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Abstract: This disclosure includes mutagenized strains of microalgae that have improved tolerance to a biocide. These mutagenized strains can be grown in liquid systems, such as pools, ponds, and the like. The mutagenized strains improved tolerance to a biocide results in an increase in the biomass yield and/or biomass productivity of the strains. The disclosure also provides methods for growing algae in liquid culture systems and methods for suppressing, inhibiting, or reducing the growth of a fungus in such systems. Biomass produced using the mutagenized strains and methods described herein can be used to produce a variety of useful products, including, but not limited to, transportation fuels.

Declarations under Rule 4.17:

— as to applicant’s entitlement to apply for and be granted a patent (Rule 4.17(i))
— as to the applicant’s entitlement to claim the priority of the earlier application (Rule 4.17(iii))

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BIOCIDE TOLERANT ALGAE STRAINS

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

FIELD

This disclosure includes mutagenized strains of microalgae that have improved tolerance to a biocide and in particular a fungicide. These mutagenized strains can be grown in liquid systems, such as pools, ponds, and the like. The mutagenized strains' improved tolerance to a biocide results in, for example, an increase in the biomass yield and/or biomass productivity and/or growth of the strains. This disclosure also includes methods for suppressing, inhibiting, or reducing the growth of a fungus in such systems. Such systems are useful for the production of biomass such as microalgae, for example, a Desmodesmus species. A Desmodesmus species can be, for example, Desmodesmus armatus. Biomass produced using the mutagenized strains and methods described herein can be used to produce a variety of useful products. In one embodiment, the biomass produced is used for the production of oil which can be refined into a variety of products, including, but not limited to, transportation fuels.

BACKGROUND

Microalgae are unicellular non-vascular photosynthetic organisms, producing oxygen by photosynthesis. One group, the microalgae, are useful for biotechnology applications for many reasons, including their high growth rate and tolerance to varying environmental conditions. Use of microalgae in a variety of industrial processes for commercially important products has been reported. For example, microalgae have uses in the production of nutritional supplements, pharmaceuticals, natural dyes, a food source for fish and crustaceans, biological control of agricultural pests, production of oxygen and removal of nitrogen, phosphorus and toxic substances in sewage treatment, and pollution controls, such as biodegradation of plastics or uptake of carbon dioxide.

Microalgae have received increasing attention for the production of fuel products. Fuel products, such as oil, petrochemicals, and other substances useful for the production of petrochemicals are increasingly in demand.
Microalgae can produce 10 to 100 times as much mass as terrestrial plants in a year. Microalgae also produce oils (lipids) and starches that may be converted into biofuels. These microalgae can grow almost anywhere, although are most commonly found at latitudes between 40 N and 40 S. With more than 100,000 known species of diatoms (a type of microalgae), 40,000 known species of green microalgae, and smaller numbers of other microalgae species, microalgae will grow rapidly in nearly any environment, with almost any kind of water, including marginal areas with limited or poor quality water.

Microalgae can store energy in the form of either oil or starch. Stored oil can be as much as 60% of the weight of the microalgae. Certain species which are enhanced in oil or starch production have been identified, and growing conditions have been tested. Processes for extracting and converting these materials to fuels have also been developed.

Microalgae, such as a Desmodesmus species (for example, Desmodesmus armatus), grown in outdoor ponds are exposed to a diversity of natural predators including, for example, ehytrids, rotifers, and amoebas. Infestations with certain predators are common and often have adverse effects on the pond's biomass productivity, while extreme infestations may even decimate ("crash") an outdoor pond culture. One method to protect the microalgae from natural predators is to use commercially available fungicides, such as OMEGA®500F (Syngenta; USA) (hereinafter "OMEGA®"). Although the therapeutic doses of biocides are effective to control predators, the biocide itself can have toxic effects on the microalgae and may reduce biomass productivity and/or biomass quality. Thus, microalga strains with improved (increased) tolerance to biocides may help improve field productivity overall, since biocide-tolerant microalga! strains should produce more biomass (improved biomass productivity) than a non-tolerant strains (e.g. a wild type microalga! strain) upon routine pesticide treatments. Thus, there exists a need for the creation, isolation, and characterization of microalgae! strains with increased biocide-tolerance that can result in an increased yield of commercially important products, such as fuel products.

**SUMMARY**

**OM27 (SE70179)**

In one embodiment, the invention is directed to an isolated microorganism (OM27) of the species deposited under ATCC Accession No. PTA-120600. The isolated microorganism (OM27) is also known herein as a Desmodesmus armatus ATCC No. PTA-120600. The isolated microorganism (OM27) associated with ATCC Accession No. PTA-120600 was deposited under the Budapest Treaty on September 24, 2013, at the American Type Culture Collection, Patent
Depository, 10801 University Boulevard, Manassas, VA. 20110-2209. In some embodiments, the invention is directed to an isolated microorganism(s) having the characteristics of the microorganism deposited under ATCC Accession No. PTA 120600 (OM27). The characteristics of the species deposited under ATCC Accession No. PTA-120600 include its growth rate, biomass productivity, in situ productivity, doubling rate, photosynthetic efficiency, photosynthetic health, lipid profiles, FAME content, and its nucleotide sequence or sequences.

OM65 (SE70181)

[0009] In one embodiment, the invention is directed to an isolated microorganism (OM65) of the species deposited under ATCC Accession No. PTA-120599. The isolated microorganism (OM65) is also known herein as a Desmodesmus armatus ATCC No. PTA-120599. The isolated microorganism (OM65) associated with ATCC Accession No. PTA-120599 was deposited under the Budapest Treaty on September 24, 2013, at the American Type Culture Collection, Patent Depository, 10801 University Boulevard, Manassas, VA, 20110-2209. In some embodiments, the invention is directed to an isolated microorganism(s) having the characteristics of the microorganism deposited under ATCC Accession No. PTA-120599 (OM65). The characteristics of the species deposited under ATCC Accession No. PTA-120599 include its growth rate, biomass productivity, in situ productivity, doubling rate, photosynthetic efficiency, photosynthetic health, lipid profiles, FAME content, and its nucleotide sequence or sequences.

[0010] Provided herein is an ultraviolet (UV) mutagenized Desmodesmus strain selected from the group consisting of OM65 and OM27, with an increased tolerance to a fungicide composition as compared to an unmutagenized Desmodesmus strain's tolerance to the fungicide composition, wherein the active ingredient of the fungicide composition is a Fungicide Resistance Action Committee 29 (FRAC 29) fungicide. In one embodiment, the FRAC 29 fungicide is any one or more of Fluazinam, Bmapacryl, Meptyldinocap, or Dinocap. In another embodiment, the FRAC 29 fungicide is an uncoupler of oxidative phosphorylation. In other embodiments, the FRAC 29 fungicide comprises from 10%-20% of the fungicide composition, from 20%-30% of the fungicide composition, from 30%-40% of the fungicide composition, from 40%-50% of the fungicide composition, from 50%-60% of the fungicide composition, from 60%-70% of the fungicide composition, from 70%-80% of the fungicide composition, from 80%-90%, or from 90% to 100% of the fungicide composition.

[0011] Also provided herein is an ultraviolet (UV) mutagenized Desmodesmus strain selected from the group consisting of OM65 and OM27 with an increased tolerance to a fungicide composition
wherein the active ingredient is Fluazinam, as compared to an unmutagenized *Desmodesmus* strain’s tolerance to the fungicide composition. In some embodiments, Fluazinam comprises from 10-20% of the fungicide composition, from 20%-30% of the fungicide composition, from 30%-40% of the fungicide composition, from 40%-50% of the fungicide composition, from 50%-60% of the fungicide composition, from 60%-70% of the fungicide composition, from 70%-80% of the fungicide composition, from 80%-90%, or from 90% to 100%, of the fungicide composition. In another embodiment, both the mutagenized *Desmodesmus* strain and the unmutagenized *Desmodesmus* strain are treated with one or more doses of the fungicide composition. In yet other embodiments, any one or more dose of the fungicide composition is from 0.25 ppm to 15 ppm, or 0.01 ppm to 20 ppm. In other embodiments, any one or more dose of the fungicide composition is from 0.1 ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm. In some embodiments, any one or more dose of the fungicide composition is from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm. In other embodiments, any one or more dose of the fungicide composition is about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm. In some embodiments, both the mutagenized *Desmodesmus* strain and the unmutagenized *Desmodesmus* strain are treated with two to six doses of the fungicide composition. In other embodiments, the increase in tolerance is defined as any one or more of an increase in: growth rate, biomass productivity, *in situ* productivity, doubling rate, photosynthetic efficiency, photosynthetic health, or FAME content. In one embodiment, the increase in tolerance is defined as an improved color of pigmentation. In one embodiment, the increase in tolerance is defined as an increase in growth rate. In another embodiment, the increase in tolerance is defined as an increase in biomass productivity. In another embodiment, the increase in biomass productivity is 2% to 5%, 5% to 10%, 10% to 15%, 15% to 20%, 20% to 25%, 25% to 30%, 30% to 35%, 35% to 40%, 40% to 45%, 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70%, 75% to 80%, 85% to 90%, 95% to 100%, 100% to 150%, 150% to 200%, 200% to 250%, 250% to 300%, 300% to 350%, 350% to 400%, or more than 400%. In one embodiment, biomass productivity is measured in dry weight (DW) grams per liter (g/L). In another embodiment, the increase in tolerance is defined as an increase *in situ* productivity. In some embodiments, the increase *in situ* productivity is 2% to 5%,
5% to 10%, 10% to 15%, 15% to 20%, 20% to 25%, 25% to 30%, 30% to 35%, 35% to 40%, 40% to 45%, 45% to 50%, 50% to 55%, 55% to 60%, 60% to 65%, 65% to 70%, 75% to 80%, 85% to 90%, 95% to 100%, 100% to 150%, 150% to 200%, 200% to 250%, 250% to 300%, 300% to 350%, 350% to 400%, or more than 400%. In other embodiments, the increase in in situ productivity is about a 20% increase, about a 28%, about a 30% increase, or about a 118% increase. In one embodiment, in situ productivity is measured in g/m²/day. In another embodiment, the increase in tolerance is defined as an increase in doubling rate. In yet another embodiment, the increase in tolerance is defined as an increase in photosynthetic efficiency. In one embodiment, the photosynthetic efficiency is determined by Pulse-Amplitude-Modulation (PAM) fluorometry (Fv/Fm). In another embodiment, the increase in tolerance is defined as an increase in photosynthetic health. In another embodiment, the photosynthetic health is determined by Pulse-Amplitude-Modulation (PAM) fluorometry (Fv/Fm). In one embodiment, the increase in tolerance is defined as an increase in FAME content. In other embodiments, the increase in FAME content is 1.25-fold to two-fold higher, two-fold to three-fold higher, three-fold to four-fold higher, or four-fold to five-fold higher. In one embodiment, the mutagenized Desmodesmus species is genetically engineered prior to being mutagenized. In another embodiment, the mutagenized Desmodesmus species is genetically engineered after being mutagenized. In other embodiments, a fungus on which the fungicide composition acts is a Chytridiomycotia or a Cryptomyctota. In other embodiments, the Chytridiomycotia is a Chytridiales, a Rhizopylectidales, a Spizellomycetales, a Rhizophydiales, a Lobulomycetales, a Cladochytiales, a Polychytrium, or a Monoblepharidomycetes.

[0012] Provided herein is an ultraviolet (UV) mutagenized Desmodesmus strain selected from the group consisting of OM65 and OM27 with an increased tolerance to OMEGA*, as compared to an unmutagenized Desmodesmus strain's tolerance to OMEGA*. In one embodiment, both the mutagenized Desmodesmus strain and the unmutagenized Desmodesmus strain are treated with one or more doses of OMEGA*. In other embodiments, any one or more dose of OMEGA* is from 0.25 ppm to 15 ppm, or from 0.01 ppm to 20 ppm. In yet other embodiments, any one or more dose of OMEGA* is from 0.1 ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm. In other embodiments, any one or more dose of OMEGA* is from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm. In yet other embodiments, any one or more dose of OMEGA* is about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm.
ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm. In one embodiment, both the mutagenized Desmodesmus strain and the unmutagenized Desmodesmus strain are treated with two to six doses of OMEGA®. In other embodiments, the increase in tolerance is defined as any one or more of an increase in: growth rate; biomass productivity; \textit{in situ} productivity; doubling rate; photosynthetic efficiency; photosynthetic health; or FAME content. In one embodiment, the increase in tolerance is defined as an improved color of pigmentation. In another embodiment, wherein the increase in tolerance is defined as an increase in growth rate. In yet another embodiment, the increase in tolerance is defined as an increase in biomass productivity. In some embodiments, the increase in biomass productivity is 2\% to 5\%, 5\% to 10\%, 10\% to 15\%, 15\% to 20\%, 20\% to 25\%, 25\% to 30\%, 30\% to 35\%, 35\% to 40\%, 40\% to 45\%, 45\% to 50\%, 50\% to 55\%, 55\% to 60\%, 60\% to 65\%, 65\% to 70\%, 75\% to 80\%, 85\% to 90\%, 95\% to 100\%, 100\% to 150\%, 150\% to 200\%, 200\% to 250\%, 250\% to 300\%, 300\% to 350\%, 350\% to 400\%, or more than 400\%. In one embodiment, biomass productivity is measured in dry weight (DW) grams per liter (g/L). In one embodiment, the increase in tolerance is defined as an increase \textit{in situ} productivity. In other embodiments, the increase in \textit{in situ} productivity is 2\% to 5\%, 5\% to 10\%, 10\% to 15\%, 15\% to 20\%, 20\% to 25\%, 25\% to 30\%, 30\% to 35\%, 35\% to 40\%, 40\% to 45\%, 45\% to 50\%, 50\% to 55\%, 55\% to 60\%, 60\% to 65\%, 65\% to 70\%, 75\% to 80\%, 85\% to 90\%, 95\% to 100\%, 100\% to 150\%, 150\% to 200\%, 200\% to 250\%, 250\% to 300\%, 300\% to 350\%, 350\% to 400\%, or more than 400\%. In some embodiments, the increase in \textit{in situ} productivity is about a 20\% increase, about a 28\%, about a 30\% increase, or about a 118\% increase. In another embodiment, \textit{in situ} productivity is measured in g/m²/day. In one embodiment, the increase in tolerance is defined as an increase in doubling rate. In another embodiment, the increase in tolerance is defined as an increase in photosynthetic efficiency. In yet another embodiment, the photosynthetic efficiency is determined by Pulse-Amplitude-Modulation (PAM) fluorometry (Fv/Fm). In one embodiment, the increase in tolerance is defined as an increase in photosynthetic health. In another embodiment, the photosynthetic health is determined by Pulse-Amplitude-Modulation (PAM) fluorometry (Fv/Fm). In one embodiment, the increase in tolerance is defined as an increase in FAME content. In other embodiments, the increase in FAME content is 1.25-fold to two-fold higher, two-fold to three-fold higher, three-fold to four-fold higher, or four-fold to five-fold higher. In one embodiment, the mutagenized Desmodesmus species is genetically engineered prior to being mutagenized. In another embodiment, the mutagenized Desmodesmus species is genetically engineered after being
mutagenized. In some embodiments, a fungus on which the fungicide composition acts is a
Chytridiomycota or a Cryptomycota. In yet other embodiments, the Chytridiomycota is a
Chytridiales, a Rhizophyctidiales, a Spizellomycetales, a Rhizophycales, a Lobulomycetales, a
Cladochytriales, a Polychytrium, or a Monohlepharidomycetes. In one embodiment, the
mutagenized Desmodesmus species is OM65. In another embodiment, the mutagenized
Desmodesmus species is OM65 and the species was deposited under ATCC Accession No. PTA-
120599 on September 24, 2013. In one embodiment, the mutagenized Desmodesmus species is
OM27. In another embodiment, the mutagenized Desmodesmus species is OM27 and the species
was deposited under ATCC Accession No. PTA-120600 on September 24, 2013.

Provided herein is an isolated Desmodesmus species OM65 that is tolerant to OMEGA® at a
concentration of from 0.01 ppm to 20 ppm which is deposited under ATCC Accession No. PTA
120599. In some embodiments, the Desmodesmus species OM65 is tolerant to OMEGA® at a
concentration of from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm,
from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm.

Provided herein is an isolated Desmodesmus species OM27 that is tolerant to OMEGA® at a
concentration of from 0.01 ppm to 20 ppm which is deposited under ATCC Accession No. PTA-
120600. In some embodiments, the Desmodesmus species OM27 is tolerant to OMEGA® at a
concentration of from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm,
from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm.

Also provided herein is an isolated microorganism (OM65) deposited
under ATCC Accession No. PTA-120599 on September 24, 2013 with the American Type Culture
Collection (ATCC) at 10801 University Boulevard, Manassas, CA, 20110, USA.

Also provided herein is an isolated microorganism having the characteristics of the
microorganism (OM65) deposited under ATCC Accession No. PTA-120599 on September 24, 2013
with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, CA, 20110, USA.

Also provided herein is an isolated microorganism (OM27) deposited
under ATCC Accession No. PTA-120600 on September 24, 2013 with the American Type Culture
Collection (ATCC) at 10801 University Boulevard, Manassas, CA, 20110, USA.

Also provided herein is an isolated microorganism having all the characteristics of the
microorganism (OM27) deposited under ATCC Accession No. PTA-600120 on September 24, 2013.
with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, CA, 20110, USA.

[0019] Provided herein is a method of producing algal biomass in a liquid culture system comprising: a) providing a liquid culture system; and b) inoculating the liquid culture system with at least one mutagenized *Desmodesmus* species selected from the group consisting of OM27 and OM65. In one embodiment, the method further comprises treating the liquid culture system either before or after step b) or both before and after step b) with an effective concentration of a fungicide composition to reduce the growth of the fungus, wherein the active ingredient of the fungicide composition is a Fungicide Resistance Action Committee 29 (FRAC 29) fungicide. In some embodiments the *Desmodesmus* species is mutagenized using ultraviolet (UV) radiation. In some embodiments, the FRAC 29 fungicide is any one or more of Fluazinam, Binapacryl, Meptyldinocap, or Dinocap. In one embodiment, the FRAC 29 fungicide is Fluazinam. In another embodiment, the fungicide composition is OMEGA®. In another embodiment, the FRAC 29 fungicide is an uncoupler of oxidative phosphorylation. In other embodiments, the FRAC 29 fungicide comprises from 10%-20% of the fungicide composition, from 20%-30% of the fungicide composition, from 30%-40% of the fungicide composition, from 40%-50% of the fungicide composition, from 50%-60% of the fungicide composition, from 60%-70% of the fungicide composition, from 70%-80% of the fungicide composition, from 80%-90% or from 90% to 100% of the fungicide composition. In one embodiment, the treating step is a prophylactic treating step. The liquid culture system may be treated with the fungicide composition on multiple occasions. In another embodiment, the liquid culture system is treated one or more times with one or more effective concentrations of the fungicide composition. In other embodiments, any one or more of the effective concentrations is from 0.25 ppm to 15 ppm, or 0.01 ppm to 20 ppm. In some embodiments, any one or more of the effective concentrations is from 0.1 ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm. In other embodiments, any one or more of the effective concentrations is from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm. In yet other embodiments, any one or more of the effective concentrations is about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm. In one embodiment, the liquid culture system is treated two to six
times with one or more effective concentrations of the fungicide composition. In some embodiments, the fungus in which the fungicide composition acts on is a Chytridiomycota or a Cryptomycota. In other embodiments, the Chytridiomycota is a Chytridiales, a Rhizophyldiales, a Spizellomycetales, a Rhizophyldiales, a Lobulomycetales, a Cladockytriales, a Polychytrium, or a Monoblepharidomycetes. In one embodiment, the reduction in growth is measured by any one or more of growth rate, biomass productivity, in situ productivity, or doubling rate. In other embodiments, the mutagenized Desmodesmus species is one or both of OM65 and OM27. In other embodiments, the mutagenized Desmodesmus species is one or both of OM65 and OM27 deposited under ATCC Accession No. PTA-120599 and ATCC Accession No. PTA-120600, respectively, on September 24, 2013 with the American Type Culture Collection (ATCC). In one embodiment, the mutagenized Desmodesmus species is genetically engineered prior to being mutagenized. In another embodiment, the mutagenized Desmodesmus species is genetically engineered after being mutagenized. In yet another embodiment, the mutagenized Desmodesmus species is grown in the liquid culture system. In some embodiments, the growing is for a number of days selected from the group consisting of 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 10 or more, 15 or more, 30 or more, 45 or more, 60 or more, 90 or more, 120 or more, 180 or more, 250 or more, 500 or more, 1000 or more, 1500 or more, and 2000 or more days after inoculation. In other embodiments, the growing is from 4 to 6 days, from 4 to 14 days, or is 96 hours or more, after the inoculation. In other embodiments, the growing is from 1 to 30 days, 1 to 40, from 30 to 60 days, from 60-90 days, or from 90-120 days. In additional embodiments, the algae is grown from 1 to 7 days, from 1 week to 2 weeks, from 2 weeks to 3 weeks, from 3 weeks to 1 month, from 1 month to 3 months, from 3 months to 6 months, from 6 months to 9 months, from 9 months to 12 months, from 12 months to 18 months, from 18 months to 2 years, from 2 years to 3 years, from 3 years to 4 years, from 4 years to 5 years, from 5 years to 6 years, from 6 years to 7 years, from 7 years to 8 years, from 8 years to 9 years or from 9 years and 10 years after inoculation. In one embodiment, the method further comprises providing one or more additional amounts of the fungicide composition to maintain an effective concentration of the fungicide composition during the growing. In another embodiment, the liquid culture system is an open outdoor liquid culture system. In other embodiments, the open outdoor culture system has a volume of: at least 20,000 liters, at least 40,000 liters, at least 80,000 liters, at least 100,000 liters, at least 150,000 liters, at least 200,000 liters, at least 250,000 liters, at least 500,000 liters, at least 600,000 liters, or at least 1,000,000 liters; or 10,000 to 20,000 liters, 10,000 to 40,000 liters, 10,000 to 80,000 liters, 10,000 to 100,000 liters, 10,000 to 150,000 liters,
10,000 to 200,000 liters, 10,000 to 250,000 liters, 10,000 to 500,000 liters, 10,000 to 600,000 liters, 10,000 to 1,000,000 liters, 20,000 to 40,000 liters, 20,000 to 80,000 liters, 20,000 to 100,000 liters, 20,000 to 150,000 liters, 20,000 to 200,000 liters, 20,000 to 250,000 liters, 20,000 to 500,000 liters, 20,000 to 600,000 liters, 20,000 to 1,000,000 liters, 40,000 to 80,000 liters, 40,000 to 100,000 liters, 40,000 to 150,000 liters, 40,000 to 200,000 liters, 40,000 to 250,000 liters, 40,000 to 500,000 liters, 40,000 to 600,000 liters, 40,000 to 1,000,000 liters, 80,000 to 100,000 liters, 80,000 to 150,000 liters, 80,000 to 200,000 liters, 80,000 to 250,000 liters, 80,000 to 500,000 liters, 80,000 to 600,000 liters, 80,000 to 1,000,000 liters, 100,000 to 150,000 liters, 100,000 to 200,000 liters, 100,000 to 250,000 liters, 100,000 to 500,000 liters, 100,000 to 600,000 liters, 100,000 to 1,000,000 liters, 200,000 to 250,000 liters, 200,000 to 500,000 liters, 200,000 to 600,000 liters, 200,000 to 1,000,000 liters, 250,000 to 500,000 liters, 250,000 to 600,000 liters, 250,000 to 1,000,000 liters, 500,000 to 600,000 liters, or 500,000 to 1,000,000 liters. In some embodiments, the open outdoor liquid culture system has an area selected from the group consisting of at least 0.25 acre, at least 0.5 acre, at least 1.0 acre, at least 1.5 acres, at least 2.0 acres, at least 2.5 acres, at least 5.0 acres, and 7.5 or more acres. In certain embodiments the open outdoor liquid culture system has an area selected from the group consisting of 0.25 to 0.5 acres, 0.25 to 1.0 acres, 0.25 to 1.5 acres, 0.25 to 2.0 acres, 0.25 to 2.5 acres, 0.25 to 5.0 acres, 0.25 to 7.5 acres, 0.5 to 1.0 acres, 0.5 to 1.5 acres, 0.5 to 2.0 acres, 0.5 to 2.5 acres, 0.5 to 5.0 acres, 0.5 to 7.5 acres, 1.0 to 1.5 acres, 1.0 to 2.0 acres, 1.0 to 2.5 acres, 1.0 to 5.0 acres, 1.0 to 7.5 acres, 2.0 to 2.5 acres, 2.0 to 5.0 acres, 2.0 to 7.5 acres, 2.5 acres to 5.0 acres, 2.5 to 7.5 acres, 7.5 to 10 acres, 10 to 15 acres, 15 to 20 acres, 20 to 25 acres, 25 to 30 acres, 30 to 35 acres, 35 to 40 acres, 40 to 45 acres, 45 to 50 acres, 50 to 75 acres, 75 to 100 acres, 100 to 125 acres, 125 to 150 acres, 150 to 175 acres, 175 to 200 acres, 200 to 250 acres, 250 to 300 acres, 300 to 350 acres, 350 to 400 acres, 400 to 450 acres, and 450 to 500 acres. In one embodiment, the liquid culture system is a mono-culture. In another embodiment, the growing provides a yield of mutagenized Desmodesmus species greater than 0.4 gram per liter (g/L) ash free dry weight (AFDW). In some embodiments, the yield is selected from the group consisting of greater than 0.5 g/L, greater than 0.6 g/L, greater than 0.7 g/L, greater than 0.8 g/L, greater than 0.9 g/L, and greater than 1.0 g/L AFDW. In other embodiments, the growing provides a yield of the mutagenized Desmodesmus species that is at least 5% greater or at least 10% greater than a yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. In yet other embodiments, the growing provides a yield of the mutagenized Desmodesmus species
selected from the group consisting of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, and 100% greater than the yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. In some embodiments, the growing provides a yield of the mutagenized Desmodesmus species selected from the group consisting of at least 1.25 fold, 1.5 fold, 2.0 fold, 2.5 fold, 5.0 fold, 7.5 fold, 10 fold and 15 fold greater than the yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. In one embodiment, the mutagenized Desmodesmus species in the liquid culture system is harvested. In another embodiment, a fuel product is obtained from the harvested mutagenized Desmodesmus species. In other embodiments, the fuel product is an oil, a petrochemical, or a substance useful in the production of a petrochemical.

[0020] Provided herein is a method of reducing the growth of a fungus in a liquid culture system, comprising: a) providing a liquid culture system; b) inoculating the liquid culture system with at least one mutagenized Desmodesmus species selected from the group consisting of OM27 and OM65; and c) treating the liquid culture system either before or after step b) or both before and after step b) with an effective concentration of a fungicide composition to reduce the growth of the fungus, wherein the active ingredient of the fungicide composition is a Fungicide Resistance Action Committee 29 (FRAC 29) fungicide. In certain embodiments the Desmodesmus species is mutagenized using ultraviolet (UV) radiation. In some embodiments, the FRAC 29 fungicide is any one or more of Fluazinam, Binapacryl, Meptyldinocap, or Dinocap. In one embodiment, the FRAC 29 fungicide is Fluazinam. In another embodiment, the fungicide composition is OMEGA®. In another embodiment, the FRAC 29 fungicide is an uncoupler of oxidative phosphorylation. In other embodiments, the FRAC 29 fungicide comprises from 10%-20% of the fungicide composition, from 20%-30% of the fungicide composition, from 30%-40% of the fungicide composition, from 40%-50% of the fungicide composition, from 50%-60% of the fungicide composition, from 60%-70% of the fungicide composition, from 70%-80% of the fungicide composition, from 80%-90%, or from 90% to 100% of the fungicide composition. In one embodiment, the treating step is a prophylactic treating step. In another embodiment, the liquid culture system is treated one or more times with one or more effective concentrations of the fungicide composition. In other embodiments, any one or more of the effective concentrations is from 0.25 ppm to 15 ppm, or 0.01 ppm to 20 ppm. In some embodiments, any one or more of the effective concentrations is from 0.1
ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm. In other embodiments, any one or more of the effective concentrations is from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm. In yet other embodiments, any one or more of the effective concentrations is about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm.

In one embodiment, the liquid culture system is treated two to six times with one or more effective concentrations of the fungicide composition. In some embodiments, the fungus in which the fungicide composition acts on is a *Chytridiomycota* or a *Cryptomycota*. In other embodiments, the *Chytridiomycota* is a *Chytridiales*, a *Rhizophylectidales*, a *Spizellomycetales*, a *Rhizophydiales*, a *Lobulomycetales*, a *Cladochytriales*, a *Polychytrium*, or a *Monoblepharidomycetes*. In one embodiment, the reduction in growth is measured by any one or more of growth rate, biomass productivity, *in situ* productivity, or doubling rate. In other embodiments, the mutagenized *Desmodesmiis* species is OM65 and OM27. In other embodiments, the mutagenized *Desmodesmus* species OM65 or OM27 was deposited under ATCC Accession No. PTA-120599, or ATCC Accession No. PTA-120600, respectively, on September 24, 2013 with the American Type Culture Collection (ATCC). In one embodiment, the mutagenized *Desmodesmus* species is genetically engineered prior to being mutagenized. In another embodiment, the mutagenized *Desmodesmus* species is genetically engineered after being mutagenized. In yet another embodiment, the mutagenized *Desmodesmus* species is grown in the liquid culture system. In some embodiments, the growing is for a number of days selected from the group consisting of 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 10 or more, 15 or more, 30 or more, 45 or more, 60 or more, 90 or more, 120 or more, 180 or more, 250 or more, 500 or more, 1000 or more, 1500 or more, and 2000 or more days after the inoculation. In other embodiments, the growing is from 4 to 6 days, from 4 to 14 days, or is 96 hours or more, after the inoculation. In other embodiments, the growing is from 1 to 30 days, 1 to 40, from 30 to 60 days, from 60-90 days, or from 90-120 days. In still another embodiment, the growing is from 1 to 7 days, from 1 week to 2 weeks, from 2 weeks to 3 weeks, from 3 weeks to 1 month, from 1 month to 3 months, from 3 months to 6 months, from 6 months to 9 months, from 9 months to 12 months, from 12 months to 18 months, from 18 months to 2 years, from 2 years to 3 years, from 3 years to 4 years, from 4 years to 5 years, from 5 years to 6
years, from 6 years to 7 years, from 7 years to 8 years, from 8 years to 9 years or from 9 years and 10 years after inoculation. In one embodiment, the method further comprises providing one or more additional amounts of the fungicide composition to maintain an effective concentration of the fungicide composition during the growing. In another embodiment, the liquid culture system is an open outdoor liquid culture system. In other embodiments, the open outdoor culture system has a volume of: at least 20,000 liters, at least 40,000 liters, at least 80,000 liters, at least 100,000 liters, at least 150,000 liters, at least 200,000 liters, at least 250,000 liters, at least 500,000 liters, at least 600,000 liters, or at least 1,000,000 liters; or 10,000 to 20,000 liters, 10,000 to 40,000 liters, 10,000 to 80,000 liters, 10,000 to 100,000 liters, 10,000 to 150,000 liters, 10,000 to 200,000 liters, 10,000 to 250,000 liters, 10,000 to 500,000 liters, 10,000 to 600,000 liters, 10,000 to 1,000,000 liters, 20,000 to 40,000 liters, 20,000 to 80,000 liters, 20,000 to 100,000 liters, 20,000 to 150,000 liters, 20,000 to 200,000 liters, 20,000 to 250,000 liters, 20,000 to 500,000 liters, 20,000 to 600,000 liters, 20,000 to 1,000,000 liters, 40,000 to 80,000 liters, 40,000 to 100,000 liters, 40,000 to 150,000 liters, 40,000 to 200,000 liters, 40,000 to 250,000 liters, 40,000 to 500,000 liters, 40,000 to 600,000 liters, 40,000 to 1,000,000 liters, 80,000 to 100,000 liters, 80,000 to 150,000 liters, 80,000 to 200,000 liters, 80,000 to 250,000 liters, 80,000 to 500,000 liters, 80,000 to 600,000 liters, 80,000 to 1,000,000 liters, 100,000 to 150,000 liters, 100,000 to 200,000 liters, 100,000 to 250,000 liters, 100,000 to 500,000 liters, 100,000 to 600,000 liters, 100,000 to 1,000,000 liters, 200,000 to 250,000 liters, 200,000 to 500,000 liters, 200,000 to 600,000 liters, 200,000 to 1,000,000 liters, 250,000 to 500,000 liters, 250,000 to 600,000 liters, 250,000 to 1,000,000 liters, 500,000 to 600,000 liters, or 500,000 to 1,000,000 liters, in some embodiments, the open outdoor liquid culture system has an area selected from the group consisting of at least 0.25 acre, at least 0.5 acre, at least 1.0 acre, at least 1.5 acres, at least 2.0 acres, at least 2.5 acres, at least 5.0 acres, and 7.5 or more acres. In an additional embodiment, the open outdoor liquid culture system has an area selected from the group consisting of 0.25 to 0.5 acres, 0.25 to 1.0 acres, 0.25 to 1.5 acres, 0.25 to 2.0 acres, 0.25 to 2.5 acres, 0.25 to 5.0 acres, 0.25 to 7.5 acres, 0.5 to 1.0 acres, 0.5 to 1.5 acres, 0.5 to 2.0 acres, 0.5 to 2.5 acres, 0.5 to 5.0 acres, 0.5 to 7.5 acres, 1.0 to 1.5 acres, 1.0 to 2.0 acres, 1.0 to 2.5 acres, 1.0 to 5.0 acres, 1.0 to 7.5 acres, 2.0 to 2.5 acres, 2.0 to 5.0 acres, 2.0 to 7.5 acres, 2.5 to 5.0 acres, 2.5 to 7.5 acres, from 7.5 to 10 acres, from 10 to 15 acres, from 15 to 20 acres, from 20 to 25 acres, from 25 to 30 acres, from 30 to 35 acres, from 35 to 40 acres, from 40 to 45 acres, from 45 to 50 acres, from 50 to 75 acres, from 75 to 100 acres, from 100 to 125 acres, from 125 to 150 acres, from 150 to 175 acres, from 175 to 200 acres, from 200 to 250 acres, from 250 to 300 acres, from 300 to
350 acres, from 350 to 400 acres, from 400 to 450 acres, and from 450 to 500 acres. In one embodiment, the liquid culture system is a mono-culture. In another embodiment, the growing provides a yield of mutagenized Desmodesmus species greater than 0.4 gram per liter (g/L) AFDW. In some embodiments, the yield is selected from the group consisting of greater than 0.5 g/L, greater than 0.6 g/L, greater than 0.7 g/L, greater than 0.8 g/L, greater than 0.9 g/L, and greater than 1.0 g/L AFDW. In other embodiments, the growing provides a yield of the mutagenized Desmodesmus species that is at least 5% greater or at least 10% greater than a yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. In yet other embodiments, the growing provides a yield of the mutagenized Desmodesmus species selected from the group consisting of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, and 100% greater than the yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. In some embodiments, the growing provides a yield of the mutagenized Desmodesmus species selected from the group consisting of at least 1.25 fold, 1.5 fold, 2.0 fold, 2.5 fold, 5.0 fold, 7.5 fold, 10 fold and 15 fold greater than the yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. In one embodiment, the mutagenized Desmodesmus species in the liquid culture system is harvested. In another embodiment, a fuel product is obtained from the harvested mutagenized Desmodesmus species. In other embodiments, the fuel product is an oil, a petrochemical, or a substance useful in the production of a petrochemical.

[0021] Also provided herein is a method of determining the biocide tolerance of a modified (for example, mutated, evolved, or genetically engineered) strain as compared to an unmodified strain, comprising: measuring the Fv/Fm deficit of the modified and unmodified strains, and determining that the strain with a smaller deficit has a higher biocide tolerance than the other. A strain can be genetically engineered by, for example, transforming it with a gene, and other techniques known to one skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS
These and other features, aspects, and advantages of the present disclosure will become better understood with regard to the following description, appended claims and accompanying figures.

Figure 1 is a photographic image of OM65 biomass collected from miniponds, as described in Example 3.

Figure 2A to Figure 2D show the results from Validation III (aka "Sawtooth Experiment") for WT, as described in Example 2.

Figure 3A to Figure 3E shows the results from Validation III (aka "Sawtooth Experiment") for OM15 (Figure 3A), OM25 (Figure 3B), OM27 (Figure 3C), OM65 (Figure 3D), and OM82 (Figure 3E), as described in Example 2.

Figure 4 shows the doubling rate of OM15, OM25, OM27, OM65, OM82, and WT at a dose of 2.5 ppm of OMEGA®, as is described in Example 2.

Figure 5 is a photographic image of Validation III at 3 ppm (Round 6), as described in Example 2.

Figure 6A to Figure 6F show Pulse-Amplitude-Modulation (PAM) fluorometry (Fv/Fm) results for outdoor minipotids OM15, OM25, OM27, OM65, OM82, and WT, as described in Example 3.

Figure 7 shows total suspended solids (TSS) productivity of ponds comprising WT, OM15, OM25, OM27, OM65, and OM82, as described in Example 3.

Figure 8 shows in situ biomass productivity for WT, OM15, OM25, OM27, OM65, and OM82, as is described in Example 3.

Figure 9 shows in situ biomass productivity for WT, OM15, OM25, OM27, OM65, and OM82, normalized to WT, as is described in Example 3.

Figure 10 shows the data from Figure 8 analyzed by OneWay ANOVA, as is described in Example 3.

Figure 11 shows the PAM yield (photosynthetic health) of OM15, OM25, OM27, OM65, OM82, and WT during the in situ biomass productivity experiment, as is described in Example 3.

Figure 12 shows the "Fv/Fm deficit" for WT, OM15, OM25, OM27, OM65, and OM82, following OMEGA® treatment, as is described in Example 3.

Figure 13 is a photographic image of OM65 biomass collected from miniponds, as is described in Example 3.
[0036] Figure 14A to Figure 14C show fluorescence measurements for OM15, OM25, GM27, OM65, QM82, and WT, as is described in Example 3.

[0037] Figure 15 shows fatty acid methyl ester (FAME) analysis of biomass from cultures of OM27, OM65, and WT, as is described in Example 3.

[0038] Figure 16 shows PAM measurements from a "control" WT *Desmodesmus* species culture that was not subject to a continuous dosing scheme of OMEGA*, as is described in Example 4.

[0039] Figure 17 shows PAM measurements from a control WT *Desmodesmus* species culture and cultures of OM27 and OM65, as described in Example 5.

[0040] Figure 18 shows biomass productivity data analyzed by One way ANOVA, as is described in Example 5. WT is on the left, OM27 in the middle, and OM65 on the right.

[0041] Figure 19 shows a markedly visual difference between an OM27 culture (left) and a control WT *Desmodesmus* species culture (right), as is described in Example 5.

**DETAILED DESCRIPTION**

[0042] The following detailed description is provided to aid those skilled in the art in practicing the present disclosure. Even so, this detailed description should not be construed to unduly limit the present disclosure as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present disclosure.

[0043] As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural reference unless the context clearly dictates otherwise.

[0044] When ranges of values are given in this specification and the appended claims such a range is intended to include all values and sub-ranges of values within such range. For example and not by way of limitation a reference to a range of 5% to 10% includes the values 5%, 6%, 7%, 8%, 9% and 10% as well as subranges such as, for example, 6% to 8%.

[0045] Oxygenic photosynthetic microalgae and cyanobacteria (collectively called algae) represent an extremely diverse, yet highly specialized group of micro-organisms that live in diverse ecological habitats such as freshwater, brackish, marine, and hyper-saline, with a range of temperatures and pH, and unique nutrient availabilities (for example, as described in Falkowski, P.G., and Raven, J.A., Aquatic Photosynthesis, Maiden, MA: Blackwell Science). One genus of microalgae is *Desmodesmus*. 
Classical ultraviolet (UV) mutagenesis and laboratory selection methods were employed to create and identify non-genetically modified (GM) variants of a Desmodesmus species that have increased or improved tolerance (hyper-tolerance or increased resistance) to the fungicide OMEGA* as compared to the unmutagenized Desmodesmus species wild type (WT) strain when challenged with the same dose(s), dosing regimen, or concentrations of OMEGA*. Improved or increased tolerance to the fungicide OMEGA* can be shown by any one or more of the parameters described herein. Other parameters known to one skilled in the art can also be used in place of or in combination with any of the following parameters. The variants can, for example, exhibit an increase in productive pond lifetime, an increase in biomass productivity, an increase in in situ productivity, and/or an increase in biomass yield relative to the WT strain when challenged with the same dose(s), dosing regimen, or concentrations of OMEGA*. The variants can, for example, have increased growth rate, increased photosynthetic efficiency (Fv/Fm), increased photosynthetic health, increased FAME content, and/or increased doubling rates as compared to the WT strain when challenged with the same dose(s), dosing regimen, or concentrations of OMEGA*. Each of the different parameters to determine tolerance described herein, for example, measuring biomass productivity can be done in using any one or more assays/methods known to one skilled in the art and can be measured in a wide range of units, for example, dry weight (DW) grams per liter (g/L). Choosing the appropriate method and units of measurement are well within the ability of one skilled in the art. Growth rate, biomass productivity, and in situ productivity can all be measured using the following exemplary units: grams per liter per day, grams per meter squared per day, OD_{750}\text{nm} per day, or colony forming units (cfu) per liter per day.

Improved tolerance can also be determined by a visual inspection of the color/pigmentation of the biomass and/or quantification of the pigmentation of the biomass. A mutagenized strain is said to have an increased tolerance to a fungicide composition as compared to an unmutagenized strain or other strain if it is more tolerant to the same concentration or dose of the fungicide composition.

The term "variant" is used interchangeably throughout the disclosure with "mutant", "mutagenized strain", "variant strain", "evolved strain", and "evolved mutant strain". The WT Desmodesmus species is sensitive to therapeutic doses (0.5-2.0 ppm) of OMEGA* (a common fungicide) resulting in a decrease of in situ productivity. OMEGA* is an effective treatment for common chytrid infestations. It would therefore be desirable to have a variant Desmodesmus
species strain(s) with improved biomass productivity that is able to sustain a minimal or reduced toxic effect when dosed with OMEGA*.

[0049] Five variant strains exhibited an increased tolerance to OMEGA* as compared to WT also treated with the same dose(s), dosing regimen, or concentration of OMEGA*. These evolved strains were cultured alongside the WT Desmodesmus species strain in outdoor miniponds and chronically exposed to therapeutic doses of OMEGA*. The evolved strains exhibited reduced photosynthetic stress levels vs. WT based on PAM fSuorometry measurements. All strains, including WT, experienced a reduction in biomass yield (g/L) when treated with OMEGA* compared to untreated conditions, as may be expected. However, four strains had biomass productivity that was greater than WT. In particular, one strain OM65 had biomass productivity that was greater than twice (118%) the productivity of WT when treated with 2ppm OMEGA*. The quality of biomass for the evolved strains was significantly better than WT, when chronically treated with OMEGA*. FAME content of biomass from OMEGA*-treated WT miniponds was -50% of normal, while FAME content of select evolved strains was similar to normal FAME content. Therefore, as a result of their innate biocide tolerance, certain evolved strains exhibited improved biomass productivity vs. WT during biocide treatment periods that were required to control pest infestations.

[0050] An increase in FAME content can be, for example, 1.25-fold to two-fold higher, two-fold to three-fold higher, three-fold to four-fold higher, or four-fold to five-fold higher. An increase in FAME content can be, for example, 0.25-fold to five-fold higher, five-fold to ten-fold higher, ten-fold to twenty-fold higher, or twenty-fold to fifty-fold higher, or higher than 50-fold.

[0051] 25 vials (1.5 mis of cells) of each of parental QM27 (SE 70179) and OM65 (SE 70181) cell lines, from which the cells of the invention are clonally derived, was deposited on September 24, 2013 with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia, 20110, USA under ATCC Accession Number Nos. PTA-120600 (OM27) (SE 70179) and PTA-120599 (OM65) (SE 70181). Both parental (unmutagenized) OM27 (SE 70179) and OM65 (SE 70181) cell lines are a Desmodesmus armatus species, isolated in Las Graces, New Mexico, USA.

[0052] ATCC deposits are made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). The treaty assures maintenance of viable cultures for 30 years from the date of deposit. The OM27 and OM65 cell lines are available from the ATCC under the terms of the Budapest Treaty which assure permanent and unrestricted availability of progeny of
the cell line(s) to the public upon issuance of the pertinent U.S. patent or upon laying open to the
public of any U.S. or foreign patent application, whichever comes first. The Budapest Treaty
assures the availability of the cell line to one determined by the U.S. Commissioner of Patents and
Trademarks to be entitled thereto according to 35 U.S.C. §122 and the Commissioner's rules
pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of
the present application has agreed that if the cell line deposit should die, be lost or destroyed when
cultivated under suitable conditions, it will be promptly replaced with a viable specimen of the same
cell line(s) upon proper notification. Availability of a deposited cell line is not to be construed as a
license to practice the invention in contravention of the rights granted under the authority of any
government in accordance with its patent laws.

[0053] In one aspect, the UV-mutated Desmodesmiis species may be a transformed microalgae
having one or more exogenous DNA sequences. In a different aspect, the UV-mutated
Desmodesmiis species may have sequences that are endogenous DNA sequences in a recombinant
construct. In another aspect, the sequences may be exogenous DNA sequences in a recombinant
construct.

[0054] Fv/Fm is the photosynthetic efficiency. The "Fv/Fm deficit" described below in the
Examples section is a relatively quantitative indicator of biocide tolerance. Strains with smaller
Fv/Fm deficits have higher biocide tolerance. Since Fv/Fm can be measured rapidly in a 96-well
format, it is possible to identify biocide tolerant clones with minimal screening/validation efforts.
An "Fv/Fm deficit" assay could also potentially serve as a rapid laboratory assay to confirm the
long term stability of the biocide tolerance phenotype.

[0055] The data obtained from the UV-mutagenized Desmodesmiis strains were analyzed by
Analysis of Variance (ANOVA) and Dunnett's test, both described below.

[0056] ANOVA is a statistical test used to determine if more than two population means are equal.
The test uses the F-distribution (probability distribution) function and information about the
variances of each population (within) and grouping of populations (between) to help decide if
variability between and within each population are significantly different.

[0057] Dunnett's test (method) is a statistical tool known to one skilled in the art and is described,
for example, in Dunnett, C. W. (1955), "A multiple comparison procedure for comparing several
treatments with a control", Journal of the American Statistical Association, 50:1096-1121, and
Dunnett, C. W. (1964), "New tables for multiple comparisons with a control", Biometrics, 20:482-
491. Dunnett's test compares group means, it is specifically designed for situations where all groups
are to be pitted against one "Reference" group. It is commonly used after ANOVA has rejected the hypothesis of equality of the means of the distributions (although this is not necessary from a strictly technical standpoint). The goal of Dunnett's test is to identify groups whose means are significantly different from the mean of this reference group. It tests the null hypothesis that no group has its mean significantly different from the mean of the reference group.

[0058] The increase in productive pond lifetime, biomass productivity, in situ biomass productivity, or biomass yield can be 2-5%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-35%, 35-40%, 40-45%, 45-50%, 50-55%, 55-60%, 60-65%, 65-70%, 75-80%, 85-90%», 95-100%», 100-150%, 150-200%, 200-250%, 250-300%, 300-350%, 350-400%, or more than 400%. The increase can be about a 20% increase, about a 28%», about a 30%» increase, or about a 118% increase.

[0059] As used herein, the term "yield" refers to the number of microalgae per unit volume at harvest, and may be expressed, for example, as the number of cells per volume of culture, a mass per volume of culture, etc. Yield, used herein may also be expressed as a mass per area of culture.

[0060] A yield of a harvested mutagenized Desmodesmus species can be greater than 0.4 gram per liter (g/L) AFDW, greater than 0.5 g/L AFDW, greater than 0.6 g/L AFDW, greater than 0.7 g/L AFDW, greater than 0.8 g/L AFDW, greater than 0.9 g/L AFDW, or greater than 1.0 g/L AFDW. The yield can be from 0.1 g/L AFDW to 2.0 g/L AFDW, from 2.0 g/L AFDW to 5.0 g/L AFDW, or from 5.0 g/L AFDW to 10.0 g/L AFDW. The growing of the mutagenized Desmodesmus species can provide a yield that is at least 5% greater or at least 10% greater than a yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. The growing of the mutagenized Desmodesmus species can provide a yield of the mutagenized Desmodesmus species selected from the group consisting of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%», and 100% greater than the yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition. The growing of the mutagenized Desmodesmus species can provide a yield of the mutagenized Desmodesmus species selected from the group consisting of at least 1.25 fold, 1.5 fold, 2.0 fold, 2.5 fold, 5.0 fold, 7.5 fold, 10 fold and 15 fold greater than the yield of an unmutagenized Desmodesmus species harvested from a second liquid culture system having a fungal infection that has not been provided the effective concentration of the fungicide composition.
An increase in the "doubling rate" or "doubling time" can be used to measure the tolerance of a mutant *Desmodesmus* species. The doubling time is the period of time required for a quantity to double in size.

OMEGA® is a fungicide composition which acts to inhibit cellular energy production. The active ingredient in OMEGA® is Fluazinam: 3-chloro-A’-[3-chloro-2,6-dinitro-4-trifluoromethyl) phenyl]-5-trifluoromethyl-2-pyridinamine (CA). This active ingredient constitutes 40% of the composition. Other ingredients make up the other 60%. Fluazinam is a highly active fungicide with a broad spectrum including soil borne diseases. Fluazinam is a multi-site fungicide that is a member of the pyridinamine family. OMEGA® has a mode of action that disrupts the energy production of a fungus. It is listed in FRAC code 29 as an uncoupler of oxidative phosphorylation. OMEGA® contains 4.17 pounds fluazinam per gallon (500 grams per liter).

Possible doses of OMEGA® range from 0.00 to 0.25 ppm, 0.1 ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppsms, from 0.25 ppm to 15 ppm, from 0.25 ppm to 20 ppm. Other possible doses range from 0.5 ppm to 5.5 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm. Possible other doses of OMEGA® range from 0.25 to 15 ppm, or from 0.01 to 20 ppm.

Other exemplary doses of OMEGA® can be about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4ppm, about 5ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm.

As described below in the Examples section, the variant strains were dosed several times (one to six times) with varying ppm of OMEGA® ranging from 0.25 to 3.0 ppm. The final ppm of any culture was not determined. Although the maximum ppm of OMEGA® left at the end of any experiment would total the amount of OMEGA® put in. However this does not account for any loss of activity of OMEGA® or removal of OMEGA® during harvesting. A variant strain can be dosed, for example, 1-3 times, 3-5 times, 6-8 times, 8-10 times, or more than 10 times. Each dose can be the same or different.

Mutagenized strains of the disclosure can be treated with one or more doses of a fungicide composition, like OMEGA®. Mutagenized strains can be treated with one or more additional amounts of a fungicide composition, after the initial treatment, to maintain an effective concentration of the fungicide composition during a growing period. Any one or more dose of a
fungicide composition can be: from 0.25 ppm to 15 ppm, or from 0.01 ppm to 20 ppm; from 0.1 ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm; from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm; or about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm.

[0067] Mutagenized strains of the disclosure can be treated with other fungicide compositions, like OMEGA*, wherein an active ingredient is fluazinam. In addition, the mutagenized strains of the disclosure can be treated with a fungicide composition in which the active ingredient is a fungicide that is listed in the group Fungicide Resistance Action Committee 29 (FRAC 29) as uncouplers of oxidative phosphorylation. Members of this group include binapacryl, meptyldinocap, or dinocap, and they are grouped together by FRAC because they have been shown to have the same mode of action. One of skill in the art could easily test a range of concentrations or doses of a fungicide composition in which the active ingredient is binapacryl, meptyldinocap, or dinocap, to determine the effective concentration or dose of each fungicide composition on any of the variants of the disclosure to control the growth of fungus.

[0068] A fungicide composition useful in treating the mutagenized strain of the disclosure, can comprise as an active ingredient any one or more of fluazinam, binapacryl, meptyldinocap, or dinocap. A fungicide composition comprises at least one active ingredient. A fungicide composition can comprise, for example, 40% active ingredients and 60% other ingredients. The other ingredients can comprise one or more inactive ingredients.

[0069] Any one active ingredient can constitute about 40% of the fungicide composition. Alternatively, any one active ingredient can constitute about 10-30%, about 30-60%, about 60-90% or about 90% to 100% of the fungicide composition. Alternatively, the FRAC 29 fungicide can comprise from 10-20% of the fungicide composition, from 20-30% of the fungicide composition, from 30-40% of the fungicide composition, from 40-50% of the fungicide composition, from 50-60% of the fungicide composition, from 60-70% of the fungicide composition, from 70-80% of the fungicide composition, from 80-90%, or from 90-100% of the fungicide composition.

[0070] FRAC is a Specialist Technical Group of CropLife International (Formerly Global Crop Protection Federation, GCPF). The purpose of FRAC is to provide fungicide resistance
management guidelines to prolong the effectiveness of "at risk" fungicides and to limit crop losses should resistance, occur. The main aims of FRAC are to: 1. Identify existing and potential resistance problems. 2. Collate information and distribute it to those involved with fungicide research, distribution, registration and use. 3. Provide guidelines and advice on the use of fungicides to reduce the risk of resistance developing, and to manage it should it occur. 4. Recommend procedures for use in fungicide resistance studies. 5. Stimulate open iiason and collaboration with universities, government agencies, advisors, extension workers, distributors and farmers.

The 2013 FRAC Code List* sorts fungicides by mode of action and assigns them a numerical code (e.g. FRAC Code 29). Below is a subset of the FRAC Code list, specifically the members of FRAC code 29 that include the fungicide fluazinam used in the disclosure.

<table>
<thead>
<tr>
<th>Mode of Action (MOA)</th>
<th>Target Site and Code</th>
<th>Chemical Group</th>
<th>Common Name</th>
<th>Comments on Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C: respiration</td>
<td>uncouplers of oxidative phosphorylation</td>
<td>dinitrophenyl cretonates</td>
<td>binapacyr, meptyldinocap, dinocap</td>
<td>resistance not known, also acaricidal activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,6-dinitroanilines</td>
<td>fluazinam</td>
<td>low risk for resistance</td>
</tr>
</tbody>
</table>

MOA: Different letters (e.g. "C") are used to distinguish fungicide groups according to their biochemical mode of action (MOA) in the biosynthetic pathways of plant pathogens. Target Site and Code: The biochemical mode of action is given. A grouping can be made due to cross resistant profiles within a group or in relation to other groups. Chemical Group: Grouping is based on chemical considerations, Nomenclature is according to the International Union of Pure and Applied Chemistry (IUPAC) and Chemical Abstract Name. Comments on Resistance: Details are given for the (molecular) mechanism of resistance and resistance risk.

"Culture time", "growing time", "length of growth," or "time to harvest," are used interchangeably herein as a measurement from the date of inoculation of a liquid culture system with an algae species. Exemplary culture times can be about 30 days, from 30-60 days, or from 60-90 days. Other exemplary growing times are: 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 10 or more, 15 or more, 30 or more, 45 or more, 60 or more, 90 or more, 120 or more, 180 or more,
250 or more, 500 or more, 1000 or more, 1500 or more, or 2000 or more days after the date of inoculation. Alternatively, the growing time can be from 4 to 6 days, from 4 to 14 days, or about 96 hours or more after inoculation of the liquid culture. The growing time can be, for example, from 4 to 6 days, from 4 to 14 days, or is 96 hours or more, after the inoculation. In other embodiments, the growing is from 1 to 30 days, 1 to 40, from 30 to 60 days, from 60-90 days, or from 90-120 days. Alternatively, the growing time can be from 1 to 7 days, from 1 week to 2 weeks, from 2 weeks to 3 weeks, from 3 weeks to 1 month, from 1 month to 3 months, from 3 months to 6 months, from 6 months to 9 months, from 9 months to 12 months, from 12 months to 18 months, from 18 months to 2 years, from 2 years to 3 years, from 3 years to 4 years, from 4 years to 5 years, from 5 years to 6 years, from 6 years to 7 years, from 7 years to 8 years, from 8 years to 9 years or from 9 years and 10 years after inoculation.

[0074] As used herein, the term "liquid system," "liquid culture system," or "culture system" refers to a system for culturing a microalgae, such as a *Desmodesmus* species. A liquid system may include both a closed and an open culture system. An open liquid system may include, for example an open or semi-closed photobioreactor, semi-closed ponds, open ponds, or lakes. A liquid system can be an outdoor liquid system, an indoor liquid system, or a combination of both.

[0075] "Growing microalgae," "growing", "growing the microalgae," "microalgae growth," and "culturing the microalgae" as used herein, refer to one or more steps including microalgae in culture to when microalgae are in suspension just prior to the beginning of a harvesting step.

[0076] As used herein, "reduced growth," "reducing the growth", "inhibited growth," "growth reduction", "a reduction in growth", and "growth inhibition" relate to the decreased reproduction or division of a pest relative to the amount of reproduction or division of a pest under similar or identical conditions in the absence of any treatment. "Reduced growth," "inhibited growth," "growth reduction" and "growth inhibition" may also refer to the killing or death of the pest by the treatment (e.g. fungicide composition).

[0077] As used herein, "harvesting," relates to the removal or isolation of all, or part of microalgae in a culture system, including a liquid culture system. Harvesting may occur continuously from a growing culture, batch-wise or as a total collection of the microalgae at the end of a culture period. A liquid, as a supernatant, siphonate, flow-through or other separated form, may be returned to the liquid culture system. In some instances, relative amount harvested refers to the amount of microalgae remaining compared to the amount contained in the liquid culture system before harvesting.
As used herein, the term “treatment” refers to methods or compositions that inhibit the growth of a pest. A treatment may include methods or compositions that kill a pest.

In an aspect, pests may be detected using Polymerase Chain Reaction (PGR) to detect ribosomal sequences. In an aspect, ribosomal sequences may include DNA sequence selected from the group consisting of NC_003053 Rhizophydiium sp. 136 mitochondrion, NC_003048 Hyaloraphidium curvatum mitochondrion, NC_003052 Spizellomyces pimctatus mitochondrion chromosome 1, NC_003061 Spizellomyces pimctatus mitochondrion chromosome 2, NC_003060 Spizellomyces pimctatus mitochondrion chromosome 3, NC_004760 Harpochytrium sp. JEL94 mitochondrion, NC_004624 Monoblepharella sp. JEL15 mitochondrion, and NC_004623 Harpochytrium sp. JEL105 mitochondrion.

Methods of the present disclosure include methods of detection that may detect a pest present at a level of at least $10^5$ cells/ml. In another aspect, the methods of the present disclosure provide for the detection of a pest at a concentration $10^4$ cells/ml. In a further aspect, the concentration of pest may be detected at $10^3$ cells/ml. In another aspect, a pest present at a concentration of $10^2$ cells/ml or even $10^1$ cells/ml may be detected.

The polymerase chain reaction (PCR) is a sensitive method for the detection of the presence of an organism in a sample. Methods for performing PCR are known in the art. Nucleic acid analysis by PCR requires sample preparation, amplification, and product analysis. Although these steps are usually performed sequentially, amplification and analysis can occur simultaneously. Quantitative analysis occurs concurrently with amplification in the same tube within the same instrument. The concept of combining amplification with product analysis has become known as ‘real time’ PCR or quantitative PCR (qPCR). See, for example, U.S. Pat. No. 6,174,670, herein incorporated by reference in its entirety.

In an aspect, real-time methods of PCR may be used to detect the presence of a pest in a liquid system (e.g., quantitative PCR). In a real time PCR assay, a fluorescent signal accumulates during each amplification cycle. A positive reaction is provided when the fluorescent signal exceeds a threshold level, typically the background fluorescence. The cycle threshold ($C_t$) the number of cycles required to cross the threshold and the $C_t$ levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the $C_t$ level the greater the amount of target nucleic acid in the sample). Real time PCR assays typically undergo 40 cycles of amplification. A person of ordinary skill would recognize that the $C_t$ value may be compared to a standard curve prepared from a serially diluted pest to determine a number of pests/ml of sample.
[0083] In an aspect, a pest is detected when the C_t value is less than 35 cycles for at least one monitoring step. In another aspect, a pest is detected when the C_t value is less than 35 cycles for at least two consecutive monitoring steps. In yet another aspect, a C_t value of less than 35 cycles for three consecutive monitoring steps indicates the presence of a pest.

[0084] The present disclosure further provides for the detection of pest when there is a consistent decrease in the Ct over two or more monitoring steps. In an aspect, a consistent decrease from a C_t of 35 or higher to a C_t value of 30 or less indicates a need for crop protective action.

[0085] In an aspect, treatment may be provided to the liquid system within hours of the detection of a pest contamination. In an aspect, treatment may be provided within 2 hours of detection of a pest contamination. In another aspect, treatment may be provided within 4 hours of detection of a pest contamination. In yet another aspect, treatment may be provided within 8 hours of the detection of a need for crop protective action. In a further aspect, treatment may be provided within one day of detection of a need for crop protective action. In another aspect, treatment may be provided within 2 days of a need for crop protective action. In an aspect, monitoring and detection of pests may be continuous.

[0086] The present disclosure provides for methods of reducing the growth of a pest in a liquid culture of microalgae where a liquid system is inoculated with microalgae, the system is monitored for the presence of a pest, and an effective concentration of a fungicide is provided to inhibit the growth of the pest relative to the growth of the pest without the fungicide, and growing the microalgae. The present disclosure further provides for the reduction of viable pests in a liquid system.

[0087] In one aspect, the present disclosure provides for a method of reducing the growth of a pest where a reduction of the growth of a pest in the presence of an inhibitor is measured relative to the growth of a pest under similar conditions in the absence of an inhibitor. In one aspect, a reduction of the growth of a pest is achieved by the death of the pest. In another aspect, a reduction of the growth of a pest is achieved by the inhibition of division of the pest. In an aspect, growth of the pest is reduced by 99%, or more. In another aspect, the growth of the pest is reduced by 95%, or more. In yet another aspect, the growth of a pest is reduced by 90%, or more. In another aspect, the growth of a pest is reduced by at least 80%. In another aspect, the growth of a pest is reduced by at least 70%. In another aspect, the growth of a pest is reduced by at least 60%. In another aspect, the growth of a pest is reduced by at least 50%. In another aspect, the growth of a pest is reduced by at least 40%.
least 90 to 99%, at least 95 to 99%, at least 80 to 95%, at least 80 to 99%, or 75 to 99%. In yet another aspect, the growth of a pest is reduced no less than 90%, 95% or 99%.

[0088] In an aspect, the pest may be a member of the fungi kingdom. In another aspect, the pest may be a member of the division Chytridiomycota or Cryptomycota. In yet another aspect, the pest may be a member of the class Chytridomycetes. In a further aspect, the pest may be a species of Chytridium spp.

[0089] Examples of pests of microalgae cultures are members of the fungi kingdom and include the division Blastocladiomycota, Chytridiomycota, Cryptomycota, Glomeromycota, Microsporidia, Neocallimastigomycota, Ascomycota, or Basidiomycota. A fungus, as used herein, includes members of the classes Chytridiomycetes and Monobiepharidomycetes as well as species of Chytridium spp. In an aspect, pests that are members of the fungi kingdom may be identified by molecular phylogeny, for example, using the methods of James et al. "A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota)," Mycologia 98(6):860-71 (2006), herein incorporated by reference in its entirety.

[0090] In another aspect, a pest may be a member of the Rozella genus of Chytridiomycota. In an aspect, a pest may be a member of the Chytridiales/Rhizophydiun clade of Chytridiomycota. In yet another aspect, a pest may be a member of the Amoebaphelidium genus. In another aspect, a pest may be phylogenetically related to a clade of the Chytridiomycota division including the Chytridiales, Rhizophyctidales, Spizellomycetales, Rhizophycales, Lobulomycetales, Cladochytriales, Polychymium and Monobiepharidomycetes orders. In an aspect, the pest may be phylogenetically related to a Rozella spp..

[0091] As used herein, the term "pest" relates to any undesired biological organism in a sample culture, such as a Desmodesmus culture. Non-limiting examples of pests are bacteria and fungi. A pest may be undesired because it decreases the growth rate of a Desmodesmus culture. Alternatively, a pest may be undesired because it decreases the overall extent of Desmodesmus growth or the total yield of Desmodesmus per volume of culture. A pest may be undesired because it leads to the death of a Desmodesmus culture. A pest may be undesired because it changes the gene expression of the cultured Desmodesmus. A pest may be population of a single organism or a mixed population.

[0092] A "fungus," as used herein, is a member of the fungi kingdom and the division Blastocladiomycota, Chytridiomycota, Glomeromycota, Microsporidia, Neocallimastigomycota, Ascomycota, or Basidiomycota. A fungus, as used herein, includes members of the classes
Chytridiomycetes and Monoblepharidomycetes as well as species of Chyridium spp., or any combination of fungi. A fungus as used herein includes members of Chyridium species included in the Chytridiomycota division of the fungi kingdom including the orders Chytridiales, Rhizophyldiales, Spizellomycetales, Rhizophyldiales, Lobulomycetales, Cladochytriales, Polychytrium and Monoblepharidomycetes.

As used herein, the term "effective concentration" or "effective dose" refers to a concentration of a biocide or fungicide that is sufficient to control the growth, or kill, a pest while providing for the continued growth, or survival, of the growing culture, for example, a Desmodesmus culture in the liquid system. Exemplary ranges of doses of a fungicide concentration are provided herein.

Examples of fungi that infect Desmodesmus cultures are members of the class Chytridiomycetes and members of the Chyridium spp. Chytrids are primitive fungi and are mostly saprophytic (degrading chitin and keratin). Some species are unicellular. As with other fungi, the cell wall in a chytrid is composed of chitin. Many chytrid species are aquatic (mostly found in fresh water). There are approximately 1,000 chytrid species, in 127 genera, distributed among 5 orders. Some chytrid species are parasitic and may infect plants, including microalgae.

Specific non-limiting examples of chytrids included in the present disclosure include Achlyogeton, Allochytridium, Allochytridium expandens, Allochytridium luteum, Allomyces, Allomyces (subgenus), Allomyces atomycyes, Allomyces catenoides, Allomyces reticulatus, Amoeboaphelidium protococcarum, Alphamycetaceae, Alphamycyes, Alphamycyes chaetiferum, Amphicypellus, Amphicypellus elegans, Anaeromyces, Anaeromyces elegans, Anaeromyces mucronatus, Angulomycetaceae, Angulomyces, Angulomyces argentinensis, Aquamycetaceae, Aquamycyes, Aquamycyes chlorogonii, Arnaudovia, Arnaudovia. hyponeustonica, Asterophlyciis irregularis, Asterophlyciis sarcoptoides, Batrachochytrium, Batrachochytrium dendrobatidis, Blastocladia arborai, Blastocladia cadiica, Blastocladia cornonata, Blastocladia cristata, Blastocladia didyma, Blastocladia elegans, Blastocladia excelsa, Blastocladia filamentosa, Blastocladia fruticosa, Blastocladia fusiformis, Blastocladia globosa var. Minutissima, Blastocladia heirosporangia, Blastocladia mammilata, Blastocladia picaria, Blastocladia pileota, Blastocladia pusilla, Blastocladia sessilis, Blastocladia spiciformis, Blastocladiella, Blastocladiella anahaenae, Blastocladiella hritarinica, Blastocladiella colombiensis, Blastocladiella nova-zeyllandiae, Blastocladiomycota, Blastocladiopsis elegans, Blyttiomycyes bartsch, Blyttiomycyes aureus booth, Blyttiomycyes conicus, Blyttiomycyes exuiae, Blyttiomycyes gregarum, Blyttiomycyes
harden, Bfyttiomyces laevis, Blyttiomyces lenis, Blyttiomyces rhizophlyctidis, Blyttiomyces spinosus, Blyttiomyces vaucheriae, Blyttiomyces verrucosus, Boothiomyces, Boothknnyces macroporosum, Caecomycyes, Caecomycyes communis, Caecomycyes equi, Caecomycyes sympodialis, CalUinastixfrontalis, Canteria, Canteria apophysata, Catenaria auxiliaris, Catenaria indica, Catenaria ramosa, Catenaria spinosa, Catenaria uncinata, Catenaria vermicola, Catenaria verrucosa, Catenochytridium hemicysti, Catenochytridium marinus, Catenochytridium oahuense, Catenophlyctis, Catenophlyctis peltata, Catenophlyctis variabilis, Catenophlyctis variabilis var. Olduaviensis, Caulochytriaeae subramanium, Caulochytrium, Caulochytrium gloeosporii, Caulochytrium protostelioides, Caulochytrium protosteloides var. Vulgaris, Chytridiaceae, Chytridiales, Chytridiomycetes, Chytridiomycota, Chytridium, Chytridium adpressum, Chytridium aggregatum, Chytridium apophysatum, Chytridium brevipes, Chytridium cepii, Chytridium chlorobotryis, Chytridium citriforme, Chytridium closterii, Chytridium codicola, Chytridium coleochaetes, Chytridium confervae, Chytridium comiculatum, Chytridium crescentum, Chytridium deltanum, Chytridium fusiforme, Chytridium gibbosum, Chytridium hemicysta, Chytridium horariumforme, Chytridium hyperparasiticum, Chytridium inflatum, Chytridium isthmiophilum, Chytridium koltanum, Chytridium lagenaria, Chytridium latipodium, Chytridium malloweenadis, Chytridium marylandicum, Chytridium mucronatum, Chytridium neopapillatum, Chytridium oedogonii, Chytridium ottariense, Chytridium parasiticum, Chytridium pilosum, Chytridium proliferum, Chytridium reniforme, Chytridium schenckii, Chytridium schenckii var. Dumontii, Chytridium scherffeli, Chytridium sexuale, Chytridium sparrowii, Chytridium stellatum, Chytridium telmatoskenae, Chytridium turbinatum, Chytriomyces, Chytriomyces angularis, Chytriomyces annulatus, Chytriomyces confervae, Chytriomyces cosmarii, Chytriomyces elegans, Chytriomyces gilgaiensis, Chytriomyces heliozoicola, Chytriomyces hyalinus, Chytriomyces hyalinus var. Granulatus, Chytriomyces laevis, Chytriomyces macro-operculatus, Chytriomyces macro-operculatus var. Hirsutus, Chytriomyces mamilifer, Chytriomyces mortierellae, Chytriomyces multi-operculatus, Chytriomyces nagatoroensis, Chytriomyces poculatus, Chytriomyces reticulatus, Chytriomyces reticulosporus, Chytriomyces rhizidioymycetis, Chytriomyces rotoruaensis, Chytriomyces suberceolatus, Chytriomyces vallesiacus, Chytriomyces verrucosus, Chytriomyces willoughbyi, Cladochytriaceae, Cladochytrium aureum, Cladochytrium granulatum, Cladochytrium indicum, Cladochytrium novogueineense, Cladochytrium replicatum, Cladochytrium salsuginosum, Clydea, Clydea vesicula, Coelomomycetaceae, Coelomycidium, Coralloidiomyces, Coralloidiomyces digitatus, Cylindrochytrium endobioticum,
Cystocladiella, Dangeardia appendiculata, Dangeardia echinulata, Dangeardia molesta,
Dangeardia sporapiculata, Dangeardia sporapiculata var. Minor, Dangeardiana, Dangeardiana
apiculata, Dangeardiana eudoririae, Dangeardiana leptorrhiza, Dangeardiana sporapiculata,
Dictyomorpha, Dictyomorpha dioica, Dictyomorpha dioica var. Pythiensis, Diplochytridium,
Diplochytridiium aggregation, Diplochytridium hrevipes, Diplochytridium cejpii, Diplochytridium
chlorobotryis, Diplochytridium citriforme, Diplochytridium codicola, Diplochytridium gibbosum,
Diplochytridium inflaxum, Diplochytridium isthmiophilum, Diplochytridium kolianum,
Diplochytridium lagenarium var. Japonense, Diplochytridium lagenarium, Diplochytridium
mallononadis, Diplochytridium mucronatum, Diplochytridium oedogonii, Diplochytridium
schenkii, Diplochytridium scherffelii, Diplochytridium sexuale, Diplochytridium stellatum,
Diplochytridiium Turbinatum, Diplophyctis asteroidea, Diplophyctis buttermersensis, Diplophyctis
chitinophila, Diplophyctis complicata, Diplophyctis nephrochytroides, Diplophyctis
sarcoptoides, Diplophyctis sexualis, Diplophyctis versiformis, Endochytrium cystarum,
Endochytrium midigutiulatum, Entophlyctis, Entophlyctis apiculata, Entophlyctis bulligera,
Entophlyctis bulligera var. Brevis, Entophlyctis caudiformis, Entophlyctis conjervae-glomeratae,
Entophlyctis crenata, Entophlyctis fdamentosa, Entophlyctis helioformis, Entophlyctis
hohata, Entophlyctis luteolus, Entophlyctis mammilliformis, Entophlyctis molesta, Entophlyctis
obscura, Entophlyctis reticutospora, Entophlyctis rhizina, Entophlyctis sphaerioides, Entophlyctis
texana, Entophlyctis variabilis, Entophlyctis variabilis, Entophlyctis vaucheriae, Entophlyctis
willoighbyi, Gaertneriomycetes, Gaertneriomycetes semiglobiferus, Gaertneriomycetes
tenuis, Globomyctaceae, Globomyces, Globomyces pollinis-pini, Gonopodya terrestris, Gorgonomycetaceae,
Gorgonomyces, Gorgonomyces haynaldii, Hapalopera, Hapalopera acnanthis, Hapalopera
difficilis, Hapalopera fragilariae, Hapalopera melosirae, Hapalopera piriformis, Harpocythriaceae,
Harpocythriales, Harpocythrium, Harpocythrium adpressum, Harpocythrium cystarum,
Harpocythrium batiyococci, Harpocythrium hedenii, Harpocythrium hyalothecae, Harpocythrium
intermedium, Harpocythrium monae, Harpocythrium natrophilum, Harpocythrium
ornithocephalum, Harpocythrium tenuissimum, Harpocythrium viride, Kappamyces, Kappamyctaceae,
Kappamyces laurelensis, KarUngia, Karlingia aurantiaca, Karlingia exo-operculata, Karlingia
expandens, Karlingia granutata, Karlingia locustris, Karlingia. lobata var. Microspora, Karlingia
potonica, Karlingia spiriosa, Karlingiomyces, Karlingiomyces laevis, Kochiomyces, Kochiomyces
dichotomus, Krispiromycyes, Krispiromycyes discoide.es, Lacustromyces, Lacustromyces
hiemalis, Lobulomycetales, Lobulomycetaceae, Lobulomyces, Lobulomyces angularis, Lobulomyces
poculatus, Lyonomyces, Lyonomyces pyriformis, Macrochytrium botrydiella, Macrochytrium botrydioides van Minutum, Maunachytrium, Maunachytrium keaense, Mesochytrium, Mesochytrium penetrans, Microallomyces, Microallomyces dendroideus, Micromyces furcata, Micromyces grandis, Micromycopsis, Mitteromyces, MiUeromyces rhyniensis, Mitochytridium regale, Monoblepharidomycetes, Monoblepharidales, Monoblepharidaceae, Monoblepharella, Monoblepharis micrandra, Monoblepharis thalassinosus, Monophagus, Monophagus blackmanii, Monophagus bruhlii, NeocalUmastigales, NeocalUmastigomycota, NeocaUimastigomycetes, NeocaHimastix, NeocaHimastix frontalis, NeocaHimastix hurleyensis, NeocaHimastix joyonii, NeocaHimastix patriciarum, NeocaHimastix variabilis, Nephrochytrium bipes, Nephrochytrium buttermerense, Nephrochytrium complicatum, Nephrochytrium sexuale, Nowakowskiella crassa, Nowakowskiella delica, Nowakowskiella elegans, Nowakowskiella granulata, Nowakowskiella keratinophila, Nowakowskiella methistemichroma, Nowakowskiella moubasherana, Nowakowskiella multispora, Nowakowskiella multispora, Nowakowskiella pitcairnensis, Nowako wksiella profusa, Nowakowskiella profusa forma constricta, Nowakowskiella sculptura, NowakowskieHaceae, Obelidium megarhizum, Oedogoniomycetaceae, Olpidium, Olpidium appendiculatum, Olpidium bornovanus, Olpidium brassicae, Olpidium cucurbitacearum, Olpidium entophlyctoides, Olpidium fulgens, Olpidium incognitum, Olpidium indicum, Olpidium indicum, Olpidium indum, Olpidium longum, Olpidium nematodae, Olpidium paradoxum, Olpidium poreferum, Olpidium pseudoeuglena, Olpidium radicale, Olpidium rostriferum var. Indica, Olpidium sparrowii, Olpidium synchytrii, Olpidium vermicola, Olpidium virulentus, Olpidium wildemani, Olpidium zopfianus, Orpinomyces, Orpinomyces bovis, Orpinomyces intercalaris, Orpinomyces joyonii, Pateramycetaceae, Pateramycetes, Pateramyces, Pateramyces corrientinensis, Phlyctidium, Phlyctidium anatropum, Phlyctidium apophysatum, Phlyctidium brevipes var. Marinum, Phlyctidium bumilleriae, Phlyctidium globosum, Phlyctidium keratinophilum, Phlyctidium keratinophilum var. Savulescui, Phlyctidium marinum, Phlyctidium mycetophagum, Phlyctidium olla, Phlyctidium scenedesmi, Phlyctidium spinulosum, Phlyctidium tenue, Phlyctidium tubulatum, Phlyctochytrium acuminatum, Phlyctochytrium aextuarii, Phlyctochytrium africanum, Phlyctochytrium apophysatum, Phlyctochytrium arcticum, Phlyctochytrium aureliae, Phlyctochytrium californicum, Phlyctochytrium chandleri, Phlyctochytrium circulidentatum, Phlyctochytrium cystoferum, Phlyctochytrium dichotomum, Phlyctochytrium dissolutum, Phlyctochytrium furcatum, Phlyctochytrium hirsutum, Phlyctochytrium incrustans, Phlyctochytrium indicum, Phlyctochytrium irregularare, Phlyctochytrium kniepii,


A liquid of a liquid culture system of the present disclosure may be a defined or undefined media. In one aspect, the liquid may include untreated water. In another aspect, the untreated water may be water obtainable from a natural source such as a river, lake, aquifer, ocean or a pond. In yet another aspect, the liquid may be brackish water having an osmolarity between 0.5 and 30 grams of salt per liter. In yet another aspect, the liquid may be salt water. In an aspect, the water may be recycled water obtainable from a sewage or waste water treatment plant, or waste water from an industrial process such as power production and the like. In an aspect of the present disclosure, the untreated water may be aquifer water. In a further aspect, the untreated water may be aquifer water that is not suitable for agriculture. In yet another aspect, the aquifer water may be aquifer water with an elevated total dissolved solids (TDS).

A liquid of a liquid culture system may be supplemented with nutrients that benefit the growth of the microalgae. In one aspect, the liquid may be supplemented with CO₂ to enhance the growth of the microalgae. In an aspect the CO₂ may be introduced into the liquid system by bubbling with air or CO₂. Bubbling with CO₂ can be, for example, at 1% to 5% CO₂. CO₂ can be delivered to the liquid system as described herein, for example, by bubbling in CO₂ from under the surface of the liquid containing the microalgae. Also, spargers can be used to inject CO₂ into the liquid. Spargers are, for example, porous disc or tube assemblies that are also referred to as bubblers, carbonators, aerators, porous stones and diffusers. In an aspect the CO₂ may be introduced into the liquid system as a liquid.

In an aspect, the liquid may be supplemented with CO₂ to increase the concentration of CO₂ in the liquid to 20 parts-per-million (ppm), or more. In another aspect, the liquid may be supplemented with CO₂ to increase the concentration of CO₂ in the liquid to 25 ppm, or more. In yet another aspect, the liquid may be supplemented with CO₂ to increase the concentration of CO₂ in the liquid to 30 ppm, or more. In another aspect, the liquid of the liquid system may be supplemented with CO₂ to increase the concentration of CO₂ in the liquid to 35 ppm, or more.
[00100] In an aspect, a liquid system may be supplemented with CO₂ to maintain the pH of the liquid system. When the microalgae photosynthesize, they drive the pH of a liquid system up. If at any time the pH surpasses an upper limit of a threshold, CO₂ is added to the pond until the pH decreases to the specified range. In an aspect, a liquid system inoculated with microalgae is supplemented with CO₂ to maintain a pH of 8.8 to 9.2.

[00101] In an aspect of the present disclosure, the pH of a liquid system is monitored as a proxy for the amount of CO₂ available for photosynthesis. In an aspect, a liquid system being provided with CO₂ may have a pH defined as an upper limit. When a liquid system being provided CO₂ reaches an upper limit, CO₂ is provided to lower the pH. In an aspect, the upper pH limit may be 9.2. In another aspect, the upper pH limit may be 9.4. In another aspect, upper limit for pH may be set at 9.4. In a further aspect, the upper limit for pH may be set at 9.6. In another aspect, the upper limit for pH may be set at 9.8. In still another aspect, the upper limit for pH may be set at 10.2, 10.4 or 10.6.

[00102] In an aspect of the present disclosure, a liquid system being provided with CO₂ may have a pH defined as a lower limit. In an aspect, CO₂ supply is terminated to the liquid system when the pH drops below a pre-defined threshold in order to raise the pH. In an aspect, the threshold may be a pH of 8.8. In another aspect, the threshold may be 9.8. In yet another aspect, the threshold may be 9.0. In an aspect the threshold may be 9.2. In a further aspect, the threshold may be 9.4. In yet another aspect, the threshold may be 9.6.

[00103] It is understood that the present disclosure provides for the addition of CO₂ to maintain a pH within a range with the threshold and limit pH values being set accordingly. It is further understood that different species of microalgae have different preferred pH ranges for optimal growth. The threshold and limit pH values may be determined experimentally to maximize the photosynthesis and growth of microalgae in a liquid culture system. In an aspect of the present disclosure the pH range may be maintained between 8.8 and 9.2. In another aspect, the pH range may be maintained between 8.8 and 9.4. In a further aspect, the pH may be maintained between 8.8 and 9.6. In an aspect, the pH may be maintained between 8.8 and 9.8. In an aspect, the pH range may be between 9.8 and 10.2. In another aspect, the pH may be between 9.6 and 10.2. In an aspect, the pH may be between 9.4 and 10.2.

[00104] The present disclosure also provides for the supplementation of the liquid system with nutrients. Nutrients that can be used in the systems described herein, or known in the art, include, for example, nitrogen, phosphorus, and trace metals. In an aspect, nitrogen may supplemented in
the form of ammonia or ammonium. In one aspect ammonium is provided as ammonium sulfate or ammonium chloride. In another aspect, the nitrogen supplement may be provided as urea. In an aspect, the supplemental nitrogen may be provided as nitrate or nitric acid. In yet another aspect, the supplemental nitrogen may be provided as a mixture, for example as a mixture of urea and ammonium nitrate, also known as URAN. In an aspect, the nitrogen may be provided as potassium nitrate (KN03). In an aspect, the nitrogen may be provided as sodium nitrate (NaN03).

[00105] A liquid culture system of the present disclosure may be supplemented with trace metals. Supplements of trace metals may include salts of iron (Fe), magnesium (Mg), potassium (K), calcium (Ca), cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo), zinc (Zn), vanadium (V) or boron (B). In an aspect the trace metal may be supplied in the form of a nitrate (N03-) or ammonium (NH4+) salt. In an aspect, potassium may be added as potassium chloride or potassium sulfate. In another aspect, potassium may be added to the liquid system as potassium nitrate. The nutrients can come, for example, in a solid form or in a liquid form. If the nutrients are in a solid form they can be mixed with, for example, fresh or salt water prior to being delivered to the liquid system containing the organism, or prior to being delivered to a culture system. In an aspect, a nutrient is applied in a manner that minimizes the potential of osmotic stress to the cells. In an aspect, nutrient additions are done over an extended period of time. In a further aspect, the nutrients may be diluted prior to being applied to a pond.

[00106] A liquid culture system of the present disclosure may be maintained at a preferred pH depending on the microalgae. In an aspect, a neutral pH may be maintained. In another aspect, the pH may be maintained between a pH of 6.5 and 7.5. In another aspect, an alkaline pH may be maintained, for example, a pH of 10. In an aspect, an alkaline pH in the range of 8.0 to 11.0 may be maintained. In yet another aspect, the pH of the liquid system may be acidic, for example, a pH of 6.0. In another aspect, an acidic pH of the liquid system may be a pH from about 4.0 to about 6.5.

[00107] Microalgae can be cultured in defined media known in the art, such as min-70, M-medium, or Tris acetate phosphate (TAP) medium. Microalgae can be grown on a defined minimal medium (for example, high salt medium (HSM), modified artificial sea water medium (MASM), or F/2 medium) with light as the sole energy source. In other instances, the microalgae can be grown in a medium (for example, TAP medium), and supplemented with an organic carbon source.

[00108] Organisms, such as microalgae, can grow naturally in fresh water or marine water. Culture media for freshwater microalgae can be, for example, synthetic media, enriched media, soil water media, and solidified media, such as agar. Various culture media have been developed and used for

[00109] In an aspect, Desmid (e.g., *Scenedesmus* and *Desmodesmus*) media may be: 1.929 g/L sodium bicarbonate, 0.1 g/L urea, 2.3730 g/L sodium sulfate, 0.52 g/L sodium chloride, 0.298 g/L potassium chloride, 0.365 g/L magnesium sulfate, 0.084 g/L sodium fluoride, 0.035 niL/L 75% phosphoric acid, 0.018 g/L Librel® Fe-Lo (BASF), 0.3 mL/L 20X iron stock solution (20X iron stock solution: 1 g/L sodium ethylenediaminetetraacetic acid (EDTA) and 3.88 g/L iron chloride) and 0.06 mL/L 100X trace metal stock solution (100X trace metal stock solution: 1 g/L sodium ethylenediaminetetraacetic acid, 7.2 g/L manganese chloride, 2.09 g/L zinc chloride, 1.26 g/L sodium molybdate, and 0.4 g/L cobalt chloride.

[00110] Organisms may be grown in outdoor open water, such as ponds, the ocean, seas, rivers, waterbeds, marshes, shallow pools, lakes, aqueducts, and reservoirs. When grown in water, the organism can be contained in a halo-like object comprised of lego-like particles. The halo-like object encircles the organism and allows it to retain nutrients from the water beneath while keeping it in open sunlight.

[00111] In accordance with the present disclosure, the microalgae can be grown in open and/or closed systems that can vary in volume over a wide range. Closed systems can include reservoir structures, such as ponds, troughs, or tubes, which are protected from the external environment and have controlled temperatures, atmospheres, and other conditions. Closed systems may obtain the light required for photosynthesis artificially or naturally. For some embodiments, the microalgae may be grown in the absence of light and/or in the presence of an organic carbon source. Optionally, microalgae growth reservoirs can include a carbon dioxide source and a circulating mechanism configured to circulate microalgae within the microalgae growth reservoirs. Other examples of closed growth environments or reservoirs include closed bioreactors.

[00112] In an open microalgae culture system, at least one aspect of the liquid system is open to the environment. An open liquid system may be provided with light for photosynthesis artificially or naturally. For some embodiments, the microalgae may be grown in the absence of light and/or in the presence of an organic carbon source. In large open systems, natural light is often used. An
open system allows the free exchange of nutrients and products, for example oxygen and carbon
dioxide with the air. One way to achieve large surface growth areas is in large ponds or in a captive marine environment. In some aspects, a raceway pond can be used as a microalgae growth reservoir in which microalgae are grown in shallow circulating ponds with constant movement around the raceway and constant extraction or skimming off of mature microalgae. In other aspects, microalgae are grown in non-circulating pools.

[00113] In both open and closed systems, microalgae cultures can become host to other biological organisms that can decrease the production of microalgae by competing for nutrients. Pest organisms are a significant problem for the efficient production of commercial products of interest by microalgae. In other cases, infection of a microalgae culture can completely destroy production either by competition or by parasitism. Non-limiting examples of pests are bacteria and fungi.

[00114] In some instances, organisms can be grown in containers wherein each container comprises one or two organisms, or a plurality of organisms. The containers can be configured to float on water. For example, a container can be filled by a combination of air and water to make the container and the organism(s) in it buoyant. An organism that is adapted to grow in fresh water can thus be grown in salt water (i.e., the ocean) and vice versa. This mechanism allows for automatic death of the organism if there is any damage to the container.

[00115] Culturing techniques for microalgae include those described, for example, in Freshwater Culture Media. In R.A. Andersen (Ed.), Algal Culturing Techniques. Elsevier Academic Press, herein incorporated by reference in its entirety.

[00116] Because photosynthetic organisms, like microalgae, require sunlight, C0₂ and water for growth, they can be cultivated in, for example, open ponds and lakes. However, these open systems are more vulnerable to contamination with a pest than a closed system. One challenge with using an open system is that the organism of interest may not grow as quickly as a pest. This becomes a problem when a pest invades the liquid environment in which the organism of interest is growing, and the invading pest has a faster growth rate and takes over the system.

[00117] In addition, in open systems there is less control over water temperature, C0₂ concentration, and lighting conditions. A growing season of the organism is largely dependent on location and, aside from tropical areas, is limited to the warmer months of the year. In addition, in an open system, the number of different organisms that can be grown is limited to those that are able to survive in the chosen location. An open system, however, is cheaper to set up and/or maintain than a closed system. Open systems are generally unable to control variables such as temperature,
humidity and light. These variables will vary in accordance with the climate in which they are situated. Thus, one of ordinary skill in the art would understand that selection of the organism for growth in an open system may be determined by the local climate of the open system. In an aspect, temperatures over a season in an open system may range from below freezing to above 110 °F.

Another approach to growing an organism is to use a semi-closed system, such as covering the pond or pool with a structure, for example, a "greenhouse-type’ structure. While this can result in a smaller system, it addresses many of the problems associated with an open system. The advantages of a semi-closed system are that it can allow for a greater number of different organisms to be grown, it can allow for an organism to be dominant over an invading organism by allowing the organism of interest to out compete the invading organism for nutrients required for its growth, and it can extend the growing season for the organism. For example, if the system is heated, the organism can grow year round.

A variation of the pond system is an artificial pond, for example, a raceway pond. In raceway ponds, the organism, water, and nutrients circulate around a "racetrack." Paddiewheels provide constant motion to the liquid in the racetrack, allowing for the organism to be circulated back to the surface of the liquid at a chosen frequency. Paddiewheels also provide a source of agitation and oxygenate the system. These raceway ponds can be enclosed, for example, in a building or a greenhouse, or can be located outdoors. It will be apparent to one skilled in the art, that other designs of artificial ponds may be used in addition to raceway ponds and that other means of motivating liquid other than paddiewheels, such as pumps, may also be used.

Some of the organisms which may be grown in the liquid systems described herein are halophilic. For example, some microalgae can grow in ocean water and salt lakes (salinity from 30-300 parts per thousand) and high salinity media (e.g., artificial seawater medium, seawater nutrient agar, brackish water medium, seawater medium, etc.).

Where a halophilic organism is used, it may be transformed with any vectors known in the art. For example, a halophilic organism may be transformed with a vector which is capable of insertion into the nuclear genome and which contains nucleic acids which encode a flocculation moiety (e.g., an anti-cell-surface-protein antibody, a carbohydrate binding protein, etc.). Transformed halophilic organisms may then be grown in high-saline environments (e.g., salt lakes, salt ponds, high-saline media, etc.) to produce the products (e.g., isoprenoids, fatty acids, biomass degrading enzymes, etc.), or biomass, of interest. In some instances, a flocculation moiety may be non-functional under high salinity conditions. In such embodiments, flocculation may be induced
by lowering the salinity (e.g., by diluting the liquid environment). Alternately, the flocculation moiety may be functional under high salinity conditions and flocculation may be controlled by increasing the salinity of the medium. Isolation of any products of interest produced by the organism may involve removing a transformed organism from a high-saline environment prior to extracting the product from the organism. In instances where the product is secreted into the surrounding environment, it may be necessary to desalinate the liquid environment prior to any further processing of the product.

Large scale culture can be conducted in a photobioreactor, semi-closed ponds, open ponds, or lakes. Multiple batches of small scale culture can be seeded into one large-scale culture vessel. The ratio of seeding volume to receiving volume can be determined at the time of seeding according to parameters such as optical density and growth rate of the small scale culture(s). In preparation of media for the large scale culture, autoclaving, adding nutrients to recycled media, evaluating the condition of recycled media, and measuring the pH, salt, and conductivity of the media can be performed. During the large scale culture, quality control is performed. Quality control criteria may include sampling and screening for contamination, strain divergence, growth kinetics, oxygen level, nitrogen level, salinity of the liquid, pH of the liquid media, sampling of growing cells for oil content measurement, dry weight/wet weight ratio, and optical density of the culture.

The present disclosure also provides for liquid systems having a controlled temperature. In one aspect, the temperature of the liquid system is maintained between 15 °C and 32 °C. In another aspect, the temperature of the system is kept above 15 °C. In yet another aspect, the temperature of the system is not allowed to exceed 32 °C. In an aspect, the temperature of the system is kept below 25 °C. In an aspect, the temperature may be from 0 to 35 °C, from 5 to 35 °C, from 10 to 35 °C, 15 to 35 °C, from 20 to 35 °C, from 25 to 35 °C, and from 30 to 35 °C. In yet another aspect, the temperature may be maintained at greater than 5 °C. In an aspect, the temperature may be maintained at greater than 10 °C. In an aspect, the temperature may be maintained at greater than 15 °C. In an aspect, the temperature may be maintained at greater than 20 °C or greater than 30 °C. The present disclosure also provides for liquid systems having a temperature determined by the environment.

The microalgae may be grown in liquid culture systems of different volumes. In one aspect, the microalgae can be grown, for example, in small scale laboratory liquid systems. Small scale laboratory systems refer to cultures in volumes of less than about 6 liters. In an aspect, the small scale laboratory culture may be 1 liter, 2 liters, 3 liters, 4 liters, or 5 liters. In another aspect of the
invention, the small scale laboratory culture may be less than one liter. In an aspect, the small scale
laboratory culture may be 100 milliliters or less. In another aspect the culture may be 10 milliliter
or less. In another aspect the liquid culture may be 5 milliliters or less. In yet another aspect, the
liquid culture may be 1 milliliter or less.

In another aspect of the present disclosure, the liquid culture systems may be large scale
cultures, where large scale cultures refers to growth of cultures in volumes of greater than about 6
liters, or greater than about 10 liters, or greater than about 20 liters. Large scale growth can also be
growth of cultures in volumes of 50 liters or more, 100 liters or more, or 200 liters or more. Large
scale growth can be growth of cultures in, for example, ponds, containers, vessels, or other areas,
where the pond, container, vessel, or area that contains the culture is for example, at least 5 square
meters, at least 10 square meters, at least 20 square meters, at least 50 square meters, at least
1,500 square meters, at least 2,500 square meters, in area, or greater.

The present disclosure further provides for very large scale liquid culture systems. In one
aspect, the volume of liquid culture may be at least 20,000 liters. In another aspect, the volume of
liquid can be up to 40,000 liters. In another aspect, the volume of liquid can be up to 80,000 liters.
In another aspect, the volume of liquid can be up to 100,000 liters. In another aspect, the volume of
liquid can be up to 150,000 liters. In another aspect, the volume of liquid can be up to 200,000
liters. In another aspect, the volume of liquid can be up to 250,000 liters. In another aspect, the
volume of liquid can be up to 500,000 liters. In another aspect, the volume of liquid can be up to
600,000 liters. In another aspect, the volume of liquid can be up to 1,000,000 liters.

In yet another aspect, the very large scale liquid system may be from 10,000 to 20,000 liters.
In an aspect, the very large scale liquid system may be from 10,000 to 40,000 liters or from 10,000
to 80,000 liters. In another aspect, the very large scale liquid system may be from 10,000 to
100,000 liters or from 10,000 to 150,000 liters. In yet another aspect, the liquid system may be
from 10,000 to 200,000 liters or from 10,000 to 250,000 liters. The present disclosure also includes
liquid systems from 10,000 to 500,000 liters or from 10,000 to 600,000 liters. The present
disclosure further provides for liquid systems from 10,000 to 1,000,000 liters.

In further aspect, the liquid culture system may be from 20,000 to 40,000 liters or from
20,000 to 80,000 liters. In another aspect, the liquid system may be from 20,000 to 100,000 liters.
In yet another aspect, the liquid system may be from 20,000 to 150,000 liters or from 20,000 to
200,000 liters. In another aspect, may be from 20,000 to 250,000 liters. In another aspect, the
liquid system may be from 20,000 to 500,000 liters. In another aspect, the liquid system may be
from 20,000 to 600,000 liters. In another aspect, the liquid system may be from 20,000 to
1,000,000 liters.

[00129] In another aspect, the liquid culture system may be from 40,000 to 80,000 liters. In another
aspect, the liquid system may be from 40,000 to 100,000 liters. In another aspect, the liquid system
may be from 40,000 to 150,000 liters. In another aspect, the liquid system may be from 40,000 to
200,000 liters. In another aspect, the liquid system may be from 40,000 to 250,000 liters. In
another aspect, the liquid system may be from 40,000 to 500,000 liters. In another aspect, the liquid
system may be from 40,000 to 600,000 liters. In another aspect, the liquid system may be from
40,000 to 1,000,000 liters.

[00130] In another aspect, the liquid system may be from 80,000 to 100,000 liters. In another aspect,
the liquid system may be from 80,000 to 150,000 liters. In another aspect, the liquid system may be
from 80,000 to 200,000 liters. In another aspect, the liquid system may be from 80,000 to 250,000
liters. In another aspect, the liquid system may be from 80,000 to 500,000 liters. In another aspect,
the liquid system may be from 80,000 to 600,000 liters. In another aspect, the liquid system may be
from 80,000 to 1,000,000 liters.

[00131] In another aspect, the liquid system may be from 100,000 to 150,000 liters. In another
aspect, the liquid system may be from 100,000 to 200,000 liters. In another aspect, the liquid
system may be from 100,000 to 250,000 liters. In another aspect, the liquid system may be from
100,000 to 500,000 liters. In another aspect, the liquid system may be from 100,000 to 600,000
liters. In another aspect, the liquid system may be from 100,000 to 1,000,000 liters.

[00132] In another aspect, the liquid system may be from 200,000 to 250,000 liters. In another
aspect, the liquid system may be from 200,000 to 500,000 liters. In another aspect, the liquid
system may be from 200,000 to 600,000 liters. In another aspect, the liquid system may be from
200,000 to 1,000,000 liters. In another aspect, the liquid system may be from 250,000 to 500,000
liters. In another aspect, the liquid system may be from 250,000 to 600,000 liters. In another
aspect, the liquid system may be from 250,000 to 1,000,000 liters. In another aspect, the liquid
system may be from 500,000 to 600,000 liters, or 500,000 to 1,000,000 liters.

[00133] In an aspect of the present disclosure, the liquid system may be a pond, either natural or
artificial. In one aspect, the artificial pond may be a raceway pond. In a raceway pond, the
organism, water, and nutrients circulate around a "racetrack." Paddlewheels provide constant
motion to the liquid in the racetrack, allowing for the organism to be circulated back to the surface
of the liquid at a chosen frequency. Paddlewheels also provide a source of agitation and oxygenate
the system. CO$_2$ may be added to a liquid system as a feedstock for photosynthesis through a CO$_2$ injection system. These raceway ponds can be enclosed, for example, in a building or a greenhouse, or can be located outdoors. In an aspect, an outdoor raceway liquid system may be enclosed with a cover, or exposed.

[00134] Raceway ponds are usually kept shallow because the organism needs to be exposed to sunlight, and sunlight can only penetrate the pond water to a limited depth. The depth of a raceway pond can be, for example, about 4 to about 12 inches. In addition, the volume of liquid that can be contained in a raceway pond can be, for example, about 200 liters to about 600,000 liters.

[00135] The raceway ponds can be operated in a continuous manner, with, for example, CO$_2$ and nutrients being constantly fed to the ponds, while water containing the organism is removed at the other end.

[00136] In an aspect, the ponds may have a surface area of at least 0.25 of an acre. In another aspect, the pond may be at least 0.5 acre or at least 1.0 acre. In yet another aspect, the pond may be at least 1.5 acres or at least 2.0 acres. The liquid system may be a pond of at least 2.5 acres or at least 5.0 acres. In an alternative aspect, the pond may be at least 7.5 acres or at least 10 acres. In still other embodiments, the pond may have a surface area of at least 12 acres, at least 15 acres, at least 18 acres, at least 20 acres, at least 25 acres, at least 30 acres, at least 35 acres, at least 40 acres, at least 45 acres, at least 50 acres, at least 100 acres, at least 200 acres, at least 300 acres, at least 400 acres or at least 500 acres.

[00137] In yet another aspect, the surface area of a pond may be from 0.25 to 0.5 acres or 0.25 to 1.0 acres. In an aspect, the liquid system may be a pond of 0.25 to 1.5 acres or 0.25 to 2.0 acres. In another aspect the pond may be from 0.25 to 2.5 acres, 0.25 to 5.0 acres or 0.25 to 7.5 acres. In yet another aspect, the liquid system may be a pond of 0.5 to 1.0 acres, 0.5 to 1.5 acres, 0.5 to 2.0 acres, 0.5 to 2.5 acres, 0.5 to 5.0 acres or 0.5 to 7.5 acres. In an aspect, the liquid system may cover an area of 1.0 to 1.5 acres or 1.0 to 2.0 acres. In an aspect, the liquid system may be a pond of 1.0 to 2.5 acres or 1.0 to 5.0 acres. In yet another aspect, the liquid system may be a pond of 1.0 to 7.5 acres or 2.0 to 2.5 acres. In another aspect the pond may be from 2.0 to 5.0 acres or 2.0 to 7.5 acres. In yet another aspect, the pond may range from 2.5 to 5.0 acres, 2.5 to 7.5 acres, 2.5 to 10 acres, 5 to 12 acres, 5 to 15 acres, 5 to 18 acres, 5 to 20 acres, 10 to 25 acres, 10 to 30 acres, 10 to 35 acres, 10 to 40 acres, 10 to 45 acres, 10 to 50 acres, 10 to 15 acres, 15 to 20 acres, 20 to 25 acres, 25 to 30 acres, 30 to 35 acres, 35 to 40 acres, 40 to 45 acres, 45 to 50 acres, 50 to 75 acres, 75 to 100 acres,
100 to 125 acres, 125 to 150 acres, 150 to 175 acres, 175 to 200 acres, 200 to 250 acres, 250 to 300 acres, 300 to 350 acres, 350 to 400 acres, 400 to 450 acres, or 450 to 500 acres in area.

[00138] Alternatively, organisms, such as microalgae, can be grown in closed structures such as photobioreactors, where the environment is under stricter control than in open systems or semi-closed systems. A photobioreactor is a bioreactor which incorporates some type of light source to provide photonic energy input into the reactor. The term photobioreactor can refer to a system closed to the environment and having no direct exchange of gases and contaminants with the environment. A photobioreactor can be described as an enclosed, illuminated culture vessel designed for controlled biomass production of prototrophic liquid cell suspension cultures. Examples of photobioreactors include, for example, glass containers, plastic tubes, tanks, plastic sleeves, and bags. Examples of light sources that can be used to provide the energy required to sustain photosynthesis include, for example, fluorescent bulbs, LEDs, and natural sunlight. Because these systems are closed everything that the organism needs to grow (for example, carbon dioxide, nutrients, water, and light) must be introduced into the bioreactor.

[00139] Photobioreactors, despite the costs to set up and maintain them, have several advantages over open systems, they can, for example, prevent or minimize contamination, permit axenic organism cultivation of monocultures (a culture consisting of only one species of organism), offer better control over the culture conditions (for example, pH, light, carbon dioxide, and temperature), prevent water evaporation, lower carbon dioxide losses due to out gassing, and permit higher cell concentrations.

[00140] On the other hand, certain requirements of photobioreactors, such as cooling, mixing, control of oxygen accumulation and biofouling, make these systems more expensive to build and operate than open systems or semi-closed systems.

[00141] Photobioreactors can be set up to be continually harvested (as is with the majority of the larger volume cultivation systems), or harvested one batch at a time (for example, as with polyethylene bag cultivation). A batch photobioreactor is set up with, for example, nutrients, an organism (for example, microalgae), and water, and the organism is allowed to grow until the batch is harvested. A continuous photobioreactor can be harvested, for example, either continually, daily, or at fixed time intervals.

[00142] High density photobioreactors may be used and include those that are described in, for example, Lee, et al., Biotech. Bioeiigineering 44: 1161-1 167, 1994. Other types of bioreactors, such as those for sewage and waste water treatments, are described in, Sawayama, et al., Appl. Micro.
Biotech., 41:729-731, 1994. Additional examples of photobioreactors are described in, U.S. Appl. Publ. No. 2005/0260553, U.S. Pat. No. 5,958,761, and U.S. Pat. No. 6,083,740. Also, organisms, such as microalgae may be mass-cultured for the removal of heavy metals (for example, as described in Wilkinson, Biotech. Letters, 11:861-864, 1989), hydrogen (for example, as described in U.S. Patent Application Publication No, 2003/0162273), and pharmaceutical compounds from a water, soil, or other source or sample. Organisms can also be cultured in conventional fermentation bioreactors, which include, but are not limited to, batch, fed-batch, cell recycle, and continuous fermenters. Additional methods of culturing organisms and variations of the methods described herein are known to one of skill in the art.

[00143] The present disclosure further provides for harvesting of the microalgae grown in the liquid system. Harvesting may be accomplished by methods known to one of skill in the art including collection of the microalgae in whole or in part. In an aspect of the disclosure, harvesting may be accomplished by removing portions of the growing culture and separating the microalgae from the liquid. In another aspect, harvesting may be accomplished by continuous flow methods, for example, using a continuous flow centrifuge.

[00144] Separation of the microalgae from the liquid may be accomplished by methods known to one of ordinary skill in the art. In one aspect, the microalgae may be allowed to settle by gravity and the overlying liquid removed. In another aspect, the microalgae may be harvested by centrifugation of the microalgae containing culture. In an aspect, centrifugation of the liquid culture may be performed in batch mode, using a fixed volume centrifuge. In a different aspect, batch harvesting of the microalgae may be accomplished using a continuous flow centrifuge. In another aspect, the microalgae may be harvested continuously from the growing culture by continuous flow centrifugation.

[00145] In one aspect of the present disclosure, harvesting of the microalgae grown in the liquid system may be facilitated by flocculation. Methods for inducing flocculation include those that can be found in U.S. Patent Publication No, US 201/0159595, Application No. 13/001027, hereby incorporated in its entirety by reference. The flocculate may be separated from the culture liquid by gravity, centrifugation or other physical method known to those of skill in the art. In a particular embodiment the flocculate may be separated from the culture liquid by dissolved air flotation (DAT).

[00146] The present disclosure provides for harvesting of all or part of the liquid culture system. In an aspect, harvesting includes separating at least 90% of the microalgae from the liquid culture to
produce a microalgae depleted liquid. In another aspect, at least 95% of the microalgae are removed from the liquid culture. In another aspect, at least 97% of the microalgae are removed from the liquid culture. In another aspect, at least 99% of the microalgae are removed from the liquid culture. In other aspects, 50% or more of the microalgae are removed. In another aspect, 75% or more of the microalgae are removed from the liquid culture. In still another aspect, 80% of more of the microalgae are removed from the liquid culture. In yet another aspect, the liquid culture can have less than 30% of the microalgae remaining after harvesting. In a further aspect, less than 25% of the microalgae remained after harvesting. In a further aspect, less than 5% of the microalgae remained after harvesting. In a further aspect, less than 2.5% of the microalgae remained after harvesting. In an aspect, less than 1% of the microalgae remain after harvesting.

[00147] In an aspect, harvesting of microalgae from the growing culture may be performed on a part of the total liquid culture. In one aspect, the part of the liquid culture is removed and the microalgae are harvested. In an aspect, at least 2 percent of a total volume of a liquid culture is removed and the microalgae harvested. In another aspect, at least 2.5% of the total volume of the liquid culture containing the growing microalgae is removed and the microalgae harvested. In an aspect, at least 5% or at least 7.5% of the total volume of the liquid culture containing the growing microalgae is removed for harvesting. In yet another aspect, at least 10% or at least 12.5% of the total volume of the liquid culture containing the growing microalgae is removed for harvesting. In a further aspect, at least 15% or at least 20% of the total volume of the liquid culture containing the growing microalgae is removed for harvesting.

[00148] In a further aspect, from 2 to 5% or from 2 to 7.5%, of the total volume of the liquid culture containing the growing microalgae is removed for harvesting. In another aspect from 2 to 20% or from 2 to 12.5% of the total volume of the liquid culture containing the growing microalgae is removed for harvesting. In an aspect, the amount of liquid removed for harvesting may range from 2 to 15% or from 2 to 20% of the total volume of the liquid culture. In a further aspect, from 2.5 to 5% or from 2.5 to 7.5% of the total liquid culture volume may be removed for harvesting. In an aspect, the amount of liquid removed for harvesting may be from 2.5 to 10% or from 2.5 to 12.5% of the total growing culture volume. In an aspect, the amount removed may range from 2.5 to 15% or from 2.5 to 20%. In a further aspect, from 5 to 7.5% or from 5 to 10% of the culture volume may be removed for harvesting. In an aspect, from 5 to 12.5%, or from 5 to 15%, or even from 5 to 20% of the total volume of liquid culture may be harvested. In another aspect, the amount of harvested culture may be from 7.5 to 10% or from 7.5 to 12.5% of the total culture volume. In an aspect, the
amount of liquid removed for harvesting may range from 7.5 to 15% or from 7.5 to 20% of the culture volume. In yet another aspect, 10 to 12.5% or 10 to 15% of the culture volume may be removed from harvesting. In an aspect, 10 to 20% of the total volume of a liquid culture may be removed for harvesting of the growing microalgae.

It is further provided as part of the present disclosure that harvesting may be conducted continuously from the growing culture of microalgae. In an aspect, removal of the microalgae maintains the culture in a logarithmic phase of microalgae growth. One of skill in the art understands that the when growing in a logarithmic phase, the number of microalgae double within a time period. The time period for microalgae doubling depends on the environment of the growing microalgae. The determination of growth rates and phases of microalgae growth are known in the art. For example, in Sode et al., "On-line monitoring of marine cyanobacterial cultivation based on phycocyanin fluorescence," J. Biotechnology 21:209-217 (1991), Torzillo et al., "On-Line Monitoring Of Chlorophyll Fluorescence To Assess The Extent Of Photo inhibition Of Photosynthesis Induced By High Oxygen Concentration And Low Temperature And Its Effect On The Productivity Of Outdoor Cultures Of Spirulina Platensis (Cyanobacteria)," J. Phycology 34:504-510 (1998), Jung and Lee, "In Situ Monitoring of Cell Concentration in a Photobioreactor Using Image Analysis: Comparison of Uniform Light Distribution Model and Artificial Neural Networks" Biotechnology Progress 22:1443-1450 (2006), and Vonshak, A. Spirulina Platensis Arthrospira: Physiology, Cell-Biology And Biotechnology. 1997. CRC Press, all of which are incorporated by reference in their entireties. In an aspect, harvesting may be performed when microalgae are in logarithmic phase growth as provided further herein.

In an aspect, a portion of the liquid culture may be removed for harvesting and the portion replaced so that the total volume of the liquid culture remains within a narrow range. In one aspect, the amount of liquid removed during continuous harvesting is up to 1000 gallons per hour. In another aspect, the amount removed during continuous harvesting may be 1% of the total volume per hour. In an aspect, up to 5% of the volume per day may be removed during a continuous harvesting. In an aspect, up to 15% of the volume per day may be removed during a continuous harvesting. In an aspect, up to 33% of the volume per day may be removed during a continuous harvesting.

The present disclosure provides for liquid culture systems that are either indoors or outdoors. The advantage of an indoor system may that the environment may be more easily controlled. In an aspect, the temperature of an indoor environment may be regulated. In another
aspect, the amount and quality of the light may be controlled. In one aspect, an indoor system may be a greenhouse. In an aspect, a greenhouse may receive natural light. In another aspect a greenhouse may be artificially lighted, in yet another aspect, natural light may be supplemented by artificial light.

[00152] In an aspect, artificial light may be fluorescent light. One source of energy is fluorescent light that can be placed, for example, at a distance of about 1 inch to about two feet from the organism. Examples of types of fluorescent lights includes, for example, cool white and daylight. If the lights are turned on and off at regular intervals (for example, 12:12 or 14:10 hours of light:dark) the cells of some organisms will become synchronized.

[00153] Growth of micro-organisms in general proceeds along known phases and this is true for the microalgaes of the present disclosure. When a liquid culture is inoculated with a microalgaes, there is often a 'lag phase' during which changes in the density of the organism are not readily detectable. Following the lag phase, the organism enters and early growth phase characterized by increasing density of the microorganism.

[00154] An early growth phase is followed by a logarithmic growth phase during which many of the microorganisms are dividing. The logarithmic growth phase is characterized by log-linear growth of the organism when the density or cell number is plotted on a logarithmic scale versus time. The 'doubling' time is used to characterize this phase of growth. Both extrinsic environmental factors and intrinsic factors control the doubling time of an organisms. Those of skill in the art recognize that the rate of doubling can be limited by the necessity of initiating and completing successive rounds of DNA synthesis and genome replication. This limit on doubling time can be observed when ail extrinsic environmental factors are non-limiting. Extrinsic factors play important roles in the growth of microalgae including the presence of nutrients, the temperature, the pH, and the availability of light for photosynthesis. Methods of growing and optimizing the growth of microalgae are known in the art, for example in Vonshak, A. Spirulina Platensis Arthospira: Physiology, Cell-Biology And Biotechnology. 1997. CRC Press and M. Tredici, "Photobiology of microalgae mass cultures: understanding the tools for the next green revolution," Biofuels 1:143 (2010), both of which are hereby incorporated by reference in their entireties.

[00155] As the density increases, the rate of doubling decreases in a phase called "late log-phase." Growth decreases due to limiting nutrients (for example, lack of CO₂, lack of a carbon source etc.) or is due to factors secreted by the growing organisms (e.g., quorum sensing).
At the end of the log-phase of growth, the number of microorganisms stops increasing and the culture enters a stationary phase. In some aspects, the microorganisms may initiate developmental pathways leading, for example, a quiescent state. In another aspect, the microorganisms may have changes in gene expression including both increases and decreases in expression. Removal of microorganisms in the stationary phase and inoculation of a fresh culture often results in a lag phase prior to entry into a logarithmic growth phase.

The doubling time (e.g. doubling rate) during growth in the logarithmic phase can depend on a number of environmental conditions. Among the factors it is recognized that the nutrients and media conditions significantly affect growth. In the present disclosure, microalgae can be autotrophic and are therefore less susceptible to the presence of carbon based food sources. One of ordinary skill in the art would understand that the availability of nitrogen affects microalgae growth. Decreased nitrogen leads to longer doubling times, or even entry into stationary phases. Increased nitrogen availability may result in decreased doubling time. In an aspect, a growing liquid culture can be monitored for changes in the environmental conditions to maintain or optimize logarithmic phase growth. Production of microalgae is optimized when growth is logarithmic.

In an aspect, the growth of the culture proceeds through different growth phases. In one aspect, a liquid culture is inoculated and proceeds from a lag phase to the logarithmic phase to the stationary phase. In another aspect, logarithmically growing microalgae are provided such that there is no lag phase of growth. In another aspect, logarithmic phase is maintained by harvesting microalgae. In a further aspect, logarithmic phase is maintained by supplementing the liquid culture system that is limited for one or more nutrients.

In an aspect, a logarithmic growth phase is maintained by harvesting microalgae and supplementing the liquid culture system. In one aspect, a liquid after harvest can be monitored and nutrients added prior to returning the liquid culture system. In another aspect, a liquid culture system can be supplied with fresh media, for example water, and logarithmic phase maintained. In an aspect, a fresh media may contain nutrients necessary to maintain the logarithmic phase of microalgae growth. In a further aspect, microalgae depleted liquid can be further purified to remove contaminants to maintain logarithmic growth.

In another aspect, the liquid culture is treated with fungicide, such as Fluazinam, during the logarithmic phase. In another aspect of the invention, the liquid culture is treated during the lag phase. In an aspect, the liquid culture is treated during the stationary phase.
[00161] In an aspect, the microalgae are harvested from the liquid culture during logarithmic phase. In another aspect, the microalgae are harvested from the liquid culture during late logarithmic phase. In another aspect, the microalgae are harvested from the liquid culture during stationary phase. In an aspect, algae growth is maintained at an optimal density for logarithmic growth. In an aspect, the optimal density may be determined experimentally for a strain of microalgae.

[00162] Testing for the presence of a pest, such as a fungus (e.g. a chyrid), need not be conducted at any particular phase of growth. Thus, the present disclosure provides for testing for the presence of a pest of a liquid system at any phase of growth of a microalgae culture. In an aspect, testing for the presence of a pest may be performed before inoculation of the liquid system with a microalga. In another aspect, testing may be performed during the lag phase of microalgae growth. In yet another aspect, testing may be performed during logarithmic growth or at late logarithmic growth. In an aspect, testing may be performed at a stationary phase of a microalgae growth cycle. In yet another aspect, testing may be performed throughout each stage of a microalgae growth cycle.

[00163] The present disclosure provides for treating a liquid system contaminated with a pest at any phase of growth of a microalgae culture and at multiple stages of growth. In an aspect, treatment may be performed before inoculation of the liquid system with microalgae. In another aspect, treatment may be performed during the lag phase of microalgae growth. In yet another aspect, treatment may be performed during logarithmic growth or at late logarithmic growth. In an aspect, treatment may be performed at a stationary phase of a microalgae growth cycle. In yet another aspect, treatment may be performed throughout each stage of a microalgae growth cycle.

[00164] In one aspect, a liquid culture is grown for 15 or more days. In another aspect, a liquid culture is grown for 30 or more days. In an aspect, a liquid culture is grown for 45 or more days. In another aspect, a liquid culture is grown 60 or more, or 90 or more days. In yet another aspect, growth time may be 120 or more, or 180 or more days. In an aspect, a liquid culture may be maintained 250 or more, or 500 or more days. In yet another aspect, growth of a liquid culture may be continued for 1000 or more, 1500 or more, or 2000 or more days after inoculation of the liquid culture. The culture may be maintained, with fungicide treatments of the present disclosure for an indefinite amount of time.

[00165] The present disclosure provides for treatments of a liquid system. Treatments may include physical methods to control the growth of, or kill a pest present in a liquid system. Physical methods may include, as non limiting examples, filtration, heating, cooling and irradiation.
The present disclosure provides for treatments of a liquid system including the addition of compositions that control the growth of, or kill a pest. In an aspect, the treatment may be provided upon detecting the presence of a pest. In an aspect, the treatment may be provide upon the detection of a fungus in a liquid system. In a further aspect, the treatment may be prophylactic and the treatment may be provided during any stage of growth of the microalgae. The treatment can, for example, be applied to the liquid in the system prior to inoculation with the strain, simultaneously applied to the liquid with the strain, or after inoculation of the liquid with the strain.

Treatments of the present disclosure include adding one fungicide, wherein an active ingredient is Fluazinam, and at least one additional fungicide, to a liquid culture system. A fungicide comprising Fluazinam can be provided alone or in combination with any one or more of the fungicides listed below in Table 1, to a liquid culture system. In addition, a fungicide comprising Binapacryl as an active ingredient can be provided alone or in combination with any one or more of the fungicides listed below in Table 1, to a liquid culture system. Also, a fungicide comprising Dinocap as an active ingredient can be provided alone or in combination with any one or more of the fungicides listed below in Table 1, to a liquid culture system. Also, a fungicide comprising Meptyldinocap as an active ingredient can be provided alone or in combination with any one or more of the fungicides listed below in Table 1, to a liquid culture system. Binapacryl, Meptyldinocap, Dinocap, and Fluazinam are all FRAC29 fungicides with the same mechanism of action.

In an aspect, a fungicide may be a chemical compound. In an aspect, the fungicide may further contain non-active (inactive) ingredients that may aid in dissolving or dispensing the active ingredient. Fungicides may be known in the art or may be developed to kill or inhibit a pest. Non-limiting examples of fungicides of the present disclosure are presented in Table 1.

Treatments of the present disclosure include providing one or more fungicides presented in Table 1. In an aspect, a first effective concentration of fungicide may be provided to a liquid system upon detection of a first pest. In another aspect, an effective concentration of second fungicide may be provided to a liquid system after the effective concentration of the first fungicide and upon detection of a pest. In an aspect, a fungicide is selected to have a different mechanism of action than a first fungicide. In a further aspect, a third fungicide may be provided as a treatment of a liquid system after the effective treatment of a first and second fungicide. In yet another aspect, a first, second and third fungicide may be rotated to ensure effective control of a pest(s) in a liquid culture system and to avoid the development of fungicide resistance in a liquid culture system.
In an aspect of the present disclosure, a combination of two fungicides may be provided upon detection of a first pest. In yet another aspect, a third fungicide may be provided where the first and second fungicide combination does not control a pest of the liquid system.

**Table 1** Fungicide Sources and Mechanisms of Action

<table>
<thead>
<tr>
<th>Description</th>
<th>Sigma-Aldrich® catalog #</th>
<th>Mode of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>acibenzolar</td>
<td>32820</td>
<td>host plant defense induction; salicylic acid pathway</td>
</tr>
<tr>
<td>azoxystrobin</td>
<td>31697</td>
<td>respiration; QOI-fungicide (quinone outside inhibitors)</td>
</tr>
<tr>
<td>benodanil</td>
<td>45338</td>
<td>respiration; SDHI (succinate dehydrogenase inhibitors)</td>
</tr>
<tr>
<td>binapacryl</td>
<td>31484</td>
<td>sterol biosynthesis in membranes, respiration: uncouplers of oxidative phosphorylation</td>
</tr>
<tr>
<td>boscalid</td>
<td>33875</td>
<td>respiration; SDHI</td>
</tr>
<tr>
<td>bronopol</td>
<td>32053</td>
<td>multi-site contact activity</td>
</tr>
<tr>
<td>captan</td>
<td>32054</td>
<td>multi-site contact activity</td>
</tr>
<tr>
<td>carbendazim</td>
<td>45368</td>
<td>mitosis and cell division; MBC - fungicides (methyl-benzimidazole carbamates)</td>
</tr>
<tr>
<td>carboxine</td>
<td>45371</td>
<td>respiration; SDHI</td>
</tr>
<tr>
<td>chlorothalonil</td>
<td>36791</td>
<td>multi-site contact activity</td>
</tr>
<tr>
<td>cyazofamid</td>
<td>33874</td>
<td>respiration; QOI-fungicides</td>
</tr>
<tr>
<td>cymoxanil</td>
<td>34326</td>
<td>unknown mode of action</td>
</tr>
<tr>
<td>cyprodinil</td>
<td>34389</td>
<td>amino acids and protein synthesis; methionine biosynthesis</td>
</tr>
</tbody>
</table>
| dibromocynanoaceta
<p>| 540978               | respiration; QOI-fungicides                                      |
| dimoxystrobin       | 33499                    | respiration; QOI-fungicides                                      |</p>
<table>
<thead>
<tr>
<th>Description</th>
<th>Sigma-Aldrich® catalog #</th>
<th>Mode of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>dinocap</strong></td>
<td>45452</td>
<td>respiration; uncouplers of oxidative phosphorylation</td>
</tr>
<tr>
<td>diquat dibromide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dithianon</td>
<td>45462</td>
<td>multi-site contact activity</td>
</tr>
<tr>
<td>dodemorph</td>
<td>45465</td>
<td>sterol biosynthesis in membranes; reductase and isomerase in sterol biosynthesis</td>
</tr>
<tr>
<td>dodine</td>
<td>PS250</td>
<td>unknown mode of action; cellular membrane disruption</td>
</tr>
<tr>
<td>endothal monohydrate</td>
<td>35525</td>
<td></td>
</tr>
<tr>
<td>fenarimol</td>
<td>45484</td>
<td>sterol biosynthesis in membranes; DMI fungicides (demethylation inhibitors)</td>
</tr>
<tr>
<td>fenhexaniid</td>
<td>31713</td>
<td>sterol biosynthesis; 3-keto reductase, C4-de-methylation</td>
</tr>
<tr>
<td>fenpropidin</td>
<td>46017</td>
<td>sterol biosynthesis in membranes; D14-reductase and isomerase in sterol biosynthesis ( erg24, erg! )</td>
</tr>
<tr>
<td><strong>fluazinam</strong></td>
<td>34095</td>
<td>respiration; uncouplers of oxidative phosphorylation</td>
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<tr>
<td>fluoxastrobin</td>
<td>33797</td>
<td>respiration; QO1-fungicides</td>
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<tr>
<td>fosetyl-aluminum (100mg)</td>
<td>PS2026</td>
<td>unknown MOA</td>
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<td>respiration; QO1-fungicides</td>
</tr>
<tr>
<td>mancozeb</td>
<td>45553</td>
<td>multi-site contact activity</td>
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<tr>
<td>mepty!dinocap</td>
<td>32098</td>
<td>respiration; uncouplers of oxidative phosphorylation</td>
</tr>
<tr>
<td>metalaxyl</td>
<td>32012</td>
<td>nucleic acid synthesis; PA - fungicides (phenyl amides)</td>
</tr>
<tr>
<td>Description</td>
<td>Sigma-AJdric® catalog #</td>
<td>Mode of Action</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>methyl isothiazolin</td>
<td>73569</td>
<td>sterol biosynthesis in membranes</td>
</tr>
<tr>
<td>nystatin</td>
<td>N4014</td>
<td>sterol biosynthesis in membranes</td>
</tr>
<tr>
<td>oryzalin</td>
<td>36182</td>
<td>microtubule assembly inhibition</td>
</tr>
<tr>
<td>pencycuron</td>
<td>31118</td>
<td>mitosis and cell division; cell division</td>
</tr>
<tr>
<td>propamocarb</td>
<td>45638</td>
<td>lipids and membrane synthesis; cell membrane permeability, fatty acids, carbamate</td>
</tr>
<tr>
<td>propiconazole</td>
<td>45642</td>
<td>sterol biosynthesis in membranes; DMI fungicides</td>
</tr>
<tr>
<td>prothioconazole</td>
<td>34232</td>
<td>sterol biosynthesis in membranes; DMI fungicides</td>
</tr>
<tr>
<td>pyraclostrobin</td>
<td>33696</td>
<td>respiration; QOI-fungicides</td>
</tr>
<tr>
<td>pyrifenoxy</td>
<td>45737</td>
<td>sterol biosynthesis in membranes; DMI fungicides</td>
</tr>
<tr>
<td>sonar</td>
<td>chem service</td>
<td></td>
</tr>
<tr>
<td>spiroxamine</td>
<td>46443</td>
<td>sterol biosynthesis in membranes; reductase and isomerase in sterol biosynthesis</td>
</tr>
<tr>
<td>tebuconazole</td>
<td>32013</td>
<td>sterol biosynthesis in membranes; DMI fungicides</td>
</tr>
<tr>
<td>temefos</td>
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<tr>
<td>terbuthylazine</td>
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<td>thiopanate-methyl</td>
<td>45688</td>
<td>mitosis and cell division; MBC - fungicides</td>
</tr>
<tr>
<td>Thiram®</td>
<td>45689</td>
<td>multi-site contact activity</td>
</tr>
<tr>
<td>tolylfluanid</td>
<td>32060</td>
<td>multi-site contact activity</td>
</tr>
<tr>
<td>triadinienol A</td>
<td>45694</td>
<td>sterol biosynthesis in membranes; DM! fungicides</td>
</tr>
<tr>
<td>Description</td>
<td>Sigma-AJDrich® catalog #</td>
<td>Mode of Action</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>triclopyr</td>
<td>32016</td>
<td>respiration; QOI-fungicide</td>
</tr>
<tr>
<td>trifloxystrobin</td>
<td>46447</td>
<td>sterol biosynthesis in membranes; DMI fungicides</td>
</tr>
<tr>
<td>triflumizole</td>
<td>32611</td>
<td>sterol biosynthesis in membranes; DMI fungicides</td>
</tr>
<tr>
<td>trifluralin</td>
<td>32061</td>
<td></td>
</tr>
<tr>
<td>triforin</td>
<td>45701</td>
<td>mitosis and cell division; β-tubulin assembly in mitosis</td>
</tr>
<tr>
<td>zoxamide</td>
<td>32501</td>
<td></td>
</tr>
</tbody>
</table>

[00171] In another aspect, a first fungicide may be an inhibitor of respiration that uncouples oxidative phosphorylation (e.g. dinocap, fluazinam, meptyldinocap, and/or binapacryl) and a second fungicide may be a quinone outside inhibitor of respiration. In another aspect, a first fungicide may be an inhibitor of respiration that uncouples oxidative phosphorylation and a second fungicide may have multi site contact activity. One of ordinary skill in the art would understand that selection of fungicides based on different mechanisms of action provides methods that avoid the development of fungicide resistant pest strains.

[00172] In an aspect, the treatments may be performed at a specified time of the day. In an aspect, the treatment may be conducted in the morning. In another aspect, the treatment may be conducted at mid-day. In yet another aspect, the treatment may be performed at or near sunset. In another aspect, treatment may be performed at night. In one aspect, treatment may be performed at two periods each day, for example in the morning and again in the evening. In another aspect, treatment may occur during the day and a second monitoring may occur at night.

[00173] The present disclosure provides for the treatment of a liquid system to minimize the formation of concentration gradients. In an aspect, an amount of treatment is calculated based on the volume of a liquid system and prepared in a volume of the media (e.g. the culture media of the liquid system) to prepare a concentrated treatment stock. A concentrated treatment stock may be slowly added to a liquid system. In an aspect, concentrated treatment stock is added behind a paddle wheel of a raceway pond system. In another aspect, the concentrated treatment stock is
dispersed by spraying of a liquid system. In yet another embodiment, the concentrated treatment stock is added to a water return line of a circulation pump.

[00174] The present disclosure provides for the treatment of a liquid system with a fungicide of the pyridinamine family. In an aspect, the pyridinamine may be fluazinam (phenyl-pyridinamine or 3-chloro-N-[3-chloro-2,6-dinitro-4-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2-pyridinamine (CAS No. 79622-59-6)). In an aspect, fluazinam may be provided as a first fungicide treatment of a liquid system. In another aspect, fluazinam is provided as a second fungicide treatment. In an aspect, fluazinam may be provided as a third fungicide treatment. In yet another aspect, fluazinam may be provided as fourth treatment or a fifth treatment. In another aspect, fluazinam may be provided as sixth treatment or a seventh treatment. In other embodiments, fluazinam may be administered in combination with one or more fungicides either separately by being administered contemporaneously with the one or more fungicides, or as part of a mixture of fungicides.

[00175] Fungicide, such as Fluazinam, may be introduced by methods known in the art. In an aspect, the fungicides may be introduced as a solid. In another aspect, the fungicides may be introduced after solvation in an appropriate solvent. In an aspect, a solvent may be water. In another aspect, the fungicide may be dissolved in an alcohol. In an aspect the alcohol may be methanol. In another aspect, the alcohol may be ethanol. In an aspect, the fungicide may be prepared in acetonitrile. In yet another aspect, the fungicide may be prepared in acetone. In still another aspect the fungicide may be dissolved in the culture medium used to grow the microalgae. In an aspect, the effect of the solvent on the organism or organisms is minimized.

[00176] The present disclosure provides for the introduction of fungicides such as Fluazicam, at an effective concentration. Effective concentrations may be determined according to manufacturer's instructions or may be determined empirically. An effective concentration of a fungicide is not toxic to the microalgae being cultured in the liquid system. Methods to determine toxicity are known in the art and include serial dilutions of a test fungicide in a growing liquid culture of microalgae. Fungicides may begin to show growth effects on microalgae in the exemplar[1’]’ ranges provided in Table 2. One of skill in the art would understand that different microalgae may have different ranges of toxicity that may be determined by growth of a microalga in the presence of a serial dilution of a fungicide. The ranges provided below can easily be widened by one skilled in the art by conducting appropriate experiments.

**Table 2**
### Exemplary Ranges of Microalgae Toxicity (ppm)

<table>
<thead>
<tr>
<th>Description</th>
<th>Exemplary Ranges of Microalgae Toxicity (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benodanil</td>
<td>0.3125 - 1.25</td>
</tr>
<tr>
<td>binapacryl</td>
<td>0.125 - 0.5</td>
</tr>
<tr>
<td>captan</td>
<td>1.953 - 125</td>
</tr>
<tr>
<td>carboxine</td>
<td>0.977 - 3.906</td>
</tr>
<tr>
<td>cyazofamid</td>
<td>0.03125 - 0.125</td>
</tr>
<tr>
<td>cymoxanil</td>
<td>15.625 - 62.5</td>
</tr>
<tr>
<td>dimoxystrobin</td>
<td>0.004 - 0.0625</td>
</tr>
<tr>
<td>dinocap</td>
<td>0.0039 - 0.0625</td>
</tr>
<tr>
<td>dithianon</td>
<td>0.625-2.5</td>
</tr>
<tr>
<td>dodemorph</td>
<td>0.195 - 0.781</td>
</tr>
<tr>
<td>fenarimol</td>
<td>0.0489 - 0.195</td>
</tr>
<tr>
<td>fenhexamid</td>
<td>15.63 - 62.5</td>
</tr>
<tr>
<td>fenpropidin</td>
<td>0.00195 - 1.953</td>
</tr>
<tr>
<td>fluazinam</td>
<td>&gt; 7.5</td>
</tr>
<tr>
<td>pencycuron</td>
<td>0.781 - 12.5</td>
</tr>
<tr>
<td>propamocarb</td>
<td>3.906 - 62.5</td>
</tr>
<tr>
<td>pyraclostrobin</td>
<td>&gt; 15</td>
</tr>
<tr>
<td>pyrifenoex</td>
<td>0.004 - 0.156</td>
</tr>
<tr>
<td>spiroxamine</td>
<td>0.0625 - 1.0</td>
</tr>
<tr>
<td>Thirarn®</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>tolyfluanid</td>
<td>1.56 - 25.0</td>
</tr>
<tr>
<td>triflumizole</td>
<td>1.563 - 6.25</td>
</tr>
<tr>
<td>zoxamide</td>
<td>0.0156 - 0.250</td>
</tr>
</tbody>
</table>

**[00177]** According to the present disclosure, a fungicide may be toxic to a microalgae if the growth of a microalgae is decreased in a given concentration range. In an aspect, an effective concentration of fungicide may cause a decrease in microalgae growth but causes a greater reduction in the growth of a pest.

**EXAMPLES**
[00178] The following examples are intended to provide illustrations of the application of the present disclosure. The following examples are not intended to completely define or otherwise limit the scope of the disclosure.

[00179] One of skill in the art will appreciate that many other methods known in the art may be substituted in lieu of the ones specifically described or referenced herein.

[00180] Throughout the entire disclosure, including the claims, and figures, OM27 is the same strain as SE 70179, and OM65 is the same strain as SE 70181.

**EXAMPLE 1: Diversity Enhancement by Mutagenesis**

[00181] Random genomic mutagenesis techniques were used to create a diverse library of mutants of a *Desmodesmus* species, which could be screened for desirable traits that are distinguishable from wild type (WT). Genomic mutations were induced by ultraviolet (UV) radiation at varying intensities (50-200 mJ). Base on differential clonal survival versus WT, a 10-fold to 200-fold increase in occurrence of phenotypic mutants following UV treatments was demonstrated.

[00182] A diverse mutant library was created as follows: the UV intensity that kills 90% of the WT population (typically ~50 mJ for 2.0*10^8 cells) was determined. In addition, the UV intensity that kills 99% of the WT population (up to 200 mJ) was also determined. Batches of WT cultures at UV intensities that range between the 90% and 99% killing intensities were mutagenized. It was assumed that many of the surviving cells will carry ≥1 mutation per genome. Mutagenized cultures were allowed to recover in culture medium and an equal proportion of live cells from each UV intensity treatment were pooled to create a final library. It was assumed that the higher intensity-treatments could introduce more mutations per genome or different types of mutations compared to the lower intensity treatments, thus creating a diverse library of mutants. Downstream selective screens were then used to identify clones with desirable traits that are different from WT.

**EXAMPLE 2: Laboratory Screening and Validation for Mutants with Increased Tolerance to OMEGA®**

**Screening Methodology**

[00183] A rapid and straightforward method to isolate biocide (e.g. a fungicide-resistant mutants is to screen a genetically diverse population using an inhibitory concentration (IC) of a given biocide. By definition, 100% of WT clones will not survive the IC treatment; while in contrast, some resistant mutants may survive IC treatment thus enabling their isolation in the laboratory. It has been demonstrated that individual mutants with desirable phenotypes from a diverse mutated library may be isolated on solid media containing an IC of a particular biocide. Alternatively, clones can
also be isolated by screening in liquid medium containing an IC of a particular biocide. Screening in liquid medium, rather than solid medium, may yield mutants with growth characteristics that would be more translatable to pond conditions.

[00184] An IC of OMEGA* for the WT Desmodesmus species was identified from a dilution series experiment ranging from 1 ppm to 210 ppm in culture medium using 5 mL tubes. The IC was determined for the WT Desmodesmus species culture based on loss of pigmentation (i.e. OD750 = 0) of the culture in treated medium (containing OMEGA*) within 4 days of incubation with OMEGA*, and the absence of colony growth from the treated liquid when plated on solid culture medium. The inhibitor (i.e. concentration of OMEGA*) determined for the WT Desmodesmus species was 100 ppm with a 4-day incubation period.

[00185] Mutagenized libraries of the Desmodesmus species were incubated in flasks containing -100 ppm OMEGA* in a culture medium for 4-6 days. Following incubation, the medium appeared clear (by OD750), and aliquots of the treated medium (containing OMEGA*) were plated onto permissive (i.e. OMEGA*-free) culture medium solid plates for surviving colony isolation. Following -14 days, mutagenized cultures yielded n=94 colonies (while OMEGA®-treated unmutagenized WT culture, as a negative control, yielded only n=3 colonies). These 94 colonies were chosen as candidates for further testing.

[00186] Given the sparge distribution of the surviving colonies, each isolated colony is derived from a single surviving cell. Cultures propagated from an isolated colony are considered axenic. Also, since time of original isolation, the strain was propagated from a single clone after sorting by fluorescence activated cell sorting (FACS). Axenic describes the state of a culture in which only a single species is present, it is a biologically pure culture.

Laboratory Validation and Ranking of Candidates for Minipond Demonstration

[00187] A series of three types of lab validation experiments to confirm OMEGA*-tolerance and to rank the clones as candidates for minipond demonstration were conducted. The three types of experiments are described below.

[00188] First, a 96-well deep block assay for growth rate in the presence of OMEGA* was conducted. This biocide growth rate assay (BGRA) is a microliter growth rate assay with the inclusion of biocide in the medium. For the BGRA, the growth rate of a culture was estimated in a 1 mL volume (96-well block) of culture medium using a log dilution series of OMEGA*; a dilution concentration ranging from 0 ppm to 9,100 ppm. In brief, cultures were inoculated at OD750 = 0.05, and a series of OD measurements were taken at 6 hour intervals for -96 hours. An
exponential growth model was fit to the data to estimate the growth rate parameter \( r \). This data is not shown.

[00189] Second, a standard 96-well high throughput (HTP) microliter growth rate assay (MGRA) was conducted on the clones in permissive medium \( i.e. \) OMEGA* -free. This assay was important to determine if the clones incurred a biological trade-off between biocide tolerance and normal growth rate. A strain that would have reduced growth rate in permissive medium would be undesirable even if it had high tolerance to a biocide, since pond cultures spend most of their time in culture medium free of biocide. For the MGRA, cultures were inoculated at \( \text{OD}_{750} = 0.05 \) in 200 uL culture medium, and a series of OD measurements were taken at 6 hour intervals for 96 hours. A logistic growth model was fit to the data series to estimate the growth rate parameter \( r \) in permissive medium. Results of the 96-well HTP microliter growth rate assay (MGRA) for nominated clones and the WT Desmodesmus species is shown in Figure 1. The y-axis represents growth rate parameter \( r \) and the x-axis represents different evolved mutant strains and wild type strains (from left to right: OM15, OM25, OM27, OM65, OM82, WT1, WT2, WT3, and WT4 (WT1-4 are four separate replicates of the WT Desmodesmus species)). Each bar represents triplicate estimates for the growth rate parameter \( r \) from a logistic growth curve. All of the evolved mutant strains exhibited a normal growth rate \( r \) in permissive media in a microliter format when compared to the wild-type strain(s).

[00190] Lastly, data from both the BGRA and the MGRA were used to rank the top 55 clones that would be tested in a third and final validation experiment (the "Validation III" or "Sawtooth Experiment")

[00191] The Validation III experiment is similar to the BGRA format described above, but was modified to allow for a semi-continuous harvest scheme in a 96-well deep block. In this format, all the cultured wells were roughly maintained in mid-log phase \( \text{OD}_{750} = 0.3 \) to 0.7) by continual cutbacks of the culture to 0.3 OD when the wells reached a trigger OD of approximately 0.7. This scheme allowed for fresh biocide (OMEGA*) to be replenished at each cutback event, which was critical since the active ingredient of the biocide is expected to decay over the course of several days. The semi-continuous cutback scheme was maintained for 4 cutback events ("rounds"), with two additional dosing events (rounds 5 and 6). As is shown in the legend of Figure 2.4, each round had six different doses of OMEGA*. For rounds 1 and 2 the doses were 0.0 ppm, 0.25 ppm, 0.5 ppm, 1.0 ppm, 1.25 ppm, and 1.5 ppm. For rounds 3 to 6, the doses were 0.0 ppm, 0.5 ppm, 1.0
ppm, 2.0 ppm, 2.5 ppm, and 3.0 ppm. The dosing schedule was the same for Figure 2A to Figure 2D, and for each of OM15, QM25, OM27, OM65, and OM82 (Figure 3A to Figure 3E).

[00192] Results from the Validation III experiments are shown for several WT cultures (Figure 2A to Figure 2D) and for OM15 (Figure 3A), OM25 (Figure 3B), OM27 (Figure 3C), OM65 (Figure 3D), and OM82 (Figure 3E). For Figure 2A to Figure 2D, and Figure 3A to Figure 3E, each data point represents three biological replicates in 96-well deep blocks. For Figure 2A to Figure 2D, and Figure 3A to Figure 3E, the Y-axis is OD750 and the x-axis is time in hours. After the 4th round, the WT cultures do not recover compared to, for example, OM65 at 3ppm OMEGA® (see Figure 3D).

[00193] The OD data from the Validation III experiments was used to determine the doubling rate for each clone at different doses of OMEGA®. For example, Figure 4 represents the doubling rate for each variant clone and several WT clones at a dose of 2.5 ppm OMEGA®; the y-axis represents doubling rate and the x-axis represents various evolved mutant strains and WT strains. Based on this analysis, OM15 and OM82 were selected for minipond testing (see Table 3 below).

[00194] At the end of the experiment at the highest dose that was administered (3 ppm OMEGA®), only n=5 of the clones survived the treatment scheme. All other cultures (including 4 WT replicates) had lost all pigment and died (Figure 5). Figure 5 is a photographic image of the Validation III experiment at Round 6 and 3 ppm OMEGA® with 5 surviving colonies. The five surviving "green well" clones became candidates for clones suitable for field testing in miniponds. However, for minipond testing, only three of these clones, which also had the highest doubling rates at lower doses (1.0 to 2.5 ppm) of OMEGA® (in Validation III), were chosen, and two additional clones that were not "green wells" although they had high doubling rates at low doses (1.0 to 2.5 ppm) of OMEGA® were also chosen. The five clones chosen for minipond experiments are shown in Table 3 below. Included in Table 3 is the selection rationale that drew upon the results from the three validation experiments: BGRA, MGRA (Figure 1) and the "Sawtooth Experiment" (Validation III; Figure 2A to Figure 2D and Figure 3A to Figure 3E).

**Table 3**

<table>
<thead>
<tr>
<th>Desmodesmus UV-mutated Strain</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM15 (B09 on Fig. 3)</td>
<td>high doubling rate at Validation III at 2.5 ppm</td>
</tr>
<tr>
<td>OM25 (C06 on Fig. 3)</td>
<td>green well in Validation III</td>
</tr>
<tr>
<td>OM27 (COS on Fig. 3)</td>
<td>green well in Validation I1</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>OM65 (E06 on Fig. 3)</td>
<td>green well and high doubling rate in Validation I1</td>
</tr>
<tr>
<td>OM82 (F08 on Fig. 3)</td>
<td>high doubling rate at Validation III at 2.5 ppm</td>
</tr>
</tbody>
</table>

**EXAMPLE 3: Minipond (MP) Experimental Design and Data**

**MP Experimental Design**

Miniponds (MPs) were used for each of the five evolved (mutated) strains (see Table 3) and for WT as an experimental control (Total n=18 MPs). Prior to inoculation, the MPs had an approximate culture medium volume of approximately 420 liters and were grown outdoors. The culture media was about pH 9 (in a range from about 8.8 to about 9.2).

First, all strains were grown indoor in carboys each at a volume (approximately 20 L) sufficient to inoculate three MPs for each strain. All MPs were inoculated at a starting total suspended solids (TSS) measurement of 0.3g/L. Upon initial inoculation, all MPs were treated immediately with OMEGA* at a dose of 0.5 ppm. Typically a MP would not be treated with biocide unless a chytrid infection is detected. However, for the purpose of experimental design, which was aimed to demonstrate biocide tolerance specifically, cultures were treated, at different times throughout the experiment, with predetermined concentrations of OMEGA*, without an identified chytrid infection. MP cultures were grown until the first MP of the experiment reached a TSS of 0.7g/L, and then all MPs were harvested at that time (even if some MPs have not reached 0.7g/L themselves) to a TSS of 0.4g/L. Immediately after the first harvest, all MPs were treated with OMEGA* at an increased dose of 1.0 ppm. Again, all cultures were grown until the first MP reached a TSS of 0.7g/L, and then all MPs were harvested to a TSS of 0.4g/L. Immediately after this second harvest, all MPs were treated with OMEGA* at an increased dose of 2.0 ppm. All cultures were then grown until the first MP reached a TSS of 0.7g/L. The growth-harvest-treatment cycle continued for a total of ~30 plus days including an additional final harvest and dosing at 2.0 ppm. Harvested MPs were replenished after each harvest with fresh culture media, rather than recovered culture media. Data was collected for each mutated and wild type strain throughout the course of the 30 plus day experiment. Standard field measurements were taken on a regular basis, such as dry weigh, OD, Fv/Fm, etc.).

**Photosynthetic Efficiency**

Photosynthetic efficiency was measured for 18 MPs (3 MPs per strain) by PAM fluorometry (Fv/Fm) throughout the 30 plus day experiment (see Figure 6A to Figure 6F). For example, for
OM15 (Figure 6A): MP9102, MP9104, and MP9110 were set up. For all six figures the y-axis is Fv/Fm (0, 0.2, 0.4, 0.6, and 0.8) and the x-axis is time in days (0 to 30 in one-day increments). For all six figures, each of the four vertical "dotted" lines represents a dosing event. From left to right: the first is a dose of 0.5 ppm of OMEGA®; the second is a dose of 1 ppm of OMEGA®; the third is a dose of 2 ppm of OMEGA®; and the fourth is a dose of 2 ppm of OMEGA®.

[00198] The photosynthetic efficiency of the WT Desmodesmus species cultures are normally expected to transiently suffer following OMEGA® treatment. Indeed all WT MPs exhibited the expected reduction in Fv/Fm except for MP9312 following the OMEGA® treatment at day 15 (see Figure 6F). Consequently, the results of this experiment for the WT Desmodesmus species are based on duplicate miniponds (MP9107 and MP9109), while all other UV-mutated strains are based on triplicate miniponds. MP9312 showed outlier behavior at day 15 that could be consistent with a missed dosing event due to human error. In Figure 6F, Fv/Fm data for MP9312 is highlighted with an arrow. As is shown in Figure 6.4 to Figure 6F, both WT MPs experienced reduced PAM measurements relative to all of the mutated ("evolved") strains. All variant strains showed increased Fv/Fm values as compared to WT throughout the course of the experiment.

Biomass Productivity

[00199] To determine biomass productivity of the MPs, regular measurements of TSS were recorded as dry weight (DW) (g/L) throughout the experiment (see Figure 7).

[00200] Figure 7 shows biomass productivity of ponds comprising the WT strain and the different evolved mutant strains (OM15, OM25, OM27, OM65, and OM82). The y-axis is DW (g/L) and the x-axis is days of experiment. Pigment loss was shown in the WT over time. Downward-facing arrows represent dosing events. From left to right, the first arrow is a dose of 0.5 ppm of OMEGA®, the second arrow is a dose of 1 ppm of OMEGA®, the third arrow is a dose of 2 ppm of OMEGA®, and the forth arrow is a dose of 2 ppm of OMEGA®. In Figure 7, each point represents the mean of replicate miniponds for a given strain. Different strains are represented by different shapes; WT is represented by squares. In situ productivity (g/m²/day) of each MP was calculated using the TSS measurements including a time dimension (days). In situ productivity was calculated for all MPs during the time span of the 1 ppm treatment ("Round 1": days 4-14) separately from the time span of the 2 ppm treatment ("Round 2": days 16-25). Given the experimental design, by which all MPs are harvested simultaneously at the point when the first MP of the cohort reaches trigger TSS (0.7 g/L), some MPs will have lower TSS than others at harvest time. Differential TSS values among MPs at the shared harvest times may reflect differential in situ productivities given the shared time-
span and OMEGA® treatment across the MPs. Many of the evolved strains showed higher DW (g/L) measurements relative to WT at each of the harvesting time points (for example, day 2, 14, and 25).

The in situ productivity for miniponds at 1 ppm (Round 1) and at 2 ppm (Round 2) is shown in Figure 8 and Figure 9. Figure 8 shows the absolute estimates oin situ productivity (g/m²/day)(y-axis). In Figure 8, the left hand column of each two-column set represents a 1 ppm OMEGA® dose, and the right hand column of each two-column set represents a 2 ppm OMEGA® dose. In Figure 9, the data is viewed in a relative fashion by normalizing all productivity estimates to the WT estimates. In Figure 9, the left hand column of each two-column set represents a 1 ppm OMEGA® dose, and the right hand column of each two-column set represents a 2 ppm OMEGA® dose. The y-axis is normalized in situ productivity. As shown in Figure 8 and Figure 9, OM25, OM27, OM65, and QM82 had higher in situ productivity when compared to WT. Throughout the experiment, MPs from two strains were consistently the first to reach trigger TSS in the group (i.e. OM27 at 1ppm and OM65 at 2ppm). The calculated in situ productivities reflect that OM65 exhibited higher biomass productivity compared to WT both at 1 ppm and 2 ppm (Figure 8). When dosed at 2 ppm OMEGA®, the evolved strain OM65 was more than twice as productive as WT under the same conditions. This represents 118% higher in situ productivity versus WT (p-value 0.019 by Dunnett's comparison of means test). In addition, OM65 trends towards about a 20% improvement versus WT at 1 ppm. Note, that as may be expected, all strains exhibited lower productivities in the presence of OMEGA® than would be typical for cultures that are not treated with biocide. However, for example, the significantly improved relative performance of OM65 when treated with OMEGA® validates the hypothesis that OM65, along with other mutated strains, indeed exhibits a hyper-tolerance of OMEGA® relative to WT. For example, even at 1 ppm, the in situ productivity of OM65 is ~ 28% higher than WT. Also noteworthy is that OM27 displayed in situ productivity similar to OM65 (Figure 8).

Figure 10 shows the data from Figure 8 analyzed by Oneway ANOVA. The product of OM65 is significantly greater than that of WT, with a p-Value of 0.0142. The means comparison with a control (WT) using Dunnett's Method yielded a p-Value of 0.019. Figure 10 represents a dose of 2 ppm of OMEGA®. The y-axis is in situ productivity (g/m²/day).

Photosynthetic Health

The photosynthetic health of the MP cultures throughout the experiment was also measured. Differential photosynthetic performance between strains may be an indicator of important biological characteristics that affect biomass productivity. One method to assess photosynthesis health is by
PAM fluorometry (Fv/Fm). Regular Fv/Fm measurements highlighted a transient treatment response to OMEGA® (at 1 ppm and 2 ppm) in which Fv/Fm values would significantly drop within ~48 hours following a treatment, and the Fv/Fm values would then recover to pre-treatment levels after ~6 days as shown in Figure 11. In Figure 11, the y-axis is Fv/Fm and the x-axis is days of experiment. Pigment loss was shown in the WT over time. Downward-facing arrows represent dosing events. From left to right, the first arrow is a dose of 0.5 ppm of OMEGA®, the second arrow is a dose of 1 ppm of OMEGA®, the third arrow is a dose of 2 ppm of OMEGA®, and the forth arrow is a dose of 2 ppm of OMEGA®. In Figure 11, each point represents the mean of replicates for a given strain. Different strains are represented by different shapes; WT is represented by a square. Interestingly, although all the MPs in the experiment exhibited this transient "Fv/Fm deficit", all evolved clones exhibited a smaller Fv/Fm deficit relative to WT. We quantified the Fv/Fm deficit by subtracting the Fv/Fm value at 48 hours from the pre-treatment Fv/Fm value (ΔFv/Fm48hrs). Specifically, OM65 and QM27, which were the top mutant strains based on in situ productivity, also exhibited the smallest Fv/Fm deficit among all the MPs at both 1 ppm and 2 ppm OMEGA®, while WT exhibited the largest Fv/Fm deficit (see Figure 12). As is shown in Figure 12, the Fv/Fm deficit (y-axis) of OM65 and OM27 was less than half the Fv/Fm deficit of WT. In addition, the Fv/Fm deficit (y-axis) of OM15, OM25, and OM82 was less than the Fv/Fm deficit of WT. In Figure 12, the left hand column of each two-column set represents a 1 ppm OMEGA® dose, and the right hand column of each two-column set represents 2 ppm OMEGA® dose.

Biomass Quality

[00204] As early as day 11, the WT MPs exhibited qualitative stress based on microscope observations. Cells in the WT ponds appeared to exhibit "loss of pigment" and some "hollow cells" were observed, while many of the non-WT MPs appeared much healthier. After day 30, biomass samples were collected from the MPs of WT along with OM65 and OM27 (since these experimental MPs exhibited the smallest Fv/Fm deficit). WT biomass exhibited an extremely de-pigmented and unhealthy appearance, while OM65 and OM27 exhibited a deep green pigmented appearance similar to the appearance of the untreated Desmodesmus species culture. Figure 13 shows biomass collected from MPs at day 30 from OM65 and WT. Biomass collected from OM27 appeared qualitatively similar to OM65. MP samples from all other evolved strains also showed pigmentation which appeared much healthier than WT. Therefore, the evolved strains tolerance to OMEGA® resulted in improved biomass quality which can lead to improved overall productivity in the field.
Another indicator of culture health can be inferred from fluorescence measurements that quantify pigmentation in the culture. After day 30, the WT culture failed to recover to normal levels of pigmentation as measured by fluorescence (Figure 14A, Figure 14B, and Figure 14C).

Each of the three figures has a y-axis representing Fluor Units and an x-axis representing days of experiments. Figure 14A is 430x685 nm wavelength, indicative of green algae; Figure 14B is 363/685 nm wavelength, indicative of cyanobacteria; and Figure 14C is 450/685 nm wavelength, indicative of chlorophyll. Interestingly, based on fluorescence, each of the evolved strains (regardless of their biomass productivity) appeared healthier than WT culture at the end of the experiment. Figure 14A to Figure 14C show that normal pigmentation (including chlorophyll) was severely impacted in the WT strains and to a lesser extent in the variant strains.

Finally, as shown in Figure 15 FAME content was determined for the biomass samples from OM65, OM27, and WT cultures at the end of the experiment (Day 30 is represented by the left hand column of each two-column pair, and Day 36 is represented by the right hand column of each two-column pair). The y-axis is FAME %. The WT cultures had -6% FAME compared to -12-14% FAME in OM65 and OM27 (Figure 15), indicating a possible effect of OMEGA® treatment on oil content. In Figure 15, biomass samples were only collected for three strains to conserve resources. The FAME estimate for WT Day 30 is based on a single MP sample (MP9107). The other FAME estimates are based on three MP replicates per strain, as outlined in the experimental design above. FAME content of OM27 and OM65 (-12-14%) is similar to untreated (no OMEGA®) WT (data not shown).

**EXAMPLE 4: Chytrid Infections During the Experiment**

The experimental design described above warranted a continuous dosing scheme of OMEGA® regardless of the presence of a chytrid infection. As may be expected, due to the relatively high levels of OMEGA® in the MPs, no chytrids were ever detected in the MPs during the experiment. To address by proxy the question of the ability of evolved strains to outperform WT in the presence of chytrids, data demonstrating that chytrids were indeed active in the miniponds during the same experimental time frame was needed. Data from an "independent" outdoor minipond of the WT Desmodesmus species during the same experimental time frame showed a chytrid infection. The independent outdoor MP of the WT Desmodesmus species was not treated with any herbicide. Chytrid infections are commonly seen in outdoor miniponds and are routinely treated with a therapeutic dose of OMEGA®. Upon dosing of the independent minipond culture at 1 ppm OMEGA®, an "Fv/Fm deficit" was observed in the independent minipond (see Figure 16).
reminiscent of the pattern seen in the experimental MPs. These results show that the outdoor MPs in the experiment were very likely exposed to chytrids as seen in the independent minipond and that, for example, OM65 outperformed WT cultures even in the face of these predators. Figure 16 represents PAM measurements from an independent minipond (RW100) of the WT Desmodesmus species. The y-axis is Fv/Fm (each "notch" is an increment of 0.1; 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8) and the x-axis is days of experiment (each notch is a two-day increment; 0, 2, 4, 6, 8, 10, 12, 14, and 16). A chytrid infection was detected in the independent minipond (RW100) and a 1ppm dose of OMEGA was administered.

EXAMPLE 5: Further Testing of OM27 and OM65

Experimental Setup

Raceway 100's (RWs) were used for further testing of OM27 (SE 70179), OM65 (SE 10781), and WT as an experimental control (total n=3 RWs). The experiment was initiated by transferring culture from existing RW ponds as described in Table 4 below. Upon initial inoculation, all RWs were grown using standard cultivation methods to allow for acclimation to the new environment. Typically, a RW would not be treated with biocide unless a chytrid infection is detected. However, for the purpose of experimental design, which was aimed to demonstrate biocide tolerance specifically, cultures were treated, at different times throughout the experiment, with predetermined concentrations of OMEGA*, in the absence of an identified chytrid infection. OMEGA* application was performed independent of harvest operations. RWs were grown with an intended density range of 0.45 - 0.65 g/L. Harvest was completed per standard operating methods returning culture media during biomass removal. Data was collected for each mutated (OM27, OM65) and wild type strain throughout the course of the 21 day experiment. Standard field measurements were taken on a regular basis, such as dry weight, OD, Fv/Fm, etc.).
Table 4

<table>
<thead>
<tr>
<th>Inoculum Origin</th>
<th>OM27 Daughter Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW (g/L)</td>
<td>Inoculum harvest volume (L)</td>
</tr>
<tr>
<td>LC:1019_ 0.62</td>
<td>5754</td>
</tr>
<tr>
<td>LC:1020_ 0.58</td>
<td>5754</td>
</tr>
<tr>
<td>LC:1021_ 0.54</td>
<td>5754</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dw (g/L)</th>
<th>WT Daughter Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum harvest volume (L)</td>
<td></td>
</tr>
<tr>
<td>LC:1025_ 0.69</td>
<td>4503</td>
</tr>
<tr>
<td>LC:1026_ 0.71</td>
<td>4503</td>
</tr>
<tr>
<td>LC:1027_ 0.81</td>
<td>4503</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Inoculum Origin</th>
<th>OM65 Daughter Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW (g/L)</td>
<td>Inoculum harvest volume (L)</td>
</tr>
<tr>
<td>LC:1028_ 0.64</td>
<td>5988</td>
</tr>
<tr>
<td>LC:1029_ 0.53</td>
<td>5988</td>
</tr>
<tr>
<td>LC:1030_ 0.50</td>
<td>5988</td>
</tr>
</tbody>
</table>

Photosynthetic Efficiency

[00209] Photosynthetic efficiency was measured for 3 RWs (1 RW per strain) by PAM fluorometry (Fv/Fm) throughout the 21 day experiment (see Figure 17). The y-axis represents Fv/Fm and the x-axis, time in days. The WT is represented by a "square", OM27 is represented by a "diamond", and OM65 is represented by a "triangle." The three downward facing arrows represents dosing events,
from left to right, of 1 ppm, 2 ppm, and 2 ppm respectively. WT was measured for 17 days, and
OM27 and OM65 for 21 days.

[00210] The data shows that in the presence of successive OMEGA® applications, the photosynthetic
efficiency is more stable in OM27 and OM65 compared to the WT parental strain. It is expected
that a drop in PAM transiently would be observed, but both of the mutants (OM27 and OM65)
recovered. Data collection was discontinued on day 17 for WT as the culture was determined to be
non-viable at this point.

Biomass Productivity

[00211] Biomass productivity was determined as a measure of the harvested product removed from
the pond during the course of the 21 day field trial. All three ponds, OMEGA® tolerant mutants
plus control, were harvested on day 13, two weeks after the first 1 ppm OMEGA® treatment.
Following the second treatment of 2 ppm OMEGA® on day 15, only the mutated strains were
eligible for harvest based on TSS and phenotype observations. OM27 was harvested on day 17 and
day 21 for an estimated total of 14 kg biomass produced. OM65 was harvested on day 20 for an
estimated total of 9 kg biomass produced. The evolved strains both showed significantly greater
harvested productivity (p<.001) during the 21 day experiment by Dunnett's test (see Figure 18)
and Table 5 below. For Figure 18, WT is on the left, OM27 in the middle, and OM65 on the right.

The y-axis of Figure 18 is g/m²/day weighted.

<table>
<thead>
<tr>
<th>Table 5</th>
</tr>
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<tbody>
<tr>
<td>Means for One Way Anova</td>
</tr>
<tr>
<td>Level</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>OM27</td>
</tr>
<tr>
<td>OM65</td>
</tr>
</tbody>
</table>

Std Error used a pooled estimate of error variance
Means Comparisons

Comparisons with a control using Dunnett’s Method

Control Group is WT

\[ I_{DI} = 2.26695 \]

\[ \alpha = 0.05 \]

<table>
<thead>
<tr>
<th>Level</th>
<th>( \text{Abs(Dif)-LSD} )</th>
<th>( p)-Value</th>
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</thead>
<tbody>
<tr>
<td>OM27</td>
<td>5.284</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>OM65</td>
<td>4.543</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>WT</td>
<td>-1.9</td>
<td>1.000</td>
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</table>

Positive values show pairs of means that are significantly different

One-way Anova

Summary of Fit

<table>
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<tr>
<th></th>
<th>R\text{square}</th>
<th>( \text{Adj R\text{square}} )</th>
<th>( \text{Root Mean Square Error} )</th>
<th>( \text{Mean of Response} )</th>
</tr>
</thead>
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<tr>
<td></td>
<td>0.600538</td>
<td>0.587044</td>
<td>2.714509</td>
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</table>

Observations (or Sum Wgts) 62

Analysis of Variance

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<tr>
<th>Source</th>
<th>DF</th>
<th>\text{Sum of Squares}</th>
<th>\text{Mean Square}</th>
<th>\text{F Ratio}</th>
<th>\text{Prob}&gt;F</th>
</tr>
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<tr>
<td>Culture ID</td>
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<td>653.7043</td>
<td>326.852</td>
<td>44.3577</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Error</td>
<td>59</td>
<td>434.7448</td>
<td>7.369</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>61</td>
<td>1088.4491</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Biomass Quality

During the course of the 21 day experiment, phenotypic observations were made based using standard microscopy techniques. Following the first round of 1 ppm OMEGA® application, there was a difference noted in the pigmentation and overall health of the cells by microscopy. The pigmentation and overall health of the OM27 and OM65 cultures were much better than the WT culture. By day 4, the WT cells were noted as exhibiting abnormal pigment, and by day 16 is was noted that the majority of the WT cells lost their pigment. In addition, the culture health was clearly indicated by visual observation (see Figure 19). Figure 19 is a comparison of OM27 and WT culture at the end of the 21 day trial. A culture of OM27 is shown in the left hand pond and a culture
of WT is shown in the right hand pond. OM 27 is green and healthy and WT is brown and unhealthy. QM65 had the same results and looked similar to OM27 when compared to WT.

[00213] While certain embodiments have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the disclosure. It should be understood that various alternatives to the embodiments of the disclosure described herein may be employed in practicing the disclosure. It is intended that the following claims define the scope of the disclosure and that methods and structures within the scope of these claims and their equivalents be covered thereby.
What is claimed is:

1. A culture of a mutagenized *Desmodesmus* species having the characteristics of American Type Culture Collection (ATCC) Accession No. PTA-120599.

2. A culture of a mutagenized *Desmodesmus* species having the characteristics of American Type Culture Collection (ATCC) Accession No. PTA-120600.

3. The culture of claim 1 or 2 wherein said *Desmodesmus* species has an increased tolerance to a Fungicide Resistance Action Committee 29 (FRAC 29) fungicide as compared to an unmutagenized wild type.

4. The culture of claim 3, wherein said FRAC-29 fungicide is any one or more of Fluazinam, Binapacryl, Meptyklinocap or Dinocap.

5. The culture of claim 3, wherein said FRAC-29 fungicide is an oxidative phosphorylation uncoupler.

6. The culture of claim 4, wherein said fungicide is Fluazinam.

7. The culture of claim 3, wherein said FRAC 29 fungicide is present in a liquid culture system.

8. The culture of claim 7, wherein said FRAC 29 fungicide is present at a concentration from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm.

9. The culture of claim 7, wherein said FRAC 29 fungicide is present at a concentration of about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm.
10. The culture of claim 3, wherein said increased tolerance is defined as any one or more of an increase in: growth rate, biomass productivity, \textit{in situ} productivity, doubling rate, photosynthetic efficiency, photosynthetic health, or FAME content.

11. The culture of claim 3, wherein said increase in tolerance is defined as an increase in growth rate.

12. The culture of claim 3, wherein said increase in tolerance is defined as an increase in biomass productivity.

13. The culture of claim 12, wherein said increase in biomass productivity is 2\% to 5\%, 5\% to 10\%, 10\% to 15\%, 15\% to 20\%, 20\% to 25\%, 25\% to 30\%, 30\% to 35\%, 35\% to 40\%, 40\% to 45\%, 45\% to 50\%, 50\% to 55\%, 55\% to 60\%, 60\% to 65\%, 65\% to 70\%, 75\% to 80\%, 85\% to 90\%, 95\% to 100\%, 100\% to 150\%, 150\% to 200\%, 200\% to 250\%, 250\% to 300\%, 300\% to 350\%, 350\% to 400\%, or more than 400\%.

14. The culture of claim 13, wherein biomass productivity is measured in dry weight (DW) grams per liter (g/L).

15. The culture of claim 3, wherein said increase in tolerance is defined as an increase \textit{in situ} productivity.

16. The culture of claim 15, wherein said increase \textit{in situ} productivity is 2\% to 5\%, 5\% to 10\%, 10\% to 15\%, 15\% to 20\%, 20\% to 25\%, 25\% to 30\%, 30\% to 35\%, 35\% to 40\%, 40\% to 45\%, 45\% to 50\%, 50\% to 55\%, 55\% to 60\%, 60\% to 65\%, 65\% to 70\%, 75\% to 80\%, 85\% to 90\%, 95\% to 100\%, 100\% to 150\%, 150\% to 200\%, 200\% to 250\%, 250\% to 300\%, 300\% to 350\%, 350\% to 400\%, or more than 400\%.

17. The culture of claim 16, wherein said increase \textit{in situ} productivity is about a 20\% increase, about a 28\%, about a 30\% increase, or about a \textit{\textless} 18\% increase.
18. The culture of claim 15, wherein *in situ* productivity is measured in g/m²/day.

19. The culture of claim 3, wherein said increased tolerance is defined as an increase in doubling rate.

20. The culture of claim 3, wherein said increased tolerance is defined as an increase in photosynthetic efficiency.

21. The culture of claim 20, wherein said photosynthetic efficiency is determined by Pulse-Amplitude-Modulation (PAM) fluorometry (Fv/Fm).

22. The culture of claim 3, wherein said increased tolerance is defined as an increase in photosynthetic health.

23. The culture of claim 22, wherein said photosynthetic health is determined by Pulse-Amplitude-Modulation (PAM) fluorometry (Fv/Fm).

24. The claim of claim 3, wherein said increase in tolerance is defined as an increase in FAME content.

25. The culture of claim 24, wherein said increase in FAME content is 1.25-fold to two-fold higher, two-fold to three-fold higher, three-fold to four-fold higher, or four-fold to five-fold higher.

26. The culture of claim 1 or 2 wherein said *Desmodesmus* species is *Desmodesmus armatus*.

27. A method of producing algal biomass, comprising:
   a) providing a liquid culture system;
   b) inoculating said liquid culture system with at least one of ATCC Accession No. PTA-120599 and ATCC Accession No. PTA-120600.
28. The method of claim 27, **further** comprising treating said liquid culture system at least either before or after step b) or both before and after step b) with an effective concentration of a fungicide composition comprising a Fungicide Resistance Action Committee 29 (FRAC 29) fungicide.

29. The method of claim 28, wherein said FRAC 29 fungicide is an uncoupler of oxidative phosphorylation.

30. The method of claim 28, wherein said fungicide composition comprises any one or more of Fluazinam, Binapacryl, Meptyldinocap, or Dinocap.

31. The method of claim 30, wherein said FRAC 29 fungicide composition comprises Fluazinam.

32. The method of claim 31, wherein said fungicide composition is OMEGA*.

33. The method of claim 28, wherein said FRAC 29 fungicide comprises from 10%-20% of said fungicide composition, from 20%-30% of said fungicide composition, from 30%-40% of said fungicide composition, from 40%-50% of said fungicide composition, from 50%-60% of said fungicide composition, from 60%-70% of said fungicide composition, from 70%-80% of said fungicide composition, from 80%-90% of said fungicide composition or from 90% to 100% of said fungicide composition.

34. The method of claim 28, wherein said treating of said liquid culture system occurs more than once.

35. The method of claim 28, wherein said effective concentration is from 0.25 ppm to 15 ppm, or 0.01 ppm to 20 ppm.

36. The method of claim 28, wherein said effective concentration is from 0.1 ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm.
37. The method of claim 28, wherein said effective concentration is from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm.

38. The method of claim 28, wherein said effective concentration is about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm.

39. The method of claim 27, further comprising growing said alga for a number of days selected from the group consisting of 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 10 or more, 15 or more, 30 or more, 45 or more, 60 or more, 90 or more, 120 or more, 180 or more, 250 or more, 500 or more, 1000 or more, 1500 or more, and 2000 or more days after inoculation.

40. The method of claim 27, further comprising growing said alga for from 1 to 7 days, from 1 week to 2 weeks, from 2 weeks to 3 weeks, from 3 weeks to 1 month, from 1 month to 3 months, from 3 months to 6 months, from 6 months to 9 months, from 9 months to 12 months, from 12 months to 18 months, from 18 months to 2 years, from 2 years to 3 years, from 3 years to 4 years, from 4 years to 5 years, from 5 years to 6 years, from 6 years to 7 years, from 7 years to 8 years, from 8 years to 9 years or from 9 years and 10 years after inoculation.

41. The method of claim 27, wherein said liquid culture system is an open outdoor liquid culture system.

42. The method of claim 41, wherein said open outdoor liquid culture system has a volume of 10,000 to 20,000 liters, 10,000 to 40,000 liters, 10,000 to 80,000 liters, 10,000 to 100,000 liters, 10,000 to 150,000 liters, 10,000 to 200,000 liters, 10,000 to 250,000 liters, 10,000 b 500,000 liters, 10,000 to 600,000 liters, 10,000 to 1,000,000 liters, 20,000 to 40,000 liters, 20,000 to 80,000 liters, 20,000 to 100,000 liters, 20,000 to 150,000 liters, 20,000 to 200,000 liters, 20,000 to 250,000 liters, 20,000 to 500,000 liters, 20,000 to 600,000 liters, 20,000 to 1,000,000 liters, 40,000 to 80,000 liters, 40,000 to 100,000 liters, 40,000 to 150,000 liters, 40,000 to 200,000 liters, 40,000 to 250,000 liters, 40,000 to 500,000 liters, 40,000 to 600,000 liters, 40,000 to 1,000,000 liters, 80,000 to 100,000 liters.
liters, 80,000 to 150,000 liters, 80,000 to 200,000 liters, 80,000 to 250,000 liters, 80,000 to 500,000 liters, 80,000 to 600,000 liters, 80,000 to 1,000,000 liters, 100,000 to 150,000 liters, 100,000 to 200,000 liters, 100,000 to 250,000 liters, 100,000 to 500,000 liters, 100,000 to 600,000 liters, 100,000 to 1,000,000 liters, 200,000 to 250,000 liters, 200,000 to 500,000 liters, 200,000 to 600,000 liters, 200,000 to 1,000,000 liters, 250,000 to 500,000 liters, 250,000 to 600,000 liters, 250,000 to 1,000,000 liters, 500,000 to 600,000 liters, or 500,000 to 1,000,000 liters.

43. The method of claim 41, wherein said open outdoor liquid culture system has an area selected from the group consisting of at least 0.25 acre, at least 0.5 acre, at least 1.0 acre, at least 1.5 acres, at least 2.0 acres, at least 2.5 acres, at least 5.0 acres, and 7.5 or more acres.

44. The method of claim 41, wherein said open outdoor liquid culture system has an area selected from the group consisting of 0.25 to 0.5 acres, 0.25 to 1.0 acres, 0.25 to 1.5 acres, 0.25 to 2.0 acres, 0.25 to 2.5 acres, 0.25 to 5.0 acres, 0.25 to 7.5 acres, 0.5 to 1.0 acres, 0.5 to 1.5 acres, 0.5 to 2.0 acres, 0.5 to 2.5 acres, 0.5 to 5.0 acres, 0.5 to 7.5 acres, 1.0 to 1.5 acres, 1.0 to 2.0 acres, 1.0 to 2.5 acres, 1.0 to 5.0 acres, 1.0 to 7.5 acres, 2.0 to 2.5 acres, 2.0 to 5.0 acres, 2.0 to 7.5 acres, 2.5 acres to 5.0 acres, 2.5 to 7.5 acres, 7.5 to 10 acres, 10 to 15 acres, 15 to 20 acres, 20 to 25 acres, 25 to 30 acres, 30 to 35 acres, 35 to 40 acres, 40 to 45 acres, 45 to 50 acres, 50 to 75 acres, 75 to 100 acres, 100 to 125 acres, 125 to 150 acres, 150 to 175 acres, 175 to 200 acres, 200 to 250 acres, 250 to 300 acres, 300 to 350 acres, 350 to 400 acres, 400 to 450 acres, and 450 to 500 acres.

45. A method of reducing the growth of fungus in a liquid culture system, comprising:
   a) providing a liquid culture system;
   b) inoculating said liquid culture system with at least one rautagenized *Desmodesmus* species selected from the group consisting of ATCC Accession No. PTA-120599 and ATCC Accession No. PTA-120600; and
   c) treating said liquid culture system either before or after step b) or both before and after step b) with an effective concentration of a fungicide composition to reduce the growth of fungus, wherein said fungicide composition contains as an active ingredient a Fungicide Resistance Action Committee 29 (FRAC 29) fungicide.
46. The method of claim 45, wherein said FRAC 29 fungicide is an uncoupler of oxidative phosphorylation.

47. The method of claim 45, wherein said fungicide composition comprises any one or more of Fluazinam, Binapacryl, Meptyldinocap, or Dinocap.

48. The method of claim 47, wherein said FRAC 29 fungicide composition comprises Fluazinam.

49. The method of claim 48, wherein said fungicide composition is OMEGA*.

50. The method of claim 45, wherein said FRAC 29 fungicide comprises from 10% to 20% of said fungicide composition, from 20% to 30% of said fungicide composition, from 30% to 40% of said fungicide composition, from 40% to 50% of said fungicide composition, from 50% to 60% of said fungicide composition, from 60% to 70% of said fungicide composition, from 70% to 80% of said fungicide composition, from 80% to 90% of said fungicide composition or from 90% to 100% of said fungicide composition.

51. The method of claim 45, wherein said treating of said liquid culture system occurs more than once.

52. The method of claim 45, wherein said effective concentration is from 0.25 ppm to 15 ppm, or 0.01 ppm to 20 ppm.

53. The method of claim 45, wherein said effective concentration is from 0.1 ppm to 20 ppm, from 0.25 ppm to 2.5 ppm, from 0.25 ppm to 5.0 ppm, from 0.25 ppm to 10 ppm, from 0.25 ppm to 12.5 ppm, from 0.25 ppm to 15 ppm, or from 0.25 ppm to 20 ppm.

54. The method of claim 45, wherein said effective concentration is from 0.5 ppm to 5.0 ppm, from 5 ppm to 10 ppm, from 10 ppm to 20 ppm, from 20 ppm to 50 ppm, or from 50 ppm to about 100 ppm.
55. The method of claim 45, wherein said effective concentration is about 0.5 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, about 5 ppm, about 6 ppm, about 7 ppm, about 8 ppm, about 9 ppm, about 10 ppm, about 11 ppm, about 12 ppm, about 13 ppm, about 14 ppm, about 15 ppm, about 16 ppm, about 17 ppm, about 18 ppm, about 19 ppm, or about 20 ppm.

56. The method of claim 45, further comprising growing said alga for a number of days selected from the group consisting of 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 10 or more, 15 or more, 30 or more, 45 or more, 60 or more, 90 or more, 120 or more, 180 or more, 250 or more, 500 or more, 1000 or more, 1500 or more, and 2000 or more days after inoculation.

57. The method of claim 45, further comprising growing said alga for from 1 to 7 days, from 1 week to 2 weeks, from 2 weeks to 3 weeks, from 3 weeks to 1 month, from 1 month to 3 months, from 3 months to 6 months, from 6 months to 9 months, from 9 months to 12 months, from 12 months to 18 months, from 18 months to 2 years, from 2 years to 3 years, from 3 years to 4 years, from 4 years to 5 years, from 5 years to 6 years, from 6 years to 7 years, from 7 years to 8 years, from 8 years to 9 years or from 9 years and 10 years after inoculation.

58. The method of claim 45, wherein said liquid culture system is an open outdoor liquid culture system.

59. The method of claim 58, wherein said open outdoor liquid culture system has a volume of 10,000 to 20,000 liters, 10,000 to 40,000 liters, 10,000 to 80,000 liters, 10,000 to 100,000 liters, 10,000 to 150,000 liters, 10,000 to 200,000 liters, 10,000 to 250,000 liters, 10,000 to 500,000 liters, 10,000 to 600,000 liters, 10,000 to 1,000,000 liters, 20,000 to 40,000 liters, 20,000 to 80,000 liters, 20,000 to 100,000 liters, 20,000 to 150,000 liters, 20,000 to 200,000 liters, 20,000 to 250,000 liters, 20,000 to 500,000 liters, 20,000 to 600,000 liters, 20,000 to 1,000,000 liters, 40,000 to 80,000 liters, 40,000 to 100,000 liters, 40,000 to 150,000 liters, 40,000 to 200,000 liters, 40,000 to 250,000 liters, 40,000 to 500,000 liters, 40,000 to 600,000 liters, 40,000 to 1,000,000 liters, 80,000 to 100,000 liters, 80,000 to 150,000 liters, 80,000 to 200,000 liters, 80,000 to 250,000 liters, 80,000 to 500,000 liters, 80,000 to 600,000 liters, 80,000 to 1,000,000 liters, 100,000 to 150,000 liters, 100,000 to 200,000 liters, 100,000 to 250,000 liters, 100,000 to 500,000 liters, 100,000 to 600,000 liters, 100,000 to 1,000,000 liters, 200,000 to 250,000 liters, 200,000 to 500,000 liters, 200,000 to 600,000 liters.
liters, 200,000 to 1,000,000 liters, 250,000 to 500,000 liters, 250,000 to 600,000 liters, 250,000 to 1,000,000 liters, 500,000 to 600,000 liters, or 500,000 to 1,000,000 liters.

60. The method of claim 58, wherein said open outdoor liquid culture system has an area selected from the group consisting of at least 0.25 acre, at least 0.5 acre, at least 1.0 acre, at least 1.5 acres, at least 2.0 acres, at least 2.5 acres, at least 5.0 acres, and 7.5 or more acres.

61. The method of claim 58, wherein said open outdoor liquid culture system has an area selected from the group consisting of 0.25 to 0.5 acres, 0.25 to 1.0 acres, 0.25 to 1.5 acres, 0.25 to 2.0 acres, 0.25 to 2.5 acres, 0.25 to 5.0 acres, 0.25 to 7.5 acres, 0.5 to 1.0 acres, 0.5 to 1.5 acres, 0.5 to 2.0 acres, 0.5 to 2.5 acres, 0.5 to 5.0 acres, 0.5 to 7.5 acres, 1.0 to 1.5 acres, 1.0 to 2.0 acres, 1.0 to 2.5 acres, 1.0 to 5.0 acres, 1.0 to 7.5 acres, 2.0 to 2.5 acres, 2.0 to 5.0 acres, 2.0 to 7.5 acres, 2.5 acres to 5.0 acres, 2.5 to 7.5 acres, 7.5 to 10 acres, 10 to 15 acres, 15 to 20 acres, 20 to 25 acres, 25 to 30 acres, 30 to 35 acres, 35 to 40 acres, 40 to 45 acres, 45 to 50 acres, 50 to 75 acres, 75 to 100 acres, 100 to 125 acres, 125 to 150 acres, 150 to 175 acres, 175 to 200 acres, 200 to 250 acres, 250 to 300 acres, 300 to 350 acres, 350 to 400 acres, 400 to 450 acres, and 450 to 500 acres.
FIG. 1
FIG. 2A
B09-W-OM15

FIG. 3A
FIG. 3C
FIG. 3D
FIG. 3E
FIG. 5
FIG. 6A
FIG. 6B
FIG. 6D
FIG. 6F
FIG. 7
FIG. 10
FIG. 13
FIG. 15
FIG. 16

Chytrids
1 ppm dose
A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

Google, pubmed; CAPLUS, MEDLINE, AGRICOLA, EMBASE, BIOSIS via STN; X-FULL via EPOQUE; desmodesmus, microalga, fungicide, herbicide, FRAC29, fluazinam, tolerance, resistance, fungal, contamination, mutation, irradiation, sapphire, Saunders, Hanley, Low, Lee, Yohn, Lopez; and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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[X] 1 Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
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  "&" document member of the same patent family

Date of the actual completion of the international search
24 November 2014

Date of mailing of the international search report
24 November 2014

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End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.