17°C (Figure 2, F), there was no initial population of rotifers although the cladoceran *M. tenuicornis* was present (<100 individuals/L). Rotifers began to appear mainly in the 2% and 5% CO_2 treatment mesocosms from day 30 after which their densities gradually increased but were much lower than those recorded in the summer experiment (Table 2 and Figure 2). From day 30 the average water temperature began to increase (from 11°C to 17°C, Figure 2, F), and *B. calyciflorus* and *C. catellina* developed firstly in the control and 0.5% (day 34), then in 2% (day 55), and eventually in 5% (day 62) treatment mesocosms. The treatment mesocosms with 5% CO_2 and 10% CO_2 eradicated the population of *M. tenuicornis* initially present in less than 30 and four days, respectively, and prevented the establishment of any zooplankton species (Table 2). Similar to the summer experiment, zooplankton reached the highest maximum and average densities in the treatment mesocosms with 0.5% of CO_2, and declined in the treatment mesocosms with higher % of CO_2. During the summer experiment the percentage of CO_2 injected correlated negatively with the densities of total combined rotifers and individual rotifer species. During the winter experiment the percentage of CO_2 injected correlated negatively with only the total combined rotifer density (Table 5). The control was excluded from the correlation analysis of both experiments because the lack of CO_2 promoted high daytime pH (Table 1), and it is likely that free ammonia toxicity reduced the density of rotifers. The average concentration of $N-NH^+_4$ was 5.0±4.3 mg/L during the summer experiment, and 16.5±7.7 mg/L, during the winter experiment.
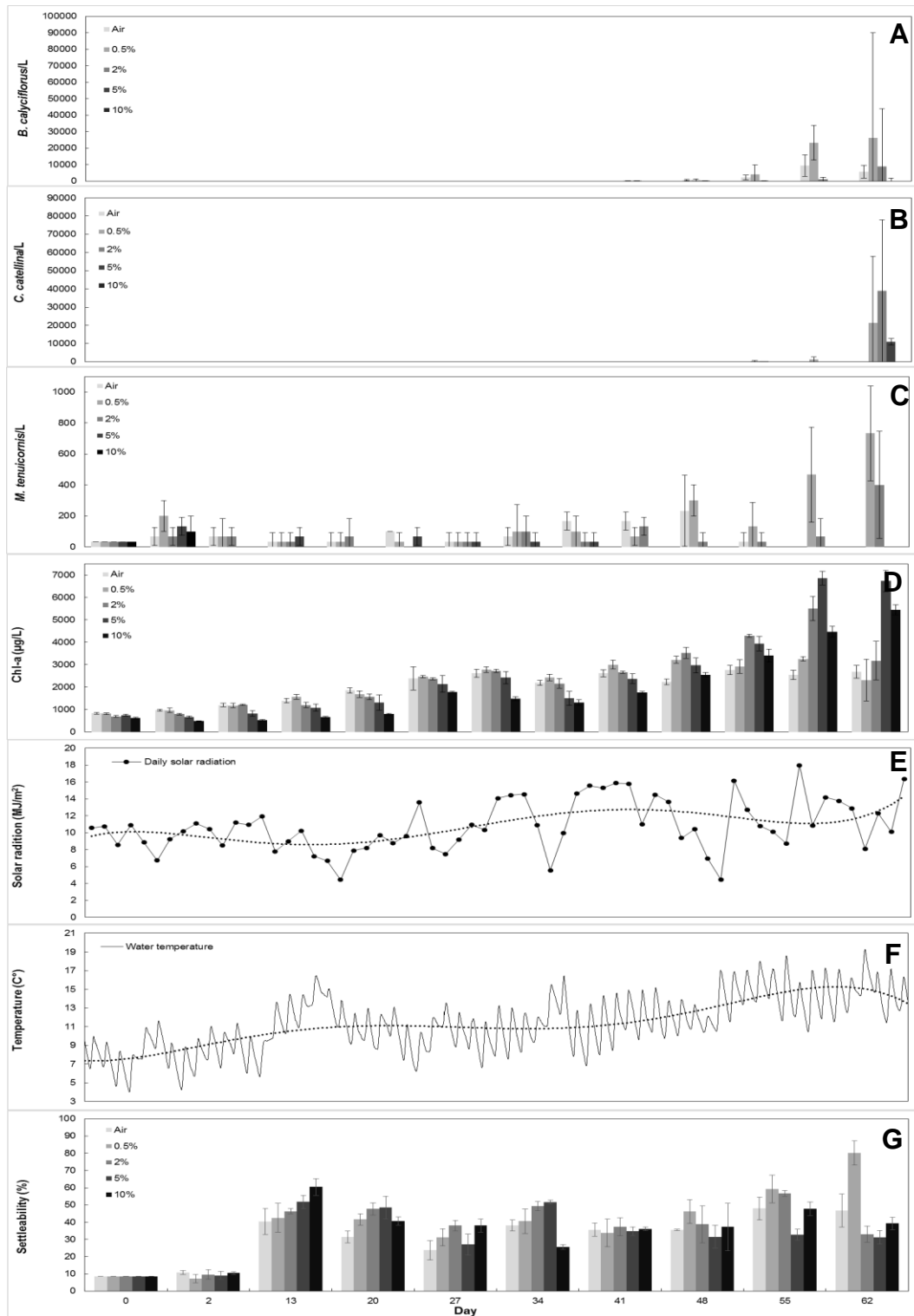
Figure 2 Densities of the rotifers *B. calyciflorus* (A), and *C. catellina* (B), the cladoceran *M. tenuicornis* (C), Chl-*a* concentration (D), average daily solar radiation (E), water temperature (F), and biomass settleability (G) in triplicate mesocosms treated with different levels of CO_2 in a CO_2/Air gas mix (0.5%, 2%, 5%, and 10%) and control mesocosms (bubbled with air), in the winter experiment. Values are averages ± standard deviation.

Microalgae concentration and biomass productivity

During the summer experiment the Chl-*a* concentration and productivity of treatment mesocosms increased after zooplankton density was reduced by CO_2 asphyxiation, and correlated positively with the percentage of CO_2 added (Table 5). The Chl-*a* concentrations of treatment mesocosms with 5% and 10% CO_2 were the highest, particularly after the zooplankton density in the control and in the mesocosms with 0.5 and 2% CO_2 treatments increased (day 7, Figure 1, D). The average Chl-*a* concentrations and productivities of the control and treatment mesocosms with 0.5% CO_2 were the lowest, and those of 10% were the highest (Table 1).

During the winter experiment, the Chl-*a* concentration of treatment mesocosms increased throughout the experimental period, and correlated positively with the average water temperature (r: 0.85, $p<0.05$, n: 14). In mesocosms where CO_2 asphyxiation reduced the density of zooplankton, the Chl-*a* concentration increased the most. The average Chl-*a* concentrations of treatment mesocosms with 5% and 10% CO_2 were the lowest of all treatments, although they became the highest after day 55, when zooplankton established in the control and treatment mesocosms with 0.5 and 2% CO_2 (Figure 2, D). The average productivities were similar for all the treatment mesocosms and slightly higher in the treatment mesocosms with 5% CO_2 (Table 1). Excluding the mesocosms with 10% CO_2, both average productivity and Chl-*a* concentration did correlate with the percentage of CO_2 added to the mesocosms (Table 5).

Microalgae dominance, settleability and MCSA

During the summer experiment mesocosms were initially dominated by the colonial algae *Pediastrum* sp. (37%), *Micractinium* sp. (52%), and *Coelastrum* sp. (10%). The control mesocosms did not show large variations of microalgae dominance, and at the end of the experiment *Pediastrum* sp. was 33%, and *Micractinium* sp. was 54% and had spines. In mesocosms with 0.5% and 2% CO_2 the relative abundance of *Pediastrum* sp. and *Micractinium* sp. were reduced to ~15% and ~5%, respectively, and they were replaced by the colonial alga *Coelastrum* sp. (up to ~55%). In mesocosms with ~5% CO_2 *Pediastrum* sp. was reduced to ~5%, and

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Micractinium sp. increased to ~84%. In mesocosms with 10% CO_2 *Pediastrum* sp. was depleted, and small colonies of *Micractinium* sp. increased to ~96%. Settleability was generally higher in mesocosms with larger densities of rotifers (Table 1, and Table 2), and peaked when the density of filter feeding rotifers such as *B. calyciflorus* was the highest (control, days 18-21; 0.5% and 2%, days 11-14; 5%, days 18-21) (Figure 1, G).

During the winter experiment, all mesocosms were initially dominated by *Micractinium* sp. (91%) which was gradually replaced by the colonial algal *Mucidosphaerium* sp. in the control, and mesocosms with 0.5%, 2% and 5% CO_2. During the last two weeks of the experiment, the algal relative abundance in mesocosms with 2% CO_2 decreased for *Mucidosphaerium* sp. (to 19%), and increased for the unicellular alga *Ankistrodesmus* sp. (to ~60%). The average settleability of mesocosms were similar (Table 1), although settleability increased in mesocosms with 0.5% and 2% CO_2 and to a lesser extent in the control, after zooplankton had established moderate populations (from day 48) (Figure 2, G). In both the summer and winter experiments, the average MCSA correlated negatively with the percentage CO_2 injected (Table 5).

Rotifer control using the cladoceran *M. tenuicornis*

When incubated with *M. tenuicornis* (treatment) the average densities of total combined rotifers and *C. catellina*, *B. calyciflorus*, and *F. longiseta* were respectively 13.8%, 7.3%, 56.7%, and 63.7% of the control mesocosms. The maximum peak density of *B. calyciflorus, C. catellina*, and *F. longiseta* in the control mesocosms were higher than in the treatment mesocosms (Figure 3, B-C-D). The average densities of the ostracod *H. incongruens* and the copepod *P. fimbriatus* in control and treatment mesocosms were similar at 778 individuals/L and 783 individuals/L; and 504 individuals/L and 479 individuals/L, respectively.
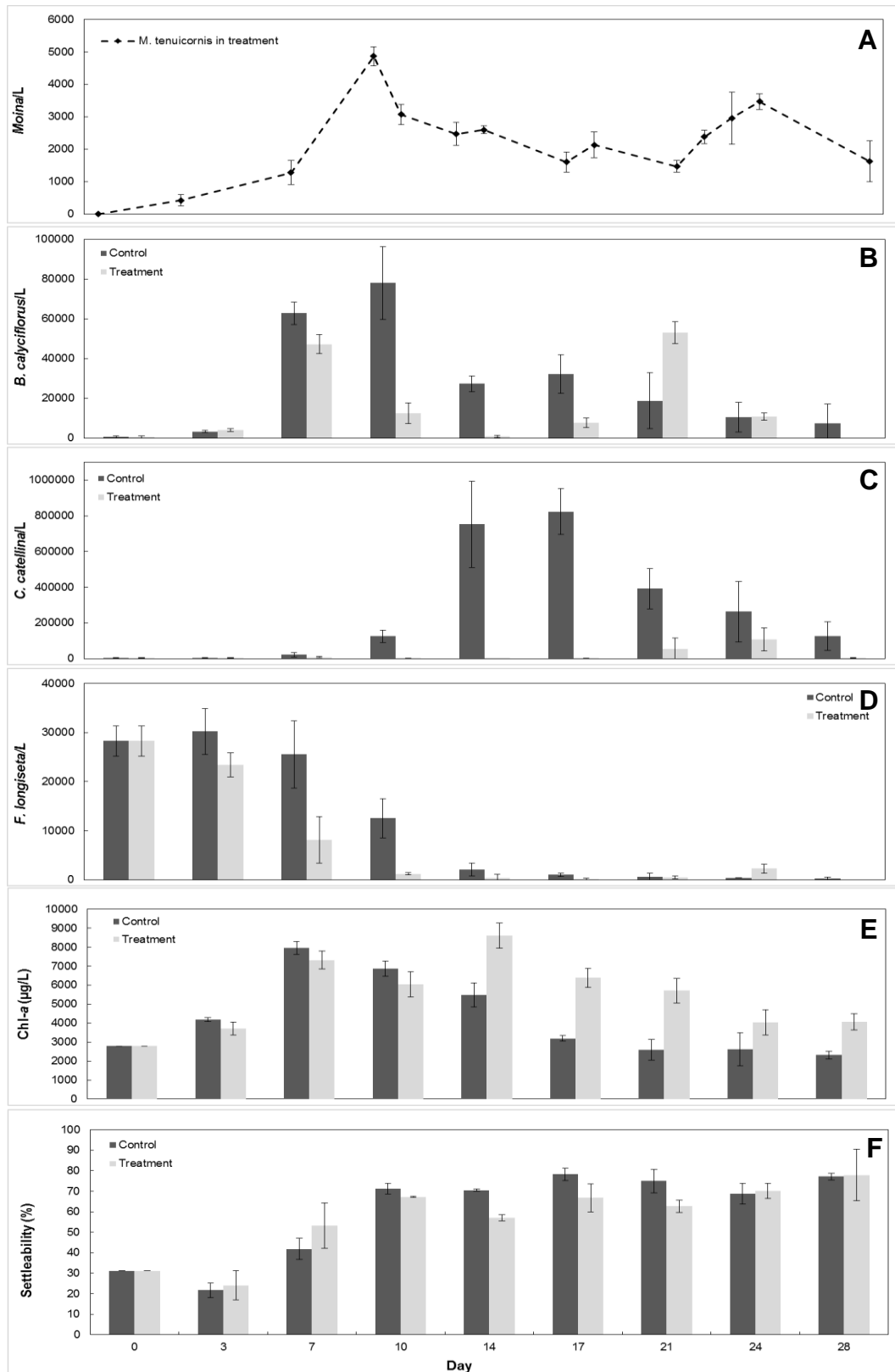
Figure 3 Density of *M. tenuicornis* in the treatment (A), and *B. calyciflorus* (B), *C. catellina* (C), *F. longiseta* (D), Chl-*a* concentration (E), and biomass settleability (F) in control and treatment (incubation with *M. tenuicornis*) triplicate mesocosms. Values are averages ± standard deviation.

Microalgae concentration and biomass productivity

The average Chl-*a* concentration of the control mesocosms (4,223±2,077 µg/L) was lower than that in treatment mesocosms (5,409±1,895 µg/L), and was greatly reduced after the rotifer density increased (day 14, Figure 3, E). The average productivity of the control mesocosms (11.9±3.3 g/m^2/d) was also lower than that in the treatment mesocosms (16.4±3.8 g/m^2/d).

Microalgae dominance, settleability and MCSA

In the control mesocosms the relative abundance of *Mucidosphaerium* sp. decreased from ~81% to 28%, and those of *Micractinium* sp. and the filamentous diatom *Staurosira* sp. increased from 2% to 37%, and from 1.5% to 17%, respectively. Small single-celled algal species always had a relative abundance of <10%. In the treatment mesocosms the relative abundances of *Mucidosphaerium* sp., the large single-celled algae *Closterium* sp., and *Micractinium* sp. remained at ~85%, ~5%, and ~5%, respectively, and smaller algal species were absent. The average settleability of the control cultures (60±22%) was similar to that of the treatment mesocosms (57±18%) throughout the entire experiment (Figure 3, F). The average MCSAs of the control mesocosms was 156±59 µm^2, and that of the treatment mesocosms was 172±50 µm^2.

Rotifer control using the ostracod *H. incongruens*

The average densities of total combined rotifers, *C. catellina*, *B. calyciflorus*, and *F. longiseta* incubated with the ostracod *H. incongruens* were 1.9%, 0.1%, 0.3%, and 48.8% of the control mesocosms. The average density of total rotifers in the control mesocosms was 316,800±308,815 individuals/L, and *B. calyciflorus, C. catellina*, and *F. longiseta* developed in high densities only in the control mesocosms (Figure 4, A-B-C). In contrast, the average density of total rotifers in the treatment mesocosms was only 5,896±10,391 individuals/L, and all species had been removed after seven days. The cladoceran *M. tenuicornis* had an average density of 114 individuals/L in the control mesocosms and did not establish in the treatment mesocosms. The average densities of the copepod *P. fimbriatus* in control and treatment mesocosms were 504 individuals/L, and 202 individuals/L.
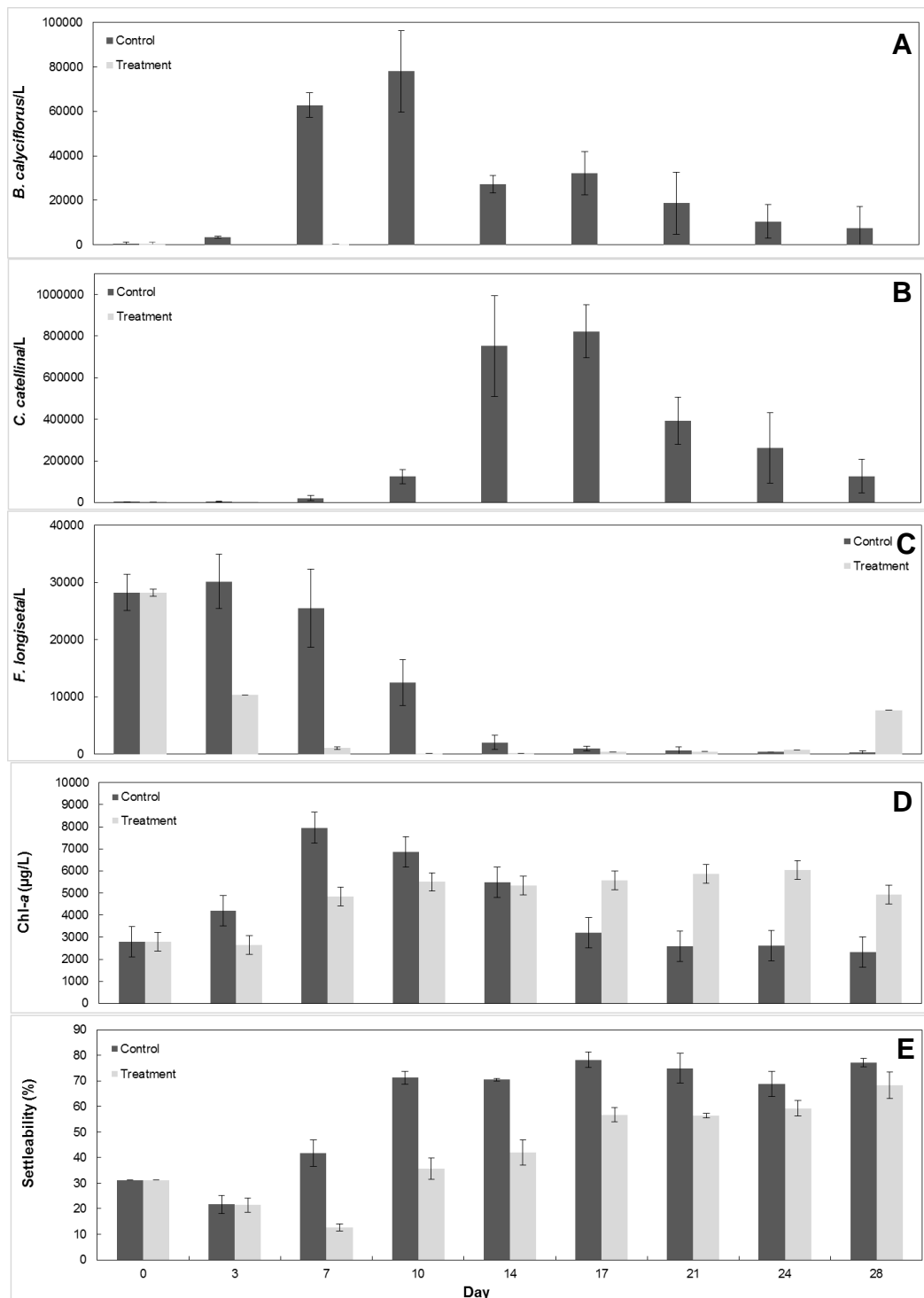
Figure 4 Densities of *B. calyciflorus* **(A),** *C. catellina* **(B),** *F. longiseta* **(C), Chl-*a* concentration (D), and biomass settleability (E) in control and treatment (incubation with** *H. incongruens***) triplicate mesocosms. Values are averages ± standard deviation.**

Microalgae concentration and biomass productivity

The average Chl-*a* concentration of the control (4,223±2,077 μg/L) and treatment (4,835±1,266 μg/L) mesocosms were similar. During the first 14

days the treatment mesocosms had lower Chl-*a* concentrations than the control mesocosms. However, after day 14 the rotifer density in the control mesocosms increased and the Chl-*a* concentration was reduced to 43% of that in the treatment mesocosms (Figure 4, D). The average productivity of the control and treatment mesocosms were 11.9±3.3 g/m^2/d and 11.7±2.2 g/m^2/d, respectively.

Microalgae dominance, settleability and MCSA

In the control mesocosms the relative abundance of *Mucidosphaerium* sp. decreased from ~81% to 28%, *Micractinium* sp. increased from 2% to 37%, and *Staurosira* sp. increased from 1.5% to 17%. The treatment mesocosms were dominated by *Mucidosphaerium sp.* (>95%), and *Staurosira* sp. was rapidly depleted. The average biomass settleability of the control mesocosms (60±22%) was higher than that of the treatment mesocosms (43±19%) (Figure 4, E). The average MCSAs of the control mesocosms (156±59 µm^2) was higher than in the treatment mesocosms (114±27 µm^2).

M. tenuicornis control using filtration

The average densities of *M. tenuicornis* in mesocosm cultures filtered with 300 µm, 500 µm, and 800 µm mesh (Figure 5, A) were 2.6%, 6.5%, 76.7% of the unfiltered control mesocosms, and the average density of *M. tenuicornis* correlated positively with filter size (Table 5). By day 4 *M. tenuicornis* had been completely removed in mesocosm cultures filtered with 300 µm and 500 µm mesh. In the no-*Moina* control mesocosms *M. tenuicornis* was absent throughout the entire experiment. In the unfiltered control mesocosms the density of *M. tenuicornis* rapidly increased up to ~4,000 individuals/L (day 15), and subsequently decreased to ~150 individuals/L (Figure 5, A). Filtration using an 800 µm mesh limited the maximum density of *M. tenuicornis* to <2,500 individuals/L (Figure 5, A) probably by removing a portion of larger (>800 µm width) adults which were also reproductive (Figure 6, E and F), but not the smaller juveniles and young adults (Figure 6, C and D). The percentage of the *M. tenuicornis* population that was carrying juveniles in the treatment mesocosms with 800 µm mesh was always slightly higher than in the unfiltered control

mesocosms, and reached a maximum of ~55% on day four in both (Figure 5, B). Filtration at 500 µm totally removed *M. tenuicornis* after four filtration events likely removing mature individuals before they reproduced (Figure 6, C). It is probable that the first filtration event removed all of the relatively large reproductive *Moina*, leaving the smaller juveniles (<500 µm) which over the next two days matured (including ~50% of them in the reproductive stage, Figure 5, B), and became large enough to all be removed in the third filtration event, before they released their juveniles. The first filtration event reduced the percentage of *Moina* with juveniles from ~16% to 0%, but by day 3 it had increased to ~48%, and was reduced to 0% again by the 4th filtration event on day 4 (Figure 5, B). Filtration using a 300 µm filter removed all *M. tenuicornis* including juveniles (which were all larger than 300 µm, Figure 6, A) after one filtration event.

When *M. tenuicornis* density was low, the mesocosms had higher densities of the rotifers *B. calyciflorus*, *C. catellina*, *F. longiseta* and bdelloid rotifers. This was detected in the no-*Moina* control, filtration at 300 µm and 500 µm during the first two weeks, and unfiltered control and filtration at 800 µm during the last two weeks (Figure 5, C).
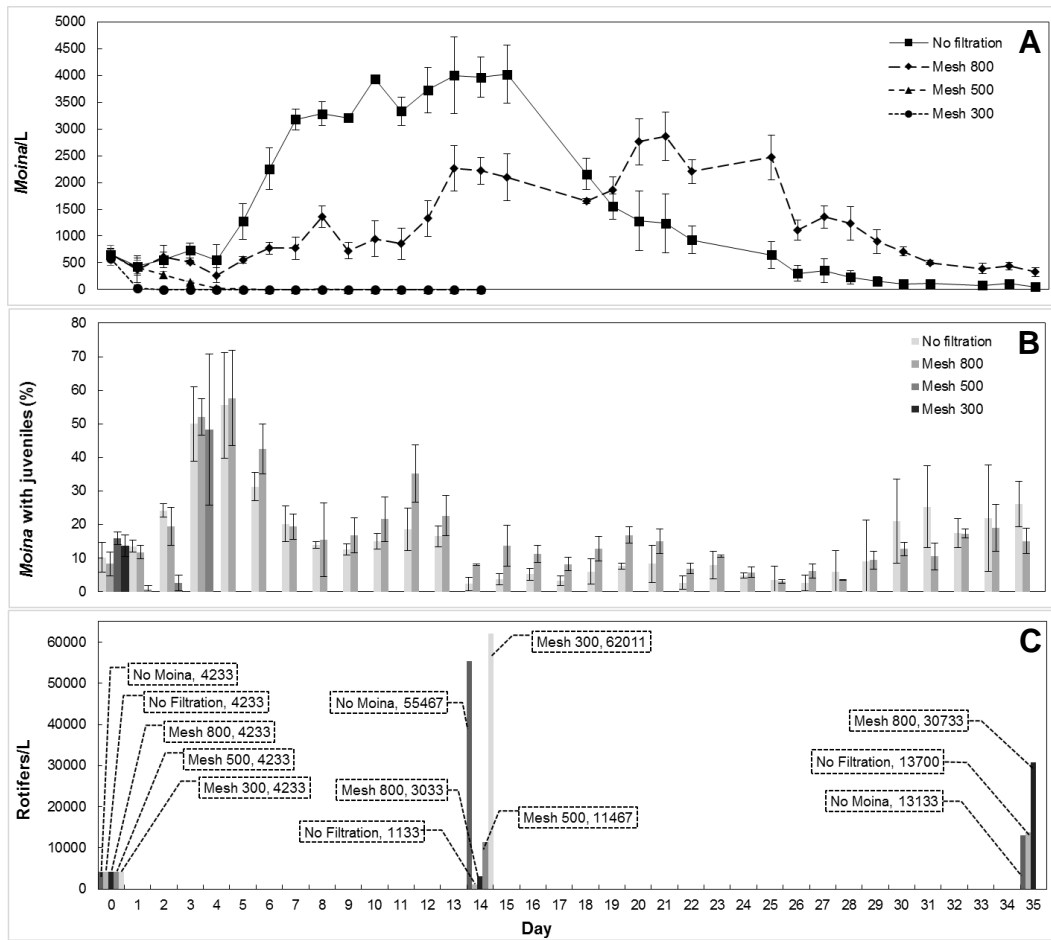
Figure 5 Density of *M. tenuicornis* (A), percentage of the total *Moina* population carrying juveniles (B), and cumulative density of rotifers (*B. calyciflorus*, *C. catellina, F. longiseta*, and bdelloid rotifers) (C) in triplicate treatment mesocosms filtered at 300 μm, 500 μm, and 800 μm, and unfiltered control. Values are averages ± standard deviation of triplicate mesocosms.
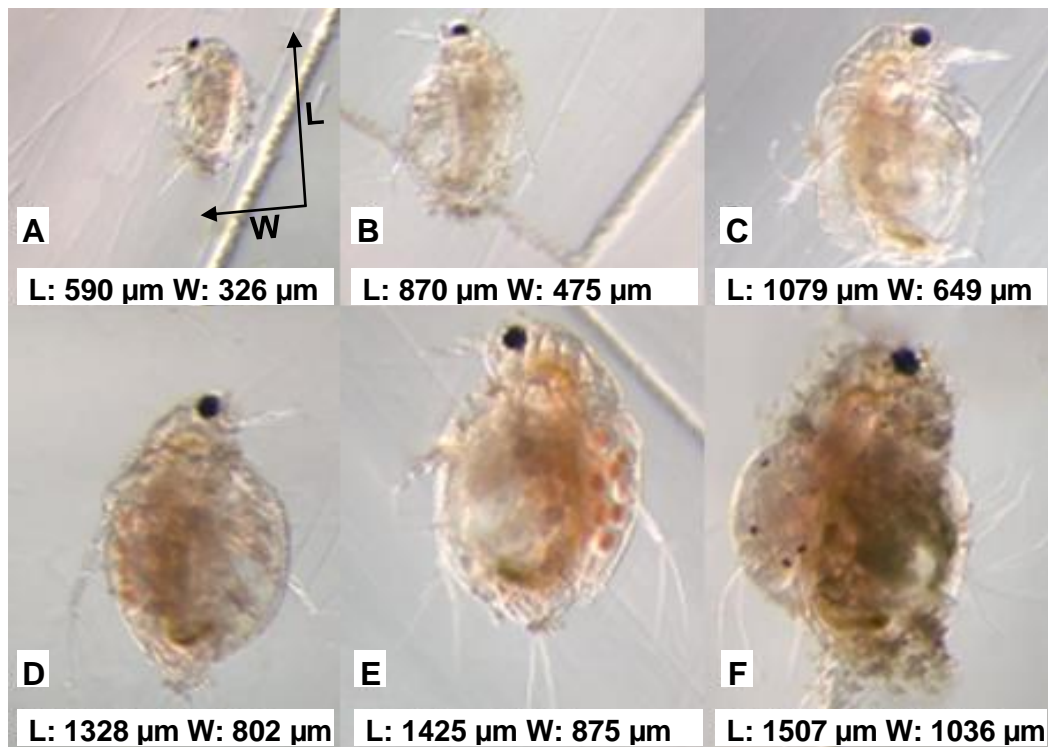
Figure 6 Different stages of *M. tenuicornis* development, showing maximum length (L), and maximum width (W). Juveniles (A-B), non-reproductive adult (C), adult carrying parthenogenetic eggs - early stage (D), adult carrying parthenogenetic eggs - late stage (E), adult with juveniles before hatching (F).

Microalgae concentration and biomass productivity

There was a negative correlation between filter size and both average Chl-*a* concentration and total productivity during the experiment (Table 5). During the first 14 days, treatment mesocosms with lower densities of *M. tenuicornis* (300 µm mesh, 500 µm mesh, and no *Moina* control) had the highest Chl-*a* concentrations and productivities (Table 3). Between day 11 and 14 the rotifer density of these treatment mesocosms and the no *Moina* control increased, resulting in a rapid reduction in the concentration of Chl-*a* from ~5,300 µg/L to ~2,700 µg/L, and leaving a large amount of detritus in suspension (visual observation).

Table 3 Average density of *M. tenuicornis*, percentage of *M. tenuicornis* population with juveniles, cumulative rotifer density, VSS concentration, productivity, Chl-*a* concentration, MCSA, and settleability in triplicate treatment mesocosms filtered at 300 µm, 500 µm, and 800 µm, and unfiltered and no-*Moina* controls. Values are averages for the first 14 days and include standard deviations in brackets (n: 15).

Treatment	M. tenuicornis density (individuals/L)	M. tenuicornis with juveniles (%)	Cumulative rotifers density (individuals/L)	VSS (mg/L)	Productivity (g/m^2/d)	Chl-a (µg/L)	MCSA (µm^2)	Settleability (%)
Control (no *Moina*)	-	-	29850 (36227)	199.3 (70.5)	7.1 (2.4)	2964 (1610)	237 (121)	55 (19)
Control (unfiltered)	1562 (1465)	15 (12.9)	2683 (2192)	172.3 (39.6)	6.0 (1.3)	2160 (674)	224 (102)	55 (14)
800	1198 (778)	17 (13)	3633 (849)	190.6 (48.9)	6.8 (1.6)	2561 (974)	247 (135)	62 (18)
500	101 (193)	5 (13)	7850 (5115)	220.3 (76.3)	8.0 (2.5)	3220 (1547)	223 (101)	54 (14)
300	41 (150)	1 (3.5)	33122 (40855)	212.7 (65.6)	7.6 (2.0)	3016 (1416)	222 (100)	50 (15)

Microalgae dominance, settleability and MCSA

Treatment mesocosms showed similar changes in microalgae relative abundance (data for 300 µm, and 500 µm filters were only measured for the first 14 days) with reductions of *Micractinium* sp. (from 79% to 0%), establishment of *Staurosira* sp. (up to ~50%), and increases of *Pediastrum sp.* (from 17% to ~45%). The only exception was the no *Moina* control where *Pediastrum sp.* decreased to 8%, and *Staurosira* sp. increased to 87%. Unicellular microalgae species were nearly absent in all the treatment mesocosms. The average settleability efficiency and MCSA were slightly higher in the 800 µm (Table 3).

The capacity of smaller mesh sizes to reduce the settleability was assessed in a laboratory test. The settleability of a microalgae culture composed of *Micractinium* sp. (60%), *Mucidosphaerium* sp. (22%), and *Pediastrum* sp. (13%) was measured after filtration at 200, 300, 500, 800, 1,000 µm. The settleability and MCSA were higher in treatments filtered at 300 µm and 500 µm, although the VSS and Chl-*a* of treatments were similar (Table 4). Moreover, there was no correlation between filter size and settleability efficiency or Chl-*a* concentration of the filtrate (Table 5).

Table 4 Average VSS concentration, Chl-*a* concentration, MCSA, and settleability of a microalgae culture filtered at 200 µm, 300 µm, 500 µm, 800 µm, and 1000 µm. Values are averages of three replicates and include standard deviations in brackets.

Filter mesh (µm)	VSS (mg/L)	Chl-*a* (µg/L)	MCSA (µm^2)	Settleability (%)
Blank	263 (4.6)	2829 (32)	145 (1342)	28 (1)
200	252 (6.9)	2838 (56)	111 (536)	21 (0.2)
300	259 (1.2)	2836 (52)	136 (1049)	24 (2)
500	264 (5.3)	2848 (97)	130 (981)	28 (3)
800	263 (4.2)	2811 (85)	97 (421)	22 (1)
1000	264 (4.2)	2829 (85)	119 (735)	23 (1)

Zooplankton control using hydrodynamic shear stress

Hydrodynamic shear stress removed all microcrustaceans from the treated mesocosm cultures within 5 days. Dismembered bodies and damaged individuals were clearly visible by microscopic analyses. In contrast, microcrustaceans grew well in the control mesocosms (Figure 7, A) and *H. incongruens*, *P. fimbriatus*, and *M. tenuicornis* reached maximum densities of ~2,000 individuals/L, ~1,000 individuals/L, and ~600 individuals/L, respectively. The average cumulative density of rotifers (450,563±561,191 individuals/L), and of *B. calyciflorus* (14,541±11,460 individuals/L), *C. catellina* (421,007±563,070 individuals/L), and *F. longiseta* (15,000±10,424 individuals/L) in the treatment mesocosms were 142%, 54%, 151%, and 134% of the control mesocosms, respectively. The maximum peaks of *B. calyciflorus* were higher in the control mesocosms than in the treatment mesocosms (Figure 7, B), although those of *C. catellina*, and *F. longiseta* were higher in the treatment mesocosms than in the control mesocosms (Figure 7, C-D). Hydrodynamic treatment promoted the removal of most of the parthenogenetic eggs attached to *B. calyciflorus* and *F. longiseta*. For example, 53% of the *B. calyciflorus* carried eggs before treatment, but this was reduced to only the 1.5% after treatment.
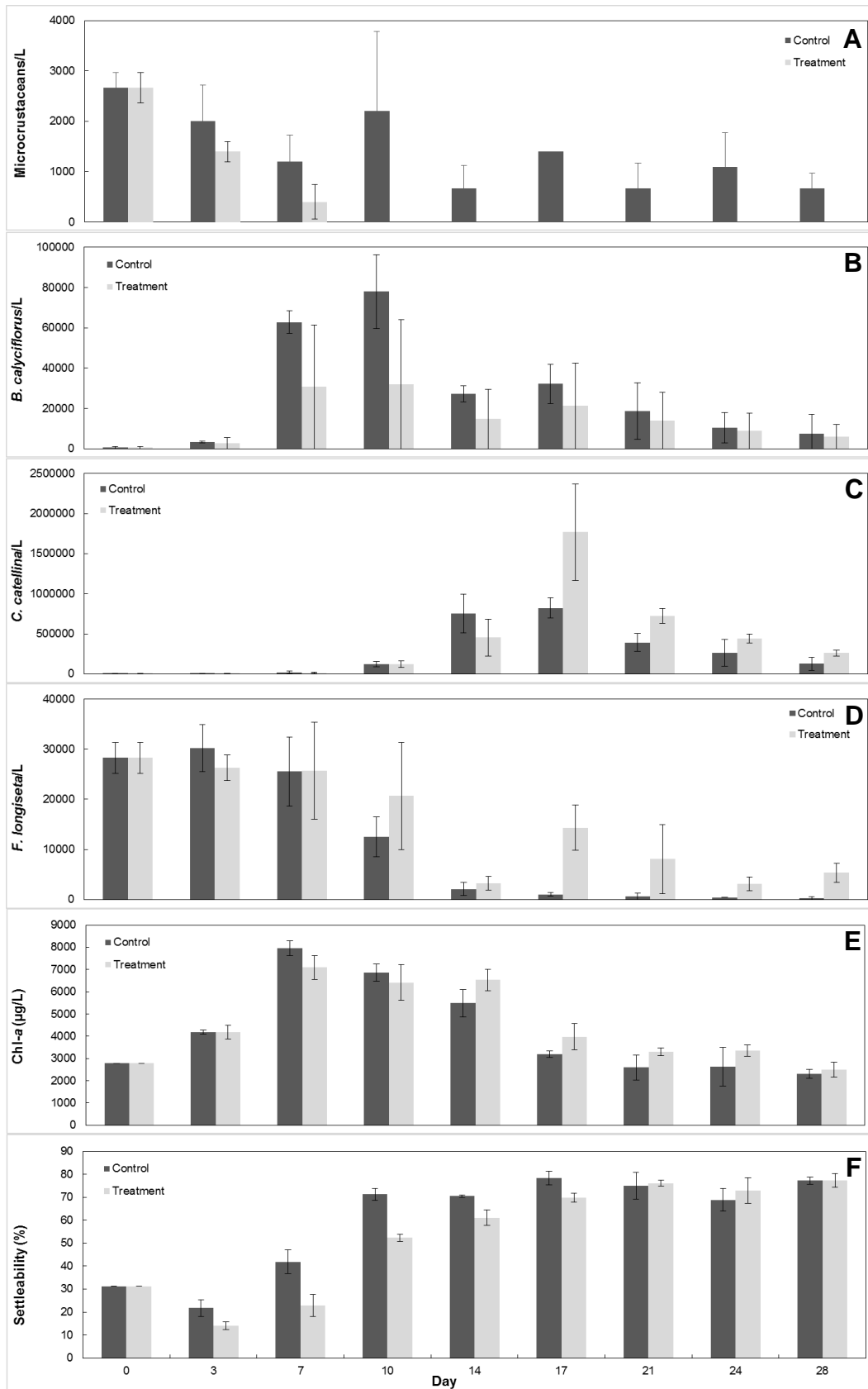
Figure 7 Cumulative density of microcrustaceans (A), densities of *B. calyciflorus* (B), *C. catellina* (C), *F. longiseta* (D), Chl-*a* concentration (E), and biomass settleability (F) in control and treatment (hydrodynamic stress) triplicate mesocosms. Values are averages ± standard deviation.

Microalgae concentration and biomass productivity

The average Chl-*a* concentration of the treatment mesocosms (4,460±1,752 µg/L) was similar to that of the control mesocosms (4,223±2,077 µg/L), and after an initial increase, it decreased throughout the experiment (Figure 7, E). The average productivity of the treatment mesocosms (12.6±3.1 g/m^2/d) was also similar to that of the control mesocosms (11.9±3.3 g/m^2/d).

Microalgae dominance, settleability and MCSA

Changes in algal relative abundance in the treatment mesocosms varied with species. That of *Mucidosphaerium* sp. was reduced from 81% to 7%, while that of *Staurosira* sp. increased to 53%. Spherical unicellular microalgae, *Scenedesmus* sp. and *Desmodesmus* sp. initially had a low relative abundance of <4% and this increased to ~40%, except for days 7 to 10 when the density of *B. calyciflorus* was >30,000 individuals/L and this rotifer likely consumed these smaller microalgae species. In the control mesocosms *Mucidosphaerium* sp. decreased from 81% to 30%, and *Micractinium* sp. increased from 2% to ~37%, while spherical unicellular microalgae were always <4%. The average biomass settleability in the control mesocosms (60±22%) was moderately higher than that of the treatment mesocosms (53±24%). During the first 17 days of the experiment, the control mesocosms had higher settleability than the treatment mesocosms, although from day 21 they were similar (Figure 7, F). The average MCSAs of the control mesocosms (156±59 µm^2) was higher than that of the treatment mesocosms (132±40 µm^2).

M. tenuicornis control in an 8 m^3 HRAP using hydrodynamic stress

Mild hydrodynamic stress rapidly reduced the density of *M. tenuicornis* in an 8 m^3 HRAP. The initial density of *M. tenuicornis* was ~1,100 individuals/L, and the number of live individuals decreased proportionally with the treatment time. The death rate of *M. tenuicornis* correlated positively with the number of pumping events (Table 5), with each event (one event corresponds to the treatment of the whole HRAP volume) causing a mortality rate of ~45% (Figure 8).
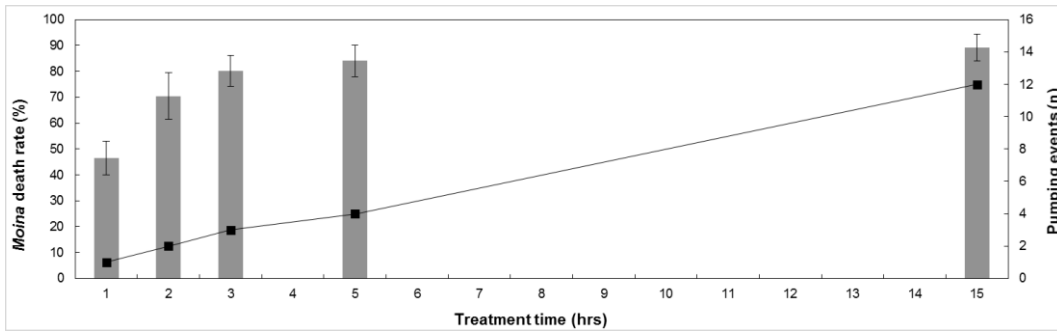
Figure 8 *M. tenuicornis* death rate during continuous mild hydrodynamic stress in an 8 m³ HRAP. One pumping event corresponds to the treatment of 8000 L (the pond volume). Values are averages of three measurements ± standard deviation.

M. tenuicornis vertical distribution in the water column of an 8 m³ HRAP

From dusk to early dawn, between 20.5% and 24.0% of *M. tenuicornis* total biomass was concentrated in the upper 50 mm of the water column of an 8 m³ HRAP. During daylight hours, especially when solar radiation was intense, *M. tenuicornis* migrated into deeper water (Figure 9), leaving only 14% of the total biomass in the upper 50 mm of the water column. Therefore, there was a negative correlation between the density of *M. tenuicornis* in the upper 50 mm of the water column and the intensity of solar radiation (Table 5).
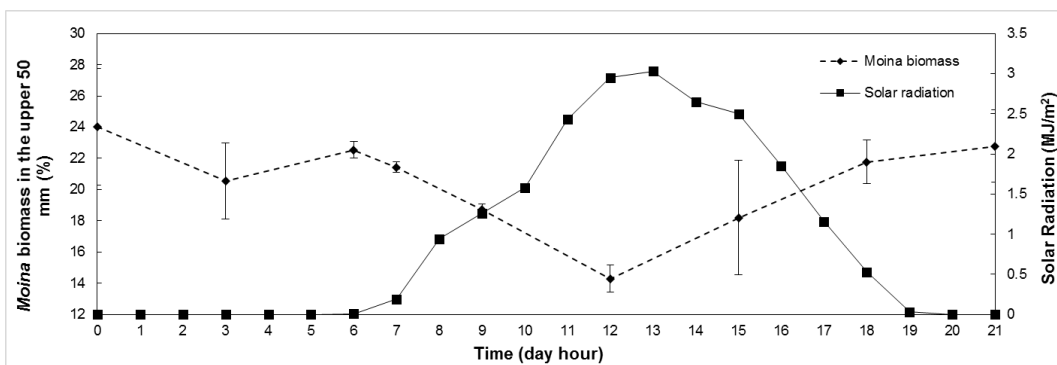
Figure 9 Percentage of *M. tenuicornis* total biomass in the upper 50 mm of the water column of an 8 m³ pilot HRAP with depth of 300 mm, and intensity of the solar radiation, during 24 h. Values are averages of triplicate measurement ± standard deviation.

Table 5 Pearson`s correlation coefficients (r) between the intensity of different treatments and zooplankton density. Positive values denote positive linear correlation, negative values denote negative linear correlation, *p<0.05 (statistical significance), **p<0.1 (moderate statistical significance), ***p>0.1 (no statistical significance). CO_2 treatment (n: 4), filtration (n: 4), hydrodynamic stress (n: 5), migration to the upper 50 mm of the water column (n: 9). Correlations not related to the discussion are indicated with "-".

	Total rotifers	Total crustaceans	B. calyciflorus	C. catellina	F. longiseta	Bdelloid rotifers	M. tenuicornis	Productivity	Chl-a	Moina in 50 mm (%)	MCSA	Settleability
CO_2 concentration (%) (summer)	-0.94*	-0.99*	-0.97*	-0.78**	-0.92**	-0.73**	-	0.88*	0.94*	-	-0.95*	-0.6***
CO_2 concentration (%) (winter)	-0.92**	-0.5***	-0.72***	-0.83***	-	-0.61***	-0.86**	0.09***	0.19***	-	-0.81**	-0.3***
Filter mesh size (µm) (*Moina* control)	-	-	-	-	-	-	0.92**	-0.95*	-0.92**	-	-	-
Filter mesh size (µm) (settleability test)									0.22***	-	-	0.46***
Pumping events (n)	-	-	-	-	-	-	-0.92*	-	-	-	-	-
Solar radiation (intensity)	-	-	-	-	-	-	-	-	-	0.81*	-	-

Comparison between treatments and their effect on microalgae concentration

The effect that treatments had on microalgae concentration (measured as Chl-*a* concentration) and settleability was estimated by comparing the Chl-*a* concentration and settleability of treatment and control mesocosm cultures during the first seven days of each experiment (when both had low densities of zooplankton). The effectiveness of each treatment in reducing the density of microcrustaceans and rotifers were also compared and summarized (Table 6).

Table 6 Comparative effectiveness of treatments on microcrustacean and rotifer reduction, and the influence of treatments on microalgae concentration (Chl-*a*) and biomass settleability, during the first seven days of treatment. During the first seven days zooplankton occurred in very low concentrations and changes of Chl-*a* and settleability were likely caused by the sole treatments. The number of "+" or "-" indicate the capacity of the treatment to reduce or not the density of zooplankton species, and to increase or not the Chl-*a* concentration and settleability. The lack of effect is indicated with "=".

Treatment	Microcrustacean reduction	Rotifer reduction	Variation of Chl-*a* concentration	Variation of biomass settleability
CO_2 asphyxiation summer	+++	++	+	-
CO_2 asphyxiation winter	+++	++	-	=
M. tenuicornis biocontrol	- - -	++	=	=
H. incongruens biocontrol	+	+++	- -	-
Filtration	+++	- - -	=	-
Hydrodynamic shear stress	+++	+/-	=	- -

DISCUSSION

Zooplankton control using CO_2 asphyxiation

Chronic elevated CO_2 concentrations <100 mg/L (treatments with 2% and 5% CO_2) reduced the average density of zooplankton, and CO_2 concentrations of ~180 mg/L (treatment with 10% CO_2) completely eradicated them. CO_2 asphyxiation of zooplankton was likely promoted by lowering the O_2 binding capacity of haemoglobin in microcrustaceans, and inhibition of intra body CO_2 release in rotifers [17], [18], [8]. The lower zooplankton density in the control mesocosms than the treatment mesocosms with 0.5% and 2% CO_2 was probably caused by the inhibition of rotifer growth due to the free ammonia toxicity that resulted from high daytime pH (up to ~10) [19]. The treatment mesocosms with 0.5% CO_2 addition had the highest average and maximum zooplankton densities, which was likely due to the non-inhibitory concentration of CO_2 and near neutral mesocosm pH (Table 1) that prevented free ammonia toxicity, and promoted zooplankton growth [20]. Cladocerans did not establish populations in mesocosms during the summer experiment likely because the HRT (4 days) was shorter than their average generation time (5-7 days) for the water temperature (21 C°) [21], [22], [23], [4]. Zooplankton also established later and were controlled to lower levels in all other CO_2 addition

treatment mesocosms in the winter experiment. This was likely due to the lower growth rate of zooplankton at the <12°C temperature, and resulting longer zooplankton generation times than the 8 day mesocosm HRT [4]. During the summer experiment Chl-*a* concentration and algal productivity were higher in mesocosms with higher concentrations of CO_2 which had a dual benefit of inhibiting zooplankton growth as well as promoting algal growth [24]. CO_2 addition also promoted higher average productivity during winter but to a lesser degree and excluding the 10% CO_2 addition mesocosms. This can be explained with the relatively low density of zooplankton and consequent grazing during winter, the low pH (~6) that likely inhibited the growth of microalgal species that prefer neutral to alkaline environment such as *Pediastrum* sp. [25], and the low solar radiation that limited algal use of the additional carbon. Maximum Chl-*a* concentrations were highest in treatment mesocosms with 5% and 10% CO_2 only after zooplankton established in mesocosms, likely because the higher CO_2 concentrations prevented the growth of zooplankton and limited the grazing losses. The overall increase of Chl-*a* concentration in all mesocosms throughout winter was likely due to the faster growth of microalgae promoted by increasing intensities of solar radiation and water temperature. Mesocosms developed biofilms on the container walls during the last 10 days of the winter experiment. However, this was not expected to affect the zooplankton development as the concentration of Chl-*a* increased and the system was not food limited.

 Average biomass settleability and algal MCSA were higher in treatment mesocosms with higher zooplankton densities (control, 0.5% and 2% CO_2). This can be explained with the capacity of grazers to promote the dominance of large colonial microalgal species [26], [27], and induce changes in the structure of microalgal cells and colonies (e.g., protective spines) [28], [29], [30], that can increase the microalgal settleability [31]. The treatment mesocosms with 10% CO_2 had lower settleability efficiency and MCSA due to the dominance poorly settleable small colonies of *Micractinium* sp. It is likely that the lack of zooplankton resulted in the non-formation of structural modifications and the dominance of large colonies in microalgae, with reduced settleability as a result. Flocculation can be

augmented by bubble enhanced turbulence both via aggregation and flotation of suspended particles [32], [33]. However, in this study flotation was not observed, and bubbles were too large to affect the settleability. Moreover, the experimental mesocosms had the same bubbling rate and settleability differences between mesocosms were expected to be caused by different treatments and zooplankton densities. However, the possible effect that bubble enhanced turbulence has on microalgal cultures should be assessed by comparing the settleability of zooplankton free microalgal culture, using mesocosms with and without bubbling.

Chronic injection of CO_2 at concentrations of ~100 mg/L may be suitable to control zooplankton levels without reducing the algal productivity and settleability (~60%), because zooplankton can develop in low to moderate densities sufficient to promote assemblages with increased settleability. When high algal settleability is not required, such as in the cultivation of smaller unicellular species (e.g., *Chlorella* sp.), higher chronic CO_2 concentrations may be used, if low culture pH is not inhibitory. Acute asphyxiation can kill zooplankton more rapidly (<30 min) [8] than chronic asphyxiation, although this requires water CO_2 concentrations two to five times higher. High CO_2 concentrations requires the use of large sparging areas in hectare-scale HRAPs, and chronic asphyxiation may be more readily applied because lower concentrations of CO_2 can be generated with less-costly sparging equipment.

Injecting 0.5% CO_2 was associated with a Chl-*a* concentration and productivity only slightly higher than that of the control mesocosms without CO_2 addition. The availability of extra inorganic carbon and the exclusion of ammonia toxicity associated with the injection of 0.5% CO_2 likely enhanced microalgal growth, although the pH close to neutrality (between 7 and 8) promoted the highest densities of zooplankton and therefore grazing losses. Ammonia toxicity likely reduced the microalgae growth in the control mesocosms, but also reduced the zooplankton density and resulting grazing pressure. Hence, we suggest that the pH of WW HRAPs with artificial addition of CO_2 to promote algal growth and nutrient removal would be optimally controlled to pH 6.5 unless zooplankton are controlled by another means.

Rotifer control using the cladoceran *M. tenuicornis*

Densities of *M. tenuicornis* >2,000 individuals/L were associated with reduced average densities of rotifers, particularly smaller species such as *C. catellina* (~50 μm), which were likely affected by larger zooplankton through both competition for shared food and mechanical interference [34], [35], [36]. The reduction of larger rotifer species such as *B. calyciflorus* (~300 μm) and *F. longiseta* (up to 500 μm including setae) required higher densities (>2,500 individuals/L) of *M. tenuicornis*. This was likely due to the larger body of *B. calyciflorus* that was less affected by mechanical interference, and the capacity of *Filinia* to escape from direct contact with *Moina* by making rapid movements [37]. It is expected that *M. tenuicornis* consumed only a small portion of the microalgae biomass. The concentrations of Chl-*a* in the treatment mesocosms (with relatively high densities of *M. tenuicornis*) were only moderately lower than in the control mesocosms when rotifer densities and resulting grazing pressure in the control mesocosms were low (up to day 10). This can be explained by the initial high relative abundance (~81%) of *Mucidosphaerium* sp. with large colonies (ø ~100 μm). Smaller crustacean zooplankton such as *Moina* have preferential feeding on smaller particles (<25 μm) and the large colonies of *Mucidosphaerium* sp. were probably hardly ingestible by *Moina* [38], [39], [40]. The high average biomass settleability and algal MCSA in both the control and treatment mesocosms were likely promoted by high densities of rotifers and *Moina* that consumed smaller microalgal species.

The establishment of a permanent population of *M. tenuicornis* that is controlled to moderate densities (e.g., 500 individuals/L) and that can be allowed to grow up to ~2,000 individuals/L during rotifer blooms, is a promising option for biocontrol of rotifers in HRAPs. *Moina* individuals to seed the HRAPs can be collected from maturation ponds where a stable population is usually established permanently. *Moina* can naturally establish in pilot-scale HRAPs for long periods of time [4], population densities can be easily controlled by filtration, and probably have minimal influence on the density of colonial microalgae due to their preferential feeding on smaller particles. In addition, this *Moina* population would provide a continuous

inoculum for subsequent maturation ponds in a HRAP system, enhancing the consumption of unsettled microalgae, and if regularly harvested, would increase the nutrient removal from the system, and provide valuable biomass for aquaculture [20], or production of high quality chitin [41].

Rotifer control using the ostracod *H. incongruens*

A population of *H. incongruens* with a density of ~1,000 individuals/L eradicated rotifers from the treatment mesocosms, although the specific eradication mechanism was not clear and further studies are required. One possibility is that chemicals released by ostracods reduce or delay rotifer reproduction as a strategy to avoid this potential predator [42]. Alternatively, *H. incongruens* releases toxic metabolites that inhibit rotifer growth and reproduction, with a mechanism similar to that observed to self-limit dense rotifer populations [43]. The semi-continuous culture (8 day HRT) of mesocosm experiments promoted larger and faster reductions of rotifers than those achieved in laboratory batch cultures [8]. The HRT of a water body can affect and even prevent the establishment of zooplankton species if it is shorter than the zooplankton reproduction rate [44], [45], and the constant removal of effluent from the mesocosms likely enhanced the reduction of rotifers. *H. incongruens* prevented the establishment of the cladoceran *M. tenuicornis* and reduced the density of the copepod *P. fimbriatus*, although the densities of these microcrustaceans were too low to infer a clear inhibitory effect from the ostracod.

H. incongruens reduced the Chl-*a* concentration of the treatment mesocosms compared to the control mesocosms over the first 10 days of the experiment, and this was probably due to grazing of microalgae. However, the Chl-*a* concentrations of the control mesocosms were reduced by half due to intense grazing as rotifer densities increased. This suggests that while ostracod densities of ~1,000 individuals/L are expected to consume a portion of the microalgae biomass, this grazing loss is far lower than that caused by a rotifer bloom.

The lower initial settleability of the treatment mesocosms compared to control mesocosms was probably caused by the dominance of the poorly settleable *Mucidosphaerium* sp. However, settleability of the treatment

mesocosms increased throughout the experiment, suggesting that biocontrol of microalgae cultures using ostracods could also promote biomass harvestability (~70%).

Ostracods can reduce the density of rotifers, can be easily established in new environments [46], can resist WW HRAP's conditions, and being bottom dwelling organisms populations are easily maintained in HRAPs [4]. Hence, ostracods could be inoculated into HRAPs (if not already established) and their retention promoted by placing the HRAP outflow at the mid-depth of the ponds to reduce their removal. *H. incongruens* also feeds preferentially on settled WW solids [47] than on suspended microalgae. Therefore, the consumption of suspended microalgae in HRAPs is expected to be lower than in experimental mesocosms with young cultures and no sediments. Excess ostracods can be harvested from HRAP eddies where they accumulate [4], further removing nutrients from the pond.

M. tenuicornis control using filtration

Filtration reduced the density of *M. tenuicornis*, physically removing individuals at different reproductive stages and body sizes. The largest 800 µm filter removed only a portion of the larger adults that were in the late reproductive stage, the smaller 500 µm filter also removed mature adults prior to the reproductive stage, and the smallest 300 µm filter removed all *M. tenuicornis* including juveniles. Moreover, treatment mesocosms with lower densities of *M. tenuicornis* (300 µm, 500 µm, and no-*Moina* control) were associated with larger densities of rotifers

Similar average settleability and MCSAs of treatment and control mesocosms were probably promoted by high densities of both *M. tenuicornis* and rotifers that consumed smaller microalgal species. The settleability of treatment mesocosms filtered with the 300 µm filter was probably reduced by partial retention of larger flocs in the smaller filter. In the laboratory test, filtration at 200 µm and to a lower extent 300 µm reduced the settleability of the microalgal culture by retaining the larger flocs of organic matter but not microalgae, as the concentrations of Chl-*a* in the filtered liquid were similar to that of the control, but the VSS and MCSA were lower. Settleability was also reduced in the samples filtered at 800 µm and

1000 μm, probably as a result of mechanical disruption of flocs caused by the higher flow of the liquid through the larger filters, as the MCSA in the filtered liquid was lower than in the control, but the VSS and concentration of Chl-*a* were similar. Filtration at 150 μm has previously been used in California to partially control large zooplankton in 12 m^2 HRAPs [44], and in aquaculture systems 300 to 700 μm filters have been used to harvest small cladocerans, adult *Daphnia*, and large cyclopoid and calanoid copepods [48], [49]. Although smaller filter sizes (≤300 μm) can eradicate *Moina* with a single filtration event, the filter may become clogged by organic matter and detritus suspended in the water, so continuous filtration with larger filters (≥500 μm) would be more manageable. Moderate populations of *M. tenuicornis* may prevent rotifers from blooming, so during periods when rotifers are expected to develop in high densities, continuous filtration with an 800 μm filter, or periodic filtration with a 500 μm filter, could be used to control the *Moina* density to <2,000 individuals/L. Filtration of HRAPs can be particularly cost effective if performed using the flow and head generated by the paddlewheel (no additional pumping required), and if performed on the upper portion of the water column (e.g., top 50 mm) to minimize the head loss. Filtration is expected to be easily scaled up and automatized, and particularly suitable to control larger zooplankton species.

Zooplankton control using hydrodynamic stress

Hydrodynamic stress removed all microcrustaceans and reduced the densities of larger rotifers. Populations of *M. tenuicornis*, *H. incongruens* and *P. fimbriatus* that were established in the mesocosms were rapidly removed, probably due to their relatively large and complex body structures compared to rotifers, being more susceptible to mechanical damage. The density of larger rotifers such as *B. calyciflorus* was reduced likely due to physical disruption of individuals and removal of eggs attached to their bodies with consequent impaired hatching. The density of smaller species such as *C. catellina* and *F. longiseta* increased, and this was probably due to their greater resilience to shear stress, the reduced competition for shared food from larger rotifers, and the lack of mechanical interference from cladocerans, both reduced or eradicated by the hydrodynamic stress

treatment. Moreover, since *Cephalodella* species do not carry eggs connected to the adult body [50], [51], egg hatching was not expected to be reduced or affected by the treatment.

Although hydrodynamic stress treatment removed all microcrustaceans and reduced the density of *B. calyciflorus*, the high density of *C. catellina* remaining grazed on microalgae and reduced the Chl-*a* concentration to levels similar to those of the control mesocosms. Hydrodynamic disruption of microalgal colonies [8] likely contributed to the reduction of *Mucidosphaerium* sp. and non-establishment of *Micractinium* sp. in the treatment mesocosms.

In a pilot scale (8 m^3) HRAP, mild hydrodynamic stress reduced the density of *M. tenuicornis*, but did not totally remove them. This was probably because the treatment device was not able to receive and process the whole cross sectional flow of the HRAP and the processed liquid was mixed back into the pond water, reducing the efficiency of the treatment. Mild hydrodynamic stress could be used to control cladocerans in full scale HRAPs and the treatment efficiency could be increased by pumping the treated HRAP water into an empty pond, or by placing a pump(s) able to receive and process the whole cross sectional flow of the HRAP. Higher hydrodynamic stress could be used to also rapidly reduce the density of smaller rotifers, especially in emergency situations (e.g. following, late detection of a bloom), and is expected to be easily automatized.

M. tenuicornis vertical distribution in the water column of an 8 m^3 HRAP

In an 8 m^3 HRAP, higher intensities of solar radiation (between 9:00 a.m. and 3:00 p.m.) were associated with lower densities of *M. tenuicornis* in the upper 50 mm of the water column. The reduced densities of *Moina* were likely promoted by phototactic migration that induced the zooplankton to move to the water surface after sunset, and to return to deeper water before sunrise [52], [53]. However, the small difference in *Moina* density measured between day and night (~10%) was likely a result of the mixing of the pilot-scale HRAP provided by the paddlewheel, and the short circulation time (~90 s) of water around the pond which prevented complete migration of

individuals from occurring. In full scale HRAPs with a much longer water circulation time (~85 min) [54], *Moina* would have sufficient time to complete the migration, increasing their density in the upper 50 mm of the water column during periods of low solar radiation (e.g., dusk, dawn, and night). If treatments such as filtration and hydrodynamic stress were used to control *Moina*, they could be performed during this low light period on the upper portion (e.g., 50 mm) of the water column, reducing the amount of water requiring treatment and therefore the treatment cost.

Impact of zooplankton species and treatments on microalgae

Grazing by zooplankton resulted in complete removal of some microalgal species and changes in microalgae relative dominance that were similar in all treatment mesocosms. The rotifer *B. calyciflorus* is only able to ingest smaller particles (ø <20 µm) [55], [56]. Thus, high densities lead to the reduction of small unicellular algal species and dominance of large colonial algal species such as *Micractinium* sp., *Mucidosphaerium* sp., and *Pediastrum* sp. (ø 50-100 µm). This occurred in the 5% CO_2 treatment and control mesocosms (summer experiment), in the filtration treatment mesocosms using 300, 500 µm filters and the no *Moina* control during the first two weeks, and in the hydrodynamic stress experiment control mesocosms. In contrast, high abundance of small microalgal species occurred in mesocosms which had low densities of *B. calyciflorus* such as the 10% CO_2 treatment, and the hydrodynamic stress treatment.

    C. catellina is able to graze on the cells of colonial algal species which have weakly bound cells [4] such as *Mucidosphaerium* sp. and *Micractinium* sp. (without spines). Therefore, high densities of *C. catellina* are associated with the reduction of these colonial algae in favour of species that are protected from the grazing activity. For example, single-celled species that are too large to be ingested; filamentous species and the colonial *Pediastrum* sp. which are too resilient to be fragmented and ingested; and the colonial *Micractinium* sp. when they are protected by spines. This occurred in the 0.5% and 2% CO_2 addition treatment mesocosms during the summer experiment and the 2% CO_2 addition treatment mesocosms during the winter experiment; in the control

mesocosms of rotifer biocontrol with *M. tenuicornis* experiment; and in the hydrodynamic stress treatment mesocosms (where hydrodynamic disruption was also likely to contribute to the disruption of colonial *Mucidosphaerium* sp. and *Micractinium* sp.).

M. tenuicornis preferentially feed on bacteria and smaller particles [39], [40], and high densities were associated with a reduction in unicellular algal species and an increase in the dominance of large colonial algal species including: *Mucidosphaerium* sp., *Micractinium* sp., and *Closterium* sp. This occurred in the rotifer biocontrol with *M. tenuicornis* treatment mesocosms, and in the 800 μm filtered and unfiltered control mesocosms.

Filamentous algae established in the mesocosms after the microalgal biomass has been severely reduced by grazing. For example, *Staurosira* sp. established at the end of all the filtration treatment mesocosms, and in the hydrodynamic stress treatment mesocosms. This was probably because filamentous algae are much harder to ingest for *Moina* and rotifers [57], [58], and remained un-grazed in the culture [40].

The concentration of microalgae during the first seven days of experiment (low densities of zooplankton) was reduced by biocontrol with *H. incongruens*, likely because the ostracods consumed a portion of microalgae, and biomass settleability was reduced by hydrodynamic shear stress, likely due to disruption of biomass flocs. However, further studies are required to assess the Chl-*a* concentration and settleability of treated cultures compared with untreated control cultures, both without zooplankton.

CONCLUSIONS

Microcrustaceans and rotifers typical of New Zealand WW HRAPs were effectively reduced or eradicated using the tested chemical, physical and biological control treatments.

Populations of microcrustaceans were reduced by using chronic CO_2 concentrations between ~60 and ~100 mg/L, and eradicated in <4 days using CO_2 concentrations of ~180 mg/L. Filtration at 300 µm and 500 µm eradicated *M. tenuicornis* in one and four filtration event, respectively; and filtration at 800 µm limited the maximum density of *M. tenuicornis* to <2,500 individuals/L over a period of 35 days. Mild hydrodynamic disruption eradicated microcrustaceans within 5 days. Phototaxis induced migration promoted higher densities of *Moina* in the upper 50 mm of the water column in an 8 m^3 HRAP during periods of low solar radiation.

Populations of rotifers were reduced by using chronic CO_2 concentrations of ~100 mg/L, and eradicated in ~4 days using CO_2 concentrations of ~180 mg/L. Establishment of the cladoceran *M. tenuicornis* at concentrations between 1,500 and 5,000 individuals/L reduced the density of rotifers, especially smaller species, with only minor consumption of colonial microalgae. Establishment of the ostracod *H. incongruens* at concentrations between 1,200 and 2,000 individuals/L in microalgal cultures eradicated the rotifer population within a week. Mild hydrodynamic stress reduced the density of larger rotifer species.

Asphyxiation using CO_2 was the most versatile and effective zooplankton control treatment, and could either be used to eradicate or reduce all types of zooplankton species. Further research is required to assess the effectiveness of treatments in hectare-scale HRAPs, and to quantify the treatment effects on microalgal concentration and settleability in absence of grazers.

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CHAPTER 6

CONTROL OF ZOOPLANKTON POPULATIONS IN A WASTEWATER TREATMENT HIGH RATE ALGAL POND USING OVERNIGHT CO$_2$ ASPHYXIATION

ABSTRACT

High Rate Algal Ponds (HRAPs) with addition of CO_2 are open pond wastewater treatment systems that recover nutrients as microalgal biomass. Such ponds are vulnerable to contamination by opportunistic zooplankton species able to survive the wastewater HRAP environment. The high food availability and a near neutral pH can promote the rapid development of high densities of zooplankton that can reduce treatment performance by consuming microalgae. Zooplankton control using night time CO_2 asphyxiation treatment was selected from promising zooplankton control methods previously screened at laboratory and mesocosm scales, and used to control zooplankton densities in an 8 m^3 HRAP over 14 months. Increasingly higher flow rates (1 to 6 L/min) of pure CO_2 were tested by using 13 control treatment events. CO_2 was injected during night time, and treatment events were repeated for a number of consecutive nights sufficient to control zooplankton density to ≤10% of that before treatment. Treatments with higher CO_2 flow rates promoted more rapid reductions of zooplankton density (12 nights to 1), and were associated with higher maximum CO_2 concentrations (100 to 420 mg/L), and lower pH (~6 to ~5). Compared to the control HRAP, CO_2 treatment decreased the average population densities of some zooplankton species over the experimental period: *Moina tenuicornis* (41.3%), *Paracyclops fimbriatus* (43.9%), *Filinia longiseta* (59.8%), but was associated with higher average population densities of others: *Heterocypris incongruens* (174.4%), *Asplanchna sieboldi* (177.8%), *Cephalodella catellina* (200.0%), and *Brachionus calyciflorus* (234.9%). However, the population densities of the rotifers *B. calyciflorus* and *C. catellina* were always reduced following CO_2 treatments with flow rates ≥2 L/min. The cladoceran *Daphnia thomsoni* and the rotifer *Brachionus urceolaris* established only in the control HRAP. Zooplankton control by CO_2 asphyxiation improved the overall performance of the treated WW HRAP compared to the control in several ways, including increasing algal biomass (VSS) (150.8%), productivity (151.4%), chlorophyll-*a* concentration (161.8%), particle size (MCSA) (115.8%), and

average settleability efficiency (189.2%). Overnight CO_2 asphyxiation showed the potential to control zooplankton and to promote better WW HRAPs performance.

INTRODUCTION

High Rate Algal Ponds (HRAPs) are 200-500 mm deep closed-loop, paddlewheel-mixed ponds of up to a few hectares in size [1], used to provide economical and efficient near tertiary-level wastewater (WW) treatment [2], [3] as well as reclaim water, nutrients and energy from organic wastes. Algal biomass can be recovered in harvest ponds by gravity settling of mainly colonial microalgae associated with bacterial flocs, and can be used for biofuel production, fertilizer and animal feed [4], [5]. Before being discharged into the environment, the algal harvest pond effluent may be further treated in a series of maturation ponds where zooplankton graze on the remaining microalgae still suspended in the water. HRAPs operated with CO_2 addition for pH control and to provide additional carbon for microalgal growth have a pH between 7 and 8, and offer an ideal environment for contamination and development of high densities of zooplankton [6]. Moreover, HRAPs have a high concentration of food (mainly bacteria and microalgae), and lack higher predators such as fish that can consume zooplankton, which further contribute to the establishment of zooplankton species that can survive WW conditions. Once established, zooplankton that can ingest the dominant microalgae, often rapidly consume the microalgal biomass [7], [8], and reduce the productivity and the nutrient removal capacity of HRAPs [6].

The necessity to control zooplankton densities in WW HRAPs is widely recognized [9], [10], [11], [12], [13], [14], and required for both consistent WW nutrient removal and microalgal productivity [15]. Zooplankton control methods should not reduce microalgal growth because algal biomass is essential for the WW nutrient removal, and should not disrupt the structure of colonial algae and algae-bacterial flocs because large particles are essential for a good settleability of the suspended biomass [16]. In particular, the effect of the zooplankton control methods should be limited to the HRAPs, and not reduce the zooplankton density in maturation ponds into which they flow, where they provide an important function in further polishing the HRAP effluent. Potential options for zooplankton control such as filtration [17], [18], [19], centrifugation

[9], heating [20], cavitation [21], [22], UV radiation [23], increased concentration of CO_2 [15], deoxygenation [24], un-ionized ammonia toxicity [25], [26], [27], biocides [28], [29], [30], [31], chitinase inhibitors [32], altering hydraulic retention time (HRT) [33], and biocontrol using competing or carnivorous zooplankton [34], [35], have been previously proposed. However, only a few of these zooplankton control methods (e.g., filtration, un-ionized ammonia toxicity, and use of biocides) have been used for zooplankton control in HRAPs [33], [27], [36]. Zooplankton control methods should control zooplankton populations to low levels, maintaining them as part of a stable community rather than totally eradicating them [6]. This is because moderate populations of zooplankton are expected to reduce the potential establishment of different zooplankton species that are less easy to control. For example, biotic resistance (the ability of a native community to keep out newly arriving species) from existing zooplankton has been shown to play an important role in reducing the establishment rates of new zooplankton arriving at ponds, and promoting healthy populations of desired species may reduce the establishment rates of less desirable zooplankton [37]. Moderate densities of certain zooplankton species may also be beneficial because they can release chemicals and metabolites that can induce the formation of microalgae colonies and cells with spines [38]. This can reduce the capacity for grazers to ingest the larger food particles relative to single celled algae without spines [39], [40], [41], and increase the biomass settleability [33], [42], [43], [44]. Zooplankton eradication should be avoided also because the high costs required to remove or kill all individuals is likely to be pointless when HRAPs are contiguous with other ponds (e.g., maturation ponds), and cross contamination occurs continuously. However, when contaminant zooplankton can rapidly consume dominant microalgal species, the control methods should rapidly (within 1-2 days) reduce the density of zooplankton to prevent severe reductions of HRAP biomass.

The efficacy of zooplankton control methods such as CO_2 asphyxiation, hydrodynamic shear stress, filtration, and biocontrol using competing cladocerans and ostracods

were previously assessed in laboratory experiments using batch microalgae and zooplankton cultures [45]. Control methods were then validated under typical WW HRAP physical and chemical (nutrient concentration, pH, temperature, light radiation), and operational (HRT, mixing, CO_2 addition) conditions using outdoor mesocosms operated as semi-continuous cultures [46]. Asphyxiation using CO_2 was the most versatile, selective, and effective zooplankton control method. Other researchers have used CO_2 addition to kill zooplankton in experimental enclosures in the form of dry ice [47], to reduce the zooplankton density in 1.5 m^3 microalgae cultures bubbling pure CO_2 [48], and in a high CO_2 gas mixture (2% O_2; 12% CO_2; 84% N_2) it was used to kill copepods and crustaceans in 1.5 L experimental enclosures [49]. However, to date, successful use of CO_2 to control zooplankton in large HRAPs has not been demonstrated.

Here we compare the performance and zooplankton community dynamics of paired 8 m^3 HRAPs, where one HRAP was treated with night time injection of CO_2 to control zooplankton density, and the other HRAP was untreated as a control, over a period of 14 months. The zooplankton community, the biotic interactions between grazers and microalgae, and the performance of the HRAPs in terms of biomass productivity and settleability were monitored. Prior to this experiment, the two HRAPs had been monitored in terms of zooplankton dynamics and WW treatment performance for a period of 14 months to assess their similarity in performance and zooplankton dynamics when both were zooplankton control methods were not in place [6]. A protocol for zooplankton management in WW HRAPs is proposed based on all our experimental work.

MATERIAL AND METHODS

Operation of the paired HRAPs

The two identical WW HRAPs (West and East) were located at the Ruakura Research Centre, Hamilton, New Zealand (37°46'29.5"S - 175°18'45.4"E). Each HRAP consisted of a single-loop raceway with a central baffle, lined with black high-density polyethylene (HDPE) plastic, with semi-circular ends, a depth of 300 mm, a volume of 8 m^3, and a surface area of 32 m^2. Each pond was circulated at an average surface velocity of 0.15 m/s using a 1 m wide, steel paddlewheel with 8 blades. The HRAPs received 1 m^3/d of settled domestic WW collected from the main WW pump station at the Ruakura Research Centre, which was added at hourly intervals. In winter, when microalgal growth is reduced, 1 m^3 of settled WW was added to each HRAP daily to give a HRT of 8 days. In spring/autumn and summer the HRT was reduced to 5 days by dilution of the influent with de-chlorinated tap water to simulate recirculation of treated effluent from which the algae had been harvested [50]. CO_2 was automatically added to both HRAPs to control the pH to a maximum of 8. The CO_2 was stored in CO_2 gas cylinders (BOC Gas Ltd, New Zealand), equipped with gas regulators and flow meters (0-12 L/min range). The pond water pH was measured every five seconds with a pH probe (Sensorex mod. S265C/CD) and when the pH exceeded 8, CO_2 was bubbled into the ponds (2 L/min) using gas diffusers placed on the bottom of the HRAP downstream of the paddlewheel, until the pH was reduced to 7.8. The pH probes were calibrated monthly with pH standard solutions. The effluent flowed by gravity from a drainage outflow pipe located on the bottom of the HRAPs into 250 L settling tanks where the biomass suspended in the culture was settled and removed from the tank bottom daily using a peristaltic pump (Masterflex, Cole-Parmer, HV-07523-60, Chicago, USA). The supernatant flowed from the settling tank into a cascade of four maturation ponds where the resident zooplankton community consumed the remaining microalgae. At the beginning of the monitoring period, the two HRAPs were emptied, carefully cleaned, their sediments removed including zooplankton resting

eggs, and inoculated with the same assemblage of naturally occurring algae that had established prior to cleaning.

Sampling protocol and environmental, physical and chemical analyses

The suspended zooplankton and microalgae were sampled in front of the paddlewheel weekly at 09:00 am, using a 2.5 L bucket dipped into the water down to 50 mm from the HRAP bottom, and with the open end facing the paddlewheel. Diapausing eggs, copepods, and ostracods were collected from the HRAP bottom using 100 mL plastic cylindrical beakers with open tops (ø 60 mm), which were held in position by laboratory stands and clamps. The beakers accumulated settled material over 1 week, were placed in three low mixing (<0.1 m/s water velocity) areas with high sedimentation [51], [6], and were carefully capped with screw lids before being removed from the water. Daily solar radiation, evaporation and rainfall were downloaded from the NIWA National Climate Database (http://cliflo.niwa.co.nz/). The pH and temperature of the HRAPs were continually measured using a Datasonde 4a (Hydrolab, HACH Environment, CO, USA), and data were logged at 15 min intervals using a data logger (CR10X, Campbell Scientific Inc., UT, USA).

Biomass measurements and settleability

To determine the weight of total suspended solids (TSS), 50 mL of HRAP water was filtered through pre-rinsed, pre-combusted (450°C for 4 h), and pre-weighed 47 mm Whatman GF/F filters (nominal pore size 0.7 µm), that were then oven dried (85°C) overnight using a drying chamber (270M Digital Series, Contherm), and weighed on an analytical scale (SI-234, Denver Instruments). The filters were then combusted at 450 °C for 1 h using a muffle furnace (F.E.Kiln, RTC1000, Bartlett Instrument Company, IA, USA), and weighed again to assess the ash weight. Ash free dry weight (or volatile suspended solids: VSS) was calculated as the difference between TSS and ash concentration [52]. Biomass productivity (microalgae, and in lower extent bacteria

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and smaller zooplankton such as rotifers) was calculated based on the VSS concentration, taking into account rainfall and evaporation [16]. Detritus and large zooplankton such as cladocerans were removed by filtering the liquid with a 300 μm mesh filter.

Samples (10 mL) for chlorophyll-*a* analysis were filtered onto 25 mm Whatman GF/F filters (nominal pore size 0.7 μm) and the chlorophyll-*a* was extracted in 100% methanol at 65°C for 5 min, followed by 12 h at 4°C in the dark. Samples were then centrifuged using a Sorvall/Dupont General Centrifuge GLC-2B at 3,000 rpm (RCF: ~1,720 g) for 15 min and the absorbance of the supernatant was measured using a UV-Visible Shimadzu UV 1601 spectrophotometer. Chl-*a* concentrations were calculated using the modified trichromatic equations for methanol [53]. Biomass settleability was estimated by settling 1 L of the microalgae culture in an Imhoff cone over a 1 h period, and oven drying (85°C, overnight) the settled material in aluminium trays [54]. Settleability efficiency was calculated as % of dry settled biomass on TSS.

Algal identification, relative abundance and average Maximum Cross-sectional Area

Algal species composition was determined on subsamples of HRAP water (1 mL) that were settled in a 25 mm ø Utermöhl chamber. Three images per sample were taken in random fields of view using a Leica DM 2500 microscope (100x - field of view (ø) 1 mm), equipped with digital Leica DFC 420 camera (Leica Microsystem, Switzerland), and the software Leica Application Suite (LAS version 4.1.0). Microalgae were identified to species, where possible, according to morphological descriptions [55]. The relative abundance of microalgae was calculated by multiplying the average biovolume of each microalgal species by the total number of cells or colonies counted (depending on the species) in the three images. The average biovolume for each microalgal species was calculated using the equations proposed by Vadrucci et al. [56] and the methodology and assumptions of Montemezzani et al. [6]. Changes in

microalgal species composition and in the shape and surface area of their cells or colonies were assessed by measuring the average surface area of suspended microalgal particles in their largest cross section (Maximum Cross-Sectional Area, MCSA (μm^2)). The average MCSA was used to quantify the size of colonies and flocs, and thus to examine their growth or formation over time [6], and was calculated by measuring all the particles visible in the three images using the freeware software 'ImageJ' V 1.43u, excluding particles <5 μm^2, and non-algal particles.

Zooplankton and diapausing egg identification and enumeration

Subsamples (100 mL) of HRAP water were bubbled with pure CO_2 to asphyxiate zooplankton and species were enumerated from triplicate 5 mL aliquots in a gridded counting chamber using a Leica M50 stereo microscope. Zooplankton were identified using available taxonomic guides [57], [58]. Sediment accumulated in the three 100 mL sediment collection beakers of each HRAP were combined and the volume was measured. Copepods, ostracods, and cladoceran diapausing eggs were separated from the microalgae using a 125 μm mesh filter. The filtered particles were re-suspended in 300 mL of water and triplicate 5 mL aliquots were collected after mixing, and counted using the same methodology used for suspended zooplankton.

CO_2 treatments and sparging system

TSS, VSS, chlorophyll-*a*, productivity, MCSA, and zooplankton abundance and dynamics of the West and East HRAPs, when equally operated and without zooplankton control methods in place, were monitored during an initial period of 14 months (August 2013 - September 2014) [6]. Following this, zooplankton control using night time CO_2 asphyxiation was implemented in the East HRAP for a further period of 14 months (October 2014 - December 2015), and the West HRAP was used as a control without CO_2 treatment. HRAPs will be referred as West and East during the initial monitoring period, and as control HRAP and treated HRAP during the treatment

period. The CO_2 injection for zooplankton control was performed using a ceramic gas sparger Point Four™ Trac-Lock Micro Bubble diffuser (Point Four System Inc., Canada), that was independent from that used for daytime CO_2 injection. The sparger was connected to a CO_2 gas cylinder, and had an optimal flow rate of 3 L/min and a sparging surface of 0.018 m^2 (310 mm x 60 mm) that resulted in 0.0023 m^2 of sparging surface for every 1 m^2 of HRAP surface. The treatment was automatically performed using a solenoid valve (Bürkert 6240, Germany), and a programmable digital timer (Arlec Australia Pty. Ltd., Melbourne, AU). The treatments were performed by injecting pure CO_2 during the night time over a period of eight hours, starting 30 min after sunset. CO_2 injection was performed at night because at night algal/bacterial respiration reduces the DO and produces CO_2, the CO_2 injected is not used in the photosynthetic metabolism of microalgae, and the lower environmental temperature promotes higher solubility of CO_2 in water.

A total of 13 zooplankton treatment events were performed over the experimental period. Treatments to control zooplankton during subsequent events were performed using increasingly higher (1, 2, 3, 4, 5, and 6 L/min) flow rates of pure CO_2, repeated for a number of consecutive nights sufficient to control zooplankton density to ≤10% of that before treatment. Different CO_2 flow rates were used to assess the combination of flow rate and time that required the lowest total amount of CO_2 to control zooplankton.

CO_2 treatments were performed as shown in Table 1.

Table 1 CO$_2$ treatments performed during 14 months, treatment duration, and CO$_2$ addition flow rate.

Treatment (n°)	Period of treatment	Treatment duration (nights)	Treatment CO$_2$ flow rate (L/min)
1	13/10/2015 - 18/10/2015	6	1
2	20/10/2014 - 24/10/2014	5	2
3	13/11/2014 - 24/11/2014	12	2
4	06/12/2014 - 11/12/2014	6	3
5	29/12/2014 - 31/12/2014	3	4
6	15/01/2015 - 17/01/2015	3	4
7	30/01/2015 - 31/01/2015	2	4
8	23/02/2015 - 25/02/2015	3	4
9	21/09/2015 - 27/09/2015	7	4
10	09/10/2015 - 12/10/2015	4	5
11	20/10/2015 - 21/10/2015	2	6
12	12/11/2015 - 13/11/2015	2	6
13	04/12/2015 - 05/12/2015	2	6

The CO$_2$ was injected into the centre of the HRAP channel on the opposite side to the paddlewheel (i.e., equidistant from the inflow and outflow of the paddlewheel). The concentration of CO$_2$ in the pond water was assessed via titration of carbonic acid [54], [59]. The CO$_2$ concentrations in the control and treated HRAPs when the CO$_2$ was not injected were similar and ranged between ~3 and ~8 mg/L.

The correct execution of CO$_2$ treatments (no interruptions or reductions of the CO$_2$ flow during the night) was monitored by comparing the CO$_2$ concentration and the pH of pond water measured at 9.00 am, with those measured at 9.00 am after standard CO$_2$ treatments (see paragraph "Standard profile of CO$_2$ concentration and pH during night time CO$_2$ injection"). The effect that treatments had on the TSS, VSS, chlorophyll-*a*, productivity, MCSA, and zooplankton abundance and dynamics in the treated HRAP were assessed over the period of 14 months. Treatments were started when zooplankton reached densities that had been previously shown to rapidly reduce the microalgal biomass in the HRAPs (>~1,000 Cladocerans/L, and >~20,000 Rotifers/L)

[6]. Treatments were considered effective only when the zooplankton density was reduced to ≤10% of that before the treatment.

Standard profile of CO_2 concentration and pH during night time CO_2 injection

The profiles of CO_2 concentration and pH resulting from the injection of 1, 2, 3, 4, 5, and 6 L/min of CO_2 in the pond water during the treatment (from 8:00 pm to 4:00 am) and subsequent five hours (from 4:00 am to 9:00 am) were measured during one night for each flow rate on triplicate 100 mL samples of the treated HRAP water collected every hour upstream of the CO_2 sparger, with samples analysed immediately. The amount of total CO_2 (g) dissolved in the water during the treatment was estimated by integrating the area below the CO_2 concentration curves of different treatments (Figure 1). The total mass of CO_2 injected into the pond water was calculated using Equation 1-2, where pressure (P) = 1 atm, temperature (T) = average daily temperature during the treatment (K), volume of CO_2 injected (V) = (flow rate (L/min) x 60 (min) x 8 (hours)), CO_2 molecular weight (MW) = 44, universal gas constant R = 0.0821 (L * atm) / (mole * K).

$$MCO2(g) = \frac{MW\ CO2 * P * V}{RT}$$
(Equation 1-2)

The efficiency of CO_2 dissolution in water was calculated by dividing the total CO_2 (g) dissolved in the water by the total CO_2 (g) injected into the water with the gas sparger. The total CO_2 (g) used for treatments performed over more consecutive nights was calculated by multiplying the CO_2 (g) consumed during one night (8 h) by the number of treatment nights.

Pearson's correlation coefficients were used to statistically analyse the relationships between CO_2 flow rate, total CO_2 injected during one night, total CO_2 injected during the whole treatment, maximum CO_2 concentration reached during the treatments, lowest pH reached during treatments, and the time required to control

zooplankton density to ≤10% of that before treatment. Moreover, the relationship between the density of filter feeding zooplankton and the relative abundance of the large colonial microalgae (*Pediastrum* sp., *Micractinium* sp., *Mucidosphaerium* sp., *Coelastrum* sp.) was also assessed. Comparisons were made by using datasets (n: 6) composed of averaged data of each season, and thresholds for significance were p=0.05 (statistical significance) and p=0.01 (high statistical significance).

RESULTS

CO_2 concentration and pH during night time CO_2 injection

Higher flow rates (1, 2, 3, 4, 5, and 6 L/min) of pure CO_2 injected for 8 h into the pond water during the night time were associated with higher maximum concentrations of CO_2 dissolved in the water, and lower minimum pH (Table 2 and Figure 1). The CO_2 flow rate of the treatments correlated positively with the total amount of CO_2 dissolved into the water (r^2= 0.97, p <0.01, n: 6), and with the maximum concentration of CO_2 in the water (r^2= 0.98, p <0.01, n: 6). Conversely, there was a negative correlation between the CO_2 flow rate of the treatments and the pond water pH (r^2= 0.84, p <0.05, n: 6).

Table 2 Night time (from 8:00 pm to 4:00 am) pure CO_2 treatments at six flow rates and resulting total CO_2 injected, total CO_2 dissolved into the water, sparging efficiency, CO_2 used during the whole treatment, maximum concentrations of CO_2 in water, minimum pH, average time required to control zooplankton to ≤10% of the density prior to treatment.

CO_2 flow rate (L/min)	Total CO_2 injected/8 h (Kg)	Total CO_2 dissolved/8 h (Kg)	Sparging efficiency (%)	Total CO_2 consumption (kg)	Maximum CO_2 concentration (mg/L)	Minimum pH	Time to control zooplankton (nights)
1	0.9	0.5	58	-	97	5.92	-
2	1.8	1.0	54	9-22	167	5.47	5-12
3	2.6	1.5	55	16	255	5.28	6
4	3.5	1.8	50	7-25	314	5.16	2-7
5	4.4	2.0	46	18	405	5.09	4
6	5.3	2.3	43	5-11	420	5.05	1-2

In all treatments the CO_2 concentration in the pond water was highest at 4:00 am when the CO_2 injection was terminated, and it decreased at a similar rate during the following hours (Figure 1). Treatments with higher CO_2 flow rates had steeper absorption and desorption profiles, except the treatments with 5 L/min and 6 L/min that had similar profiles. Treatments with lower CO_2 flow rates (1, 2, and 3 L/min) generated bubbles that were not visible at the pond water surface, and therefore had a higher CO_2 sparging efficiency than those that used higher flow rates (4, 5, and 6 L/min) (Table 2). Higher CO_2 flow rates promoted the generation of larger bubbles that reached the water surface (visual observation).
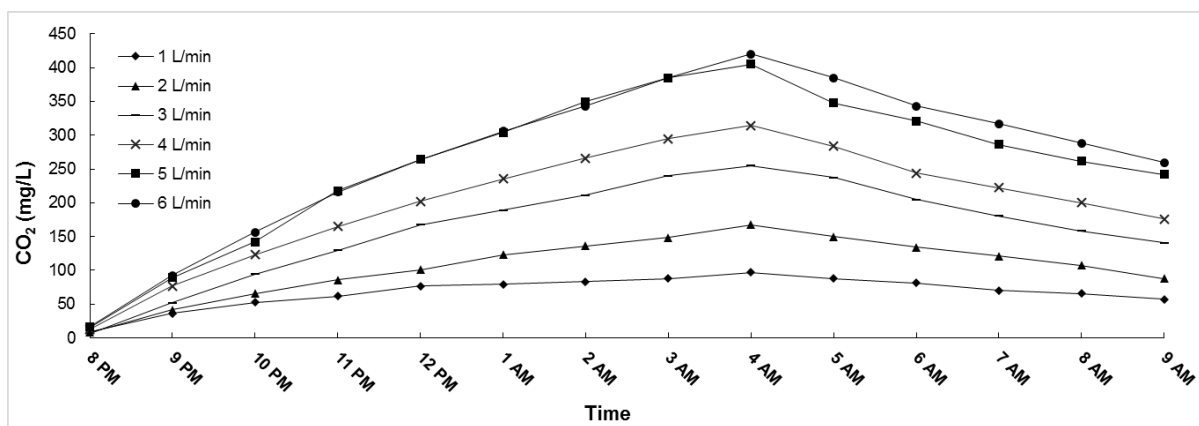
Figure 1 CO_2 concentration in the East High Rate Algal Pond water measured during six different nights (one for each flow rate) over eight hours of treatment (from 8:00 pm to 4:00 am), and during the subsequent five hours (from 4:00 am to 9:00 am).

Similarity between performance and zooplankton dynamics of the two HRAPs

The HRAPs had nearly identical average physical and chemical parameters (TSS, VSS, chlorophyll-*a* concentration, productivity, and MCSA), and similar zooplankton dynamics and average densities when they were operated without treatments to reduce the zooplankton during the initial 14 month monitoring period (Table 3). Moreover, zooplankton population dynamics were similar during the no-treatment [6] and the treatment periods (see paragraph "Zooplankton establishment"), with

cladocerans establishing from April to December (excluding July), and rotifers between September and April.

Table 3 Average and maximum total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll-*a*, productivity, Maximum Cross-Sectional Area (MCSA), zooplankton abundance and diapausing eggs of *M. tenuicornis* and *D. thomsoni* in the West and East HRAPs during 14 months of monitoring (August 2013 - September 2014), and during 14 months when the East High Rate Algal Pond (HRAP) was treated with night time CO_2 asphyxiation, and West HRAP was untreated as a control (October 2014 - December 2015). Values are averages of 14 months (n: 62) with standard deviations. Concentrations of *M. tenuicornis*, *D. thomsoni, B. calyciflorus, C. catellina, F. longiseta, B. urceolaris, A. sieboldi*, and bdelloid rotifers are given as organisms/L of water. Concentrations of *Moina* and *Daphnia* diapausing egg, *P. fimbriatus*, and *H. incongruens* are given as organisms/L of sediment. A lack of a species is indicated with "-". Bold values refer to the treated HRAP.

Variable	HRAP	Monitoring period (14 months)		Treatment period (14 months)	
		Average	Maximum	Average	Maximum
TSS (mg/L)	W	128 (53)	312	119 (95)	398
	E	131 (59)	393	**183 (81)**	**354**
VSS (mg/L)	W	121 (48)	306	112 (91)	386
	E	122 (54)	369	**169 (74)**	**324**
Chlorophyll-*a* (µg/L)	W	1713 (965)	5379	1854 (1902)	6394
	E	1720 (1105)	5175	**2999 (1750)**	**7092**
Productivity (g/m²/d)	W	5.7 (3.4)	14	4.8 (4.3)	18
	E	6.0 (3.7)	20	**7.3 (3.9)**	**16**
MCSA (µm²)	W	179 (119)	575	93 (116)	543
	E	191 (153)	608	**107 (108)**	**520**
Cladoceran, *M. tenuicornis* (ind./L)	W	392 (884)	5600	488 (799)	5133
	E	501 (712)	3400	**202 (524)**	**4667**
Cladoceran, *Daphnia* (ind./L)	W	-	-	52 (174	933
	E	-	-	-	-
Rotifer, *B. calyciflorus* (ind./L)	W	3954 (8040)	34267	1092 (3057)	14667
	E	4088 (8087)	35133	**2565 (8928)**	**62933**
Rotifer, *C. Catellina* (ind./L)	W	17850 (55021)	265600	480 (1588)	10800
	E	19583 (67889)	368800	**961 (3882)**	**31467**
Rotifer, *F. longiseta* (ind./L)	W	5018 (9976)	45067	10185 (20467)	113133
	E	3492 (7544)	44060	**6092 (14206)**	**84933**
Rotifer, *B. urceolaris* (ind./L)	W	-	-	259 (897)	5000
	E	-	-	-	-
Rotifer, *A. sieboldi* (ind./L)	W	-	-	10 (56)	400
	E	-	-	**17 (119)**	**1200**
Rotifer, Bdelloid sp. (ind./L)	W	122 (402)	1933	2 (5)	133
	E	182 (548)	3133	**6 (43)**	**467**
Ostracod, *H. incongruens* (ind./L sediment)	W	74154 (110383)	538000	79866 (114190)	441600
	E	34469 (55552)	247067	**139280 (142254)**	**551000**
Copepod, *P. fimbriatus* (ind./L sediment)	W	1245 (3603)	20667	412 (662)	3667
	E	862 (1850)	9867	**181 (352)**	**2067**
Diapauing eggs *Moina* (ind./L sediment)	W	1356 (3047)	21533	15622 (10250)	48933
	E	23434 (24226)	117400	**22056 (15350)**	**76000**
Diapausing eggs *Daphnia* (ind./L sediment)	W	-	-	1373 (3866)	14333
	E	-	-	-	-

Zooplankton establishment

Zooplankton densities were higher during spring and summer (Figure 2 and Figure 3). *Moina tenuicornis* densities were higher in the control HRAP compared to the treated HRAP (Figure 2-B). The average density of *M. tenuicornis* in the treated HRAP was the lowest during both the no-treatment and the treatment periods (Table 3). However, the average density of *M. tenuicornis* diapausing eggs in the treated HRAP was higher than that in the control HRAP and similar to that of the East HRAP during the monitoring period (Table 3). *Daphnia thomsoni* was not detected during the initial monitoring period (Table 3) and during the treatment period only established in the control HRAP following a period when *M. tenuicornis* had high densities (Figure 2-D). *D. thomsoni* progressively replaced the population of *M. tenuicornis* (Figure 2-B-D). The ostracod *H. incongruens* persisted throughout the treatment period in both HRAPs (excluding the control HRAP during August, October, and December 2015, Figure 2-F), and its average density in the treated HRAP was the highest recorded during both the no-treatment and the treatment periods (Table 3). The copepod *P. fimbriatus* always occurred at low density (Table 3, Figure 2-G), and its average density in the treatment HRAP was the lowest of all HRAPs during both the no-treatment and the treatment periods (Table 3).
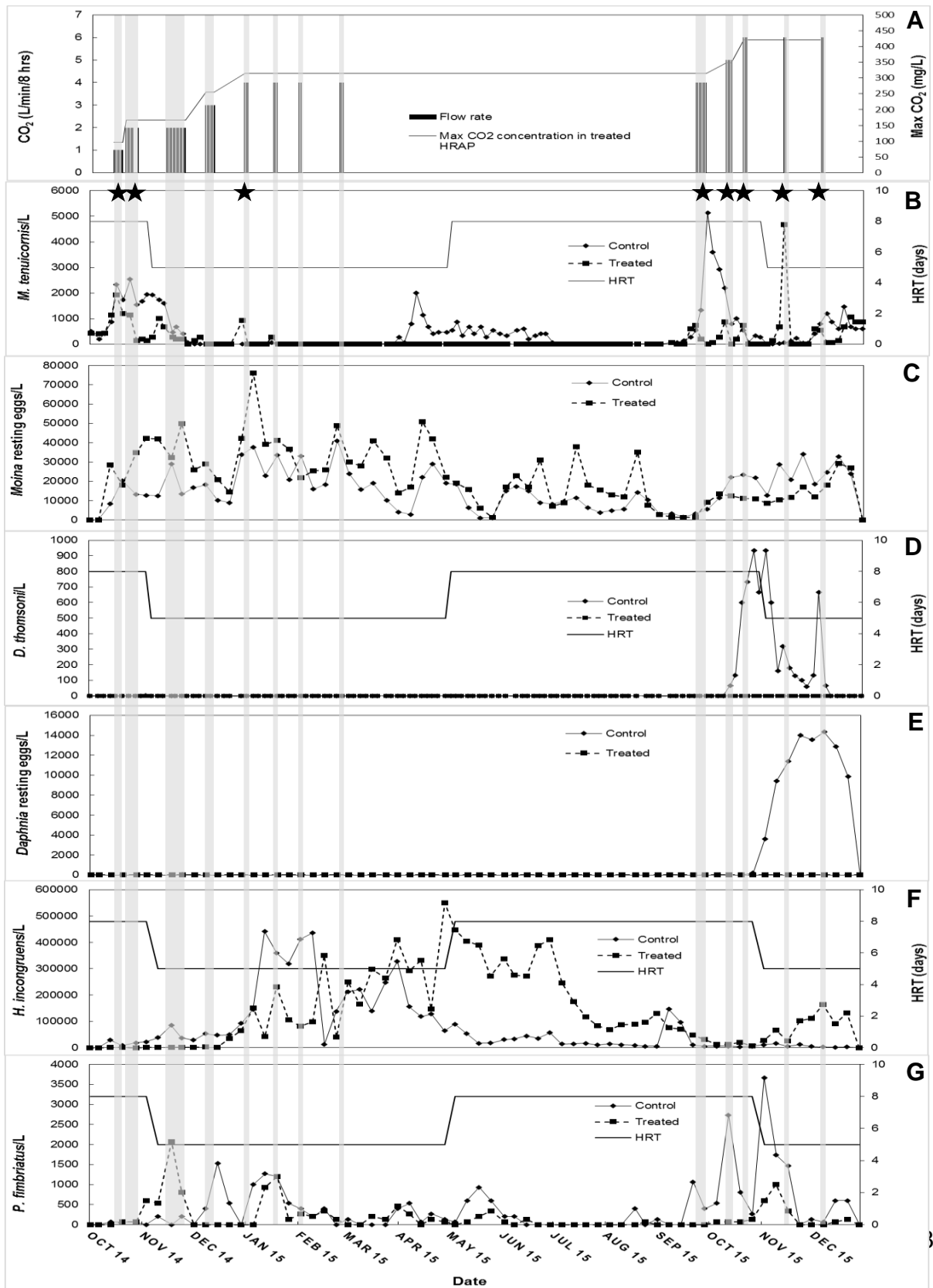
Figure 2 Treatments (13) performed during the experimental period with six CO$_2$ flow rates (vertical bars of plot A) and resulting maximum CO$_2$ concentrations in the pond water (horizontal line of plot A), densities of *M. tenuicornis* (B), *M. tenuicornis* diapausing eggs (C), *D. thomsoni* (D), *D. thomsoni* diapausing eggs (E), *H. incongruens* (F), and *P. fimbriatus* (G) in control and CO$_2$ treated High Rate Algal Ponds. The horizontal lines in B, D, F, and G indicate the hydraulic retention time (HRT). Vertical grey bands across the plots indicate the CO$_2$ treatments and the black stars indicate the relevant species responsible for initiating the CO$_2$ treatment.

The rotifers *B. calyciflorus*, *C. catellina* and *A. sieboldi* reached higher average and maximum densities in the treated HRAP than in the control HRAP (Table 3 and Figure 3-B-D-F). Conversely, *F. longiseta* reached lower average and maximum densities in the treated HRAP than in the control HRAP (Table 3, Figure 3-E). *B. urceolaris* established only in the control HRAP (Table 3 and Figure 3-C), and bdelloid rotifers occurred only occasionally in both HRAPs (Table 3).

Zooplankton control using CO₂ asphyxiation

Treatments performed using higher CO_2 flow rates promoted faster reductions of zooplankton density (Table 4). The number of treatment nights required to control zooplankton (*M. tenuicornis* and rotifers) to densities ≤10% of that before treatment correlated negatively with all of the following: CO_2 flow rate ($r^2 = 0.65$, $p < 0.05$, n: 6); the maximum CO_2 concentration reached during treatment ($r^2 = 0.70$, $p < 0.05$, n: 6); the total amount of CO_2 injected during one night (8 h) ($r^2 = 0.64$, $p < 0.05$, n: 6); and the total amount of CO_2 injected during the entire treatment (over consecutive nights) ($r^2 = 0.76$, $p < 0.05$, n: 6). Treatments with a CO_2 flow rate of 1 L/min were insufficient to reduce the density of both cladocerans and rotifers.

CO_2 asphyxiation was particularly effective in the control of microcrustaceans, with the exception of the ostracod *H. incongruens*. The average populations of the cladoceran *M. tenuicornis*, copepod *P. fimbriatus*, and ostracod *H. incongruens* in the treated HRAP were 41.3%, 43.9 %, and 174.4% of that recorded in the control HRAP, while the cladoceran *D. thomsoni* established only in the control HRAP (Figure 2-D). The control of *M. tenuicornis* to densities ≤10% of those before treatment required up to ~12 nights at a CO_2 flow rate of ~2 L/min, ~3-7 nights at a CO_2 flow rate of 4-5 L/min, and ~1-2 nights at a CO_2 flow rate of 6 L/min (Table 4). Treatments with a CO_2 flow rate of 6 L/min usually eradicated *M. tenuicornis* in only one night, but the second night of treatment was conducted to reduce the chance of individuals surviving the first night of treatment and re-establishing in the HRAP. The densities of both the ostracod *H. incongruens* and the copepod *P. fimbriatus* declined after ~1 week following the CO_2 treatments with a flow rate ≥2 L/min (Figure 2-F-G), although similar reductions

were also detected in the control HRAP. The density of *H. incongruens* always increased shortly after (~2 weeks) the CO_2 treatments (Figure 2-F).The densities of the rotifers *B. calyciflorus* and *C. catellina* were allowed to increase to over 20,000 individuals/L before starting treatments n° 3, 4, 6, 7 and 8, and reached much higher maximum densities than those observed in the control HRAP (Table 4, Figure 3). However, the maximum densities of *B. calyciflorus* and *C. catellina* were higher than those in the control HRAP only before the CO_2 treatments were initiated, and always decreased after CO_2 treatments with flow rates ≥2 L/min were performed. This resulted in higher overall average densities of *B. calyciflorus* and *C. catellina* across experiments in the treated HRAP compared to the control HRAP. The rotifer *A. sieboldi* was only present for a short time (< two weeks) in both control and treatment HRAPs, and did not need to be treated with CO_2 asphyxiation. CO_2 flow rates of 2 and 3 L/min were sufficient to reduce maximal densities of rotifers to levels similar to those of the control in 4-5 days, but required ~2 weeks to reduce the population density to ≤10% of that before the treatment. Flow rates of 4 L/min eradicated dense populations of rotifer within ~3 days. Treatments with CO_2 flow rates of 5 and 6 L/min prevented rotifers from establishing in the pond. The average densities of *F. longiseta*, *A. sieboldi*, *C. catellina*, and *B. calyciflorus* in the treated pond during the entire experimental period were 59.8%, 177.8%, 200.0%, and 234.9% of densities in the control, respectively, while *B. urceolaris* only established in the control HRAP. Control of *B. calyciflorus* required ~12 nights of CO_2 addition at a flow rate of 2 L/min, compared with ~5 nights at a flow rate of 3 L/min. Control of *C. catellina* required ~3 nights of CO_2 addition at a flow rate of 4 L/min, and control of *F. longiseta* required ~7 nights of CO_2 addition at a flow rate of 2-3 L/min, compared with ~3 nights at a flow rate of 4 L/min (Table 4). The time required to control rotifers at CO_2 addition flow rates of 5 and 6 L/min could not be assessed as rotifers were unable to establish in the treated pond.

Table 4 CO$_2$ treatments performed during 14 months, maximum concentration of CO$_2$ reached during the treatment, the hydraulic retention time (HRT) during the treatment, average water temperature over the treatment period, and zooplankton densities at the beginning and at the end of the treatment. The zooplankton species in the table are the only that rapidly consumed the microalgae in HRAPs during the study (*M. tenuicornis, B. calyciflorus, C. Catellina, and F. longiseta*).

Treatment (n°)	Max [CO$_2$] (mg/L)	HRT (days)	Average water T (°C)	*Moina* density (ind./L)	Rotifer density (ind./L)
1	100	8	18	None	*B. calyciflorus*: 15,000 - 4,700
2	170	8	18	1,100 - <200	*B. calyciflorus*: 5,000 - <200 *F. longiseta*: 18,000 - <200
3	170	5	18	1,000 - 0	*B. calyciflorus*: 66,000 - 5,500
4	255	5	21	-	*B. calyciflorus*: 43,000 - 4,500 *F. longiseta*: 85,000 - 1,200
5	315	5	23	1,000 - 0	*F. longiseta*: 32,000 - 800
6	315	5	25	300 - 0	*C. catellina*: 24,000 - 0 *F. longiseta*: 16,000 - 0
7	315	5	24	-	*C. catellina*: 14,600 - 1,100
8	315	5	22	-	*C. catellina*: 31,500 - 3,300 *F. longiseta*: 50,600 - 38,500
9	315	8	15	1,300 - 0	-
10	405	8	16	900 - 0	-
11	420	8	16	800 - 0	-
12	420	5	18	4,600 - 0	-
13	420	5	19	600 - 0	-

CO$_2$ treatment performed when the pond HRT was shorter resulted in faster reduction or eradication of zooplankton. For example, *M. tenuicornis* was eradicated in one to two nights when the pond HRT was five days, compared with three to seven nights when the pond HRT was 8 days (for treatments of both 4 and 6 L/min of CO$_2$, Table 2).

Effect of zooplankton control on productivity, microalgae concentration, microalgae dominance, and MCSA

Overall, the CO_2 treated HRAP had higher average settleability efficiency, chlorophyll-*a* concentration, productivity, VSS, and MCSA than those of the control HRAP and both HRAPs during the initial monitoring period (Table 3, Table 5).

Table 5 Percentage of total suspended solids (TSS), volatile suspended solids (VSS), productivity, chlorophyll-*a*, Maximum Cross-Sectional Area (MCSA), and settleability efficiency in the High Rate Algal Pond (HRAP) with CO_2 treatment compared with those in the control HRAP. Percentages were calculated using seasonal averages. Spring 2014 (n: 9), summer 2014-15 (n: 13), autumn 2015 (n: 13), winter 2015 (n: 13), spring 2015 (n: 13), summer 2015 (n: 5). *High value due to a low biomass concentration and lack of settling particles in the control HRAP, and resulting low settleability efficiency and MCSA.

% of treated HRAP on the control HRAP	TSS (%)	VSS (%)	Productivity (%)	Chlorophyll-*a* (%)	MCSA (%)	Settleability (%)
Spring 2014 (01/10 - 24/11)	132.9	130.7	127.9	132.1	61.7	189.3
Summer 2014-15 (24/11 - 23/02)	97.5	97.0	97.2	89.9	125.0	144.7
Autumn 2015 (23/02 - 25/05)	252.2	231.9	235.7	344.2	251.0	333.2
Winter 2015 (25/05 - 24/08)	126.6	122.3	123.1	114.5	103.8	118.0
Spring 2015 (24/08 - 23/11)	341.0	340.8	394.5	391.0	295.2	1375.7[*]
Early summer 2015 (23/11 - 28/12)	309.8	311.6	313.7	892.8	104.3	511.7
Experimental period (14 months)	**154.4**	**150.8**	**151.4**	**161.8**	**115.8**	**189.2**

The average productivity and Chl-*a* concentration in the treated HRAP were higher than those in the control HRAP during autumn, spring, and early summer (Table 5), and particularly from March to May 2015, and from October to December 2015 (Figure 4-C-D). However, during summer and winter the differences between the treated and untreated HRAPs were small, despite the treated HRAP having higher densities of rotifers than the control HRAP (Figure 3, October 2014 - February 2015).

When colonial microalgae (*Mucidosphaerium* sp., *Micractinium* sp., and *Pediastrum* sp.) were dominant in the HRAPs, blooms of filter feeding zooplankton only reduced the Chl-*a* concentration to 50% of pre-bloom levels (October to mid-February 2014-15 in both HRAPs, November to December 2015 in the treated HRAP, and December 2015 in the control HRAP) (Figure 2-B-D, Figure 3-B-C-E-F, Figure 4-D, and Figure 5-A-B). In contrast, when smaller microalgal such as round unicellular species, *Scenedesmus* sp., and *Desmodesmus* sp. were dominant in the HRAP, zooplankton blooms rapidly reduced the microalgal biomass (Chl-*a* close to zero) (March - May 2015 and October - November 2015 in the control HRAP, mid-October 2015 in the treated HRAP).

The average biomass settleability and MCSA were also higher in the treated HRAP than in the control HRAP (Table 3, Table 5, and Figure 5-D-E). The high biomass settleability of both HRAPs during July and August (Figure 5-D) was associated with a lower Chl-*a* concentrations, dominance of poorly settleable smaller microalgae species, and a higher concentration of suspended biomass (indicated by microscopic analysis and comparatively high VSS) (Figure 4 B-D). This was caused by a malfunction of the WW addition system that pumped a portion of WW solids together with the liquid inflow. Microalgal dominance showed similar variations during both the treatment and the no-treatment periods. During the treatment period HRAPs were dominated by colonial microalgae such as *Mucidosphaerium* sp. and *Micractinium* sp. from October 2014 to April 2015, then unicellular species dominated from May to October 2015, and eventually the dominance changed back to colonial species (October - December 2015, Figure 5-A-B-C). Similarly, during the monitoring period colonial species such as *Micractinium* sp., *Pediastrum* sp. and *Mucidosphaerium* sp. dominated HRAPs from September 2013 to June 2014, and smaller species such as *Desmodesmus*, *Ankistrodesmus* sp. and *Monoraphidium* sp. dominated from June to August 2014 [6]. Generally, the dominance of colonial microalgae and larger MCSAs (October 2014 - March 2015) were associated with periods of high densities of filter feeding zooplankton. The density of filter feeding *M. tenuicornis*, *D. thomsoni*, *B. calyciflorus*, *B. urceolaris*, *A. sieboldi*, and *F. longiseta* had a moderate positive correlation with the abundance of the large colonial microalgae *Pediastrum* sp., *Micractinium* sp., *Mucidosphaerium* sp., and *Coelastrum* sp. (r^2= 0.15 (control), r^2= 0.15 (treated), $p < 0.01$, n: 65), and with the average MCSA (r^2= 0.19 (control), r^2= 0.43 (treated), $p < 0.01$, n: 65).
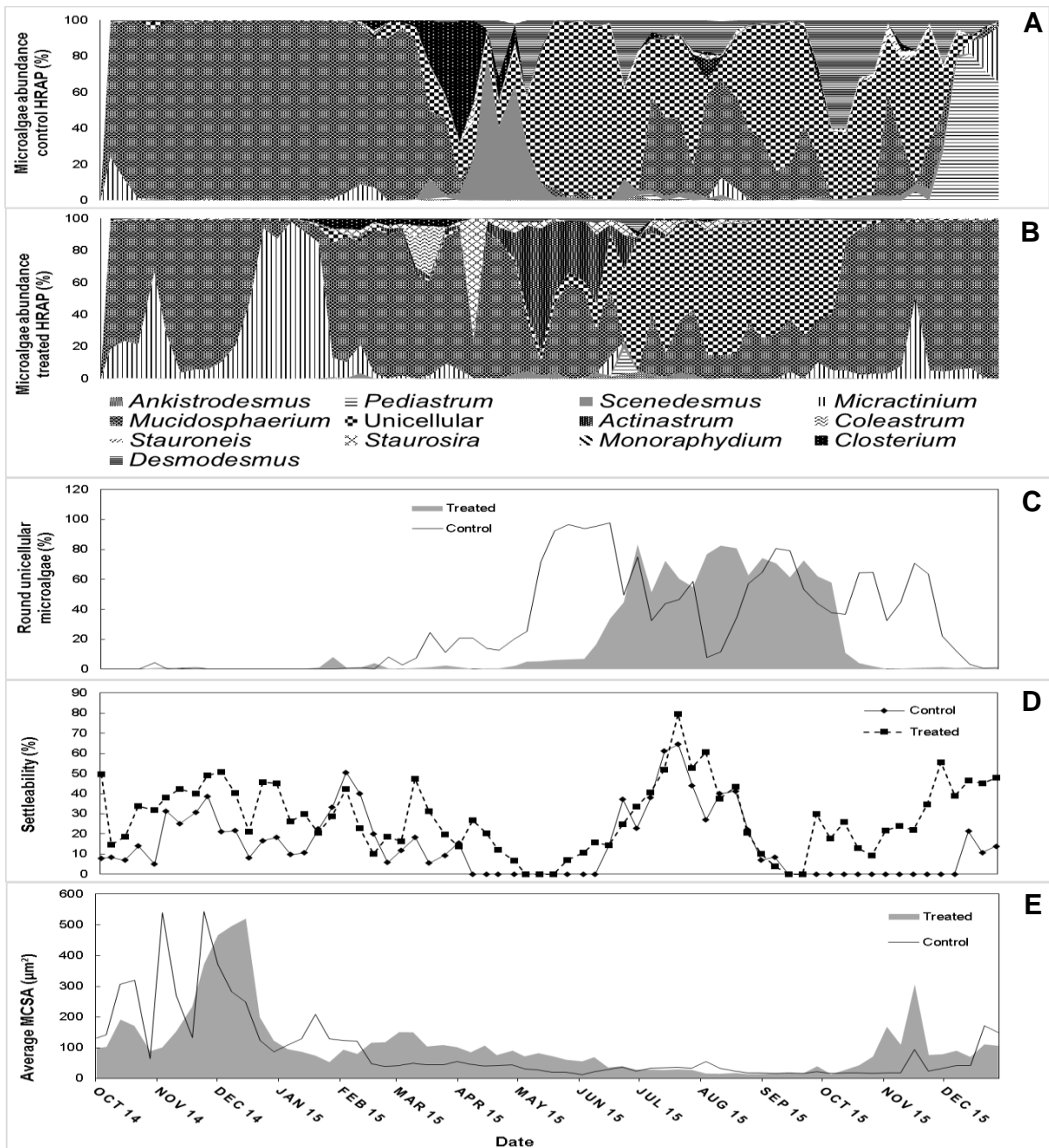
Figure 5 Comparative variation of the relative abundance of the main microalgal species, relative abundance of round unicellular microalgae (C), biomass settleability (D), and average Maximum Cross-Sectional Area (MCSA) (E), in control High Rate Algal Ponds (HRAP) (A) and treated HRAP (B), over 14 months.

DISCUSSION

Zooplankton control using CO_2 asphyxiation

CO_2 asphyxiation was found to be a versatile and effective treatment to reduce the density of cladocerans and rotifers in the 8 m^3 WW HRAP. The injection of CO_2 at higher flow rates promoted faster reductions of zooplankton densities and showed that different concentrations of CO_2 can be used to selectively control different zooplankton groups. The higher maximum concentration of CO_2 and the higher total quantity of CO_2 dissolved into the pond water at higher CO_2 flow rates were expected as a result of the higher volume of CO_2 injected and the increased gas/liquid interface for gas (CO_2) exchange with the water. However, the optimal operational CO_2 flow rate of the sparger (3 L/min, according to the manufacturer) reduced the sparging efficiency at CO_2 flow rates >3 L/min. Assuming night-time CO_2 absorption profiles similar to those of this study, treatments resulting in maximum pond water CO_2 concentrations between 180 mg/L and 250 mg/L can reduce the density of cladocerans and rotifers to ≤10% of that before treatment over 6-12 nights, and should be used to prevent harmful zooplankton blooms. Treatments resulting in maximum CO_2 concentrations of ~315 mg/L can markedly reduce and even eradicate zooplankton over 2 to 6 nights, and should be used when zooplankton can rapidly consume the dominant microalgal species; for example, when *B. calyciflorus* can graze on *Ankistrodesmus* sp., or *M. tenuicornis* can graze on *Monoraphidium* sp. [6]. Treatments that resulted in maximum CO_2 concentrations between 405 mg/L and 420 mg/L can eradicate *M. tenuicornis* over 1 to 3 nights and prevent the establishment of rotifers, and should be used in case of unexpected blooms of both cladocerans and rotifers to rapidly eliminate zooplankton populations. Conversely, treatments resulting in maximum CO_2 concentrations of ~100 mg/L are insufficient to control zooplankton because density reductions occurred over long periods of time (~4 weeks), and could have also been the result of natural dynamics of the zooplankton population. Moderate densities of filter feeding zooplankton can promote increased settleability of microalgae by

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consuming smaller microalgal species and promoting colonies formation and aggregation [6]. Hence, when increased microalgal settleability is beneficial for the HRAP performance the CO_2 concentration and the treatment time should be sufficiently low to preserve a moderate density of these zooplankton species. A previous study that investigated the control of zooplankton using laboratory experiments proposed that *M. tenuicornis* and *B. calyciflorus* can be eradicated overnight using ~340 mg/L, and ~530 mg/L of CO_2, respectively [45], assuming a 100% efficient transfer of CO_2 to the water. This is comparable with our findings given that with ~50% of CO_2 transfer efficiency, ~315 mg/L of CO_2 eradicated *M. tenuicornis* and rotifers in 2 to 6 nights. Chronic (continuous) CO_2 injection could be used as an alternative to overnight CO_2 injection. For example, a chronic CO_2 concentration of ~100 mg/L could be used to promote very low densities of zooplankton over few weeks, and a chronic CO_2 concentration of ~180 mg/L could be used to eradicate or prevent the establishment of all zooplankton species in less than four days [46].

CO_2 asphyxiation is not expected to increase the production of cladoceran diapausing eggs because the density of *M. tenuicornis* diapausing eggs in the treated HRAP sometimes increased and other times decreased after CO_2 treatments, and the average density of diapausing eggs in the treated HRAP was similar to that of East HRAP during the no-treatment period. The density of the ostracod *H. incongruens* usually decreased after the CO_2 treatments, especially after longer periods of treatment, although the population always recovered shortly after. It is expected that the capacity of ostracods to enclose themselves within their shells to survive unfavourable events [58], permitted their survival during the short lasting (few hours) maximum CO_2 concentrations. Moreover, CO_2 asphyxiation is not expected to reduce the density of ostracods in HRAPs over long periods of time because the sediment of the treated HRAP had the highest average density of ostracods during both the no-treatment and the treatment periods. The ostracod *H. incongruens* can promote reductions in rotifer density [45], [46], and their establishment in HRAPs should be

beneficial to overall performance. Reductions of the copepod *P. fimbriatus* were detected in both control and treated HRAPs and could not be directly associated with CO_2 asphyxiation during the treatment period. However, copepods are expected to be vulnerable to CO_2 asphyxiation given that a chronic CO_2 concentration of ~100 mg/L eradicated *P. fimbriatus* in less than one week in outdoor mesocosms [46].

The faster reduction time of zooplankton densities at shorter HRTs (five days instead of eight days) given equal CO_2 flow rates, was likely a consequence of the faster removal of individuals from the HRAP at shorter HRTs. However, shorter HRTs could also reduce the concentrations of CO_2 dissolved in the pond water more quickly due to higher dilution with inflow WW. Different environmental and biological parameters are also expected to affect the efficacy of CO_2 asphyxiation. For example, higher water temperature can decrease the treatment efficacy due to reduced solubility of CO_2 in the pond water, and shorter generation times of zooplankton [60], [61].

Zooplankton succession in HRAPs and influence of treatments on zooplankton population dynamics

The similar zooplankton species successions between the East and West HRAPs during both the no-treatment [6] and the treatment periods were likely promoted by similar opportunistic zooplankton species inhabiting the water bodies of the region [62]; the same local climate that promoted comparable zooplankton dynamics [63]; and the selective pressure of the WW HRAP environment that limited the contaminant species to those able to survive these conditions [64], [65], [66]. Moreover, the concurrent establishment of the same species of zooplankton in HRAPs was probably favoured by cross contamination between the two adjacent ponds. From September to May, frogs, ducks, their feathers and droppings were visually detected in 42% (control HRAP) and 34% (treated HRAP) of the total days of observation. The low density of zooplankton throughout the winter season (June - August) during both the no-treatment and the treatment periods was probably the result of lower water

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temperature, and increased generation time of zooplankton. Zooplankton with longer reproduction times were likely flushed out of the HRAPs before completing their reproductive cycle within the HRT (8 days) [6].

The higher average densities that *B. calyciflorus* and *C. catellina* reached in the treated HRAP compared to the control HRAP during periods of CO_2 treatments with flow rates ≤ 4 l/min could have been the result of the lower densities of *M. tenuicornis* in the treated HRAP compared to the control HRAP. The rotifers probably increased in density due to the reduced competing pressure of *Moina*. For example, the maximum peaks of *B. calyciflorus* (mid-November and early-December) were associated with reductions of *M. tenuicornis* densities from ~2,000 individuals/L and ~1,000 individuals/L to zero, respectively, in the treated HRAP. Cladocerans have a competitive advantage over rotifers in terms of shared food and mechanical damage [28], [67], [68], [69], [35], and *M. tenuicornis* was previously shown to reduce the densities of *B. calyciflorus* and *Brachionus rubens* in laboratory [45] and outdoor mesocosm cultures [46]. The establishment of the predatory rotifer *A. sieboldi* was associated with the eradication of *B. calyciflorus* in both HRAPs, probably due to predation by *Asplanchna* on *B. calyciflorus* [70], [71], [72]. *C. catellina* established when competing larger zooplankton such as cladocerans and *B. calyciflorus* were absent and did not exert competitive pressure.

D. thomsoni established in the control HRAP only after a period when *M. tenuicornis* showed high densities, and grazed on microalgae reducing their concentration (Chl-*a* close to zero). The lack of microalgae reduced night time algal respiration, and the resulting higher levels of dissolved O_2 in water likely aided the establishment of *D. thomsoni,* known to have a lower tolerance to low DO concentrations compared to *Moina* spp. [73], [74], [75]. The establishment of the larger *D. thomsoni* was associated with a reduction in the densities of the smaller *M. tenuicornis*, probably because the higher efficiency that larger zooplankters have in ingesting food particles compared to smaller ones [76], resulted in a competitive

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advantage of *Daphnia* over *Moina*. The decline and successive absence of *D. thomsoni* after the pond HRT was reduced from eight to five days (September 2015) was likely due to an insufficient time for *Daphnia* to conclude their reproductive cycle. At 20 °C (average temperature of HRAP water when the *Daphnia* population established) the reproductive cycle of *Daphnia* spp. typically takes ~7-8 days [77], [78], which is longer than the 5 day HRAP HRT. The microalgal population of the control HRAP recovered only after *D. thomsoni* disappeared, likely because *Daphnia* spp. can ingest particles up to 80 μm [39], which would have prevented the growth and establishment of most microalgae species.

Effects of zooplankton control on productivity, microalgae concentration, species, settleability and MCSA

The higher productivity and chlorophyll-*a* concentrations in the treated HRAP compared to the control HRAPs can be explained by the lower zooplankton population and grazing pressure in the treated HRAP. Overall, the additional carbon deriving from the CO_2 injected to control zooplankton was not expected to significantly increase the microalgal productivity due to reduced carbon limitation, as additional CO_2 was already provided for pH control [79]. However, a certain increase of productivity in the treated HRAP may have occurred during months with higher solar radiation (January - February). If high CO_2 flow rates (5-6 L/min) had been used for zooplankton control from the beginning of the experimental period, it is likely that the treated HRAP would have had higher average biomass concentrations, productivities and Chl-*a* concentrations, due to faster and more effective control of rotifers.

High densities of zooplankton reduced the microalgal biomass to low levels, particularly when smaller microalgae such as unicellular species were dominant in the HRAPs. This can be explained by the higher clearance and ingestion rates that filter feeding zooplankton have with smaller (<10 μm) food particles [80], [41], [81]. The similar productivity and Chl-*a* concentration of HRAPs during winter (treatment period)

(Table 5) was probably due to the lack of zooplankton and resulting low grazing pressure in both HRAPs.

CO_2 asphyxiation did not appear to directly affect microalgae dominance, settleability efficiency or MCSA, since consistent increases or reductions of these parameters were not measured during or immediately after CO_2 treatment. As discussed in Chapter 5, increased settleability due to bubble enhanced turbulence was not expected. Although CO_2 bubbles injected in the pilot HRAP were much smaller than those in the mesocosm experiments, CO_2 injection in the HRAP was performed on a very small portion of the pond area (~0.06 % of the total), and for a limited amount of time (~13 % of the total).

The high abundance of unicellular microalgae and the low MCSA in both HRAPs during the winter months (May - September 2015) were likely a consequence of insufficient densities or lack of zooplankton to consume these faster growing and smaller unicellular microalgal species [82], [83], [84].

Implications for zooplankton management

The eradication of zooplankton from a full scale HRAP of 5,000 m^3 and with a surface area of 20,000 m^2 would require ~11 m^2 of sparging surface and ~3.2 tonnes of CO_2. This calculation is made assuming a night time treatment (8 hours) with the same ratio of sparging area/HRAP surface used in the current study, ~43% sparging efficiency, a maximum pond water CO_2 concentration of ~0.42 Kg/m^3, and the reductions in zooplankton densities that were achieved in this study. This is similar to what was previously proposed in a study based on laboratory experiments where the amounts of CO_2 required for overnight eradication of *M. tenuicornis* and *B. calyciflorus* in a 3,000 m^3 HRAP, were estimated to be ~1.0 tonnes and ~1.5 tonnes of CO_2 respectively (i.e., 0.3 and 0.5 kg CO_2/m^3 HRAP respectively), although these calculations assumed 100% CO_2 transfer efficiency [45]. Assuming an average cost of bulk CO_2 captured from waste industrial streams of 17.5 US$/tonne [85], the cost of a single overnight

treatment is estimated in ~60 US$. However, the use of commercial food grade CO_2 would have a cost ~30 times higher [86]. Sparging efficiency could be increased by using more efficient sparging devices such as nano bubble generators that can have ~100% CO_2 transfer efficiency into the water [87], [88]. In particular, the use of nano-bubble generators could be used to inject the CO_2 in a limited portion of the HRAP because the CO_2 bubbles would reside in the water for days before reaching the surface and outgas in the atmosphere [89], [90], [91]. Control of zooplankton using nano-bubbles (100% CO_2 transfer efficiency and negligible CO_2 outgassing) over a period of 14 months, using overnight CO_2 treatments and assuming six blooms of rotifers and five blooms of *M. tenuicornis* (as occurred during the monitoring period [6], and the current study) would require ~36 tonnes of CO_2, with an estimated cost of 630 US$.

Alternatively, chronic (permanent) injection of CO_2 at concentrations of ~100 mg/L could be used to reduce or eradicate zooplankton populations over a few weeks [46], although this strategy is unsuitable to control rapid zooplankton blooms especially in the case of late detection. Assuming nano-bubbles with a life time of 48 hours, a permanent CO_2 concentration of 100 mg/L in a 5,000 m^3 HRAP over a period of 14 months would require ~100 tonnes of CO_2, with a total cost of 1,750 US$.

Overall, overnight CO_2 injection requires a lower total consumption of CO_2 to control zooplankton compared to chronic injection, and it is recommended when zooplankton control is the only aim of the treatment. However, chronic injection should be used when additional CO_2 is required to increase the microalgal productivity [92], or when very high concentrations of CO_2 cannot be achieved in the HRAP.

The similar seasonal zooplankton community dynamics in the HRAPs over more than 2 years of operation including both the treatment period (current study) and the previous monitoring period [6] indicate that for a particular HRAP system (geographical location, design, and operation), an assessment of zooplankton population dynamics

over at least one year could provide sufficient information to predict future seasonal zooplankton population dynamics from which to design an effective treatment protocol.

Chronic and overnight CO_2 asphyxiation could be also combined. Chronic treatment could be used to prevent or reduce zooplankton blooms when they are expected (e.g., spring and autumn), and overnight treatment could be used to control rapid and unexpected zooplankton blooms. CO_2 asphyxiation could be particularly effective for controlling zooplankton contamination in closed photobioreactor microalgal cultivation systems [93], [94], where CO_2 off gassing would be negligible. Zooplankton control using CO_2 asphyxiation would be particularly beneficial in systems culturing microalgae for food or pharmaceutical purposes as the treatment does not leave chemical residues.

Proposed protocol for zooplankton management in HRAPs

This study is a portion of a wider research aimed to develop control treatments for zooplankton that was conducted in pilot-scale HRAPs located at the Ruakura Research Centre (North Island, New Zealand). A zooplankton protocol for zooplankton management (Table 6) has been derived from data acquired during the study including: a literature review of potential zooplankton control treatments [15]; an initial 14 month monitoring period of zooplankton and microalgae dynamics in two 8 m^3 HRAPs [6]; and assessments of zooplankton control methods using laboratory experiments [45], outdoor mesocosms [46] and the two 8 m^3 HRAP (current study). The proposed protocol should be suitable for HRAPs with construction, operational, zooplankton species and dynamics similar to the HRAPs of this study.

The protocol includes treatments such as CO_2 asphyxiation, mechanical filtration, mild hydrodynamic stress, and biocontrol using cladocerans, ostracods and carnivorous rotifers. Zooplankton species that established in high densities during the study are classified according to their capacity to graze on microalgal species typical of WW HRAPs in the North Island of New Zealand. Zooplankton density thresholds

expected to cause severe reductions in pond microalgal biomass are also provided. Harmful blooms of zooplankton can occur in < 7 days ([6], and current study). Hence, the pond zooplankton density must be assessed at ~3-4 day intervals to avoid the risk that rapid zooplankton blooms would occur undetected.

Zooplankton type	Ciliates	Filter feeder cladocerans	Filter feeder rotifers (large)	Filter feeder rotifers (small)	Grasping rotifers	Carnivorous rotifers	Benthic ostracods
Example species	*Paramecium* sp.	*M. tenuicornis - Daphnia thomsoni*	*B. calyciflorus*	*B. urceolaris - B. rubens*	*C. catellina*	*A. sieboldi*	*H. incongruens*
Food preferences	Graze on bacteria and smaller microalgae species	Preferential consumption of smaller microalgae species and bacteria Capacity to ingest colonial microalgal species	Consumption of microalgae species with ø or thickness <20 µm Consumption of bacteria	Consumption of unicellular microalgae species Consumption of bacteria	Consumption of colonial microalgal species with cells weakly connected together	Consumption of all microalgal species and smaller rotifers	Consumption of detritus and settled organic matter
Benefits for HRAP	Possible reduction of unicellular microalgae	Promote the dominance of colonial microalgal species Promote the formation of larger microalgae colonies Reduce the density of rotifers	Promote the dominance of colonial microalgal species Promote the formation of larger microalgae colonies	Promote the dominance of larger microalgal species	Reduction of poorly settleable microalgae such as *Mucidosphaerium* sp. Promote the dominance of resilient colonial microalgae such as *Pediastrum* sp.	Reduction of smaller rotifer population density	Reduction of rotifer population density
Beneficial density (ind./L)	Unknown	1,000 < 1,500	10,000 < 20,000	Unknown	15,000 < 40,000	500 < 1,000	1,000 < 2,000
Detriments for HRAP	Not detected	Consumption of colonial microalgae species	Rapid consumption of smaller microalgal species	Unknown	Consumption of unspined *Micractinium* sp.	Consume all microalgal species	Not detected
Threshold for control (ind./L)	Unknown	> 2,000	> 20,000	Unknown	> 50,000	> 1,000	-
Prevention options	Permanent CO_2 injection at ~100 mg/L	Permanent CO_2 injection at ~100 mg/L Permanent filtration with meshes 500 ≤ 800 µm	Permanent CO_2 injection at 100-180 mg/L Permanent establishment of ~3,000 *M. tenuicornis*/L Permanent establishment of ~2,000 *H. incongruens*/L Establishment of carnivorous rotifers	Permanent CO_2 injection at 100 mg/L Permanent establishment of ~2,000 *M. tenuicornis*/L Permanent establishment of ~1,000 *H. incongruens*/L	Permanent CO_2 injection at 100 mg/L Establishment of ~1,000 *M. tenuicornis*/L Establishment of ~1,000 *H. incongruens*/L Establishment of carnivorous rotifers	Permanent CO_2 injection at 100-180 mg/L	Permanent CO_2 injection at ~180 mg/L
Control options	CO_2 injection at ~420 mg/L for 1-2 nights Establishment of larger competing zooplankton species	CO_2 injection at ~315 mg/L for 2-3 nights Single filtration event with mesh of 300 µm Four filtration events with mesh of 500 µm One pass through hydrodynamic disruption for ~50% mortality	CO_2 injection at 315 mg/L for 2-3 nights One pass/day through hydrodynamic disruption for 2 weeks Establishment of ~2,000 *M. tenuicornis* /L for ~2 weeks Establishment of ~1,000 *H. incongruens* /L for ~1 week	CO_2 injection at 315 mg/L for 3-4 nights Establishment of ~2,000 *M. tenuicornis*/L for ~10 days Establishment of ~1,000 *H. incongruens*/ L for ~7 days	CO_2 injection at 315 mg/L for 2-3 nights Establishment of ~2,000 *M. tenuicornis*/L for ~3 days Establishment of ~1,000 *H. incongruens*/ L for ~3 days	CO_2 injection at ~315 mg/L for 2-3 nights Mild hydrodynamic disruption	One pass through mild hydrodynamic (including sediments)

Table 6 Zooplankton control protocol for zooplankton management in wastewater High Rate Algal Ponds (HRAPs). Zooplankton food preferences, the beneficial and detrimental effect they can have on HRAP performance, the population density required for a noticeable beneficial effect and the threshold population density that can cause detrimental effects are given. Zooplankton prevention and control options are suggested. Data are based on the current study and [15], [6], [45], [46]. For details about treatments refer to the specific papers.

An alternative strategy to control zooplankton?

The results obtained during the two 14 months monitoring periods of pilot HRAPs suggested that the reduction of the pond HRT is a potential option for zooplankton density reduction. The modification of the HRT for zooplankton management was reported in Chapter 2 but was not assessed later in the study. Park et al. showed that *Pediastrum* sp. (microalgae species desired in the HRAPs) requires 4-5 days (warm season) and 8 days (cold season) to complete the reproductive cycle [95]. Reduction of HRT below optimum is expected to negatively affect the HRAPs performance in terms of nutrient removal/recovery and biomass productivity. However, we think that a moderate reduction of HRAP performance can be acceptable to prevent zooplankton blooms. In the present study the productivity of the treated HRAP was the 150% of the control HRAP over 14 months (Chapter 6), suggesting that a moderate reduction of HRAPs performance is likely to be compensated by the increased productivity.

Future research should investigate the effects that moderate reductions of HRT in HRAPs have on zooplankton densities. Here we propose a possible research strategy: 1) Published data for reproductive age and embryonic development of cladocerans and rotifers at different temperatures (from 10 to 25 °C) (Chapter 2), can be used to plot the minimum time required for the first hatching (reproductive age + the embryonic development) at different water temperatures (Figure 1) 2) the trend line that describes the minimum time required for the first hatching curve can be extended to cover the temperature range of a given HRAP system (here from 5 °C to 28 °C) 3) the resulting formula can be used to calculate the minimum time required for the first hatching of cladocerans and rotifers during a full year (Figure 2) 4) three or four different HRTs that can prevent rotifers hatching during a full year should be

identified. In our example, 3-4 days should be used during summer (average water temperature >25 °C), 5-6 days during spring and autumn and beginning/end of summer, and 6-7 days during winter. Rotifers have lower generation times than cladocerans and HRTs that prevent the establishment of rotifers are also expected to prevent the establishment of cladocerans.
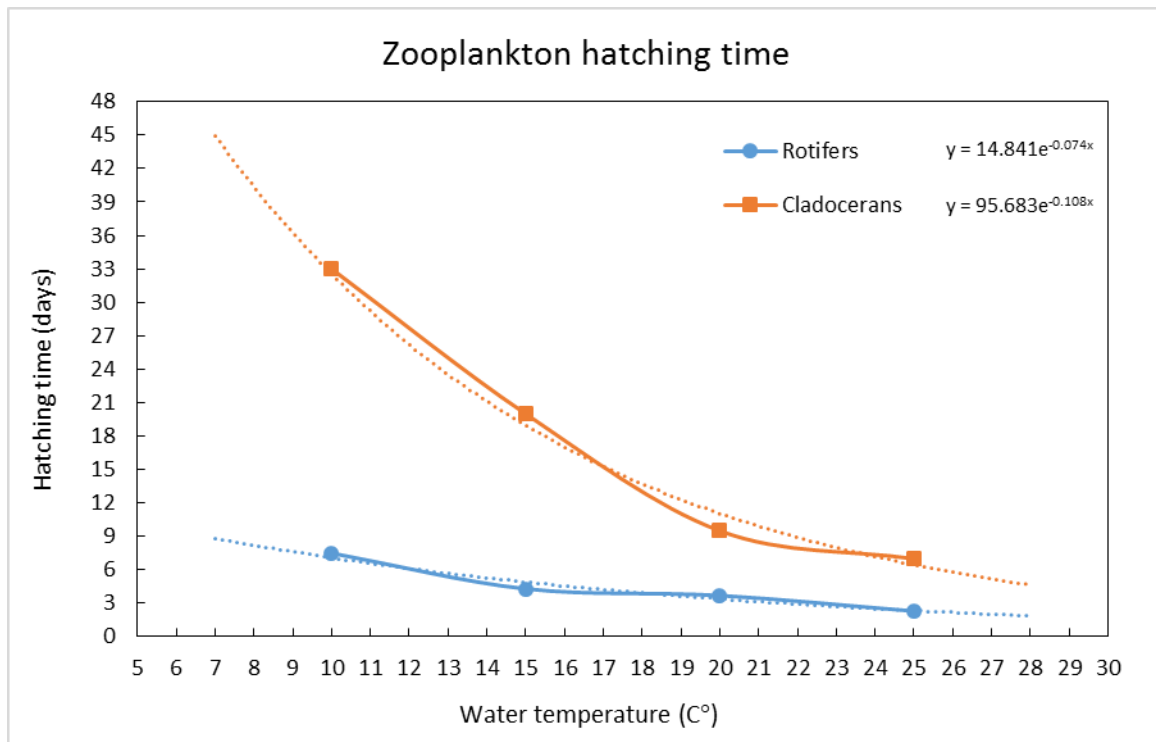

Figure 1 Theoretical minimum time required for the first hatching (reproductive age + the embryonic development) of zooplankton at different water temperatures. Data point have been sourced from Tables 1 and 2 of Chapter 2. Trend lines have been extrapolated to cover the temperature range occurring in the example HRAP. Trend lines equations have been plotted.
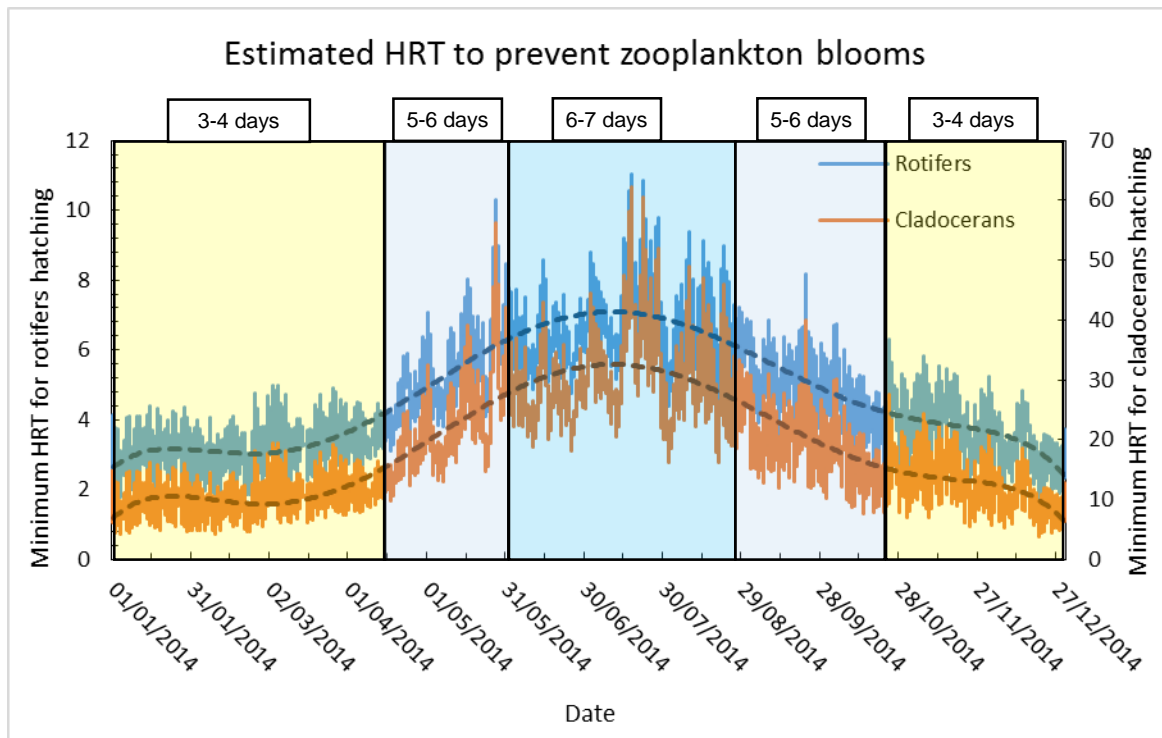
Figure 2 Minimum time required for the first hatching of cladocerans and rotifers at typical temperatures of HRAP water during 12 months (Chapter 3) calculated by using the trend line equations of Figure 1. Rotifers have lower hatching times than cladocerans, and HRTs that can prevent the establishment of rotifers are expected to prevent also the establishment of cladocerans.

Visual observations in pilot HRAPs during the two 14 month monitoring periods showed that at combination of HRT and temperature similar to those of our example, rotifers did not establish or their density rapidly decreased. Conversely, cladocerans established at HRTs much lower (6-8 days) compared to those resulting from our calculations (10 to 35 days) (Figure 2). Cladocerans can stratify in the water column (See phototaxis in Chapter 2, and Figure 9 in Chapter 5), and the short time spent at depth during the 24 h (where the HRAPs` outflow is located) resulted in increased retention of individuals into the HRAP.

Reduced HRT could be implemented by constantly adjusting the inflow rate depending on the water temperature. The control of cladocerans should be achieved by using simple mechanical treatments such as filtration, or combining the use of lower

HRT with an HRAP outflow that varies its position in the water column depending on the solar radiation intensity. The cladocerans density at different levels of the water column should be assessed during 24 h and the position of the HRAP outflow should be automatically regulated to maximize the removal of cladocerans from the water. The decreased HRAPs performance in term of microalgal growth at shorter HRT could be mitigated by reducing the water column height and increase the light penetration available for the photosynthetic process. Given that the average reduction of HRT expected to prevent zooplankton blooms is ~10-20% of that currently used, we propose a reduction of the water column of the same % (height: ~240-270 mm).

CONCLUSIONS

Night time CO_2 asphyxiation showed the potential to control different zooplankton species at different magnitudes: from moderate density reduction, to rapid population eradication and even prevention of establishment. However, the lack of competition from cladocerans in the treated HRAP promoted the development of higher densities of some rotifer species which resulted in a productivity similar to that of the control HRAP. Lower rates of CO_2 addition required longer periods of time to control zooplankton, and were more effective in controlling populations of microcrustaceans than rotifers. Higher rates of CO_2 addition promoted more rapid reductions in zooplankton densities, prevented the establishment of rotifer species and rapidly eradicated microcrustaceans, although they were associated with lower CO_2 sparging efficiencies. CO_2 addition promoted higher average VSS, productivity, Chl-*a* concentration, MCSA and microalgae settleability efficiency in the treatment HRAP compared to the control HRAP over the 14 months of the experiment. Overall, CO_2 asphyxiation effectively controlled zooplankton density, increased biomass production, and was easy to implement, perform and control.

Further research should investigate the use of nano bubble generators to inject the CO_2 for zooplankton control, and assess the efficacy of overnight CO_2 asphyxiation for zooplankton control in full scale HRAPs.

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CHAPTER 7

GENERAL CONCLUSIONS

Summary

My thesis research aimed to understand and measure the invertebrate dynamics in WW HRAPs with CO_2 addition, and to develop and validate cost effective and environmentally friendly zooplankton control methods for hectare scale HRAPs. The life histories of zooplankton inhabiting two 8 m^3 WW HRAPs for a period of 14 months, and extensive literature research on existing technologies to control zooplankton in laboratory cultures, experimental ponds, ballast waters, and aquaculture systems were used as a starting point to develop a number of zooplankton control treatments. Reduction of zooplankton densities were achieved using chemical, biological, and mechanical treatments that were validated with experiments performed on laboratory and field-scale microalgae cultures, and field-scale HRAPs.

The paired HRAPs used for this study had similar zooplankton dynamics at equivalent times of the year, suggesting that the assessment of zooplankton succession in HRAPs in similar geographical locations, and of similar design and operation, may be sufficient to predict seasonal zooplankton population dynamics. The extreme physicochemical conditions of the WW HRAPs likely limits the variety of zooplankton species able to establish, but also promoted very high population densities of those that could survive, due to the high food availability, the control of pH to near neutral, and the reduced competition from other species. The establishment of large populations of zooplankton species able to efficiently consume the dominant microalgae was associated with rapid reductions in productivity, total suspended solids, volatile suspended solids, chlorophyll-*a* concentration, a decreased capacity of HRAPs to remove nutrients from the pond water, and changes in microalgal dominance. Episodes of inhibition were observed among grazer species that established in HRAPs. For example, large populations of cladocerans, copepods and

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an ostracod appeared to reduce the densities of rotifer species. Grazing pressure, and possibly infochemicals released from the grazers into the pond water, were associated with an increased size and density of algae colonies, increases in the number of cells in colonies, and the formation of protective spines in microalgae. The settling of biomass in HRAPs was higher when colonial algae were dominant, and when microalgae had structural modifications induced by grazing pressure.

The results of my study confirmed the necessity to undertake zooplankton control measures to prevent reductions in microalgal density and WW performance in HRAPs. Moreover, my study indicated that any zooplankton control treatment should keep population densities to low levels, and maintain them as part of a stable community rather than completely eradicating them. The complete eradication of zooplankton established in HRAPs can promote the establishment of different or less desirable zooplankton species that are less easy to control. Zooplankton eradication should be avoided also because the high energy input required to remove or kill all individuals is likely to be futile when HRAPs are contiguous with other ponds (e.g., maturation ponds), and cross contamination occurs continuously. Certain zooplankton species were beneficial by selecting larger microalgal species and promoting the formation of colonies and spines in microalgae. This had the dual benefit of increasing the biomass settleability, and reducing the capacity of grazers to ingest them relative to single celled algae without spines. Hence, zooplankton control treatments that can be managed to select desired zooplankton species are preferred. However, treatments should also be able to rapidly reduce the density of zooplankton when contaminant zooplankton can rapidly consume dominant microalgal species.

A number of zooplankton control treatments potentially applicable to WW HRAPs were identified. These included increasing the pond night-time CO_2 concentration by gas injection; promoting temporary lethal un-ionized ammonia toxicity during daytime high pH periods; continuous filtration of the upper 50–80 mm of the water column; mechanical hydrodynamic cavitation and shear stress;

application of chemicals such as cypermethrin, permethrin, carbaryl, and commercial products such as PeracleanOcean™, SeaKleen™, and the chitinase inhibitor chitosan; application of infochemicals naturally occurring in zooplankton laden maturation ponds to promote algal colony and spine formation and inhibit the grazing activity; biocontrol of zooplankton grazers using competitor or predatory organisms. Of these treatments, chemical CO_2 asphyxiation, biocontrol using the cladoceran *M. tenuicornis* and the ostracod *H. incongruens*, mechanical filtration, and mild hydrodynamic stress were selected for laboratory and pilot scale testing because they were novel, environmentally friendly, and potentially cost effective options. Treatments were performed on laboratory cultures first, then outdoor mesocosm cultures with physicochemical conditions similar to those of HRAPs, and their efficacy assessed. The various treatment options promoted different magnitudes of zooplankton reductions. Further, treated cultures were associated with higher microalgal concentration, productivity, and culture stability compared to control cultures. Acute injection of CO_2 killed all zooplankton species in laboratory cultures, and chronic injection of CO_2 caused mortality of cladoceran and rotifer species in outdoor mesocosms. CO_2 injection during night periods of 8 hours controlled zooplankton densities in pilot HRAPs over a period of 14 months, and showed the potential to control different zooplankton species at different magnitudes. Acute CO_2 injection required a lower total quantity of CO_2 to control zooplankton compared with chronic injection, and it is recommended when zooplankton control is the only aim of the addition. However, chronic injection can be used when CO_2 is required as an additional carbon source to increase the microalgal productivity, or when very high concentrations of CO_2 cannot be achieved in the HRAP. Biocontrol of rotifers was achieved using populations of the cladoceran *M. tenuicornis* and was particularly effective on smaller species. Moderate densities of *Moina* can be permanently established in HRAPs, population densities can be controlled by filtration and can be allowed to grow during rotifer blooms. A permanent establishment of populations of

Moina may be difficult to achieve. However, individuals established in maturation ponds can be used to seed the HRAP. Biocontrol of rotifers was also achieved using populations of the ostracod *Heterocypris incongruens* established in the sediment. Ostracods were more effective in reducing rotifer densities than *Moina*, were easily retained in HRAPs, and thrived in the WW environment. More research is required to investigate the environmental, physicochemical, and biological conditions that can enhance ostracods reproduction and growth, and promote high population densities in of ostracods in HRAPs. Filtration rapidly controlled *M. tenuicornis* and it is expected to be easily scaled up and automatized, although it is only suitable to control larger zooplankton species. Hydrodynamic shear stress eradicated *M. tenuicornis* and reduced the density of larger rotifers. However, the density of smaller rotifer species increased. Mild hydrodynamic stress is easy to implement and operate, and could be used for a rapid and cost effective control of cladocerans in hectare scale HRAPs. Reductions of smaller zooplankton species may be achieved by increasing the intensity of hydrodynamic stress, although the disruptive effect on microalgal structure may prevent the use of this treatment at higher intensities. Phototaxis induced migration and low concentrations of CO_2 in water promoted higher densities of *Moina* in the upper 50 mm of the water column. Induced migration can be used to perform treatments such as filtration and hydrodynamic stress to control *Moina* on the upper portion of the water column, reducing the amount of water processed and the overall treatment costs.

Asphyxiation using CO_2 was the most versatile and effective zooplankton control treatment because it could be dosed to achieve desired reductions in zooplankton densities, and showed a high selectivity on zooplankton species. To date, the cost effective supply of CO_2 is the main drawback for the implementation of CO_2 asphyxiation at a hectare scale, although CO_2 can be derived from waste streams such as biogas, exhaust or flue gases, or as a by-product from industrial fermentation. From a fundamental perspective, the use of CO_2 to control zooplankton could convert one

of the most concerning waste stream of our time (CO_2) into valuable biomass. Moreover, CO_2 asphyxiation could be particularly efficient in controlling zooplankton in closed microalgal cultivation systems such as photobioreactor where CO_2 off-gassing would be negligible. Further research should explore the use of more efficient sparging systems such as nano bubble generators to reduce the CO_2 required for treatments.

Treatments upscaling and limitations

Overall, the capacity of different treatments to control zooplankton was similar at different scales (flask, mesocosm, and pilot HRAP). However, the efficacy of these treatments when used for zooplankton control at hectare scale has to be proven. CO_2 asphyxiation promoted higher reduction rates of zooplankton density when higher amounts of CO_2 were injected in water. However, the generation of high CO_2 concentrations in the pilot HRAP required longer CO_2 injection times, and the increase of CO_2 concentration in hectare scale HRAPs is expected to be challenging. Strategies for the effective injection of CO_2 at hectare scale must be developed, and future experiments must include the use of microbubble generators to rapidly and efficiently increase the CO_2 water concentration. Biocontrol of rotifers using *M. tenuicornis* was achieved using similar *Moina* densities both in flasks and mesocosm experiments, and in pilot HRAPs reductions of rotifer densities occurred at comparable *Moina* concentrations. However, the establishment of cladocerans populations in HRAPs for long periods of time is expected to be difficult to achieve. Future experiments should establish and maintain *M. tenuicornis* in HRAPs for long periods of time by using regular seeding to increase their density and filtration to reduce it. Biocontrol of rotifers using the ostracod *H. incongruens* was achieved at densities comprised between 1,000 and 2,000 ostracods/L both in laboratory flasks and outdoor mesocosms. Higher densities of the ostracod *H. incongruens* were also associated with lower densities of the rotifer population in outdoor HRAPs. However,

it was not possible to compare the ostracod concentrations of HRAPs with those used in flasks and mesocosms due to different methodologies used for the density assessment. Future experiments should assess the possibility to seed a large amount of ostracods into HRAPs and assess their population density over at least one year. Mechanical hydrodynamic stress eradicated microcrustaceans in flasks, mesocosms, and pilot HRAPs. Future experiments should test mild hydrodynamic disruption at full scale HRAPs by using a large agricultural pump, quantify the energy cost for the treatment, and assess the effects on flocs disruption and biomass settleability. Mechanical filtration has been tested only at mesocosm scale as it is expected to perform similarly at larger scales. Future experiments should aim to the design and application of a cost effective and easy to use passive filtration system for hectare scale HRAPs.

The main limitations expected in the application of the proposed strategies to control zooplankton are: 1) gas transport and availability in CO_2 asphyxiation 2) capital and energy cost of mechanical disruption of rotifers 3) need of skilled personnel and labour cost for biocontrol. These zooplankton control strategies are expected to be applicable globally, with the only exclusion of biocontrol, and in a minor extent CO_2 asphyxiation. Different climates/geographical areas are associated with different zooplankton species that can contaminate HRAPs or that are available for the biocontrol. Hence, different locations should be assessed for potential biocontrol options and tailored protocols should be developed. CO_2 asphyxiation efficiency could be reduced in warmer water due to increased zooplankton development, reduced solubility of CO_2 in water, and shorter pond HRT generally associated with the warmer weather.

Practical implications

My study has provided a protocol for zooplankton management for HRAPs. Effective control treatments can also be combined to design optimized strategies to control

specific zooplankton species. For example, when cladocerans are the contaminant zooplankton, filtration is cheaper and easier to implement compared to CO_2 injection, and it could be used to partially remove *Moina* and indirectly reduce the density of rotifers. However, the CO_2 option is available if blooms of rotifers are expected during periods when *Moina* is not established in the HRAP system. The protocol is expected to be a valuable tool for any large-scale algae farming system. When employees able to identify zooplankton are not available, a standardized control protocol that does not require a constant monitoring of the HRAP community composition should be used. Possible options include: 1) the operation of HRAPs without pH control so that the high daytime pH (particularly during summer) would promote ammonia toxicity with resulting reduction of zooplankton density 2) the filtration of cladocerans and mechanical disruption of rotifers 3) the use of chronic CO_2 asphyxiation 4) the modification of the HRT coupled with variable outflow height (if proved to be effective). However, excluding the option n° 1, the other zooplankton control strategies would require an initial set up performed by skilled employees.

Cost effective and reliable treatments for zooplankton control can support the worldwide use of hectare scale open cultivation ponds for microalgae by simplifying their management and reducing the cost for their operation. Moreover, the proposed treatments also offer a starting point to develop zooplankton control treatments for any microalgal cultivation system. Future research should now be aimed at determining the effectiveness of the proposed control treatments at full operational (hectare) scale of existing HRAP systems. Particularly, the assessment of the performance, the physicochemical and biological parameters of HRAPs operated with permanent higher concentration of CO_2 warrant further investigation. If the low zooplankton density and high productivity of microalgae culture assessed in the high CO_2 concentration mesocosms experiment would be demonstrated at the hectare scale, the use of open raceway ponds both for WW treatment and microalgae production would significantly increase their profitability. Sourcing large amounts of CO_2 is expected to be the main

cost/limit to the application of CO_2 asphyxiation at hectare scale, particularly in developing countries. However, we believe that it is worthwhile to invest in CO_2 delivery technologies, as from a fundamental perspective, it would provide a mean to capture, immobilize, reuse CO_2, and reduce the emission of a greenhouse gas. The cost of infrastructures to deliver CO_2 in areas where it can be converted/fixed into valuable biomass could be paid by industrialized countries as the CO_2 is mostly generated by their economies. These costs could be lower compared to that of future carbon tax schemes and technologies such as storing CO_2 under the ground.

The possibility to control zooplankton density and, to a certain extent, species, opens up a number of potential applications for zooplankton control treatments other than zooplankton reduction or eradication: 1) selected zooplankton species could be seeded and controlled in microalgae production systems to improve biomass settleability and to consume unwanted microalgal species; 2) moderate populations of larger zooplankton permanently established in HRAPs could be used to recover the WW nutrients in the form of zooplankton biomass, as larger zooplankton such as cladocerans are more easily removed from water than microalgae. Larger zooplankton that graze preferentially on smaller food particles can consume suspended microalgae, including poorly settleable species, and can be regularly harvested using filtration. WW nutrients recovery in the form of zooplankton biomass would also eliminate the need to grow highly settleable microalgal species, simplifying the operation of HRAPs, reducing the biomass settled on the bottom of the pond, and improving microalgae mixing in the water column; 3) zooplankton control strategies can be used to construct combined systems for WW treatment and zooplankton growth. For example, unicellular and smaller microalgae species could be cultured in zooplankton free HRAPs (e.g., by operating the HRAPs with high concentration of CO_2), and used to feed dense cultures of zooplankton in subsequent ponds. Then, filtration could be used to harvest zooplankton in these ponds. Alternatively, effluents with high concentration of zooplankton could be released in

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natural waters where the resulting consumption of microalgae could mitigate eutrophication and provide a food source for resident fish populations, promoting their development.