Operational, Prophylactic, and Interdictive Technologies for Algal Crop Protection

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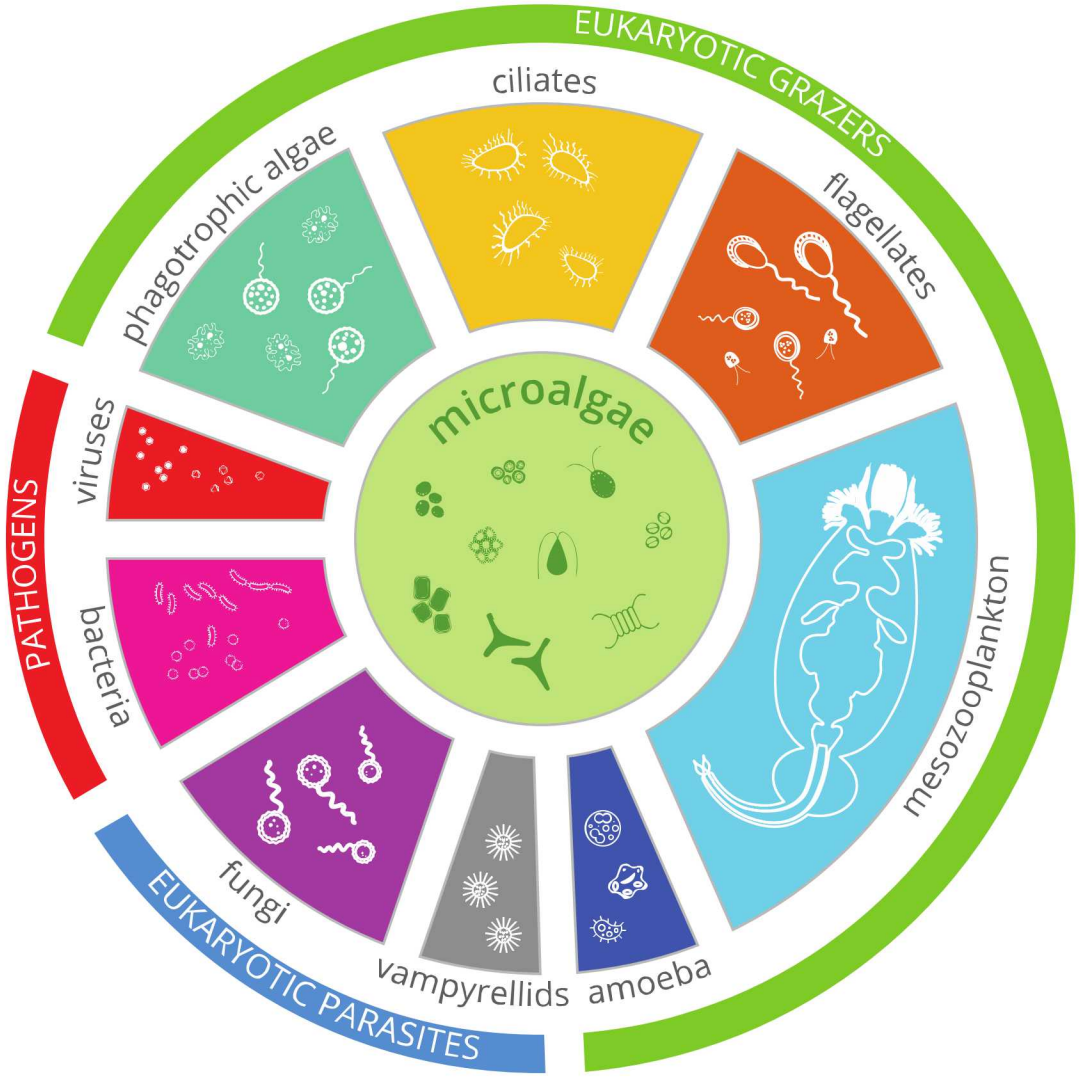
ABSTRACT

Both open and closed algal mass culture systems, for aquaculture, biofuel, and biotechnology applications, are subject to contamination with deleterious species such as grazers, parasites, and pathogens. In open cultivation systems, pond crashes due to infection with deleterious species can result in the loss of 30% of annualized production. More expensive closed photobioreactors are less prone to infection, but nonetheless do fail and can be more difficult to decontaminate than open systems. The development of effective methodologies and technologies to interdict or prevent crashes of algal mass culture systems is necessary to increase biomass production, drive down costs, reduce the risk involved with algal cultivation, and ultimately make it more favorable for investment by entrepreneurs and biotechnology companies. A variety of chemical, biological, and technological controls have been proposed and developed for algae crop protection. We review these previously developed crop protection strategies, along with examining novel strategies and areas of ongoing research and development.

INTRODUCTION

The variety of deleterious species that can lead to biomass loss in algal production systems is extensive, diverse, and not completely understood. There are a few systematic studies of biological agents that infect production systems and several important culprits have been identified (Figure 1). Carney *et al* (2016b) used second generation sequencing to determine that rotifers (*Brachionus*), gastrotrichs (*Chaetonotus*), parasitic vampyrellids (*Vampyrella*), ciliates (*Acineta*), and free-living amoebae (*Nolandella*) were most commonly identified in crashed ponds, suggesting that these grazers played a critical role in the demise of the algal mass culture systems. Eukaryotes are not the only benefactors of algal cultures as a carbon and nutrient source. Bacteria have spent millennia co-evolving with microalgae; some in symbiotic mutualism and some as parasitic hosts of specific microalgal strains. Bacteria are, to a great degree, morphologically indistinguishable, and in some cases difficult to cultivate, and therefore can prove challenging to identify and characterize. Thus, demonstrating that a particular bacterial species is responsible for failure of algal mass culture (essentially satisfying Koch's postulates) is challenging. Although algicidal bacteria are also well known in natural systems, only two bacteria, *Vampirovibrio chlorellavorus* (Ganuza *et al* 2016) and the pleomorphic bacterial strain FD111 (Lee *et al* 2018) have been implicated in the demise of algal mass cultures. Thus, recent work has identified etiological agents of pond crashes, and that can be grouped into eukaryotic grazers, eukaryotic parasites, and bacterial pathogens.

Figure 1: Diverse array of microorganisms are known to feast upon algae.



Algae are plagued by diseases caused by eukaryotic parasites and grazers (for review see Gachon *et al* 2010), but information on how detrimental these organisms are to algal mass culture systems is limited. Major classes of eukaryotic parasites include vampyrellids and fungi (for review see Carney & Lane 2014). Chytrid fungi are a well-established fungal parasite known to induce disease in host plants as well as freshwater (Atkinson 1909) and marine algae (Wei *et al* 2010). Calcofluor white was used by Rasconi *et al* (2009) for the detection of chytrids in environmental samples and could easily be applied to mass culture systems. Within mass cultures of *Graesiella* sp., Ding *et al* (2018) identified the epibiotic fungal parasite *Rhizophydium scenedesmi* as the biological agent of reduced growth and lipid accumulation. Karpov et al (2017) identified novel strains of *Amoeboradix gromovi*, a chytrid-like parasite of the alga *Tribonema gayanum*. Additional pests include eukaryotic parasites, *Amoeboaphelidium protococcarum*, isolated from an outdoor algal culture of *Scenedesmus dimorphus* (Letcher *et al* 2013), and *Paraphysoderma sedebokerense*, an amoeboid parasite also isolated from *S. dimorphus* in outdoor culture (Letcher *et al* 2016). Microzooplanktonic grazers are also a devastating threat to algae. Rotifers, ciliates, flagellates, and

amoeba are all known to cause detrimental effects to open and closed algal mass cultures (for review see Day *et al* 2017). Additionally, amoeboid grazers have been identified for cultures of the cyanobacteria, *Leptolyngbya* and *Synechococcus* (Ma *et al* 2016). In a closed photobioreactor system, Troschl et al (2017) found that the ciliated protozoa *Colpoda steinii* was frequently found, despite bleaching, and would regularly clear dense cultures of the cyanobacterium *Synechocystis* sp. in 2-3 days.

In addition, algal viruses are ubiquitous in natural systems and have been implicated in the demise of algal blooms (Van Etten & Duningan, 2012). Viruses are well-known for being highly abundant in marine environments with estimates reaching 10^{10} virions mL^{-1} of surface ocean water (Villain *et al* 2016). Despite their staggering presence in aquatic communities, there are currently no published reports of algal viruses causing pond crashes. However, it is possible that this could be due to the difficulty of detecting or identifying viral agents. The impact of algal viruses on algae culture has been implicated in the report by Evans & Wilson (2008) showing that the dinoflagellate *Oxyrrhis marina* preferentially grazed virus-infected *Emiliania huxylei* compared to uninfected controls. One possible explanation of this could be an evolutionarily-driven preference for algal grazers to obtain higher quantities of carbon per meal, as algal viruses are some of the largest known in the world with genomes as high as 2.5 Mb and capsids as large as 1.5 um (Villain *et al* 2016). Or, perhaps virus-infected algal cells are manipulated to produce less chemical deterrents, thus promoting algal grazing and virus dispersal. Although not currently implicated in the demise of mass algal culture systems, in light of the important role that viruses play in natural systems, it does seem likely that viral agents would have a significant impact on algal production systems.

There is a long history of research and development for physical, chemical, and biological algal crop protection strategies (for review see Wang *et al* 2013 and Montemezzani *et al* 2015) and it is still an area of significant interest, innovation, and active research. Operational strategies, such as higher salinity and pH, have been investigated as methods for mitigating contamination by understanding the optimal environmental conditions of deleterious species and employing cultivation methods that antagonize grazer health, reproduction, and viability (Wang *et al* 2016; von Alvensleben *et al* 2013). Use of supportive or protective algal strains in polyculture have recently been investigated as biological methods for mitigating contamination. Several species of cyanobacteria have known biocidal properties and can be used to provide an algal defence in polycultures for production strains without an innate defensive system (see review in Renuka *et al* 2018). Operational controls can sometimes overlap with prophylactic strategies meant to prevent contamination. Prophylactic strategies are applied to all production units in advance of infection, designed to prevent infection by deleterious species or reduce the impact of such infection. For example, higher salinity, pH, or CO_2 levels minimize contamination by generating an environment unsuitable for invasive species (see review Day *et al* 2012a). Mechanical hydrodynamic stress and filtration has been shown to effectively kill or capture grazers and reduce pond contamination (Montemezzani *et al* 2017b). In many cases, a compound or technique can be used in either a prophylactic or interdictive strategy, such as with sodium hypochlorite. It can be used in low doses to deter contamination from susceptible grazers or in higher doses to treat algal cultures with an alarming level of detrimental organisms present (Zmora *et al* 2013). Although often costly, prophylactic and operational strategies can be effective measures to prevent devastating losses of algal crop and profits.

Ultimately, contamination is a serious problem in both open algal ponds and closed photobioreactor systems and thus the economic need for crop protection is substantial (Hannon *et al* 2010). It has been estimated that crashes can result in the loss of up to 30% of annualized production in open systems (McBride *et al*, 2014). A primary key to successful crop protection in algal production systems is early detection. There are several strategies for the detection of deleterious species (see summary in Carney *et al* 2016a). Early detection enables effective interdiction strategies. Interdictive strategies, targeted applications of a biocide in response to detection of a deleterious species, are common in the production of low-value products where economic demands are the highest (McBride *et al* 2014). This limits the expense associated with the treatment of the production units that need it. Perhaps the most common interdictive strategy is the 'rescue harvest', where the infected production unit is harvested immediately upon detection of a deleterious species to prevent total loss through a culture crash.

In this review/chapter, we will focus on providing an overview on the various operational, prophylactic, and interdictive methodologies that have been developed and tested for mass cultivation of algae. Additionally, we will highlight some advanced, novel methods for detection and algal crop protection. Our goal is to define the state of the art for algal crop protection in order to facilitate further innovation and development within the field to help eliminate a significant technical and economic barrier to algal biotechnology including production of biofuel, commodity chemicals and high value products.

CHEMICAL AND BIOCHEMICAL INTERVENTIONS

Some of the oldest and best studied methods for crop protection include the use of relatively simple chemical compounds to treat infected systems. The overall goal is to identify compounds that can be added to production systems at concentrations that kill, inhibit, or mitigate contamination by deleterious species. Ideally, chemical treatments will not be detrimental to algal production, adversely impact downstream use of the biomass, or add excessive additional cost. This balance can be difficult to achieve and limit the utility of these approaches. Although many chemical treatments can both be used in prophylaxis and interdiction, extensive and continued use of chemical agents can lead to the desensitization and resistance in the targeted pest species. Here, we discuss the variety of chemical treatments and their effectiveness on prophylaxis and interdiction of algal cultures.

Copper

Heavy metals are often found in wastewater treatment facilities, for which bioremediation via algal cultivation is a tempting prospect. Pradeep *et al* (2015) tested the effectiveness of copper treatment on the rotifer *Brachionus calyciflorus* in *Chlorella kessleri* cultures. The lethal concentration for 50% of the population (LC50) of Cu for isolated *B. calyciflorus* was <0.1ppm and 1.5 ppm (about 0.2 and 24 µM, respectively) when 7 rotifers per mL were in cultivation with the algae at a starting algal biomass concentration of approximately 100mg L^{-1} cell dry weight.. This type of result is not atypical; often the effective dose is found to be higher in dense biomass cultures. Dissolved organic carbon and sunlight can also reduce the effectiveness of many agents. Thus, the effectiveness of any crop protection strategy needs to be tested under realistic cultivation conditions. The need to test under realistic conditions was also demonstrated by Wang *et al* (2017b). In outdoor cultivation,

10.0 mg L^{-1} (about 157 µM) of copper in raceways eradicated all of the *Colpoda sp.* ciliates found in *Chlorella vulgaris* and the *Vorticella sp.* ciliates found in *Scenedesmus acutus* cultures, but also inhibited algal growth which was not observed in 24-h flask/column tests with copper sulfate. The effects of heavy metal toxicity on algal culture was further investigated by Huang *et al* (2016). In the presence of nanomolar levels of free copper II ions, the innate grazer-defense mechanism of *S. obliquus* of colony formation was inhibited along with photosynthetic efficiency and algal growth. However, this effect might only be limited to algal cultures that form colonies or *Scenedesmus* sp. cultures, and other algal species might respond significantly better or worse. These results do corroborate what was observed by Wang *et al* 2017b after treatments of micromolar to millimolar levels of copper were added to algal raceways. Lower concentrations of copper also limited ciliate populations and did not inhibit algal growth. However, these results need to be further tested on other algal cultures, raceway conditions, and grazer populations. Additionally, environmental caution and the potential for accumulation in algal biomass. should be considered whenever adding heavy metals to a water system.

Bleach

Sodium hypochlorite (NaOCl) is a commonly utilized oxidant for the interdiction of pond crashes. Bleach, defined as 3-8% sodium hypochlorite, is readily available and commonly used in production facilities for the pretreatment of source water and decontamination or disinfection of equipment. Park *et al* (2016) evaluated the effectiveness of sodium hypochlorite (bleach) against the rotifer *Brachionus calyciflorus* in co-culture with *Chlorella kessleri*. Although the 24-h LC50 for *B. calyciflorus* was determined to be 0.198 mg L^{-1} (5.59 µM) of chlorine, pond treatments of 0.45 mg L^{-1} (13 µM) of chlorine, the rotifer population was uninhibited for 96 h and ended up crashing the entire algal culture. Park *et al* 2016 increased chlorine dosage to 0.45 – 0.6 mg L^{-1} (13 – 17 µM) every 2 h and were able to kill *B. calyciflorus* without harming *C. kessleri*, despite its 24-h LC50 of 0.321 mg L^{-1} (9.06 µM) to chlorine. Higher concentrations of chlorine were tested by Wang *et al* (2017b) in controlling ciliate populations in algal cultures. While 10.0 mg L^{-1} (282 µM) of chlorine eliminated 95% of the *Colpoda* sp. ciliate in *Chlorella vulgaris* LRB-1201 ponds, this same chlorine dosage only eliminated 65% of *Vorticella* sp. ciliates in *Scenedesmus acutus* ponds and severely inhibited the growth of the alga. Although no dosage information is provided, Lee *et al* (2018) similarly indicate that sodium hypochlorite is an effective treatment strategy for the abatement of the new pleomorphic bacterial strain, FD111, and prevents the culture crash of *Microchloropsis salina* (note: *Nannochloropsis salina*, CCMP 1776, has recently been reclassified and renamed *Microchloropsis salina*). However, timing of bleach treatment, in addition to concentration, might be key since some alga might not be able to recover after bleach treatment if their numbers are greatly reduced from grazing. When outdoor culture systems of *Microchloropsis salina* started to diminish due to grazing on day 9, Carney *et al* (2016b) reported that sodium hypochlorite treatments were performed on day 10 at 2.5 ppm (0.034 mM), day 12 at 5 ppm (0.067 mM), and day 14 (0.13 mM) with little success. Perhaps the addition of bleach starting at low concentrations on day 10 of the culture cycle was insufficient to rescue the pond. Weissmann *et al* (2010) took the opposite approach and found that highly concentrated "shock" treatments of sodium hypochlorite at 40 – 80 mg L^{-1} (0.5 – 1 mM) at the beginning of the culture run significantly helped control contamination. Zmora *et al* (2013) recommend doses of 4-10 ppm (112 – 282 µM) of active chlorination in order to eliminate grazers from algal cultures. Although bleach treatment has previously succeeded as a selective biocide against some zooplankton cultures, further outdoor

trials are required to determine effective dosages, biological sensitivity, and if prophylactic or interdictive measures are optimal.

Other oxidants

Many other oxidants have been tested for their effectiveness as an algal crop protectant with limited success. Karuppasamy *et al* 2018 tested a number of oxidants, along with other chemicals, for effectiveness against grazers in microalgal cultures. found that sodium perborate and sodium percarbonate, oxidizers typically found in laundry detergent, were effective against *Oxyrrhis sp.* grazers with an LD50 of 50 ppm (0.5 and 0.3 mM, respectively), but were ineffective against *Euplotes sp.* and not tested for any negative effects on their choice alga, *Chlorella vulgaris*. Potassium permanganate, a well-established oxidizer used in a variety of applications ranging from human medicine to biological control of aquaculture, was effective at inhibiting *Oxyrrhis*, *Euplotes*, as well as the alga *C. vulgaris* at 1 ppm (7 μM). The most promising oxidant tested was peracetic acid, which had an LD50 of 25ppm (0.35 mM) against both grazers and 50 ppm (0.7 mM) against the algae. Peracetic acid has been previously analyzed and utilized for its antimicrobial effects. However, the United States Environmental Protection Agency warns against handling concentrations of peracetic acid that exceed 1.3 ppm (0.017 mM) as it can be hazardous to human health. Additionally, the cost for routine utilization of peracetic acid was not deemed to be favorable per the technoeconomic model generated and referenced within Karuppasamy *et al* (2018). These mixed results suggest that even low concentrations of oxidants could be lethal to all living organisms within pond systems. Further studies are required to determine what algal strains are tolerant of oxidants in order to optimize interdictive methods for pond contaminants.

Ozone, chlorine dioxide, and hydrogen peroxide are also effective disinfectants for aquaculture and they have been explored as treatment options for algal mass culture systems as well. Kamaroddin *et al* (2016) found that ten minute treatments with 8 mg L^{-1} (0.2 μM) of ozone reduced bacterial contamination by *Halomonas* sp. within *Dunaliella salina* cultures. Chlorine dioxide, in comparison to liquid chlorine treatment, has been found to be more effective against viruses and mesoplankton, but similarly effective toward algae (Junli *et al* 1977). At 3.0 mg L^{-1} (44.5 μM) of chlorine dioxide, the algae tested were mostly unaffected, but the viruses and several animal planktons were impacted. These results suggest that additional tests could determine an appropriate interdictive dosage for chlorine dioxide for treatment of mass algal cultures.

Originally classified as an algicide (Kay *et al*, 1982), hydrogen peroxide treatment at 150-200 mg L^{-1} (4.4 – 6 mM) was lethal to ciliate populations present within *Dunaliella salina* mass culture, however the algae was also susceptible (Moreno-Garrido & Canavate, 2001). Drábková *et al* 2007 found that the cyanobacterium *Microcystis aeruginosa,* the green alga *Pseudokirchneriella supcapitata,* and the marine diatom *Navicula seminulum* were all susceptible to pulsed hydrogen peroxide exposure, but at different levels. The rapid chemical decomposition and light sensitivity of hydrogen peroxide also were found to play a significant role in the growth of all the algae. For the most sensitive algae tested, cyanobacterium *M. aeruginosa*, high hydrogen peroxide concentrations inhibited photosynthetic oxygen evolution and may have inhibited growth. Although it is currently unclear why different algae are susceptible at different dosages of hydrogen peroxide, understanding the impacts of temperature, light, salinity, and pH on toxicants

added to treat algal production systems is necessary. Future work to elucidate the impacts of abiotic factors on chemical efficacy will further advance available treatment options for algal ponds.

Natural compounds

In addition to ozone, other naturally occurring chemicals have been investigated for their use in generating and maintaining axenic algal cultures. Xenic cultures of the freshwater *Ettlia* sp. YC001 were treated for three days with an antibiotic and antifungal cocktail of chloramphenicol, imipenem, carbendazim, rifampicin, and tetracycline by Lee *et al* (2015). The antibiotic cocktail was initially toxic to the algae, but the cultures recovered and were confirmed to be axenic after serial dilutions, gel electrophoresis, and SEM imaging. Karuppasamy *et al* (2018) tested the effect of several antimicrobial compounds on the grazers *Euplotes* and *Oxyrrhis.* Several of the quaternary amines were found to be extremely potent against grazers, but some also inhibited the *Chlorella vulgaris* algal growth. Benzalkonium chloride (BAC) was optimal in price and toxicity towards grazers and was further assessed in an outdoor culture trial where *C. vulgaris* was infected with *Euplotes*. Only the BAC-treated algal culture, and not the un-treated control, successfully recovered. Although successful, the use of antibiotics and antimicrobials are broadly considered to be environmentally harmful and largely unscaleable for mass algal culture systems. Antibiotics and antimicrobials remain an effective laboratory tool in order to specifically exclude bacteria and facilitate studies of algal physiology and genetic engineering.

Rotenone is a naturally occurring insecticide, piscicide, and pesticide that is found in the stems and roots of the *Fabaceae* family, such as the North American jicama plant, *Pachyrhizus erosus*. Van Ginkel *et al* (2015) first tested the effectiveness of rotenone as a rotifer-killing agent and found that the freshwater *Brachionus calyciflorus* had a 24-h LC50 of 0.074 μM and marine rotifers, *Brachionus rotundiformis* and *Brachionus manjavacas*, had LC50s of 0.13 μM and 0.26 μM, respectively. The freshwater alga, *Chlorella kessleri*, and marine alga, *Tetraselmis suecica*, were uninhibited by all rotenone doses tested. Similarly, Van Ginkel *et al* (2016) found that *Nannochloropsis oculata* was unaffected up to 7.6 μM of rotenone while *B. manjavacas* and *B. rotundiformis* were reproductively inhibited at 0.35 and 0.18 μM, respectively. However, treatment of *T. suecica* with rotenone had an effective concentration for 50% of the population (EC50) of 0.56 μM, indicating that algae were susceptible to rotenone toxicity. El-Sayed *et al* (2018) further examined the absorptivity of rotenone by freshwater *Chlorella kessleri* in order to determine an effective dosage strategy for algal production ponds. Karuppasamy *et al* (2018) found that *Euplotes* and *Oxyrrhis* sp. were also both susceptible to rotenone with LC50 values of 5 and 1.3 μM, respectively, while the *Chlorella vulgaris* was uninhibited. Rotenone is biodegradable and will break down in 3 to 8 days depending on a variety of conditions, such as pH, temperature, and light intensity. Additionally, as a known piscicide and moderately hazardous chemical marked by the World Health Organization, concentrated stocks of rotenone can be harmful to humans and devastating to fish and insect populations if accidentally spilled into the water column and natural environment. Despite these risks, Van Ginkel *et al* (2015, 2016) assert that rotenone could readily support biofuel development for $2,000 per year for biweekly rotenone prophylaxis to support 1 million gallons of biodiesel production, assuming 50% lipid content of the algae. Interestingly, availability and cost were cited by Karuppasamy *et al* (2018) as to reasons why rotenone is not ideal for pond treatment methods.

The antimalarial compound, quinine, is found naturally in cinchona and reijia tree bark. Xu *et al* (2015) tested co-cultures of *Brachionus calyciflorus* and *Chlorella kessleri* with quinine sulfate to determine sensitivity and toxicity concentrations. Although the LC50 for *B. calyciflorus* was 17 μM, complete inhibition of the freshwater rotifers was observed at 7.7 μM when in co-culture with *C. kessleri*. In contrast, quinine sulfate concentrations up to 23 μM had no effect on the growth of *C. kessleri*. Similarly, 20.0 mg L^{-1} (26 μM) of quinine sulfate eliminated more than 85% of the contaminating *Colpoda* sp. from *Chlorella vulgaris* ponds and 90% of the *Vorticella* sp. ciliates from *Scenedesmus acutus* ponds (Wang *et al* 2017b). In both cases, neither alga was inhibited after more than 24-h. Additionally, 10 mg L^{-1} (13 μM) of quinine sulfate rapidly extinguished ciliate populations contaminating outdoor *Dunaliella salina* cultures while posing no threat to algal growth (Moreno-Garrido & Canavate 2001). These data suggest that quinine sulfate is a broadly applicable interdictive treatment option that works rapidly to destroy grazers without causing harm to algal cultures.

Several naturally-occurring botanical pesticides were tested for their toxicity against rotifers and algal strains. Huang *et al* (2014a) tested four pesticides, celangulin, matrine, toosendanin, and azadirachtin, against the marine rotifer, *Brachionus plicatilis.* The nonalkaloidal insecticide celangulin, originally isolated from a bittersweet plant found in China, *Celastrus angulatus,* inhibited rotifers with a 24-h LC50 of 0.175 mg L^{-1} (0.259 μM). Matrine, an anticancer nematicidal alkaloid found in *Sophora* genus members, was similarly toxic to *B. plicatilis* with a 24-h LC50 of 0.061 mg L^{-1} (0.22 μM). Both celangulin and matrine were about 100-times more potent than azadirachtin, a carcinogenic, genotoxic insecticide isolated from the Indian lilac (*Meliaceae*). Although azadirachtin is active against over 200 species of insects, it was the least toxic botanical pesticide tested against *Brachionus plicatilis* with a 24-h LC50 of 18.386 mg L^{-1} (25.5 μM). Toosedanin is a triterpenoid extract from *Meliaceae* family members, such as the chinaberry. Historically used as a parasiticide and agricultural insecticide in ancient China, toosendanin has been found to induce apoptosis in many cell lines and was similarly toxic to *B. plicatilis* with a 24-h LC50 value of 0.002131 mg L^{-1} (3.7 nM). Fortunately, neither *Chlorella* nor *Nannochloropsis* sp. exhibited growth or photosynthetic inhibition after 0.001755 – 0.002132 mg L^{-1} (3.1 – 3.7 nM) toosedanin treatments. Huang *et al* (2014b) further assessed the potential for botanical pesticides treatment of algal cultivation systems by testing celangulin and toosendanin together in a 1:9 ratio for toxicity on the freshwater rotifer *Brachionus calyciflorus* in co-culture with the cyanobacteria *Arthrospira platensis*. Two treatments of the 1:9 pesticide mixture over three days successfully inhibited the rotifer population without harming the *S. platensis* in outdoor mass cultivation systems. The physiological effects of toosendanin treatment on *Brachionus plicatilis* were further analyzed by Huang *et al* (2017). Toosendanin reduced body size, population size, and the reproductive ability of the marine rotifer over the course of 48-h. Pepsase and tryptase were directly inhibited by toosendanin, suggesting a mechanism for the antifeedant effect of the pesticide on *B. plicatilis*. Yun *et al* 2018 demonstrated that 6mg L^{-1} of a biosurfactant produced by *Bacillus subtilis* C9 was effective at controlling the cladoceran grazers *Daphnia pulex* and *Moina macrocopa* within 24 hours without deleterious impact on the growth or lipid content of the algal species tested.

Biocides

Synthetic biocides are commonly utilized for treating fungi, pests, insects, and parasites. Karuppasamy *et al* (2018) screened a variety of biocides to determine which would be most effective and economical for treatment of mass culture systems. Fungicides captan, pyraclostrobin, fluoxasttrobin, and propiconazole were found to be potent inhibitors of the grazers *Euplotes* and *Oxyrrhis*, and unharmful to *Chlorella vulgaris*. At dosage concentrations of 1.0 – 2.0 ug L^{-1} (3.4 - 6.8 nM), the antifungal Benomyl was effective at killing *Chytridium* sp. within cultures of *Scenedesmus obliquus* (Abeliovich and Dikbuck 1977). Woo & Kamei (2003) identified an anti-*Pythium* protein (referred to as 'SAP' by the authors), isolated from a marine *Streptomyces* sp., that was an effective inhibitor of the infectious agent, *Pythium porphyrae,* known for causing red rot disease in *Porphyra sp.* The mechanism of SAP was determined to be through alteration of the fungus membrane permeability. Vital dyes, such as Lugals iodine, Methylene blue, and Toluidine blue, interact with the cellular membrane in order to penetrate living cells. Karuppasamy *et al* (2018) found that low doses of these vital dyes were effective at inhibiting *Euplotes* and *Oxyrrhis* but were also toxic to *Chlorella vulgaris*. Despite these compelling results, the fungicides and vital dyes were determined to be too costly for treatment of algal ponds (Karuppasamy *et al* 2018).

"Pesticide" is a broad term that includes insecticides, herbicides, parasiticides, and any other chemical that inhibits pests associated with aqua- and agriculture. Karuppasamy *et al* (2018) tested the effects of cypermethrin, methyl parathion, carbaryl profenofos, and triazophos on *Euplotes*, *Oxyrrhis*, and *Chlorella vulgaris.* The authors determined that all the pesticides showed robust, selective inhibition of grazers and not the algae. Similarly, Huang *et al* (2011) determined the 24-h LC50 of cypermethrin on *Brachionus calyciflorus* to be 3.38 mg L^{-1} (8.11 μM) and of deltamethrin to be 0.595 mg L^{-1} (1.18 μM). Herbicides pendimethalin, ethalfluralin, and sodium dimethyldithiocarbamate, as well as the flavonol quercetin, were also found to be quite effective against grazer populations (Karuppasamy *et al* 2018). Antiparasitic compounds have been tested for use as anti-feeding agents to prevent algal culture loss. Ivermectin, a macrocyclic lactone naturally produced by *Streptomyces avermitilis*, has been identified by the World Health Organization as the most effective and safest human treatment of parasites. Garric *et al* (2007) tested the toxicity of ivermectin on *Daphnia magna* and determined the 48-h LC50 to be 5.7 ng L^{-1} or 0.006 nM. This highly potent parasiticide was comparably ineffective at inhibiting cultures of *Pseudokirchneriella subcapitata*. Karuppasamy *et al* (2018) observed similar potency of ivermectin towards *Euplotes* and *Oxyrrhis*, although not against *Chlorella vulgaris.* Other antiparasitic compounds tested, benzylisothiocyanate, abamectin, and niclosamide, exhibited a similar trend. The potency of the biocides often came with a high environmental, and oftentimes economical, cost due to the high toxicity, likelihood of off-target effects, and lasting ecological harm. While chemical treatment strategies are some of the oldest known contaminant mitigation techniques, many additional novel methods in development have demonstrated use in abatement procedures and are worthy of review.

Table 1: Summary of various chemical interdictive procedures for contamination abatement

Chemical additive	Tested against	Advantages	Disadvantages	Reference
Copper copper sulfate	*Brachionus calyciflorus* *Colpoda* sp. *Vorticella* sp.	High heavy metal concentrations found in many wastewater treatment facilities	Inhibits algal growth, limits photosynthetic efficiency, inhibits colony formation of *S. obliquus*, heavy metal pollutant	Pradeep et al 2015 Wang et al 2017 Huang et al 2016
Sodium hypochlorite (active ingredient in bleach at 3-8% by weight)	*Brachionus calyciflorus,* *Colpoda* sp. *Vorticella* sp. Bacterial strain FD111	No long lasting residual, chlorine dissipates rapidly	Chlorine dissipates rapidly, requires multiple doses, selective activity against various zooplankton	Park et al 2016 Lee et al 2018 Carney et al 2016b Wang et al 2017
Oxidants sodium perborate sodium percarbonate peracetic acid potassium permanganate ozone chlorine dioxide hydrogen peroxide	Varying impacts on *Oxyrrhis* and *Euplotes* (see ref), ClO_2 effective against viruses and animal plankton, H_2O_2 effective against ciliates, ozone effective against bacteria *Halomonas sp.*	Degradable, several oxidizers known biocides; ozone microbubbles can spur algal CO_2 uptake and growth; ClO_2 more effective than liquid Cl_2	High cost for peracetic acid, EPA warns against use >1.3 ppm for peracetic acid; H_2O_2, ClO_2, and $KMnO_4$ are algicides; $NaBO_3$ is carcinogenic	Karuppasamy et al 2018 Moreno-Garrido & Cañavate 2001 Kamaroddin et al 2016 Drábková et al 2007 Junli et al 1997
Antibiotic/ antifungal cocktail Carbendazim Chloramphenicol Imipenem Rifampicin Tetracycline	Bacteria and fungi associated with xenic algal culture	In three days can make xenic alga axenic	Environmentally harmful due to increasing antibiotic resistance, too expensive, not scalable for outdoor cultures	Lee et al 2015
Rotenone	*Brachionus calyciflorus,* *Brachionus rotundiformis,* *Brachionus manjavacas,* *Euplotes,* *Oxyrrhis* sp.	Rapidly degrades to nontoxic chemicals, algae are largely insensitive, effective against marine and freshwater rotifers	Rapidly degrades in sunlight, extremely toxic to insects and aquatic life, harmful to humans in concentrated doses	Van Ginkel et al 2015 Van Ginkel et al 2016 El-Sayed et al 2018 Karuppasamy et al 2018
Quinine sulfate	*Brachionus calyciflorus,* *Colpoda sp.,* *Vorticella sp.,* Unknown ciliate	Effective against variety of grazers without harming algae, long-lasting in water system	Expensive, toxic to human health, environmentally harmful do to developing resistance	Wang et al 2017 Xu et al 2015 Moreno-Garrido & Cañavate 2001

Antimicrobial compounds Benzalkonium chloride (BAC) Benzethonium chloride (BEC) Cetyl trimethylammonium bromide Cetylpyridinium chloride Tetraethylammonium bromide Tetraethylammonium chloride Tetraethylammonium iodide Dichlorohexidine gluconate Imidazolidinyl urea Methylisothiazolinone	*Euplotes, Oxyrrhis sp.*	Quaternary amines potently inhibit grazers by damaging cell membrane	Environmentally harmful due to increasing antibiotic resistance, too expensive, not scalable for outdoor cultures, some antimicrobials were too potent for algae	Karuppasamy et al 2018
Botanical pesticides Celangulin Matrine Toosendanin Azadirachtin	*Brachionus plicatilis, Brachionus calyciflorus*	Biodegradable, low toxicity to mammals, safe for microalgae	Broad spectrum pesticide in environment, untested in algae raceways, biodegradable and need repeat doses, toxic to humans in highly concentrated stocks	Huang et al 2014a Huang et al 2014b Huang et al 2017
Antifungals Captan Pyraclostrobin Fluoxastrobin Propiconazole Benomyl	*Euplotes, Oxyrrhis, Chytridium sp.*	Potent grazer inhibition	Environmentally harmful due to antifungal resistance, too expensive, not scalable for outdoor cultures	Karuppasamy et al 2018 Abeliovich and Dikbuck 1977
Pesticides Cypermethrin (Cyp) Deltamethrin (Del) Methyl Parathion Carbaryl Profenofos Triazophos	*Euplotes, Oxyrrhis, Brachionus calyciflorus*	Potent grazer inhibition	Cyp decomposition increases with exposure to sun, water, and oxygen, is highly toxic to fish, bees, and insects; Del highly toxic to aquatic life	Karuppasamy et al 2018 Huang et al 2011
Vital dyes Lugals iodine Methylene blue Toluidine blue	*Euplotes, Oxyrrhis*	Potent grazer inhibition	Too expensive, too toxic for algae	Karuppasamy et al 2018
Herbicides Pendimethalin Ethalfluralin Sodium dimethyldithiocarbamate	*Euplotes, Oxyrrhis*	Potent grazer inhibition	Toxic off-target environmental effects	Karuppasamy et al 2018
Anti-feeding agent antiparasitic compounds Benzylisothiocynate Ivermectin Abamectin Niclosamide	*Euplotes, Oxyrrhis, Daphnia magna*	Potent grazer inhibition	Toxic off-target environmental effects	Karuppasamy et al 2018 Garric et al 2007

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PHYSICAL DISRUPTION OR REMOVAL

Hydrodynamic shear

The differential velocities of different layers in a fluid imparts a shear force that is referred to as hydrodynamic. Montemezzani *et al* (2017a) evaluated the effectiveness of hydrodynamic shear to disrupt the rotifer *Brachionus calyciflorus* and the cladoceran *Moina tenuicornis.* The laboratory scale system that was employed induced hydrodynamic shear by pumping cultures through perforated aluminium plates. The highest mortality rates (100% of *M. tenuicornis* and 80% of *B. calyciflorus*) were achieved by passage through a 4 mm orifice with a pressure differential across the plate of 2.5 bar. The authors described some disruption of multicellular algal aggregates and colonial forms but did not characterize the overall effect of hydrodynamic shear on algal viability

Hydrodynamic cavitation

Hydrodynamic cavitation is caused in flowing liquid that has undergone a rapid reduction of pressure causing the formation of vapor cavities (microbubbles). Upon increase in pressure, these vapor cavities implode causing an intense physical shockwave that can physically disrupt biological structures. Microalgae and grazer species are differentially sensitive to cavitation, a phenomenon that can be leveraged in crop protection applications. Kim *et al* (2017) demonstrated that hydrodynamic cavitation physically disrupted the rotifer *Brachionus rotundiformis* in cultures of *Microchloropsis salina.* Four passes of the culture through the cavitation systems resulted in a 99.9% disruption of the rotifers. It was unclear if the rotifer eggs retain viability after treatment, thus periodic treatments were suggested to fully protect the cultivation system from rotifer infestation. Little apparent reduction was observed in the viability of *M. salina* by this treatment. The differential sensitivity of the algae and the rotifers to cavitation is due to the differences in cell sizes, with *M. salina* averaging 2-3μm in length and *B. rotundiformis* averaging 90-150 μm in length. This also suggests that grazer species larger than rotifers, such as *Daphnia,* will be even more susceptible to disruption by cavitation. Sawant *et al* (2008) demonstrated that with a combination of shear forces and cavitation induced by their system, 80% of zooplankton (copepods, bivalve, gastropod, decapod and polychaete larvae, (*Favella* and nematodes) were destroyed in a single pass. Thus, hydrodynamic cavitation is a compelling prophylactic and interdictive method for contamination control in algal mass culture.

Ultrasonication

In ultrasonication, sound energy at frequencies in excess of 20 kHz, is introduced into an aqueous system resulting in both physical and chemical effects within the sample. Physical effects include hydro-mechanical shear forces. Ultrasonication induces chemical effects, referred to as sonochemistry, through the formation and implosion of microbubbles resulting in highly localized high temperature regimes (Suslick 1990). The combination of these effects is known to result in the disruption of cellular membranes (sonoporation). Holm *et al* (2008) characterized the effectiveness of ultrasonication on the disruption of bacteria, phytoplankton, and zooplankton in ballast water. In general, the effectiveness of the treatment in terms of energy and time required varied directly with the size of the organism, with bacteria being the least susceptible and the zooplankton being the most. The differences in susceptibility that were observed indicated that it could be possible to disrupt grazer species without causing undue harm to algal species. Wang *et*

al (2018) tested the effectiveness of ultrasonication on the disruption of a range of deleterious species, including the flagellate *Poterioochromonas,* in outdoor raceway cultures of *Chlorella* sp. Despite its relatively small size, *Poterioochromonas* was found to be sensitive to sonication; likely due to its lack of a cell wall. Additional contaminating species present in the pond, including an unknown fungus, *Acanthamoeba,* and ciliates, were disrupted by the treatment, but a number of species, including the flagellate *Bodomorpha* sp., the ciliate *Colpoda* sp., and the amoeba *Paradermamoeba* sp., were unaffected by ultrasonication, as was *Chlorella* sp. Ultrasonication would provide an effective interdictive method, but only towards those species that are susceptible.

Pulsed electric fields

Pulsed electric fields (PEF) are known to cause disruption in biological membranes (electroporation). This method works by inducing an electrical potential across the cellular membrane resulting in a rearrangement in the structure of the lipid bilayer and resulting in the formation of a pore. It is well known that electroporation is limited by the conductivity of the medium and may not be effective for marine systems. Rego *et al* 2015 demonstrated the effectiveness of PEF in the control of rotifers found in outdoor, closed, tubular photobioreactor (PBR) cultures of *Chlorella* sp. without impacting algal growth rate. The PEF system was integrated directly into the PBR so that the culture was treated as it circulated through the system. Over a 6-h test, it was calculated that each cell in the system received 36 electrical pulses (900 V/cm, 65 μs pulses of 50 Hz) and six hours of treatment was sufficient to disrupt 85% of the rotifer population. Encouragingly, continuous treatment over a period of days resulted in 100% disruption without a deleterious effect on the algal growth rate.

Foam flotation

Umar *et al* (2018) tested the efficacy of foam flotation for the removal of the ciliate *Tetrahymena pyriformis* from laboratory scale PBR cultures of *Chlorella vulgaris.* In this process, algae were cultivated in a columnar PBR sparged with air from the base (effectively a bubble column). The air was passed through a gas permeable membrane to create bubbles at the millimeter scale. The addition of sodium dodecyl sulfate (SDS) in combination with the sparging created a fine foam that differentially lifted the ciliates (*Tetrahymena pyriformis* CCAP 1630/1W) to the top of the column for removal. Efficiency of ciliate removal was directly dependent on SDS concentration, air flow rate, and number of treatment cycles. At 10 mg L^{-1} SDS and an air flow rate of 1 L min^{-1} approximately 20% of ciliates were removed in a single treatment cycle. At 40 mg L^{-1} SDS and an air flow rate of 2 L min^{-1} 96% of ciliates were removed after three treatment cycles. This treatment did not affect algal growth but did provide an effective way of removing the deleterious species from the cultivation system.

Hydrocyclone

Hydrocyclone separation technology is based on the flowing of particle-containing liquid through a vortex thus inducing a centripetal force that results in the removal of suspended particles. This technology has been applied for the removal of exotic species from ballast water (Abu-Khader *et al* 2011). Although hydrocyclone technology has not been reported for the differential removal of

grazers or other deleterious species from algal cultures, it may be useful to evaluate this technology for this application.

Filtration

Many grazer species are large enough that they can be removed by passage of the culture through a mesh or net (Borowitzka 2005) however the author does not give any specific examples. Montemezzani et al (2017b) reported 100% removal of the cladoceran *Moina tecunicornis* after a single passage through a 300 □m filter. Effectiveness of this technique is, of course, dependent on the relative sizes of the algae and grazers and the volume of culture to be treated.

NUTRITIONAL CONTROL

Phosphate

Phosphate limitation has been shown to reduce the rate of infection of the diatom *Asterioneila formosa* by the parasitic fungus *Rhizophydium planktonicum* in turbidostat cultures (Bruning 1991). However, once an infection had been established, the growth rate of the fungus was only slightly impaired, whereas the host algae displayed a reduced growth rate due to the phosphate limitation. This reduction on algal growth rate allowed the fungal infection to eventually outpace algal growth. Phosphate limitation did, however, increase the algal density required to sustain the fungal infection. A similar decrease in susceptibility to chytrid infection was observed, at high C/P ratios, in the diatom *Synedra* in pond simulating mesocosms (Flynn *et al* 2017). The authors modelled the effect of phosphate limitation on the production of algae with a high C/N ratio resulting in biomass that was an inferior food source for zooplankton. The key was to devise phosphate limitation conditions that changed the biochemical makeup of the algae without reducing their growth rate. Such a balance may be achievable in closed systems with rigorous control of the physiochemical parameters, but seems unlikely to be achieved in open and/or outdoor cultivation systems.

Nitrogen source; urea and ammonia versus nitrate

Addition of reduced nitrogen compounds can be an effective way to control contamination of algal cultures with deleterious species. Mendez & Uribe (2012) grew both indoor and outdoor cultures of *Arthrospira sp.* in the presence of urea and ammonium bicarbonate and at a pH of 9.5-10.5. At concentrations of 60 mg L^{-1} (1 mM) of urea or 100 mg L^{-1} (1.3 mM) of ammonium bicarbonate, *Arthrospira* growth was not affected, but both *Brachionus sp.* and *Amoeba sp*. were inhibited. Reduced nitrogen compound concentrations above this were detrimental to *Arthrospira* growth. Free ammonia in the form of ammonium hydroxide has also been used to control grazers. Lincoln *et al* (1983) used ammonium hydroxide to control the zooplankton, *Brachionus rubens* and *Diaphanosoma brachurum*, in cultures being grown on facultative lagoon effluent from swine wastewater (pH 7.8-8.0). Laboratory and field testing showed that the addition of 16-18 mg L^{-1} (0.52 – 0.58 mM) free NH_3-N was lethal to *Brachionus* and 20 mg L^{-1} (0.64 mM) killed *Diaphanosoma,* but algal growth was unaffected in both cases. Schluter & Groeneweg (1985) showed that free NH_3-N at concentration below 5 mg L^{-1} (0.16 mM) controlled, but did not kill *Brachionus rubens*, and that the effect was reversible. At free NH_3-N concentrations above 5 mg

L^{-1} (0.16 mM), the rotifers were dead after 48 hours. In laboratory scale experiments of *N. oculata* infected with *Brachionus plicatilis*, Thomas *et al* (2017) demonstrated that exposure to NH_4Cl at 42-53 mg L^{-1} (1.4 – 1.7 mM) free NH_3-N for at least six hours was effective at killing the rotifers while not affecting *N. oculata* growth. Tests were done in artificial sea water medium and ASW supplemented with dairy anaerobic digester effluent. Free ammonia concentrations were manipulated by altering pH over a range from 7 to 9. Some of these studies show that algal growth was not affected by addition of ammonia compounds, but caution should be taken. Many algal species are sensitive to reduced nitrogen compounds and free NH_3-N concentrations and growth may be reduced or inhibited. (See reference Gutierrez *et al* 2016).

Other inorganic salts

Interestingly, Abeliovich and Dikbuck (1977) found that *Chytridium sp.* were inhibited from infecting *Scenedesmus obliquus* cultures when Na^+ and Ca^{2+} cations were replaced with K^+ and Mg^{2+} cations at concentrations of 0.01 – 0.1 M within the growth medium. The authors also show that while this cation replacement inhibits chytrid infection, algal growth of *S. obliquus* is unaffected. This promising cation-replacement strategy requires further trials in mass culture systems to determine the efficacy of cation replacement for large scale algal production.

CULTIVATION SYSTEM OPERATION STRATEGIES

Cultivation under alkaline pH

Cultivation of algae at alkaline pH has been shown to be an effective method of reducing contamination by deleterious species. Moheimani & Borowitzka (2006) reported the semi-continuous outdoor cultivation of *Pleurochrysis carterae* over a period of 13 months. The ponds ranged from a pH of 8 at the end of dark phase to a maximum of pH 11 during daylight. This extreme range in pH is credited with reducing contamination by deleterious species. Wang *et al* (2017b) reported that cultivation of *Scenedesmus acutus* LRB-1201 under CO_2-limiting conditions resulted in an increase of culture pH from 7.63 to 10.88. These high pH conditions resulted in an 80% decrease in *Vorticella*. Peng *et al* 2015 reported that growth of *Neochloris oleoabundans* at high sodium bicarbonate (160 mM) containing medium at pH 9.5 was effective in limiting the growth of unspecified protozoa. Vadlamani *et al* 2017 reported the cultivation of a high pH adapted strain of *Chlorella sorokiniana* SLA-04 at a pH in excess of 10 in both laboratory and outdoor open ponds. Outdoor ponds were challenged with an experimental infection with the grazer *Daphnia magna.* Under high pH conditions *D. magna* were rapidly killed, further demonstrating the effectiveness of utilizing high pH-tolerant systems and algal strains.

Transient pH shifts

Transient low pH treatments either through acid addition or CO_2 treatment have been shown to control deleterious species in production systems. Ganuza *et al* 2016 demonstrated that a 15-minute shift of *Chlorella* mass cultures to pH 3.5, through HCl addition, in the presence of 0.5 g L^{-1} acetate killed 90 percent of the bacteria *Vampirovibrio chlorellavorus* and was well tolerated by the algae. Similarly, Becker (1994) determined that shifting culture pH to 3 for a period of 1 to 2 hours effectively eliminates rotifers.

CO_2 asphyxiation and anoxia

Long term growth of pilot scale cultures of *Chlorella sorokiniana* cultures maintained at pH 6 or pH 6.5 through CO_2 sparging (but not through acid addition) resulted in the death of *Poterioochromonas malhamensis*, and death or inhibition of the ciliates, *Tetrahymena thermophila*, *Colpoda* sp., and *Vennalla* sp., and the amoeba *Sterkiella* sp. (Ma *et al* (2017)). Montemezzani *et al* (2017a) demonstrated the effectiveness of long-term CO_2 sparging in controlling grazers in 20L outdoor mesocosm cultures derived from wastewater treatment high rate algae ponds. Continuous sparging with 5% and 10% CO_2 was determined to be effective in controlling multiple species of rotifers. Limiting CO_2 asphyxiation to overnight was also demonstrated to be successful in eradicating grazers in outdoor ponds (Montemezzani *et al* (2017c). In an $8m^3$ high rate algal pond, nightly 100% CO_2 injections of 1-6L/min was successful in reducing the populations of some, but not all, zooplankton species present. Affected species included *Moina tenuicornis*, *Paracyclops fimbriatus*, and *Filinia longiseta*. Recalcitrant species included *Heterocypris incongruens*, *Asplanchna sieboldin*, *Cephalodella catellina*, and *Brachionus calyciflorus*. Despite the reported success of CO_2 asphyxiation, the protozoa *Colpoda steinii* is unaffected by this treatment, as well as high ammonia (Troschl *et al* 2017). However, partially anoxic conditions did inhibit growth of this protozoa in *Synechocystics sp.* cultures, suggesting that this could be a promising avenue for further investigation.

BIOLOGICAL CONTROL

Polyculture, natural assemblages, and crop rotation

Most algal cultivation systems are predicated on the production of a single algal species with the optimal characteristics (e.g. biochemical makeup or the production of a specific product). A few species have been identified with innate resistance to specific grazers which may make them better choices for mass culture. These include strains of *Chlorella* sp. that have pronounced resistance against protozoa (Yang and Kong, 2011) and *Tetreselmis* sp. (Erkelens *et al*., 2014). In cases where monocultures can be avoided, the cultivation of deliberate polycultures or natural assemblages can have significant advantages in crop protection. A number of research groups (Hillebrand & Cardinale 2004, Corcoran & Boeing 2012, Roelke 2017, Smith & Crews 2014, Cho *et al* 2017, Godwin *et al* 2018) have determined that polycultures can increase the resilience and production rate of a cultivation system in the presence of grazers. This increase in resistance to grazing may be due to an "interference effect" where prey diversity decreases effective grazing. For a given set of growth conditions, polycultures may not out perform a monoculture of the optimal species, but under grazing pressure they will have higher rates of survival and production. Under appropriate circumstances, polyculture can be a highly effective crop protection strategy. Furthermore, crop rotation strategies have also been proposed to limit grazer or pathogen carry-over in batch strategy production systems (Kagami *et al*., 2007, Smith *et al* 2015).

Trophic control

Trophic cascades have been proposed as an alternative approach to controlling grazer populations. Such mechanisms work by altering the concentrations of organisms that prey upon or infect grazer species, thus reducing their number and impact on algal populations. Montemezzani *et al* (2017b)

demonstrated the trophic control by the addition of rotifer-specific predators that displayed limited grazing on microalgae. In these experiments, inoculation with 2500 individuals per L of the cladoceran *Moina tenuicornis* or with 1000 individuals per L of the ostracod *Heterocypris incongruens,* decreased rotifer populations by 70-80% compared to the un-treated controls. Thom *et al* 2018 reported the trophic control of the cladoceran *Daphnia* by predators such as dragonfly larvae [Odonata: Libellulidae] and back-swimmers [Hemiptera: Notonectidae] in outdoor tanks containing *Chlorella*. Predation of *Daphnia* reduced grazing losses of the algae.

Allelopathy and natural defenses of microalgae

In nature, competition for resources and survival has evolutionarily driven the natural selection of organisms with the ability to generate secondary metabolites as "defense" molecules that chemically inhibit predators and competitors. These chemicals, often referred to as "allelochemicals" to reference their allelopathic purpose, have been considered and tested as a method of algal protection in mass culture systems (for review, see Mendes & Vermelho 2013). Relative to frequent, toxic, and costly chemical or physical treatments for pond maintenance, utilizing the natural defence systems of microalgae is a novel, innate, and natural method for bolstering algal culture protection. Polycultures including allelochemical-producing algae strains and/or genetic modification to engineer defense-less algae to generate their own chemical defense system are two applications for current work in this field. The low cost of growing algae that continually generate their own chemical defense system is worth further investigation.

Dimethylsulfoniopropionate (DMSP) is a well-known chemical signal produced by marine algae, such as *Phaeocystis* and *Emiliania huxleyi*, when they are under attack. Glycine betaine is a similar molecule to DMSP and has been shown to be negatively allelopathic. During algal grazing, oxidation of cellular membrane fatty acids generates several aldehydes, including trans,trans-2,4-decadienal. Hue *et al* (2018) tested these established allelochemicals, along with the amino acid proline and the chemical analog of DMSP known as methyl 3-(methylthio)propionate (MMP), on several ciliate populations in the presence of the microalga *Chlamydomonas* sp. All chemicals inhibited ciliate populations of *Sterkiella*, *Stylonychia notophora*, *Oxytricha sp.,* and two different *Paramecium* species, but this effect required very high concentrations of proline and glycine betaine (250-300 mM) compared to <10 mM concentrations needed for the other chemical treatments. Grazer populations were most sensitive to 0.13 mM of decadienal, but the *Chlamydomonas* culture was also inhibited at this concentration. DMSP and MMP were preferred for their potency (4.75 mM and 10 mM, respectively), but the authors suspected that their addition to mass culture systems might be problematic in that both are volatile compounds that could dissipate quickly in large, open, outdoor cultures. Introduction of the necessary gene cluster for innate algal synthesis of DMSP would mitigate this concern.

Differences in species and strain responses to grazers suggest the presence of allelochemicals and require further study. Yuan *et al* (2017) found that both the culture suspension and 0.45-um filtrates of *Didymogenes* sp. HN-4 were able to suppress *Rataria* sp. rotifer grazing rates. The authors postulate that the alga must be generating a chemical defense to deter rotifer grazing since control rotifers feasted upon *Chlorella* sp. normally. *Rataria* sp. rotifers were found to swim more slowly and exhibited morphological differences in the presence of *Didymogenes* sp. HN-4. Although a definitive chemical or mechanism has yet to be determined, the cultivation of microalgae capable

of producing defensive allelochemicals is a novel avenue for algal crop protection strategies. Allewaert *et al* (2018) found that of the 44 strains of *Haematococcus* analysed, ten strains were determined to have lower susceptibility to the blastocladialean fungal parasite, *Paraphysoderma sedebokerense* PS1. These differences in susceptibility were not correlated with phylogeny or sampling location, but several did have flagellated morphologies that seemed to be dominant. Possibly, these select strains have inherited morphological and chemical defenses that mitigate fungal infection. Better understanding the mechanism of defense can be used for genetic modification of *Haematococcus* sp. to resist the detrimental fungal pathogen.

Plants, including microalgae, are known to trigger immunological responses as a result of activation of microbe-associated molecular patterns (MAMPs). Oligosaccharides, polysaccharides, chitin, and other microbially-derived molecules are recognized by algae and innate defence systems are activated to mitigate or inhibit the nearby pathogen (for review see Burketova *et al* 2015). In order to better understand the attraction of chytrid zoospores to their algal hosts, Scholz *et al* (2017) assessed the attraction of eight carbohydrates, five fatty acids, six amino acids, and three compatible solutes, both individually and combined, in chemotaxis experiments of four chytrid species. Of these, the combined carbohydrate solution was the best attractant for chytrid pathogens. Interestingly, carbohydrates, polyunsaturated fatty acids, and aldehydes were the chemical classes present more often in the resistant diatom strains tested. In addition to chemical defenses, morphological changes can also aid in algal survival. Verschoor *et al* (2009) found that *Scenedesmus obliquus* form colonies when in co-culture with the freshwater rotifer *Brachionus calyciflorus*. *S. obliquus* is known to rapidly form algal colonies in the presence of rotifers, but their disintegration was slowed under continuous light conditions and due to "lingering infochemicals of herbivory past", even after all rotifers were removed from the culture. The lengthy disintegration of *S. obliquus* colonies could be explained by the high cost of such a defensive morphological change or could point to highly-sensitive putative chemical sensors of *S. obliquus* that keep the alga fully "armored" until the threat has passed. Interestingly, if a competitive algal strain is present in culture with *S. obliquus*, colony formation is inhibited. Such is the case Zhu *et al* (2015) observed when *S. obliquus* cultures were grown with the grazer *Daphnia* (or in the presence of *Daphnia* filtrate) along with the competitive alga, *Microcystis aeruginosa*. The authors observed that when given the choice between colony formation to reduce grazing rates and maintaining a high competitive ability, the *S. obliquus* cultures do not form colonies. This could be because *S. obliquus* colonies do not grow and compete as well, or it is possible that chemicals from *M. aeruginosa* are inhibiting the infochemicals from grazing and *S. obliquus* does not respond normally. Regardless of mechanism, this shows that defense mechanisms are inducible and different environmental stresses result in different responses by the algae.

Various chemical treatments, nutrient limitations, and environmental changes can influence the use of defense molecules used by an alga. Abscisic acid is a plant hormone known for its role in development, growth, and stress tolerance. Pouneva (2006) discovered that both higher endogenous abscisic acid levels and ~100 μM treatments of abscisic acid prevented infection by the fungal parasite *Phlyctidium scenedesmi* on cultures of *Scenedesmus acutus*. Absence of vital nutrients can be a signal that competition is high, and the algae metabolically responds by generating chemicals that might help capture the necessary nutrients to allow for survival. Bagwell *et al* (2016) found that algal cultures, *Scenedesmus* sp. strain 18B and *Chlorella* sp. strain 15, in

an iron-limited environment were more cytotoxic. The authors hypothesized that the secondary metabolites produced by the algae were meant to scavenge iron. What was found, however, was that the *Chlorella sp.* grown in an iron-limited environment was incredibly resistant to devastation by the *Vampirovibrio chlorellavorus* bacteria; only a slight loss (0-9%) of the iron-limited *Chlorella* sp. culture was observed compared to nutrient-replete control cultures that had 72% loss of algal biomass. In this case, nutrient depletion led to an increase in cytotoxicity and defense molecules, but environmental changes can also lead to loss of algal defense systems. Demott & McKinney (2015) observed that after three years of semi-continuous culture of *Oocystis* in light (i.e. 24 h of fluorescent light, exposed daily for three consecutive years) in the absence of grazers, the *Oocystis* culture lost innate defense molecules. Control *Oocystis* cultures grew more slowly but were more resistant to *Daphnia* grazing. The authors postulate that there might be a "defense vs. growth" tradeoff, similar to the results of colony formation inhibition by *S. obliquus* (Zhu *et al*, 2015). These studies indicate that algae likely have a suite of unknown chemical defenses that need to be better understood for use in algal production systems.

ADVANCED METHODS

Genetic engineering strategies

The genetic engineering of algal production strains to resist deleterious species is an approach that, although confronted by both ecological and regulatory hurdles, is likely to be an area of active research and innovation. Larkum *et al* (2011) and Qin *et al* (2012) review the status of genetic engineering technology in a variety of microalgal species. A significant barrier to the deployment of genetically modified (GM) algae is the potential environmental risk if such strains were to be cultivated in open systems. In one such outdoor cultivation study by Szyjka *et al* (2017), a GM strain of *Acutodesmus dimorphus* was developed to contain exogenous genes for enhanced fatty acid biosynthesis and green fluorescence protein (GFP). It was found that the strains retained the exogenous genes and their associated phenotypes for the length of a 50-day cultivation trial. When the cultivation systems were inoculated in water containing natural assemblages from the local aquatic environment, the GM strain proved incapable of out competing native strains.

In order to control for any potentially deleterious effects of GM algae on the natural environment, a number of different strategies have been proposed for their biocontainment. A common strategy for the biocontainment of GM microalgae is to cripple the production strain so that it is dependent on the pond environment and unable to compete in the natural environment. An example of this is to genetically knockout the gene for nitrate reductase. Most production-scale ponds utilize ammonium as a nitrogen source as it is much cheaper than nitrate salts. Nitrate reductase deficient mutants are able to grow in ammonium-fed production ponds but suffer a severe fitness deficit in the natural environment (Navarro *et al* 2005). Another example is the genetic knockout of high-affinity trace metal transporters, as trace metals are usually replete in the pond environment but present at low concentrations or even limiting in the natural environment (Blaby-Haas & Merchant, 2012).

GM algae strains have been developed to out compete weed species and to create algal strains that are resistant to chemical agents that may be used for the control of grazer populations. Loera-Quezada *et al* (2016) developed a GM strain of *Chlamydomonas reinhardtii* capable of using

phosphite as a sole phosphorus source and thus outcompeting the growth of exogenous weed species. Bruggeman *et al* (2014) developed a *C. reinhardtii* strain engineered to contain genes conferring resistance to the herbicides glyphosate (Vick 2010), oxyfluorfen, and norflurazon. Along with serving as selectable markers for genetic engineering, these genes confer resistance and thus facilitate the use of broad-spectrum herbicides to control contamination by weed species. Corcoran *et al* (2018) took an alternative U.V. mutagenesis and selection approach to the development of strains of the green algae *Desmodesmus armatus* able to tolerate a broad spectrum fungicide.

Industrial microbial ecology

With the advent of modern methods for microbiome and metagenome analysis, there is now a focus on manipulating the microbial ecology of the production system for purposes of crop protection, growth enhancement and control of biomass composition (e.g. lipid content) (Beyter *et al* 2016, Mooij *et al* 2015, Smith & McBride 2015). Algal polyculture is an example of a preliminary form of industrial ecology where the deliberate co-culture of multiple algae species can yield improved grazer and disease resistance (Shurin *et al* 2014). More challenging is the control of the species composition and function of the underlying microbiome. Identification of and co-cultivation of algae with probiotic bacterial species has been shown to increase the growth and productivity of algal culture systems. Fisher et al (2019) have shown that algae associated microbial consortia can be selected that protect algae from rotifer grazing. It is postulated that by maintaining a healthy normal microbial flora, niches that could be exploited by deleterious species would be otherwise occupied. The effective leveraging and utilization of industrial ecology to enhance stability of production systems is an area of active research where considerable work is needed and advances are possible.

DETECTION METHODOLOGIES

Although not a direct means of crop protection, identification and detection of deleterious species is an important aspect of any crop protection strategy. Early and accurate identification of deleterious species is important for any interdictions strategy: in many cases treatment is too expensive to apply generally as a prophylaxis and therefore is only utilized when there is evidence of infection. Light microscopy is a standard and essential method for monitoring ponds (Borowitzka 2005), however the method has its limitations. It takes some level of training to identify grazer species and the method is unable to discriminate between morphologically-identical bacteria and viruses. Furthermore, microscopic evaluation can be labor-intensive, especially as production operations increase in scale. A number of alternative means have been developed to solve this problem, including semi-automated imaging flow cytometry and a variety of molecular techniques.

Day *et al* (2012b) and Wang *et al* (2017a) demonstrated the use of a FlowCAM imaging flow-cytometer to detect a wide variety of grazer species, such as *Euplotes vannus, Oxyrrhis marina, Uronema marinum, Brachionus calyciflorus*, *Cohnilembus reniformis, Paramoeba eihardii, Hartmanella* sp*,* and *Poterioocrhromonas,* in indoor and outdoor cultivation of *Chlorella* and *Nannochloropsis oculata* at sensitivities of less than 10 cells per mL Once the image recognition system is trained, the FlowCAM is capable of operation in a semiautonomous fashion. A number

of nucleic acid-based methods, including Polymerase Chain Reaction (PCR), and nucleic acid hybridization-based assays have been developed for detection of deleterious species (for review see Carney *et al* 2016a). Such assays have the advantage of being potentially less time consuming than microscopy and having the ability to detect and identify bio-contaminants, such as bacteria and viruses, that are not amenable to microscopy analysis.

CONCLUSION

Decades of research and innovation have generated a litany of operational, prophylactic, and interdictive procedures for mitigating and treating biological contaminants in algal mass cultivation systems. Currently proven technologies are summarized in Table 2. Here, we have provided an unbiased summary of both established and novel techniques (Figure 2) in hopes of providing the reader with sufficient background knowledge of the field and opportunities for further investigation. Cultivation of algal cultures in open versus closed systems is one of the first decisions that algal production facilities need to answer. Both options are further implicated in downstream operational strategies, economical parameters, and possible contamination issues. While open outdoor cultures seem to be the most susceptible to contamination and culture crashes, they do remain the most inexpensive method for algal culture production and financial considerations. Closed photobioreactors are considerably more expensive to set up, maintain, and operate, but are less susceptible to infestations of detrimental species. However, if a closed system does become contaminated, they can be extremely difficult to disinfect completely, leading to additional expensive decisions to either scourge the system with highly toxic, expensive chemicals, or purchase a new one.

After the choice of an open or closed culture system is made, many operational considerations can prevent infection by deleterious species. Some algal strains with high salinity, pH, and CO_2 tolerance are less likely to be infected by microorganisms, zooplankton, parasites, and other harmful contaminants that are unable to thrive in such extreme environments. Wang *et al* (2016) determined than an integrated pest management strategy in tuning the pH and salinity parameters away from the ecologically optimal values for rotifers, ciliates, and amoebae was an effective strategy in mitigating contamination in large-scale raceway ponds. Nightly CO_2 asphyxiation is another promising strategy that purges sensitive organisms intolerant of temporarily acidic conditions. While this could impact algal growth and cellular respiration, the benefits might outweigh this risk. Similarly, routine pH “shock” treatments have been shown to mitigate grazers while causing no lasting harm to algal cultures. Polycultures, crop rotation, and natural assemblages are biological control methods that bolster the aquaculture community and defend against invasive species, in some cases. Some algal cultures are known to generate defense molecules that could aid in their own or other community members’ protection against grazing. Thus, choice of algal strain(s) and the operational set up of the mass cultures could tremendously impact the productivity and stability of algal cultivation.

Prophylactic measures can overlap with operational strategies, but can also be a separate, additional layer of protection for protecting mass culture systems. Nutrient limitation or alteration is one example of how an operational strategy for culture set up can have prophylactic effects on the culture system. Many biological organisms can easily uptake phosphate and nitrate as critical P and N nutrients. Some algae, however, can also metabolize urea and ammonia as nitrogen

sources and can survive on lower phosphate. However, lowering phosphate will impact algal growth and productivity, so these choices are not made without important downstream repercussions. Ammonia and urea are toxic to some algal grazers, thus high levels of these chemicals can be readily used by some algae as a nitrogen source and simultaneously deter algal contaminants. Allelopathy and natural chemical defenses of some algal strains alone or in polycultures can also act as prophylaxis for contamination. Ecological and trophic level control are biological control mechanisms that are currently breaking ground in their novelty and whole-

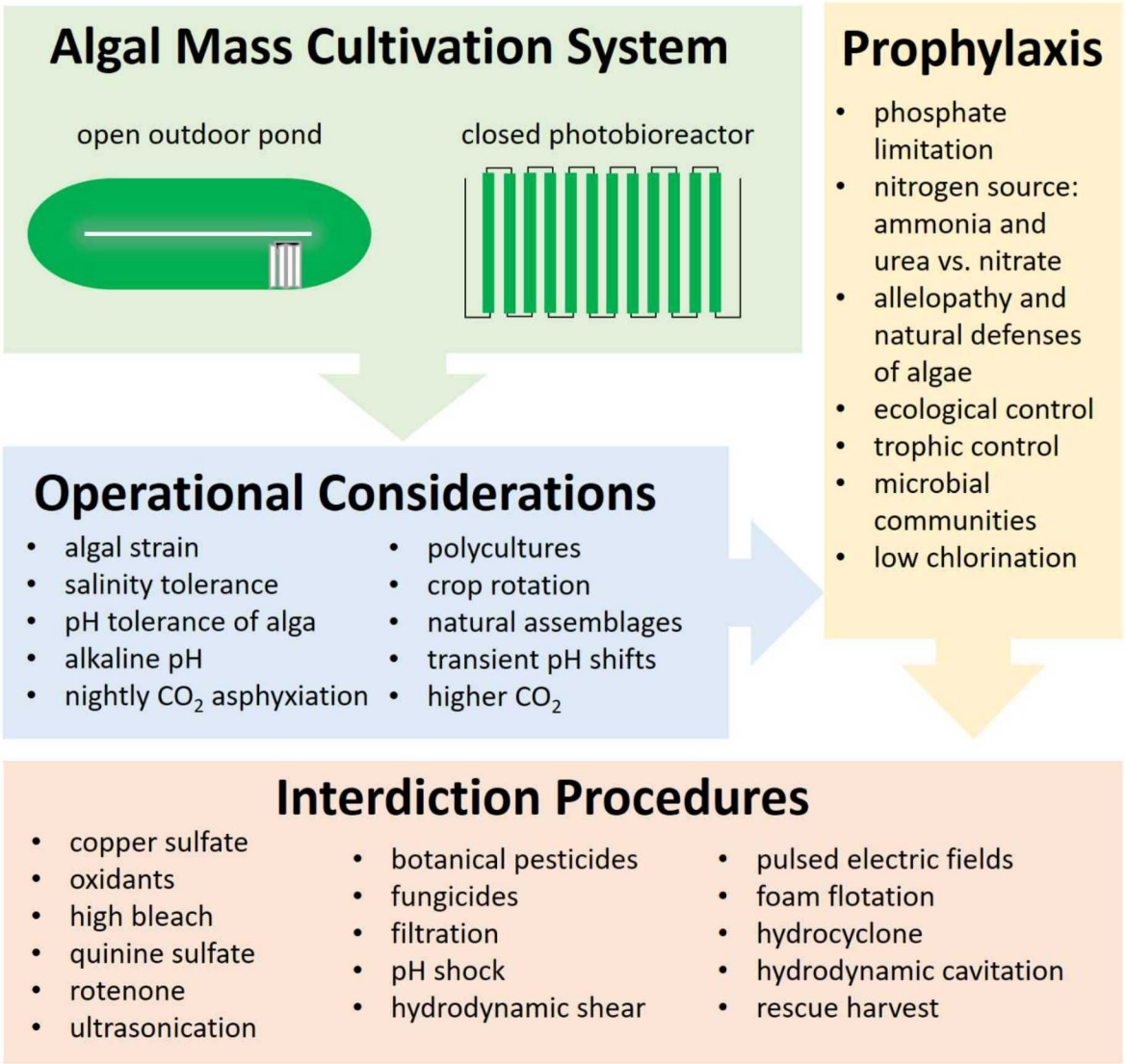

Figure 2: Summary of the various operational strategies, prophylactic methods, and interdiction procedures that can aid in preventing, mitigating, and rescuing algal mass culture systems from devastating contamination and culture crashes.

system approach to prevent culture crashes. Monocultures of algae are more susceptible to infection and harsh changes in abiotic factors (temperature, rain, pH, light), while systems with diverse taxonomic makeups, including microbes, other algal strains, and predators of known algal grazers, are generally more stabilized and resistant to devastating culture crashes. In general,

Table 2: Summary of current treatment options for biological contaminants of algal cultures

algae	treatment	grazer or pathogen	reference
Arthrospira sp.	urea, ammonium bicarbonate	*Branchionus sp. and Amoeba sp.*	Mendez & Uribe 2012
Chlorella sp.	pH shock	*Vampirovibrio chlorellavorus*	Ganuza et al 2016
Chlorella sp., Nannochloropsis sp.	four botanical pesticides	*Brachionus plicatilis*	Huang et al 2014a
Chlorella sp., Scenedesmus sp.	Fe	*Vampirovibrio chlorellavorus*	Bagwell et al 2016
Chlorella kessleri	chlorine	*Brachionus calyciflorus*	Park et al 2016
Chlorella kessleri	copper	*Brachionus calyciflorus*	Pradeep et al 2015
Chlorella kessleri	quinine sulfate	*Rotifer Brachionus calyciflorus*	Xu et al 2015
Chlorella kessleri, Tetraselmis suecica	rotenone	*Brachionus calyciflorus, Brachionus rotundiformis, Brachionus manjavacas*	Van Ginkel et al 2015
Chlorella sorokiniana	CO_2-mediated low culture pH	*Poterioochromonas malhamensis*	Ma et al 2017
Chlorella sp.	ultrasonication	*Poterioochromonas sp.*	Wang et al 2018
Chlorella vulgaris	benzalkonium chloride	*Oxyrrhis, Euplotes*	Karuppasamy et al 2018
Chlorella vulgaris, Scenedesmus acutus	copper, chlorine, quinine sulfate	*Vorticella sp.*	Wang et al 2017a
Chlorella vulgaris	foam flotation	*Tetrahymena pyriformis*	Umar et al 2018
Dunaliella salina	ozone	*Halomonas sp.*	Kamaroddin et al 2016
Dunaliella salina	hydrogen peroxide	ciliates	Moreno-Garrido & Canavate JP 2001
Nannochloropsis oculata	rotenone	*B. manjavacas, B. rotundiformis*	Van Ginkel et al 2016
Nannochloropsis oculata	pesticides	*Brachionus plicatilis*	Huang et al 2017
Nannochloropsis oculata	ammonia	*Brachionus plicatilis*	Thomas et al 2017
Microchloropsis salina	hydrodynamic cavitation	*Brachionus rotundiformis*	Kim et al 2017
natural assemblage	ammonia hydroxide	*Brachionus rubens, Diaphanosoma brachyurum*	Lincoln et al 1983
natural assemblage	CO_2 asphyxiation, hydrodynamic shear	*Moina tenuicornis, Brachionus calyciformus*	Montemezzani et al 2017a
natural assemblage	CO_2 asphyxiation, biological control using competitor species, filtration, and mechanical disruption using hydrodynamic shear stress	*Brachionus spp.*	Montemezzani et al 2017b
natural assemblage	CO_2 asphyxiation	microcrustaceans, rotifers, and copepods	Montemezzani et al 2017c
natural assemblage	sonication	cladocerans and rotifers	Holm et al 2008
Pseudokirchneriella subcapitata	parasiticide ivermectin	*Daphnia magna*	Garric et al 2007
Scenedesmus costato-granulatus	ammonia	*Brachionus rubens*	Schluter & Groeneweg 1985
Scenedesmus obliquus	nutrient substitution, Benomyl	*Chytridium sp.*	Abeiovich and Dikbuck 1977
Scenedesmus obliquus	pyrethroids insecticides	*Brachionus calyciflorus*	Huang et al 2011
Spirulina platensis	pesticides	*Brachionus calyciflorus*	Huang et al 2014b
Synechocystis sp.	anoxia	*Colpoda steinii*	Troschl et al 2017

advanced planning to incorporate prophylactic measures into algal mass culture operational strategies can go a long way in mitigating grazer-induced culture crashes.

Prophylactic procedures can also overlap with interdictive strategies, to some extent. Chlorination, via sodium hypochlorite or chlorine dioxide application, can be used in low doses as prophylaxis or high doses as a treatment option for algicidal species. Similarly, pH, copper sulfate, some oxidants, quinine sulfate, and rotenone have been used as effective chemical treatment options shown to selectively impact only the algal grazers and not the algae. Several biocides have also been assessed for their impact on algae cultures, including fungicides, botanical pesticides, and parasiticides. Although most of the tested biocides were highly effective at eliminating contaminants, they were also often too expensive, environmentally harmful, or detrimental to algal strain productivity and thus are currently of limited utility. Further research is required to ascertain tolerant chemical concentrations, generate resistant genetically-engineered algal strains, and conduct additional broad chemical screens against both grazer and algal species to identify new chemical treatments worth further study. Physical interdictive strategies are another category of algal pest remediation. Hydrodynamic shear and cavitation, filtration, pulsed electric fields, foam flotation, hydrocyclone, and ultrasonication are all demonstrated techniques for mitigating and eliminating grazer species without changing the chemical constituents or operational parameters of the aquaculture. Oftentimes, these physical abatement procedures come with a high energy, equipment, and economical cost, thus are often neglected as a rational prophylactic and treatment regime. Finally, the "rescue harvest" is an interdiction of last resort – should operational, prophylactic, and treatment options all fail, the culture can be immediately harvested to avoid a complete culture crash and negative profit margins. Hence, advanced detection methodologies for determining the species and concentration of contaminants present within mass cultures is crucial to culture maintenance and generating a profit. Furthermore, early detection procedures can facilitate targeted, effective treatment procedures for specific deleterious species. With adequate operational, prophylactic, and interdictive methods in place, algal pond culture systems can be productive, profitable, and effective industrial-scale operations.

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