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Published in:
Algal Research
Publication date:
2017
Publisher rights:
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10.1016/j.algal.2017.08.024

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Download date: 25. Mar. 2020
Microzooplanktonic grazers – a potentially devastating threat to the commercial success of microalgal mass culture

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ABSTRACT

Eukaryotic microalgae and prokaryotic cyanobacteria are globally the most important primary producers, forming the base of food web in aquatic ecosystems. As such, they are eaten by a huge diversity of protistan taxa (e.g., amoeba, flagellates and ciliates), as well as zooplanktonic and larger metazoan grazers. As in terrestrial agriculture, grazing has the potential to devastate the microalgal “crop” and this has obvious implications to the commercial success of the developing microalgal industry. Whilst in conventional agriculture thousands of years of exploitation of a relatively small number of crop plants, has resulted in tools, knowledge and strategies that can manage this issue, in the case of microalgal mass culture this is relatively undeveloped. This review explores our current understanding of the issue and where further research is needed, focusing on the diversity of grazers and how microalgae under various environmental regimes and culture conditions avoid being annihilated. In addition, the implications of algal mass culture, where the objective is to maintain a virtual monoculture, are discussed in the context of how infection could be prevented/minimised and if infection occurs, how this may be managed to prevent
excessive losses in productivity or quality of the algal crop. The ultimate objective would be the development of robust methodologies for the early detection of “infection” of microalgal mass-cultures. This would allow the timely implementation of best management practices to prevent/reduce, damage caused by grazing. In reality, whilst there will be areas of commonality, as in terrestrial agricultural crops, methods will be need to be specifically tailored for each algal taxon, cultivation system and location.

Keywords:
- Algal biofuel
- Amoeba
- Biological constraints
- Ciliates
- Grazers
- Rotifers

1. Introduction

Microalgae are sunlight-driven cell factories that photosynthetically convert carbon dioxide, water and mineral nutrients to potential products such as: biofuels, human foods, animal feeds and high value compounds. The advantage of using microalgae rather than other higher plants have been well documented and include factors such as: many taxa have very high photosynthetic efficiencies and biomass productivities and can grow in conditions that are not favourable for terrestrial biomass production [1]. Thus, microalgae can provide an alternative to current unsustainable over-exploitation of natural resources, with possibility of providing a solution to the environmental dilemma of food versus energy production on high quality arable land. Whilst man has used microalgae, in particular *Spirulina/Arthrospira*, as a food for hundreds if not thousands of years [2, 3], the origins of the current development of the microalgal biotech
sector may be traced to the 1940’s, where attempts to grow microalgae were focussed on finding alternative sources of chemicals for use in munition manufacturing during the Second World War, by examining the production of lipids by various micro-algae [4, 5]. Later, during the oil-crisis of the 1970’s, when the price of crude oil was high, microalgae were “revisited” for their potential in biofuels based on their ability to accumulate oil, usually in the form of triacylglycerol’s [6]. Over the past ten years there has been an upsurge in interest in the commercial potential of microalgae stimulated by factors including: concerns associated with anthropogenic climate change, energy supply and security and increased interest in higher value metabolites for use in the food, pharma and wellness sectors. A variety of strategies have been proposed including coproduction of biofuels with high-value products [7]. To date, technologies have been developed for the production of a range of high and intermediate-value products at a commercial scale, such as health foods, aquaculture feeds and niche-market “healthy” oils and industrial oils, as well as specific high-value products. The latter are mostly lipid-based nutraceuticals or cosmeceuticals such as carotenoids and omega-3 polyunsaturated fatty acids [8]. Some of the algae producing products, such as beta-carotene, are cultivated profitably at large scale in artificial, saline lagoons, and raceway pounds [9]. Other microalgae cultivated under less harsh environmental conditions, such as *Haematococcus pluvialis* for astaxanthin production, are more susceptible to being outcompeted by other microalgae and are most commonly grown in more enclosed photobioreactor systems [10, 11].

Key to the commercial success is the cost of production at the commercial scale. However, there is relatively limited public domain knowledge about costs of microalgal cultivation and processing at the commercial scale. It has been suggested that model-based simulations, combined with pilot-plant production data, can fill this gap and a recent study by Ruiz et al. [12] performed a techno-economic evaluation of the whole process chain including cultivation, biorefinery and market exploitation for a 100 hectares facility in six locations. Their projections indicated a current cost per unit of dry biomass of 3.4 Euro kg\(^{-1}\) for microalgae cultivation in Spain (excluding biorefining products), with an expected reduction to 0.5 Euro kg\(^{-1}\) in the next ten years. At the current production costs a range of high-value products (e.g., polyunsaturated fatty acids and pigments) would be currently profitable, but products aimed at the food and bulk chemical commodities markets require further production cost reductions to become economically viable. Efficient and sustainable microalgal cultivation is only likely to be commercially
profitable if conducted in either photobioreactors, or open pond systems sited on land, within which growth conditions can be controlled and optimised. For most if not all envisaged products, a large number of factors require optimisation including choice of microalga(e), nutrients, pH etc., in addition to the engineering aspects of microalgal production and downstream processing. However, a key factor commonly overlooked is the issue of “crop” loss due to grazing. The implications of a reduction in productivity and quality of harvested biomass, due to removal of the microalgae by grazers, at best reduces profitability and where a catastrophic culture “crash” results from grazing pressure this could be an industry threatening issue.

2. Grazers

Algae are crucial to the “health” of the planet, contributing approximately 50% of the total global photosynthetic activity [13] and forming the basis of the food chain for over 70% of the world's biomass [14]. In almost all natural aquatic environments top down control, i.e., grazing by ciliates, amoeba, rotifers and other zooplankton form a key aspect of the food web and as such have significant influence on ecosystems and are critical to the effective functioning of the microbial loop [15]. Although there is much variability from site to site worldwide, on average meiofauna, i.e., organisms with a body size of <1mm, alone graze at a rate of 0.01 h⁻¹, or 1% of the standing stock of both heterotrophs and autotrophs per hour [16]. There is a considerable body of literature on microzooplanktonic grazers, which includes reviews on freshwater and marine ecosystems [17, 18], mesocosm studies [19] and the roles of grazers in manmade environments such as wastewater treatment plants [20]. Clearly, their capacity to ingest microalgae has major implications to the development of algal mass culture systems, with respect to productivity, sustainability and commercial viability. The key factors that require consideration are the impact that grazers have on algal productivity and, in mixed populations, competition between different algal taxa. This largely depends on the mode of grazing and the selectivity of the grazer(s). It is known that an individual grazer taxon may exhibit preference for certain food items and selection has been shown to be influenced by prey size [21, 22], motility [23], as well as the chemical characteristics of the food particle/alga [24]. Furthermore, some species are able to discriminate against inert particles [25]. However, the morphological variety of the both grazers and their potential food (Fig. 1) is such that in reality most potentially commercially exploitable algae are at risk of being eaten by grazers.
Industry threatening grazers range from macroscopic insects to microflagellates, barely larger than the alga(e) they ingest. Aquatic insect larvae have been reported to graze in Spirulina ponds [26, 27] and are effectively unavoidable in open freshwater pond systems. Larger zooplankton such as the Brine shrimps Artemia and Paraartemia may be a significant problem in marine, or even hypersaline pond systems where the salt levels drop below 15% (w/v) NaCl [6]. In freshwater, brackish and marine media-based production systems rotifers and cladocerans may be the major grazing zooplankton having the potential to reduce algal concentrations and production to low levels within just a few days or weeks [28, 29]. For example, rotifers and cladocerans at high densities (>10^5 L^-1) have been reported to reduce algal cell density by 90% within 2 days [30] and Cauchie et al. [31] measured a 99% reduction in algal chlorophyll-a due to Daphnia grazing over several days in an open pond system. Debatably protozoa, because of their size, diversity and speed of reproduction, pose the largest threat to commercial exploitation of algae. The authors have observed a 90% reduction in the cyanobacterium Oscillatoria within 5 to 6 days, with a corresponding 100-fold increase in the grazing ciliate Nassula [32]. In a recent study by the authors, scale-up of Chlorella production in an open pond system was severely constrained by repeated contamination and grazing by one species of chrysophyte, which was identified as Poterioochromonas malhamensis [33]. Additionally, we have observed on cultivation of Scenedesmus that the microalga usually grew well, however, culture quality and productivity deteriorated when it was invaded by vampyrellids [34]. This effect is not restricted to freshwater taxa, grazing ciliates have been observed to clarify dense outdoor mass cultures of Dunaliella salina within 2 days [35]. Furthermore, in Dunaliella ponds when the salinity drops below 20% (w/v) NaCl, amoeba and ciliates can rapidly decimate the algal culture [36]. In the context of ensuring consistency of productivity, grazing is a widespread problem and there is a growing literature on the topic (Table 1). It is worth noting that many of the microzooplanktonic grazers listed are capable of forming resistant resting stages, cysts spores etc. that may remain viable for many years. These may remain dormant in sediments or biofilms within production facilities, or be spread by wind currents [37] and as such form a major threat to open pond production systems in particular. Defences of algae against predation and the prevention of infection of production facilities are discussed in the following sections.

3. Natural defences

3.1 Morphological adaptation
Planktonic microalgae, especially nanoplanktonic species with dimensions of 2 - 30 µm are highly susceptible to zooplankton grazing [69, 70]. However, algae with larger cell sizes, 20 - 30 µm or more in longest dimension, are generally less susceptible to being ingested by microzooplanktonic grazers, simply because of their size [69]. This morphological “solution” of becoming too large to be consumed conflicts with the selection pressure of resource acquisition, which generally favours algae with smaller cell sizes and short doubling times [71]. The capacity for rapid resource acquisition and growth is a major factor in the selection of biotechnologically exploitable algal strains [8] and to some extent explains the focus on organisms with small cell size including *Chlorella* and *Nannochloropsis*.

Many algal taxa have relatively plastic phenotypes, with different cell sizes and morphological features being observed at different points in their life cycle, or under different environmental conditions. The prokaryotic cyanophyte *Microcystis aeruginosa*, a common bloom-forming organism, on cultivation in the lab invariably grows as a unicellular suspension. In nature and under experimental conditions, including when a culture of the flagellate grazer *Ochromonas* sp. was placed in dialysis tubing in a culture of the cyanobacterium, *M. aeruginosa* colony formation was induced [72]. This behaviour has also been reported by Yang et al. [73] as a response to direct grazing pressure on *M. aeruginosa* by *Ochromonas* sp. In both cases the colonies formed exceeded the upper size limit for ingestion by the flagellates, thus protecting the algae from predation. Grazing protection has also been observed in filamentous cyanobacteria, for example *Aphanizomenon* has been observed to form flake-like assemblages in the presence of the grazer *Daphnia*, but grew as single filaments in their absence [74]. Similar responses have been reported for eukaryotic microalgae, for example the green alga *Chlamydomonas reinhardtii* usually occurs as single, bi-flagellated cells in culture. However, as for other members of the Volvocales, under appropriate environmental conditions, *C. reinhardtii* can also form palmelloid life stages. In a study on exponentially growing unicellular *C. reinhardtii*, cultured in the presence of a grazer, the rotifer *Brachionus calyciflorus*, the alga formed mucoid, palmelloid colonies within 25 hours [41]. A similar response, also attributed to info-chemicals released by grazers, has been observed in *Scenedesmus subspicatus* and *S. acutus* where colonial forms are less vulnerable to grazing, but they have higher sinking rates and, thus, may be more likely to suffer from light limitation than single-celled forms [63]. A further example of grazer-induced mucilaginous colony production is by the bloom-forming prymnesiophyte...
Phaeocystis globosa which may be induced to form colonies by the heterotrophic dinoflagellates Noctiluca scintilans and Gyrodinium dominans [75]. The poor nutritional quality of the colonies can result in low or inefficient feeding by copepods [76]. To date, there has been a limited amount of work on the specificity of microzooplanktonic grazer-induced phenotypic plasticity, although the study by Luo et al. [77] indicates that there is a specific interaction, at least at the genus level, although it was observed that different strains of the same species demonstrated different levels of response. In field samples of Micractinium pusillum, the alga forms characteristic cuboidal or tetrahedral colonies with long and strong bristles, much more frequently than in cultured samples, which on long-term routine maintenance in culture collections lose their bristles/spines [78]. Medium from cultures of B. calyciflorus was demonstrated to induce long bristles (50—100 µm) in M. pusillum; however, medium from Daphnia cultures failed to induce bristle formation [77]. On testing a related chlorophyte, Chlorella vulgaris, no effect was observed on challenging cultures with samples of either grazer. Further work is needed to gain greater understanding of the nature and function of info chemicals and their capacity to induce protective phenotypic change in algae, but the implications to their use to protect algal crops has obvious potential.

3.2 Chemical antipredator defence

It has been known for many years that microalgae are capable of producing bioactive metabolites, with one of the first reports being on the production of an antibacterial fatty acid, Chlorellin, isolated from cultures of C. vulgaris [79]. The bioactive metabolites produced by algae encompass a wide range of chemical entities, with activities against viruses, bacteria, fungi, protozoa, as well as potential use in the treatment of cancers and other health problems [80, 81, 82]. It may be speculated that algae produce these chemicals to give them an ecological advantage and it is known that in natural ecosystems substances can be released by cyanobacteria into the waterbody that influence feeding rates of zooplankton, as demonstrated by Ostrofsky et al. [83] who reported inhibition of feeding rates by compounds released by Anabaena flos-aquae under both lab and field conditions. This phenomenon appears to be relatively widely spread across both cyanobacterial and microalgal taxa and it would appear logical to assume that toxin production is specifically related to grazing inhibition. However, published evidence suggests otherwise, for example the systematic study undertaken by Wang et al. [84]. This study investigated the effects on
the rotifer *B. plicatilis* of 10 strains of *Alexandrium* including eight toxic (PSP-toxin producers) and three non-toxic isolates, their media and material from lysed cells. The feeding experiments demonstrated that the rotifers readily ingested cells of a non-toxin containing isolate of *A. tamarense*, as well as *A. lusitanicum*, and *A. minutum*, both of which contain PSP-toxin, but grazed relatively less, or not at all, on the other seven *Alexandrium* strains tested. Ingestion of PSP toxin containing cells from these two known toxic species had no impact on rotifer mortality; however, even though no PSP toxins were found in *Alexandrium* sp1 and *Alexandrium* sp2, these resulted in a collapse of the rotifer populations, as did the other five toxin containing isolates tested. It thus appears likely that toxic mechanism(s) other than PSP were responsible for causing the lethal effects on the rotifers.

To date, in most cases, there is little published on either the structure or functional mode of algal-produced antipredator chemicals. However, a significant body of work has been undertaken on the model grazer, the dinoflagellate *Oxyrrhis marina*, which on ingestion and subsequent lysis of the alga *Emiliania huxleyi*, converts dimethylsulphoniopropionate (DMSP) from the alga, to dimethyl sulphide (DMS) via the enzyme DMSP lyase [85]. In a subsequent study [86] it was demonstrated that this reaction deters protozoan herbivores, presumably through the production of highly concentrated acrylate, and that *O. marina* differed in its ability to ingest and survive on algae with high-activity DMSP lyase, but preferentially select strains with low enzyme activity when offered prey mixtures. This response was not restricted to a single grazer, as addition of DMSP has also been reported to reduce grazing on *E. huxleyi* by the dinoflagellates *Amphidinium longum* and *Gymnodinium* sp., as well as the ciliate *Coxliella* sp. [87].

An alternative protective strategy involves the release of chemicals that do not prevent, or reduce, grazing on the alga, but reduce fecundity of the grazer. Diatoms are major primary producers at the base of the marine food web and their main predators include the herbivorous copepods. Secondary metabolites, including defensive oxylipins, released by these algae immediately after grazing-induced cell damage, are targeted not against the predators themselves, but rather at interfering with their reproductive success [88]. This strategy is obviously slower acting than directly killing the grazer, or deterring it from ingestion of algal cells, but has the potential to facilitate managed coexistence of the algae and grazers.

A significant amount of additional research is required to identify the range of anti-grazer compounds that are produced by algae, how their production can be controlled, or stimulated, and elucidation of their
mode(s) of action. The possibility of harness the potential of the natural “chemical warfare” between the
algae and their grazers has commercial implications and could be a crucial component in the management
of future algal production facilities. Clearly, this is a future area of research that warrants further
investigation.

4. Microalgal mass culture

In the context of commercial exploitation the scale of production in algal biotechnology can vary
hugely with 10’s or 100’s of litres being appropriate in the context of the needs for small-scale aquaculture
applications, but for other products considerably larger volumes/foot-prints are needed. For example, the
β-carotene production facility in Hutt Lagoon Australia is ~740 hectares and future algal biofuel facilities,
in order to be economically viable, will need to be significantly larger again. Irrespective of the
volume/size of production plant, or whether the algae are being cultivated in closed photobioreactors, or
open pond-based systems, infection by microzooplanktonic grazers poses a threat that could potentially
devastate the algal crop, or at best reduce productivity and hence commercial viability.

4.1 Detection

By the time an algal culture is being heavily grazed the effects may be obvious by eye as a gross
change in colour, with a reduction in the green colouration as the algal cell density reduces and in extreme
cases an increase in brown colouration as the grazers “bloom” and the algae die and lyse. Whilst changes
in colouration, optical density, or turbidity could all form the basis of an automated monitoring system,
these are unlikely to be sufficiently sensitive to detect the early stages of infection. Therefore, by the time
the grazers have been detected it may be too late to apply any strategies that may be available to control or
manage the infection. It is, therefore, important to be able to have an “early warning system” one can
potentially prevent grazing becoming a problem.

Microscopy is the most obvious approach to detection and enumeration. Sample size may vary, but is
restricted in the case of Haemocytometers to <1.0 µl, or if a larger chamber such as a Sedgwick Rafter cell
[89] is employed to 1.0 ml. Additionally, the size and configuration of the Haemocytometer may preclude its use in detecting or enumerating large grazers including rotifers and copepods. An alternative to examine larger volumes (50-100 ml) is to fix samples and then employ sedimentation chambers, where the samples can be observed using an inverted microscope [90]. This approach has the advantage that much lower densities of grazers may be detectable directly from the sample. However, the samples need a settlement phase, generally 12 - 20 h, so there is a significant delay in being able to detect, or enumerate, any grazers present. Furthermore, from the authors experience it is problematic when algal cell densities are high (> 1 x 10⁶ cells ml⁻¹) as the algae may “bury” the grazers and although it works well for larger zooplankton, many protozoans will lyse on being treated with some fixatives. However, in many cases employing Lugol’s (2%, final fixative concentration) is effective and if cells are too dark a few drops of sodium thiosulfate can be added to bleach the sample.

More recently cytometric approaches have been trialled using a FlowCAM [91, 92] that can readily detect a wide range of grazers including: amoeba, flagellates, ciliates and zooplankton. This method has a better detection capability than microscopy, with reported detection limits of <1 individual ml⁻¹ for B. calyciflorus in an algal culture (Chlorella sp.) with a cell density of 10⁷ cells ml⁻¹ [92] and <10 cells ml⁻¹ for both “large” and “small” ciliate grazers, Euplotes vannus (~80 x 45 µm) and an unidentified holotrichous ciliate (~18 x 8 µm) respectively, in N. oculata cultures with a cell density of 10⁷ cells ml⁻¹ [91]. Furthermore, small ciliates could be detected in cultures with algal densities of up to 1.4 x 10⁸ cells ml⁻¹ (>0.5 g l⁻¹ dry wt.) [91]. This approach has a number of further advantages in so far as it has the potential to be fully automated and, although samples are still relatively small, flow rates of between 0.1 – 1.0 ml min⁻¹ are readily achievable. However, there are limitations to the system, as it was not technically possible under the configurations employed, to use the system to directly examine cultures with >2.0 x 10⁸ N. oculata cells ml⁻¹, as this was beyond the computational/image capturing capacity of the FlowCAM [91]. Furthermore, at cell densities >3.0 x 10⁸ N. oculata cells ml⁻¹, using the flow-cells available, the system invariably became blocked [91].

An alternative strategy that could be employed is to use a targeted molecular approach whereby known individual grazers, or groups of grazers could be identified employing multiple polymerase chain reaction (PCR)-based tools. On using allele-specific probes and monitoring contaminants using
Quantitative PRC (QPCR) “weedy” invader algae may be detected at levels as low as one in $10^8$ cells in a culture [93]. Furthermore, this study demonstrated that the QPCR method developed was $10^4$ times more sensitive than flow cytometry in the detection of Tetraselmis striata cells serially diluted in Nannochloropsis salina culture [93]. The approach has also been employed to detect the parasitic ciliate Cryptocaryon irritans, which causes “white spot disease” in marine fishes, from the natural environment at cell densities of $<1$ cells ml$^{-1}$ [94]. Furthermore Carney et al. [95] have demonstrated that microbiome analysis by Second Generation Sequencing has the capacity to identify both eukaryotes and bacteria agents associated culture crashes in raceway mass cultures of Nannochloropsis salina. In addition to known grazers, such as Brachionus and the gastrotrich Chaetonotus, additional organisms with the potential to ingest N. salina including the suctorian ciliate Acineta, the potentially parasitic vampyrellid, Vampyrella and the amoeba Nolandella were identified [95]. The rapid developments in the use of high-throughput community sequencing and the availability and increasing sophistication of bioinformatic tools suggest to the authors that ultimately molecular approaches will be the most probable way forward for the early detection of grazers in algal crops.

Irrespective of the detection method that may be employed, unless the option for on-line continuous monitoring is available, the key to early detection will be the instigation of a suitable sampling regime. This will need to be tailored to the requirements of the individual production system, but it is suggested that a minimum of daily sampling at a number of points in each production unit (pond, bioreactor, etc.) is needed. Furthermore, sampling of sediments and/or any biofilms, if present, should be included, as these may be potential sources of inocula that could result in infection. In addition, effective grazer DNA/RNA sample preparation is critical, particularly in high-density microalgal cultures where the cell density of microalgae may be much greater than that of the invader grazers at early stages of infection. This makes it difficult to get enough grazer DNA/RNA for an effective gene-level analysis, as the grazer DNA/RNA materials may be lost or masked by the overwhelming amount of microalgal DNA/RNA isolated from an infested sample. A method for selectively separating or enriching grazer DNA/RNA from an algae-dominant background needs to be developed.

4.2 Prevention of infection
There is a limited number of publications specifically suggesting how to reduce, or prevent, infection of algal crops by grazers. The most obvious approaches involve engineering orientated solutions and/or having housekeeping procedures in place that reduce the risk of initial infection. For closed photobioreactors these are more readily achievable. An appropriately high specification photobioreactor, ideally with the possibility of sterilizing both the unit and medium, with all manipulations undertaken aseptically will theoretically not become infected. However, such ideals are not practicable on scale-up, due to their cost implications, unless the product is of high commercial value. In reality, cleaning and sanitisation rather than sterilization is achievable and these procedures must be tailored to the unit with the objective of minimising the possibility or introduction of cysts/eggs of microzooplanktonic grazers. At the planning stage engineering options that minimise connections between separate production units to avoid cross-contamination should be included. Furthermore, on planning and construction it should be ensured that there are no areas where detritus can remain between production batches, as they might retain live or resting stages of the grazers. In open systems the challenge is even greater and in reality little can be done to avoid infection beyond approaches like using netting to prevent the loss of algae associated insect larvae grazing, which has been successfully implemented in in *Spirulina* ponds [6]. In addition, employing physical barriers, such as the use of green houses, or “Poly-tunnels” may reduce the risk of wind-borne infection by cysts and eggs of grazers [32]. In reality, management protocols and the possible use of treatments or other approaches to manage infection and subsequent grazing will provide the only practicable solutions in production scale facilities.

4.3 Treatment

A wide variety of approaches, with varying degrees of success, have been trialled to control grazers of microalgae and cyanobacteria (Table 2). Direct engineering solutions to prevent or reduce algal grazing are fraught with difficulties and physical separation is not always possible, as in many cases both microalgae and their grazers are similar in size (preventing size-fractionation or centrifugation approaches). These techniques are in addition relatively expensive and time consuming, so some tested engineering approaches will not be practicable above lab-scale. However, cavitation induced by ultrasonication has
significant potential and has been demonstrated to be effective in inactivating large zooplankton, such as *Ceriodaphnia dubia*, *Brachionus plicatilis* and *B. calyciflorus*, with little effect on microalgae [96].

Chemical treatment, analogous to the use of pesticides in agriculture, has been considered, but these present issues associated with efficacy, cost and environmental damage. The use of many broad-spectrum metabolic inhibitors, such as Cycloheximide, is inappropriate as in most cases both the algal crop and the potential grazers are eukaryotes. There are a few targeted anti-grazers pesticides, for example the protozoan specific inhibitor Cytochalasin [97], or quinine sulphate, which has been demonstrated to rapidly destroy ciliates in an outdoor pond when dosed at 10 mg l$^{-1}$, whilst not damaging the alga *D. salina* [35]. Additionally, the anti-protozoan drug Metronidazole has been successfully used in treating *Scenedesmus* sp. cultures invaded by the zooflagellate *Amphelidium* sp. [59]. However such chemicals, where they are available, would be prohibitively expensive to use at production scale, in addition to any collateral environmental damage that may result from their application. It is worth noting that various approaches have been used successfully in small-scale cultures, for example reasonable success was obtained with formalin, methylene blue and malachite green at low concentrations in *Chlorella* and *Dunaliella* cultures respectively [42, 98]. Other strategies that have been trialled for their suitability for use in open ponds include: the use of copper to selectively inhibit *B. calyciflorus* growth in a mass culture of *Chlorella kessleri* [99]; hyperchlorite in *Nannochloropsis* cultures to control protozoa and disadvantage invasive strains [100]; treatment with the use of ammonia to treat rotifers and cladocerans in open ponds; and pH adjustments (decrease to pH 3 for 1–2 hours, followed by adjustment to pH 7.5) have been used to target rotifers [101, 102] and heterotrophic flagellates [66]. Glyphosate and ozone have been used to maintain *Nannochloropsis* cultures [99, 103]. Whilst the use of conventional pesticides including: Dipterex, Parathion, and dichlorodiphenyltrichloroethane (DDT) have been historically demonstrated to control zooplankton in laboratory-scale *Chlorella* sp. cultures, these have not been used in large-scale culture [44] and today would no longer be considered environmentally acceptable.

The above treatments are not likely to be applicable at scale and alternative more readily scalable and cost-effective approaches are required. Options that involve temporarily changing environmental conditions such as osmotic potential, pH, or temperature, all have significant engineering and cost implications. However, where systems have been installed to increase CO$_2$ levels to boost algal
productivity these could in parallel be employed to control the grazer population. Increasing pond night-
time CO$_2$ concentration by gas injection of pure CO$_2$, has been demonstrated to result in the rapid control
of a zooplankton bloom in a 1.5 m$^3$ microalgal cultures [104]. Furthermore, the authors [33] have recently
investigated the effect of culture pH (i.e., 6.0, 6.5, 7.0 and 7.5), maintained by supply of compressed air
bubbles containing various concentrations of CO$_2$, on Chlorella sorokiniana culture stability in the
presence of a grazing threat by the flagellate *P. malhamensis* and several other protozoa. When CO$_2$ levels
were low, culture crashes due to grazing were observed, and it was speculated that increased CO$_2$ partial
pressure in the culture medium enhanced diffusion of CO$_2$ into the cytoplasm of *P. malhamensis*, reducing
the intracellular pH leading to cell death [33]. This approach has been successfully trialled in the field in a
100 l raceway pond where increasing the CO$_2$ supply to a *C. sorokiniana* culture reduced the pH from 6.8
to 6.0 leading to a significant reduction in *P. malhamensis* numbers and elimination of other grazers within
8 h of initiating the treatment [33].

An alternative approach that is included here for completeness is to employ top-down biological
control, such as the introduction of higher trophic predators. This could include larger copepods or
predatory rotifers. This approach has been employed by Mitchell and Richmond [105] to use rotifers to
remove microalgal contaminants from *Spirulina*; however, for many potential algal crops using this
approach to target grazers is likely to be problematic as they will invariably consume both microalgae and
their grazers. The alternative bottom up biological control approach of introducing targeted grazer
pathogens is equally problematic. Furthermore, the authors are unaware of any known, cultivatable, target-
specific protozoan or other microzooplanktonic pathogens, or viruses, which could be introduced to
instigate biological control.

5. Further approaches to crop protection

It is certain that there is no “one size fits all” solution(s) to the prevention and control of grazers in
microalgal mass culture. Furthermore, there is a great deal of work needed before effective strategies are
available and the likelihood is that bespoke solutions will be required for each alga and production plant.
Prevention of infection is invariably more likely to be effective than treatment, or the management of
contaminant grazers, but there are constraints with respect of the economic viability of many potential
products if high cost, sterilisable, bio-secure bioreactors have to be employed. A number of important gaps
in our current knowledge and opportunities have been identified above and these are synthesised in the
three paragraphs below.

Firstly, “know your enemy”, although there is a considerable body of work on different grazer taxa,
there is still, to some extent, a lack of coordinated research that interconnects all aspects of this topic in the
context of algal biotechnology. Montagnes et al. [122] started this process but were specifically discussing
natural populations focussing on the needs for research to understand: searching, contact, capture,
processing, ingestion, digestion at community, population, and individual levels, and the need to develop
linkages in research focussing on food selection, food webs and modelling. Understanding the interactions
between the organisms involved and the capacity to manage mixed populations to the extent that grazing
of the crop is not at a level that results in either catastrophic crop loss, or a reduction in economic viability,
will be crucial to sustainability in the microalgal industry.

Secondly, breeding strain selection and the potential of modern molecular methods. Understanding
susceptibility to predation at the molecular and metabolomic levels will potentially allow the manipulation
of cultures or use of mutants that are less susceptible to grazing. For example, it has been hypothesized
that the SwmB protein defends *Synechococcus* from ingestion by *O. marina* by interfering with attachment
of dinoflagellate prey capture organelles or cell surface receptors [123]. Work has also been undertaken to
explore grazing resistance of *Synechococcus* by a heterolobosean amoeba. Challenging a library of mutants
of *S. elongatus* by an amoeba led to the identification of a grazer-resistant knockout mutant of the wzm
ABC O-antigen transporter gene, SynPCC7942_1126 and three other genes involved in O-antigen
synthesis and transport conferred resistance to being grazed [124]. Better understanding of how specific
protective traits, such as spine formation, may be induced at the molecular level could lead to the
development of GM algae that are commercially exploitable. Similarly, the capacity to grow in extremes of
osmotic potential could become the basis of a platform of products and processes, as the molecular
mechanisms become understood and transformation systems are developed. All of the options involving
the future industrial-scale cultivation and exploitation of transgenic algae raise issues with respect to
ethics, public acceptability, safety and stability that are beyond the scope of this review. However an
alternative, although less targeted, approach involving strain selection, potentially in conjunction with
conventional mutagenesis may also provide a platform to develop grazer resistant algal isolates.
Finally, integrated solutions that incorporate pest identification, tracking and management, as developed by Sapphire Energy Inc. to address the challenge of *Scenedesmus dimorphus* infection by *Amoebophidium protococcarum* [125], will be required for each facility where a significant threat of grazers occurs. In reality, this will involve all algal production facilities even those extremophiles where the threat may be readily managed via osmotic potential or pH. The implementation of solutions that can prevent crashes and offers the capacity to manage contamination in open ponds is crucial to the future economic viability of algal biotechnology.

Authors contributions

The conception and design of the review were carried out by all authors. Data acquisition was primarily performed by JGD and YG. JGD performed the initial drafting of the article, this and subsequent versions were reviewed and revised by HQ and YG. YG prepared Fig 1. All authors contributed to the final approval of the article.

Acknowledgements

JGD acknowledges National Capability funding for the Culture Collection of Algae and Protozoa (CCAP) from the UK Natural Environment Research Council (NERC). This work was partially funded by the State Development & Investment Corporation and China Electronics Engineering Design Institute, China (No. Y34115-1-Z01-0007).

References


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[17]


[33] M.Y. Ma, D.N. Yuan, Y. He, M. Park M, Y.C. Gong, Q. Hu, Effective control of *Poterioochromonas malhamensis* in massive culture of *Chlorella sorokiniana* GT-1 by maintaining CO2-mediated low culture pH. Algal Res. (Accepted)


### Table 1. Exemplar reports on microzooplanktonic grazers of potentially biotechnologically exploitable algae

<table>
<thead>
<tr>
<th>Target algae</th>
<th>Predator</th>
<th>Culture system</th>
<th>Volume/ size/ location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena flos-aquae</em></td>
<td>Rotifer</td>
<td><em>Brachionus calyciflorus</em></td>
<td>Laboratory culture 125 mL flasks</td>
<td>[38]</td>
</tr>
<tr>
<td><em>Anacystis nidulans</em></td>
<td>Ciliate</td>
<td><em>Colpoda ateinii</em></td>
<td>Open raceways for algal mass cultivation N/A</td>
<td>[39]</td>
</tr>
<tr>
<td><em>Arthrospira sp.</em></td>
<td>Rotifers &amp; Amoebae</td>
<td><em>Branchionus sp.</em> &amp; <em>Amoeba sp.</em></td>
<td>Ponds 1000 m²</td>
<td>[40]</td>
</tr>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>Rotifer</td>
<td><em>B. calyciflorus</em></td>
<td>Laboratory culture 100 mL flask</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Chlorella</em></td>
<td>Ciliate</td>
<td><em>Euplotes sp.</em></td>
<td>Culture tank 1500 L</td>
<td>[42]</td>
</tr>
<tr>
<td><em>Chlorella</em></td>
<td>Flagellate</td>
<td><em>Poterioochromonas</em></td>
<td>Laboratory culture 2 L flask</td>
<td>[43]</td>
</tr>
<tr>
<td>Species</td>
<td>Life Form</td>
<td>Organism</td>
<td>Culture Conditions</td>
<td>Volume</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------</td>
<td>-----------------------------------</td>
<td>-------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Chlorella</td>
<td>Copepod</td>
<td>Copepodans</td>
<td>Open ponds for algal mass cultivation</td>
<td>6000 L</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>Rotifers</td>
<td><em>B. calyciflorus</em> &amp; <em>B. patulus</em></td>
<td>Laboratory culture</td>
<td>2 L flask</td>
</tr>
<tr>
<td>Cyanobacterium</td>
<td>Ciliate</td>
<td><em>Colpoda ateinii</em></td>
<td>Laboratory culture</td>
<td>100 mL glass vessels</td>
</tr>
<tr>
<td>Cyanobacterium</td>
<td>Amoeba</td>
<td><em>Naegleria</em></td>
<td>Laboratory culture</td>
<td>500 mL flask</td>
</tr>
<tr>
<td>Cyanobacterium</td>
<td>Rotifer</td>
<td><em>B. calyciflorus</em></td>
<td>Laboratory culture</td>
<td>250 mL flask</td>
</tr>
<tr>
<td>Chlamydomonas reinharidii</td>
<td>Rotifers</td>
<td><em>B. calyciflorus</em></td>
<td>Laboratory culture</td>
<td>Six-well culture plate</td>
</tr>
<tr>
<td>Chlamydomonas</td>
<td>Copepod</td>
<td><em>Diaptomus sicilis</em></td>
<td>Laboratory culture</td>
<td>500 mL flask</td>
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<td>Anacystis nidulans</td>
<td>Ciliate</td>
<td><em>Colpoda ateinii</em></td>
<td>Laboratory culture</td>
<td>100 mL glass vessels</td>
</tr>
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<td>Cylindrospermum</td>
<td>Amoebae</td>
<td><em>Naegleria</em> sp.</td>
<td>Natural systems</td>
<td>500 mL flask</td>
</tr>
<tr>
<td>Dunaliella salina</td>
<td>Amoebae</td>
<td><em>Naegleria</em> sp. &amp;</td>
<td>Open ponds</td>
<td>Hutt Lagoon,</td>
</tr>
<tr>
<td>Species</td>
<td>Type</td>
<td>Description</td>
<td>Location</td>
<td>Volume (L)</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------------------</td>
<td>-------------------------------------------</td>
<td>--------------------</td>
<td>--------------------</td>
</tr>
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<td>unidentified ciliates</td>
<td>Western Australia</td>
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<tr>
<td>D. salina</td>
<td>Ciliates</td>
<td>Open ponds</td>
<td>400 L and 2000 L</td>
<td>[35]</td>
</tr>
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<td>Microcystis viridis</td>
<td>Flagellate</td>
<td>Poterioochromonas malhamensis</td>
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<td>2 L flask</td>
</tr>
<tr>
<td>Nannochloropsis oculata</td>
<td>Rotifer</td>
<td>B. plicatilis</td>
<td>Laboratory culture</td>
<td>250 mL flask</td>
</tr>
<tr>
<td>Oscillatoria agardhii</td>
<td>Ciliate</td>
<td>Nassula ornata</td>
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<td>500 mL flask</td>
</tr>
<tr>
<td>Phaeodactylum</td>
<td>Flagellate</td>
<td>Monas sp.</td>
<td>Open tanks for algal mass cultivation</td>
<td>1200 L and 2400 L</td>
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<td>Flagellate</td>
<td>Gymnodinium sp.</td>
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<td>Prorocentrum minimum</td>
<td>Ciliates</td>
<td>Tintinnids &amp; nonloricate ciliates</td>
<td>Laboratory culture</td>
<td>six-well culture plates</td>
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<tr>
<td>Scenedesmus</td>
<td>Ciliate</td>
<td>Loxodes magnus</td>
<td>Natural systems</td>
<td>1 Ha pond</td>
</tr>
<tr>
<td>Species</td>
<td>Organism Type</td>
<td>Co-organism</td>
<td>Culture Type</td>
<td>Vessel Size</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
<td>------------------------------</td>
<td>----------------------</td>
<td>-------------</td>
</tr>
<tr>
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<td><em>Amphelidium sp.</em></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>&amp; open ponds for</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>algal mass</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cultivation</td>
<td></td>
</tr>
<tr>
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<td><em>B. calyciflorus</em></td>
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<td>300 mL flask</td>
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<td><em>Ceriodaphnia dubia</em></td>
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<td>100 mL flask</td>
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<tr>
<td><em>Scenedesmus</em></td>
<td>Cladocerans</td>
<td><em>Daphnia</em></td>
<td>Laboratory culture</td>
<td>100 mL flask</td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td>Cladocerans</td>
<td><em>Daphnia magna</em></td>
<td></td>
<td>100 mL flask</td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td>Cladocerans</td>
<td><em>Daphnia magna</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td>Cladocerans</td>
<td><em>Daphnia magna</em></td>
<td>Laboratory culture</td>
<td>100 mL flask</td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td>Rotifers</td>
<td>*B. calyciflorus &amp; B.</td>
<td>Laboratory culture</td>
<td>2 L flask</td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td></td>
<td><em>patulus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td>Ciliate</td>
<td><em>Loxodes magnus</em></td>
<td>Natural systems</td>
<td>1 Ha pond</td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td>Amoeba</td>
<td><em>Vernalophrys sp.</em></td>
<td>Open raceways for</td>
<td>600 L</td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td></td>
<td></td>
<td>algal mass</td>
<td></td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td></td>
<td></td>
<td>cultivation</td>
<td></td>
</tr>
<tr>
<td><em>Scenedesmus</em> dimorphus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Organism Type</td>
<td>Organism Name</td>
<td>Source of Cells</td>
<td>Volume</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------</td>
<td>------------------------</td>
<td>-----------------------</td>
<td>------------</td>
</tr>
<tr>
<td><em>Synechococcus</em></td>
<td>Amoeba</td>
<td>Unspecified amoebae</td>
<td>Natural systems</td>
<td>Esthwaite, UK, Lake District</td>
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<tr>
<td>Various</td>
<td>Ciliate</td>
<td><em>Lohmanniella</em> sp.</td>
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<tr>
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<td>Rotifer</td>
<td><em>Brachionus plicatilis</em></td>
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<td>500 mL</td>
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<tr>
<td><em>Synechocystis sp.</em></td>
<td>Flagellate</td>
<td><em>Poterioochromonas</em> sp.</td>
<td>Laboratory culture</td>
<td>800 mL</td>
</tr>
<tr>
<td>PCC 6803</td>
<td></td>
<td></td>
<td>&amp; various algae &amp; various algae culture systems</td>
<td></td>
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<tr>
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<td>Amoeba</td>
<td><em>Vrihiamoeba italica</em></td>
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<td>24-well cell culture plates</td>
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<td><em>Chlorella vulgaris</em></td>
<td>Flagellate</td>
<td><em>Pseudobodo</em> sp.</td>
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<td>20 mL flask</td>
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</table>

N/A Information not available
Table 2. Approaches that have been trialled in lab-, pilot- and large-scale algal cultivation systems to control microzooplanktonic grazers

<table>
<thead>
<tr>
<th>Grazer</th>
<th>Species</th>
<th>Control measures</th>
<th>Microalga</th>
<th>Effectiveness</th>
<th>Cultivation system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladocera</td>
<td><em>Moina tenuicornis</em></td>
<td>High-concentration CO₂</td>
<td>not provided</td>
<td></td>
<td>Outdoor mass culture</td>
<td>[107]</td>
</tr>
<tr>
<td>Cladocera</td>
<td><em>Daphnia magna</em></td>
<td>Fenitrothion</td>
<td><em>Nannochloris oculata</em></td>
<td>24h-ŁC₅₀: 6.7*10⁻⁵ mg L⁻¹</td>
<td>toxicity testing</td>
<td>[108]</td>
</tr>
<tr>
<td>Cladocera</td>
<td><em>Moina micrura</em></td>
<td>Unionised ammonia</td>
<td><em>Oocystis pusilla</em></td>
<td>Mortality rates: 63%</td>
<td>waste treatment pond</td>
<td>[109]</td>
</tr>
<tr>
<td>Copepoda</td>
<td><em>Diaphanosoma brachyurum</em></td>
<td>Ammonium hydroxide</td>
<td>microalgae</td>
<td>24h LD100:&lt;20 mg L⁻¹</td>
<td>high-rate algal pond</td>
<td>[102]</td>
</tr>
<tr>
<td>Copepoda</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[106]</td>
</tr>
<tr>
<td>Rotifer</td>
<td>Rotifer</td>
<td>Filtration</td>
<td>--</td>
<td></td>
<td>Outdoor ponds</td>
<td>[6]</td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>Brachionus spp.</em></td>
<td>pH adjusting</td>
<td><em>Chlorella pyrenoidosa</em></td>
<td></td>
<td>Laboratory</td>
<td>[110]</td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>B. plicatilis</em></td>
<td>Toosendanin, Celangulin, Azadirachtin</td>
<td><em>Chlorella sp., Nannochlorop sis sp.</em></td>
<td></td>
<td>Laboratory</td>
<td>[111]</td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>B. calyciflorus</em></td>
<td>Copper Ion</td>
<td><em>C. kessleri</em></td>
<td></td>
<td>Laboratory</td>
<td>[99]</td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>B. calyciflorus</em></td>
<td>Rotenone</td>
<td><em>C. kessleri</em></td>
<td></td>
<td>Laboratory</td>
<td>[112]</td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>B. calyciflorus</em></td>
<td>Quinine Sulfate</td>
<td><em>C. kessleri</em></td>
<td></td>
<td>Laboratory</td>
<td>[113]</td>
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<td>Organism</td>
<td>Species/Compounds</td>
<td>Conditions</td>
<td>Sources</td>
<td></td>
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<td>-------------------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>---------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>Brachionus</em> sp. Urea and <em>Arthrospira</em> sp. Ammonium &amp; Bicarbonate</td>
<td>Laboratory &amp; outdoor mass culture</td>
<td>[40]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>Brachionus</em> spp. High-concentration CO2</td>
<td>Not provided mass culture</td>
<td>[114]</td>
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<td></td>
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</tr>
<tr>
<td>Rotifer</td>
<td><em>B. plicatilis</em> Toosendanin, celangulin, Asadiractin</td>
<td>*Chlorella, <em>Nannochloropsis</em> <em>sps</em></td>
<td>2.132*10^{-3} mg L^{-1}; 0.175 mg L^{-1}; 18.386 mg L^{-1}</td>
<td></td>
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<tr>
<td>Rotifer</td>
<td><em>Lecane inermis</em> Al-salts</td>
<td>24 h-LC_{50}</td>
<td>Lab-based toxicity tests</td>
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<td></td>
<td></td>
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<tr>
<td>Rotifer</td>
<td><em>Philodina roseola</em> Carbofuran</td>
<td>48h EC_{50}:</td>
<td>Lab-based toxicity tests</td>
<td></td>
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<td>Rotifer</td>
<td><em>B. calyciflorus</em> Chlorpyrifos <em>Scenedesmus obliquus</em></td>
<td>10.0 mg L^{-1}</td>
<td>Lab-based toxicity tests</td>
<td></td>
<td></td>
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<tr>
<td>Rotifer</td>
<td><em>B. calyciflorus</em> Copper <em>C. kessleri</em></td>
<td>1.5 ppm</td>
<td>Open pond</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rotifer</td>
<td><em>B. calyciflorus</em> Rotenone <em>C. kessleri</em></td>
<td>24 h-LC_{50}:</td>
<td>Lab-based toxicity tests</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotifer</td>
<td><em>B. calyciflorus</em> &amp; <em>B. rubens</em> Microcystis sensitive</td>
<td>Lab-based</td>
<td>[118]</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Amoebae</td>
<td><em>Amoeba</em> sp. Urea (U) and <em>Arthrospira</em> sp. Ammonium &amp; bicarbonate</td>
<td><em>Microcystis aeruginosa</em> sensitive or 100 mg L^{-1} mass (AB) cultures</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoebae</td>
<td><em>Rosclus</em> ithacus Chemical mixture *</td>
<td>0.002 to 0.0064 mg L^{-1}</td>
<td>Lab-based toxicity tests</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Abbreviations*: CO_{2} - Carbon Dioxide, u - ug, mg, l, * - Logically reasonable assumption
<table>
<thead>
<tr>
<th>Amoebae</th>
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<th>Salinity shift</th>
<th>Dunaliella sp.</th>
<th>Outdoor mass culture</th>
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<tbody>
<tr>
<td>Ciliate</td>
<td>unknown</td>
<td>Quinine Sulfate</td>
<td>D. salina</td>
<td>Laboratory &amp; outdoor mass culture</td>
</tr>
<tr>
<td>Ciliate</td>
<td>Unknown</td>
<td>Salinity shift</td>
<td>Dunaliella sp.</td>
<td>Outdoor mass culture</td>
</tr>
<tr>
<td>Ciliate</td>
<td>Euplotes vannus</td>
<td>NH₄Cl</td>
<td>2-h LC50: 7870 mg total ammonia-N L⁻¹</td>
<td>Conventiona open system</td>
</tr>
<tr>
<td>Ciliate</td>
<td>Predator ciliates</td>
<td>Quinine sulphate</td>
<td>D. salina 24-h LC₁₀₀:12–14 mg L⁻¹</td>
<td>Outdoor algal mass cultures</td>
</tr>
<tr>
<td>Flagellate</td>
<td>Poterioochromonas sp.</td>
<td>High pH</td>
<td>Synechocystis sp.</td>
<td>Laboratory &amp; outdoor mass culture</td>
</tr>
<tr>
<td>Flagellate</td>
<td>Unidentified</td>
<td>pH 7.5–9.5 &amp; bicarbonate 0–160 mM</td>
<td>N. oleoabundans inhibited protozoan growth</td>
<td>Laboratory</td>
</tr>
</tbody>
</table>

* include Methomyl, dimethoate, malathion, dicuran, cypermethrin, carbendazim, fenithrothion and butachlor.
Fig. 1 Exemplar microzooplanktonic grazers: A. *Poterioochromonas malhamensis* grazing on *Chlorella*; B. *Nuclearia* grazing on *Spirulina*; C. *Nuclearia* grazing on *Scenedesmus*; D. Heterolobosean amoebae grazing on *Spirulina*; E. *Saccamoeba* grazing on *Nannochloropsis*; F. *Frontonia* grazing on *Spirulina*; G. *Colpoda* grazing on *Chlorella*; H. *Euplotes* grazing on *Nannochloropsis*; I. *Sterkiella histriomuscorum* grazing on *Scenedesmus*; J. *Vorticella convallaria* grazing on *Scenedesmus*; K. *Lecane inermis* in *Chlorella* culture; L. *Brachinous plicatilis* in *Spirulina* culture; M. Vampyrellid grazing on *Scenedesmus*; N. *Brachinous calyciflorus* grazing on *Scenedesmus*. Scale bar=10µm. Except Fig. 1M was cited from Gong et al. (2015, Fig. 6E), all the others images were unpublished data from Yingchun Gong.