Contents

Testing chemical crop protection treatments

Each year, novel treatment methods are tested on current and new pests. Previous efforts have largely focused on pesticides as chemical treatment options. This year, the scope of chemicals for testing avoided pesticides due to concerns over the ability to use such chemicals in production processes in the near future. At small scale, pesticides were tested for continued data collection on their applicability to algae cultivation in the long term, but the primary goal of research was to discover novel treatments that could potentially be deployed in production processes within the next year.

Researching new methods

A paper study was initiated to list potential chemical treatment options. Patent and research publications as well as internet sources were used for source material. Pesticides were included in this literature review partly for information purposes but also with a view to finding pesticides that could be used for “on-label” applications. Other search criteria included chemicals known to be toxic to expected pests such as rotifers and amoeba, and applications that may transfer to algae cultivation such as water sanitation and swimming pool treatment methods. An initial list of approximately 150 treatments was drafted which was then shortlisted using criteria such as safety, cost, likelihood of success, likelihood of regulatory approval for use etc. A short list of 52 chemicals was the result of this effort. From this list, 17 different chemical treatments (including media components that have shown success against previous pests) were recommended for laboratory testing based on the likelihood of deployment this year. Pesticides that were already in use were also included in this list for testing at lab scale. Depending on the type of chemicals, the eventual use could be either maintenance at a set level in the media or application to the pond as a “dose”. This strategy is based on how pesticides are used in traditional agriculture, in response to pests or conditions favoring pest propagation.

Testing new methods

Laboratory toxicity

The short-listed chemicals were tested for both efficacy against known pests (rotifers and weed algae) as well as toxicity against crop algae. All treatments were also used in laboratory satellite cultures (preview tubes) and against any new pests as they were identified throughout the year (e.g. diatoms, FD111). The following figures summarize some of the results of these studies. Broad ranges of concentrations were tested in 24-well format against *Nannochloropsis* to assess toxicity and to refine the concentration ranges to be used in efficacy testing against pests. Concentration ranges were chosen based on known information for each chemical with the goal to use higher concentrations that would likely be used in the field. This should aid our understanding of what both the toxic level and potential useful dose might be. Cost and regulatory considerations are taken into account during this process but efficacy and toxicity were the main drivers in these early experiments. An example of the type of data generated in this approach is shown in Figure 1.

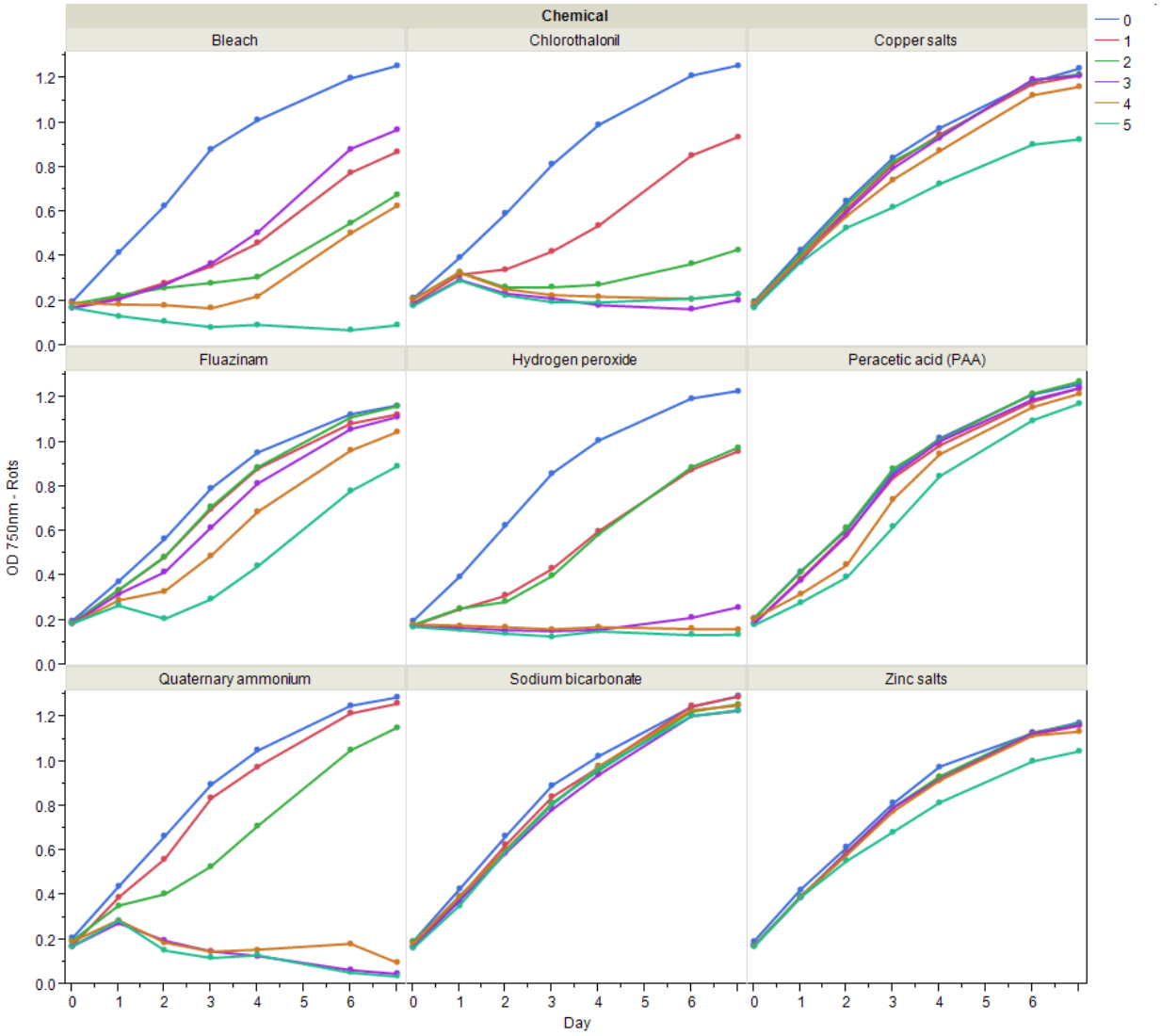

Figure 1. Example of toxicity testing against crop algae. A gradient of each chemical was added to culture in 24-well plates. OD750nm over time is shown for five different concentrations for each chemical (levels not shown). Untreated control is shown in blue (0). From this data, concentrations may be altered for future experiments.

Laboratory efficacy

Once toxicity was tested for each chemical, the concentrations for testing efficacy were refined and used within known current pest models. The panel was tested against rotifers and weed algae. Once this work concluded, promising treatments advanced to field experiments. Example data from testing against weeds and rotifers is shown in Figures 2 and 3. No chemicals were identified that showed a large enough difference between the efficacious dose against the weeds and the toxic concentration to the crop to be considered useful. However, a number of chemicals showed potential for inhibiting weeds at low levels while not significantly affecting *Nannochloropsis* in this system. Further large scale experiments over longer time periods would be required to assess the positive effect of these treatments with respect to the effects against pests vs. the potential negative affect of reducing productivity.

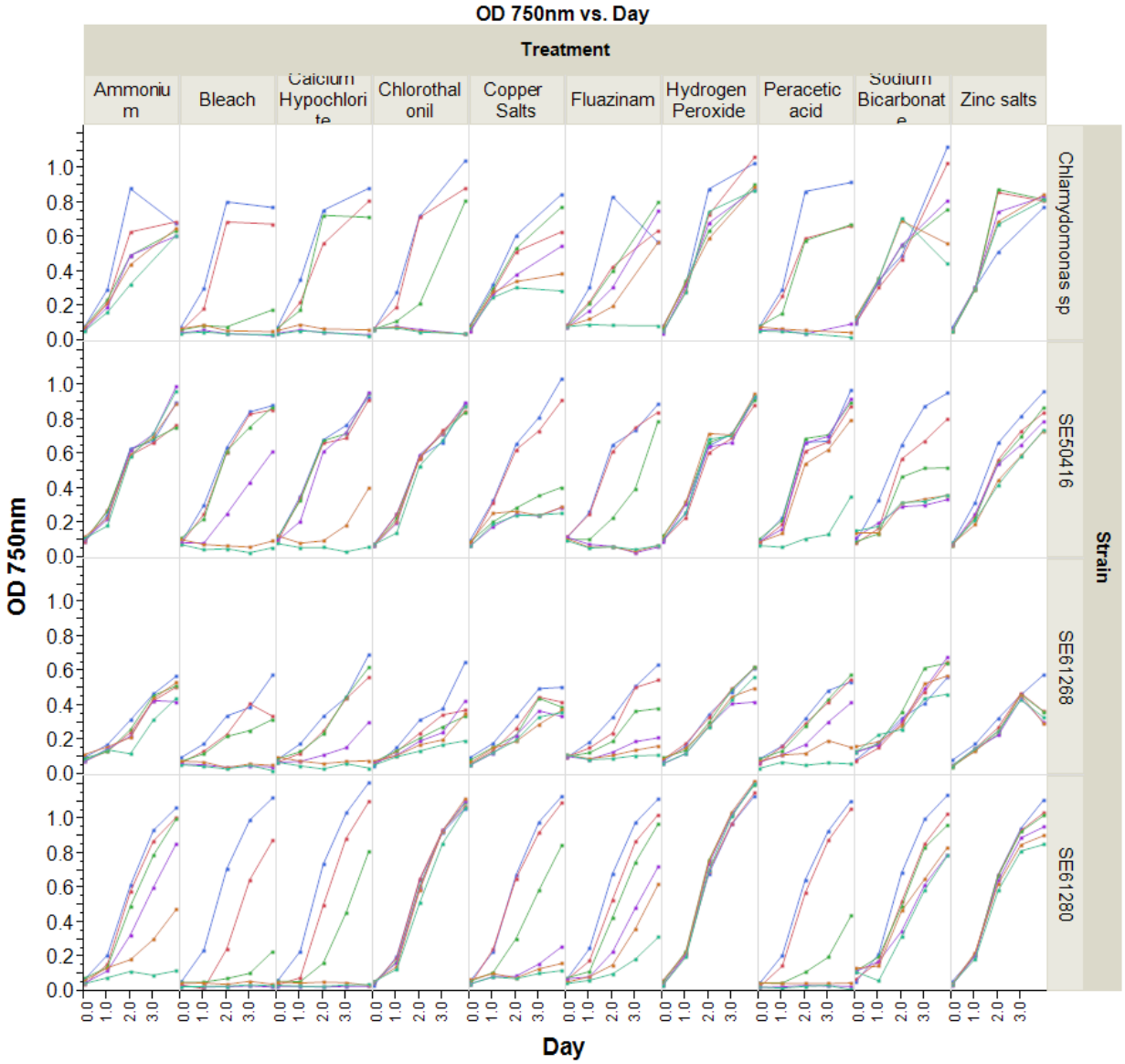

Figure 2. Example data screening efficacy of chemical treatments against four weed strains. Graphs show OD 750nm over time for weed cultures grown in 24-well plate format treated with five different concentrations of chemical. Untreated control cultures are shown in blue.

A number of the chemicals showed efficacy against rotifers at concentrations that did not impact *Nannochloropsis*. Any chemicals that showed a positive effect against pests were retested against pests with refined concentrations to repeat observations prior to choosing treatments to move forward for field testing. As expected with any screening, some false positives were identified in this approach which eventually showed no useful efficacy. An example of secondary screening against rotifers with a refined chemical list is shown in Table 1. below.

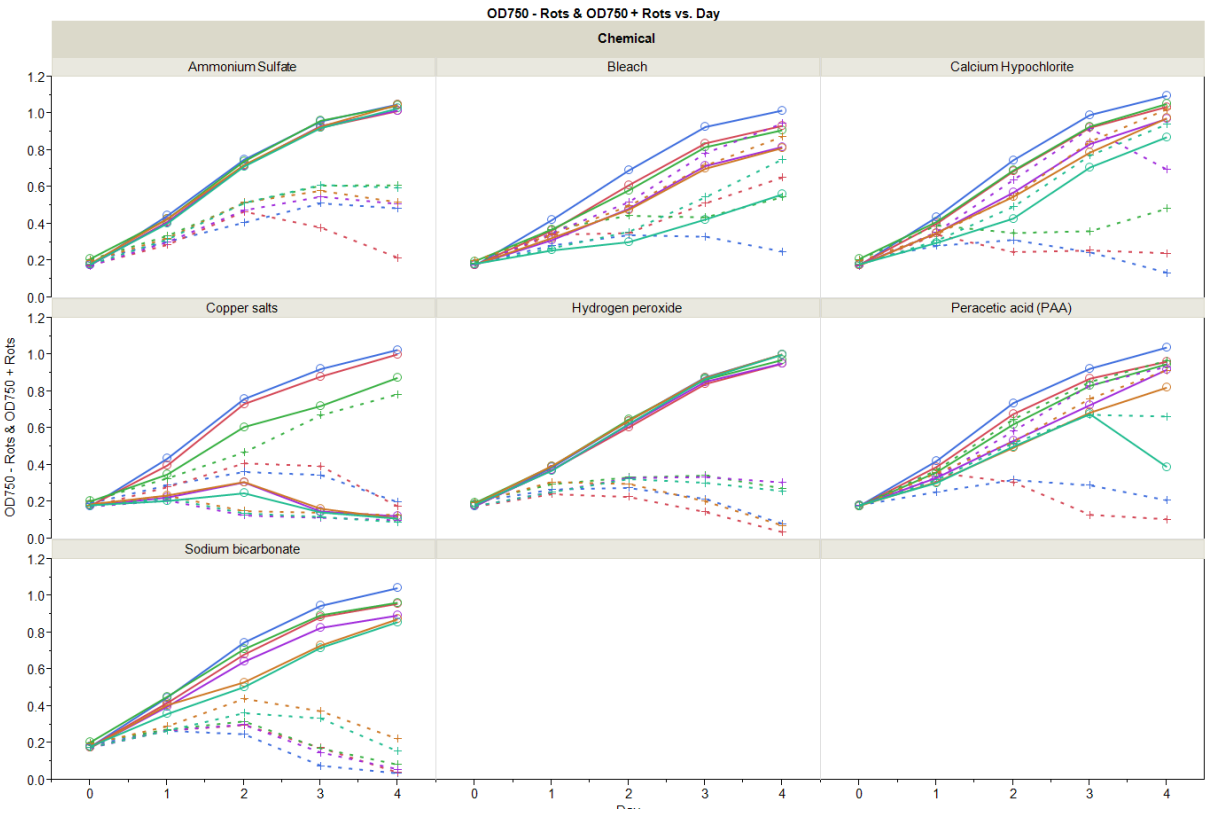

Figure 3. Example of refined efficacy screen against a known pest. Graphs show OD750nm over time for cultures with (dashed lines) or without (solid lines) rotifers. Colors represent different concentrations of chemical treatment. Control with no chemical treatment is shown in blue. In this assay rotifers cause lower and/or eventual decline in OD over time. Effective chemical treatment is shown when dashed lines are similar to solid lines. Toxicity is observed if change in OD is significantly lower than the untreated control (blue).

The most consistently successful treatments against multiple pests identified in this initial testing were oxidizing agents (PAA, H2O2, Bleach, Calcium hypochlorite). Due to this, an additional chemical was added to the screening panel. Trichlor tablets, also known as stabilized chlorine, are a slow release chlorine that also includes cyanuric acid (CYA) which acts as a stabilizer by reducing the breakdown of free chlorine by sunlight. Since this product is only available in sizes for use in swimming pools and the active ingredient should essentially be the same as bleach and calcium hypochlorite (chlorine), testing was initiated at the minipond scale without laboratory scale testing. The table below summarizes all the chemical treatments that were tested this year, the scale tested and what efficacy was observed.

Treatment	**Largest Scale tested**	**Efficacy shown against**
Zinc salts	Lab	Partial efficacy noted at high levels
Copper salts	Minipond	Partial efficacy noted at high levels

Bicarbonate / alkalinity	Lab	None
Nutrient levels (P)	Minipond	None
Nutrient levels (N, Fe, Trace)	Lab	None
TDS (NaCl, plus other salts)	Lab	None
Ammonia	Raceway	Rotifers
pH	Commercial Raceway	FD111 (possible indirect effect)
Bleach (sodium hypochlorite)	Commercial Raceway	Rotifers, FD111/2, weeds
Calcium Hypochlorite	Commercial Raceway	Rotifers, FD111/2
Trichlor / CYA	Raceway	FD111
Hydrogen peroxide	Minipond	FD111, weeds
Quaternary ammonium	Minipond	Rotifers, FD111
Ozone	Lab	None
Peracetic acid (PAA)	Lab	FD111, Rotifers, weeds
Omega 500F (Fluazinam)	Lab	Rotifers, weeds, FD111
Bravo Weatherstik (Chlorothalonil)	Lab	Weeds

Table 1. Summary of treatments tested for crop protection of *Nannochloropsis* cultures in 2015. Scale that treatments were used, and which pests' efficacy was shown for is shown.

Improving methodologies

Using 24-well plates at the Las Cruces site increased throughput and efficiency for a number of experiments. Throughput could be increased further by utilization of 96-well systems used in San Diego. These had not previously been used for testing of crop protection chemicals and thus an experiment was designed to test the utility of such a system for future studies. A selection of chemicals tested in 24-well plate format was used in similar concentration gradients in culture of four different candidate *Nannochloropsis* strains in 96-well plate MGRA. Example data from this study is shown in Figure 4. Results were positive and similar observations were made as in other growth systems. The increased throughput, replication and data handling of this method should improve future crop protection screening efforts.

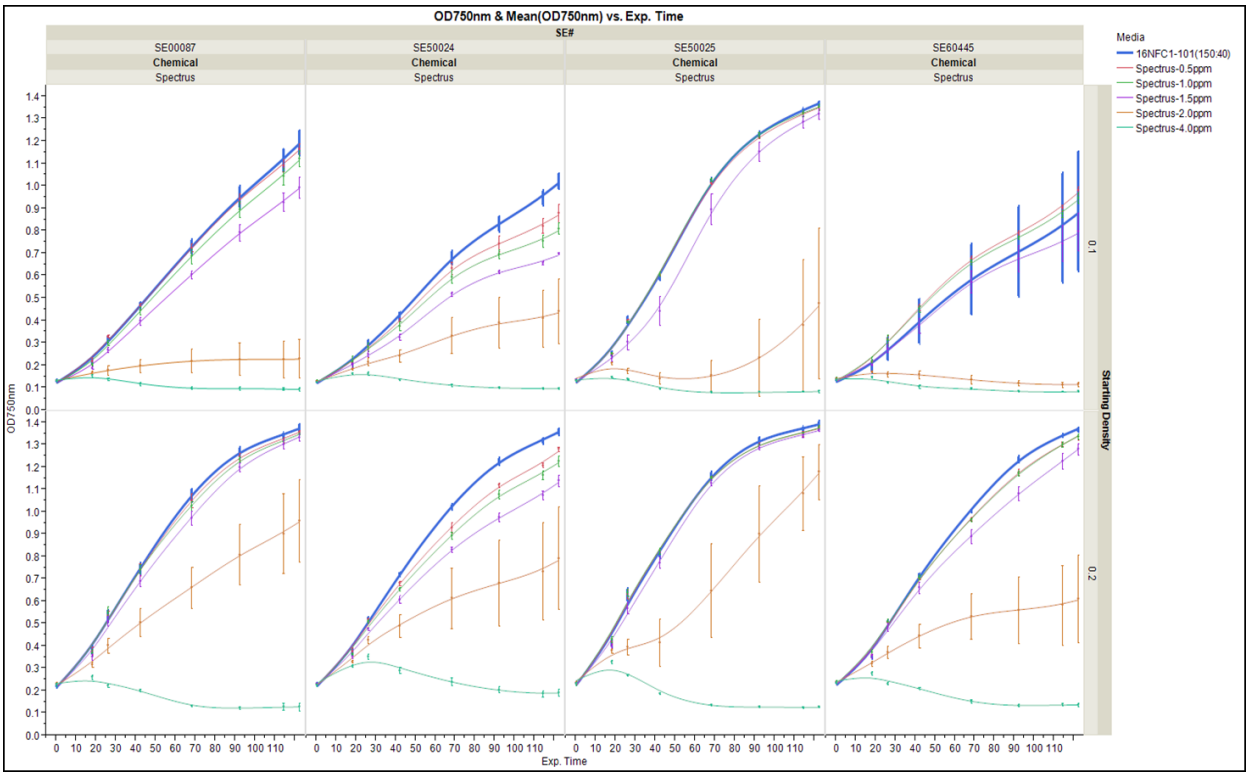

Figure 4. Use of high throughput systems for crop protection testing. Microplate growth rate assay (MGRA) was used for testing chemical toxicity. Example data is shown for four different candidate strains grown with varying amounts of Spectrus CT1300 (Quaternary ammonium) and started at two different densities (0.1 vs. 0.2 OD750nm as shown on right). Graphs show OD 750nm over time. Plates were cultivated at 25°C with 16hr/day light cycle at 150µEi.

Regulatory approval process

In order to maintain compliance with local environmental regulations on the use and application of chemicals, we have established a process taken prior to any testing of chemicals in outdoor ponds. A flow diagram of this process is shown below (Fig. 5 and 6). Once documentation is in place detailing limits of use, field testing can begin.

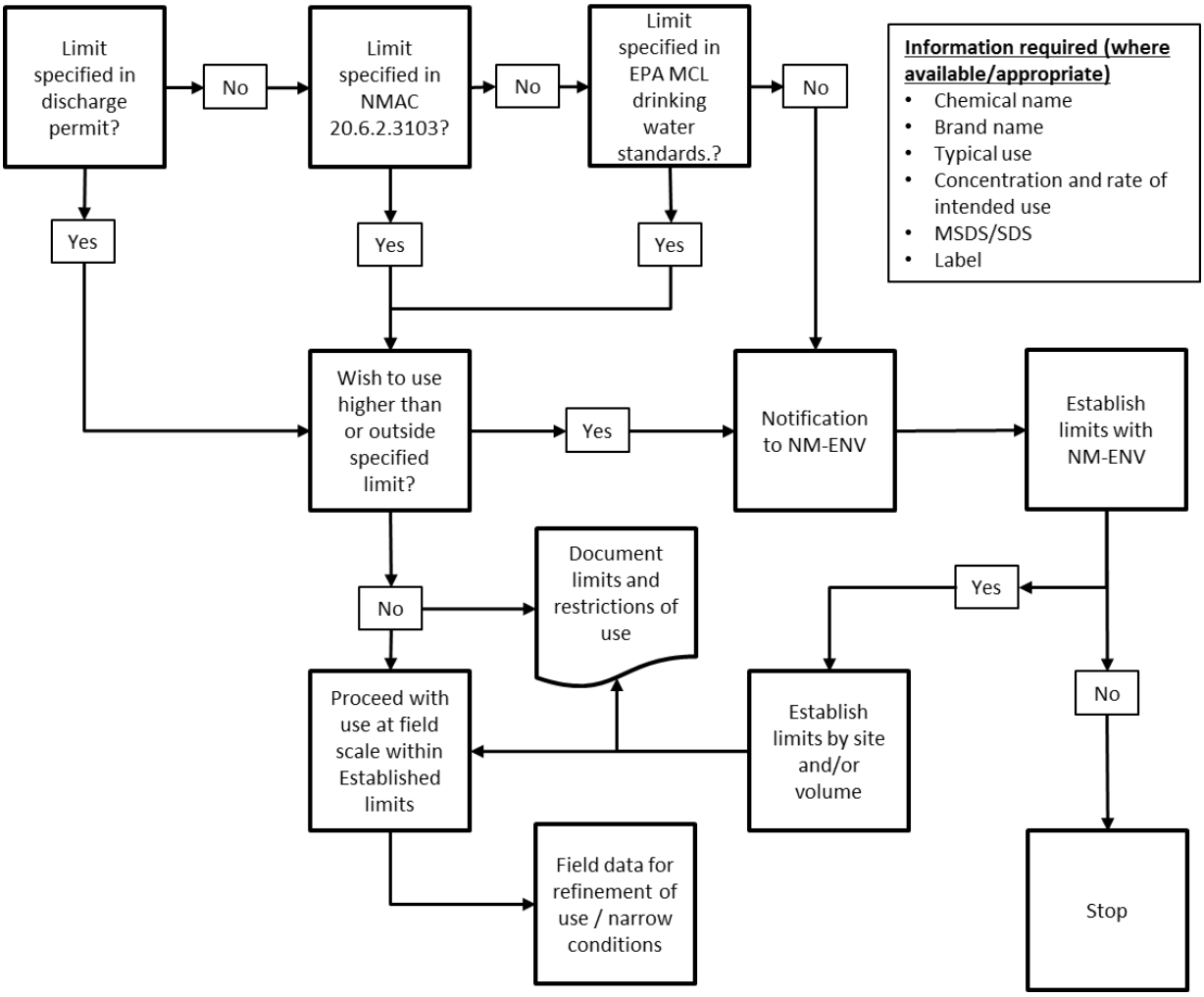

Figure 5. Process prior to outside use of simple chemicals for crop protection.

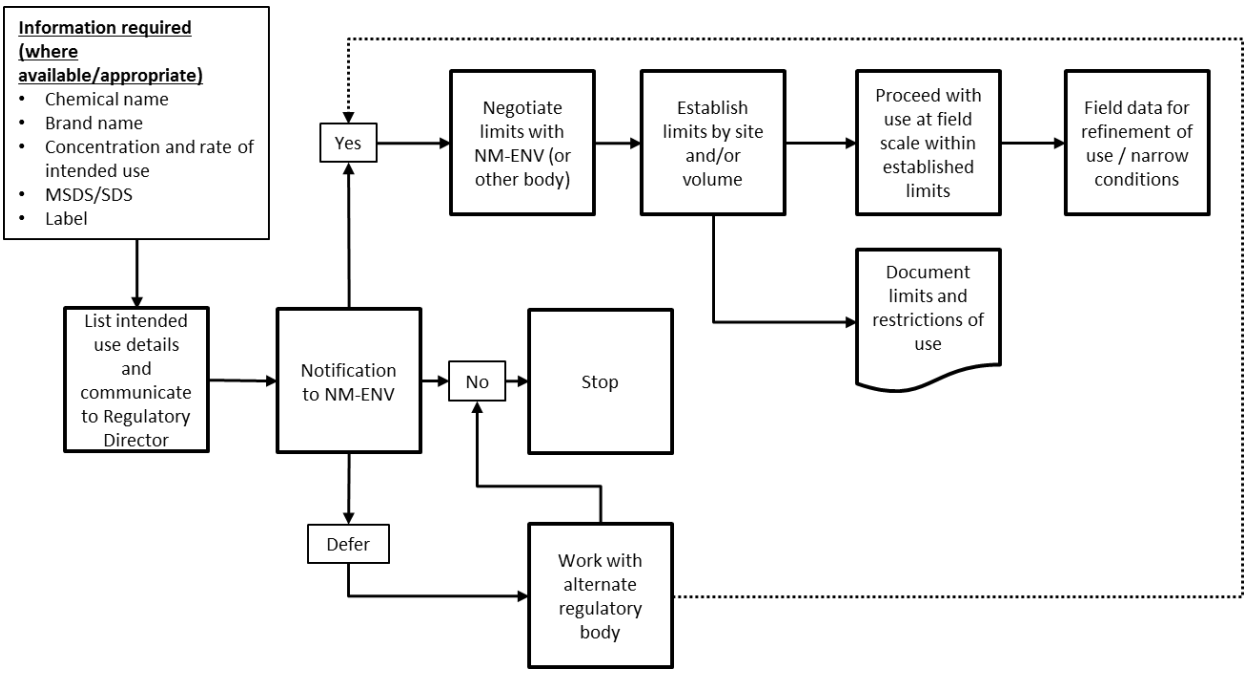

Figure 6. Regulatory approval process for non-simple chemistries. Examples of this kind of chemistry would be pesticides.

Field testing new treatments

Once approval for use outside is confirmed, the first stage of testing any new treatment is at the minipond scale (volume~200-350l). Similar to laboratory experiments, ranges of concentrations are used to establish toxicity effects in the field as compared to the lab. Differences in the field may affect the action of chemicals (light intensity/cycle, temperature and other weather conditions, presence of other organisms and organic/inorganic matter, pH) so it is important to compare the effect to laboratory observations before using chemicals for crop protection at large-scale. As soon as the opportunity arises to use new chemicals against pests, miniponds are also used to test efficacy. This could be either towards the end of a minipond experiment designed for other purposes or with satellite culture from unhealthy raceway cultures to compare the new treatment with the treatment that is applied to the raceway culture.

Minipond testing against known pests: Rotifers.

Treatments that were used this year against rotifers based on laboratory or previous experience were Chlorine (bleach, calcium hypochlorite, trichlor), Quaternary Ammonium (Spectrus CT3100), Ammonia, and Fluazinam (Omega 500F). PAA was also successful at laboratory scale but regulatory approval for using this chemical was not agreed at the time.

Growth of rotifers can be prevented by the addition of ammonia to the culture media. This is known in the field and has been an approach taken in previous algal cultures at Sapphire. Efficacy of ammonium sulfate was shown in the laboratory and thus was tested in miniponds prior to use at larger scale. As shown in Figure 7, Ammonium sulfate successfully prevented the propagation of rotifers in *Nannochloropsis* culture. However, despite the successful treatment, inhibition of *Nannochloropsis* growth was also observed (not shown). In separate strain selection experiments, the sensitivity to ammonia of different *Nannochloropsis* strains was observed to be variable and thus this approach for

crop protection may be suitable for alternative strains. The efficacy of ammonia is strongly affected by pH due to the equilibrium between ammonia and ammonium and thus the choice of this treatment against rotifers requires consideration of culture pH setpoints. Bleach, which is also affected by pH though to a lower degree, successfully prevented rotifer growth in minipond experiments when used at greater than 2ppm and this treatment was chosen for further use at larger scale.

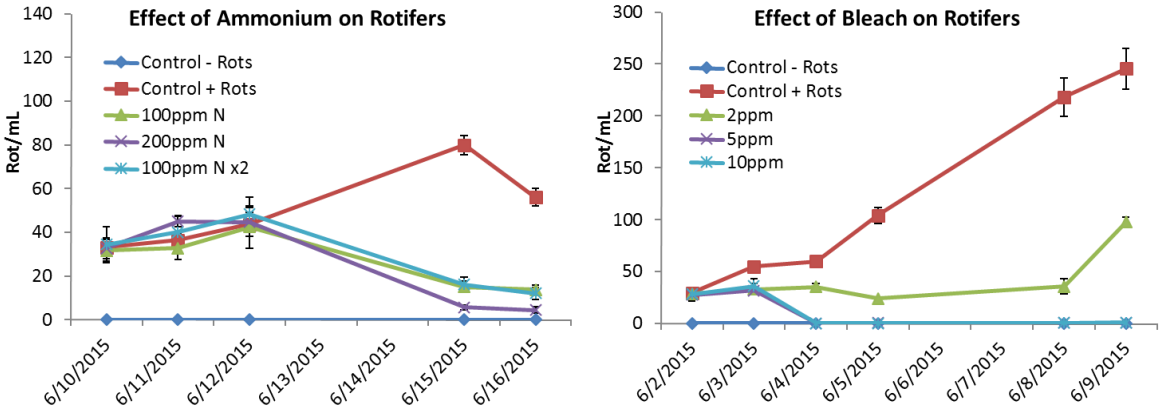

Figure 7. Effect of ammonia and bleach dosing on rotifer level. Rotifer/ml over time is shown. *Nannochloropsis* was cultivated in miniponds and infected with rotifers at approximately 30/ml. Left panel: Ammonium sulfate was added to the ponds at the levels of ppm N equivalent shown. Dosing was applied on 6/10 and then again on 6/12 for the 100ppm N x2 treatment. The drop in rotifer number in the last datapoint for the + rotifer control is a result of the rotifers depleting the food source (algae) in the culture by this time. Right panel: A single dose of bleach was added to the ponds at the levels shown.

Alternative chemicals can be used to add chlorine to the ponds. One of these alternatives is calcium hypochlorite which was shown to treat rotifers at lab scale. The advantages of calcium hypochlorite over sodium hypochlorite (bleach) is stability of the compound during storage. It is also available in powder form which may be advantageous for chemical handling in some situations. Over the course of the summer testing period, calcium hypochlorite was directly compared to bleach. Calcium hypochlorite was consistently more effective than bleach at treating rotifer infection when used at similar concentrations. It is currently unclear why this effect was observed since the active ingredient for each chemical is the same (chlorine). It is possible that the different chemical nature of the two products is impacting the efficacy. Bleach and Calcium hypochlorite were both effectively used at larger scale raceway and production pond scale for crop protection against rotifers and other pests in 2015.

Quaternary ammonium was also successfully used against rotifers at minipond scale. The specific chemical product used for these experiments was approved for use under an experimental use permit from the New Mexico Department of Agriculture (Spectrus CT3100). Since this permit does not cover use at production scale, no further testing was completed.

Minipond testing against new pests:

As expected, novel pest phenotypes were observed during field trials of *Nannochloropsis* strains. As described in sections x and y this represents an opportunity for testing new crop protection treatments, in both the laboratory and field. An example of this is shown earlier in section x for treatment of weed species that appeared in early outdoor cultures (*Amphora* diatoms and *Tetraselmis*). An example of using satellite culture in miniponds for testing new or alternative chemicals for treatment (after successful laboratory testing) for the new pest FD111 is shown below (Fig. 8). One treatment that was

shown to be successful in lab scale experiments (H2O2) was tested alongside current effective treatments (bleach) and an alternative chlorine product (trichlor). In this experiment both trichlor and hydrogen peroxide were successful at preventing culture crash (Figure x.). Some toxicity towards the crop strain was observed with both chemicals. The toxicity observed with trichlor was likely due to the size (and thus dose) of the tablet used and so further experimentation with this chemical proceeded in minipond and larger cultures. Despite showing efficacy, the toxicity of hydrogen peroxide at close to effective doses along with other factors such as cost and storage considerations meant that no further testing was completed. This experiment also highlights the power of the miniponds as satellite culture since the data for the control miniponds closely matches that of the parent raceway pond culture. The untreated control crashed within a couple of days of initiating the experiment, thus also showing that the dose applied to the raceway was effective and necessary.

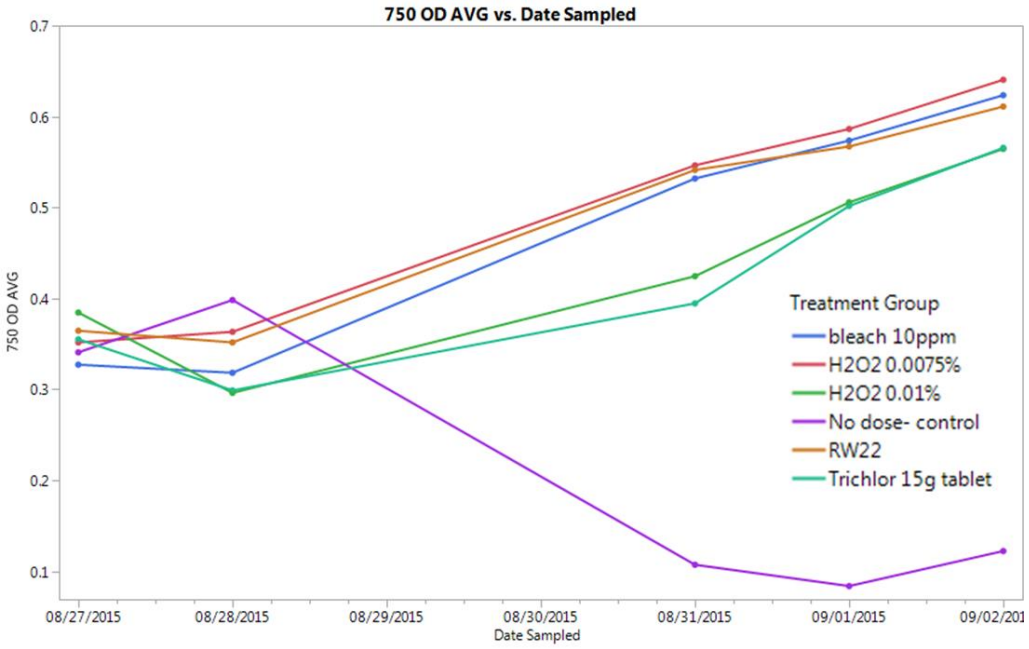

Figure 8. Field testing of New chemicals against new pests. Graph shows OD750nm over time for minipond culture of *Nannochloropsis*. Culture from unhealthy raceway culture was cultivated in miniponds and dosed with different chemicals as shown. Culture contained pest FD111. Data from the source raceway is also shown (RW22).

Improving crop protection in the future

The primary goal of screening chemicals is to identify and move forward with successful treatments to get them into use as quickly as possible. A good example this year is bleach and calcium hypochlorite that were used successfully at all scales of cultivation, allowing maintenance of active algae culture throughout the year. However, when moving through a screening process in this way, some treatments that show marginal or inconsistent success tend to not advance beyond initial field testing. A number of chemicals showed potential efficacy but were not used at or beyond minipond trials. This could be because of regulatory issues that may change in the future or due to inconsistent data. These treatments continue to be used at laboratory scale since they may become useful at a later date, for example should regulatory issues change, or cultivation conditions change. Copper is one example of this situation that was encountered this year. In multiple experiments at laboratory and minipond scale, cultures containing extra added copper were observed to take longer to crash. Since cultures still

crashed, the treatments were not immediately advanced for further testing. Further experimentation of copper addition in combination with water chemistry changes and/or changing environmental factors could improve our understanding of the delayed culture crash.

Alternatively, a small change in culture health and longevity induced by a treatment may prove financially beneficial if it reduces the number of other chemical doses required to maintain healthy ponds. A similar observation as described above was also observed for pH and paddle wheel schedule. On multiple occasions, these variables were observed to affect culture health in the presence of pests. One example of this is shown in Figure 9 (from minipond experiment 0183-II). Miniponds were inoculated from raceway cultures that were known to contain pests and were expected to crash. Ponds cultured at pH8 crashed immediately while ponds cultured at pH7 showed a delayed crash phenotype. The other variable in the experiment was paddle wheel schedule. Ponds at both pH set points appeared to crash faster with 24 hour mixing as compared to ponds that were only mixed for 14 hours during the daytime, which stayed green longer and also had reduced weed levels.

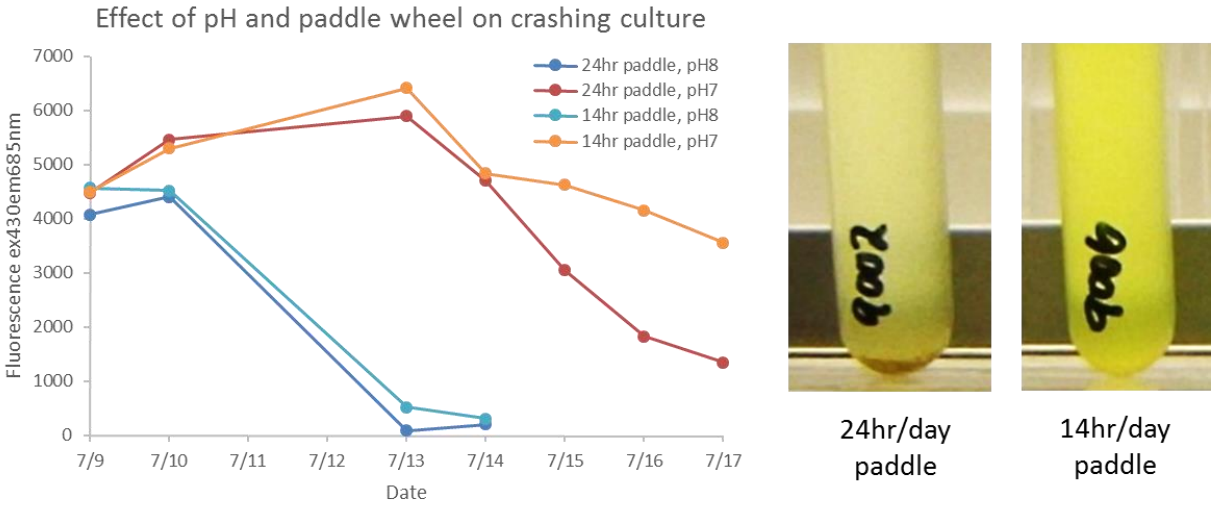

Figure 9. Effect of pH and paddle wheel schedule on pest crash. Graph shows fluorescence over time for miniponds cultivated at different pH setpoints and with different paddle wheel schedules. “14hr paddle” ponds were mixed by paddle wheel from 6am until 8pm each day. Pictures on the right show culture appearance on 7/16/15 for the ponds at pH7 with different paddle wheel schedules. The pond with 24-hour mixing appears less green, less dense, and contains more settled debris (dark matter settled at bottom of tube). Similar visual observations were made during the health decline of the pH8 ponds as well as for other ponds in similar experiments.

As mentioned above with the low efficacy of various chemicals against weed strains, these types of subtle improvements cannot be fully evaluated at small scale. Thus, these treatments remain in use at smaller scale, for example in satellite culture experiments, and may be tested at larger scale in the future should efficacy against new pests be observed or if cost benefit calculations indicate potential utility at scale.

Scheduled dosing

Once efficacy and usefulness of a new crop protection method or chemical is established in miniponds the treatment is used in raceway culture and, if successful, will eventually be incorporated into the established crop protection schedule in experimental raceway and production ponds. Treatment

practices will then be refined in later experiments which may again use small scale miniponds. For example, the use of scheduled dosing as an alternative to dosing in response to the presence of a pest was investigated this year.

A schedule for dosing was decided based on historical data and environmental conditions that are likely conducive to pest pressure. Two such experiments were completed this year with the chlorine chemicals bleach and calcium hypochlorite. The first experiment investigated the effect of different schedules of bleach dosing ranging from a total dose per week of 10-20ppm spread between 1-5 applications. This scope represents a comparison of costs (amount of product used, and labor costs to apply) and assesses the effect on crash prevention and/or productivity. As shown in Figure 10, all dose schedules were effective at preventing the culture from crashing since the untreated control culture died within the first week of the experiment. Some toxicity was observed if the highest doses were applied when the ponds were at the lowest density. Treatment groups with frequent low doses (2ppm 5x/week, 5ppm 3x/week) were the most productive and healthy but this was not borne out by statistical analysis. Despite difficulty separating treatment groups within this experiment statistically, it is clear that an opportunity to improve dosing practices, while possibly reducing costs, exists and these results will be incorporated into research plans towards the future milestones “Refining crop protection tools – improved best practices cultivation and crop protection”

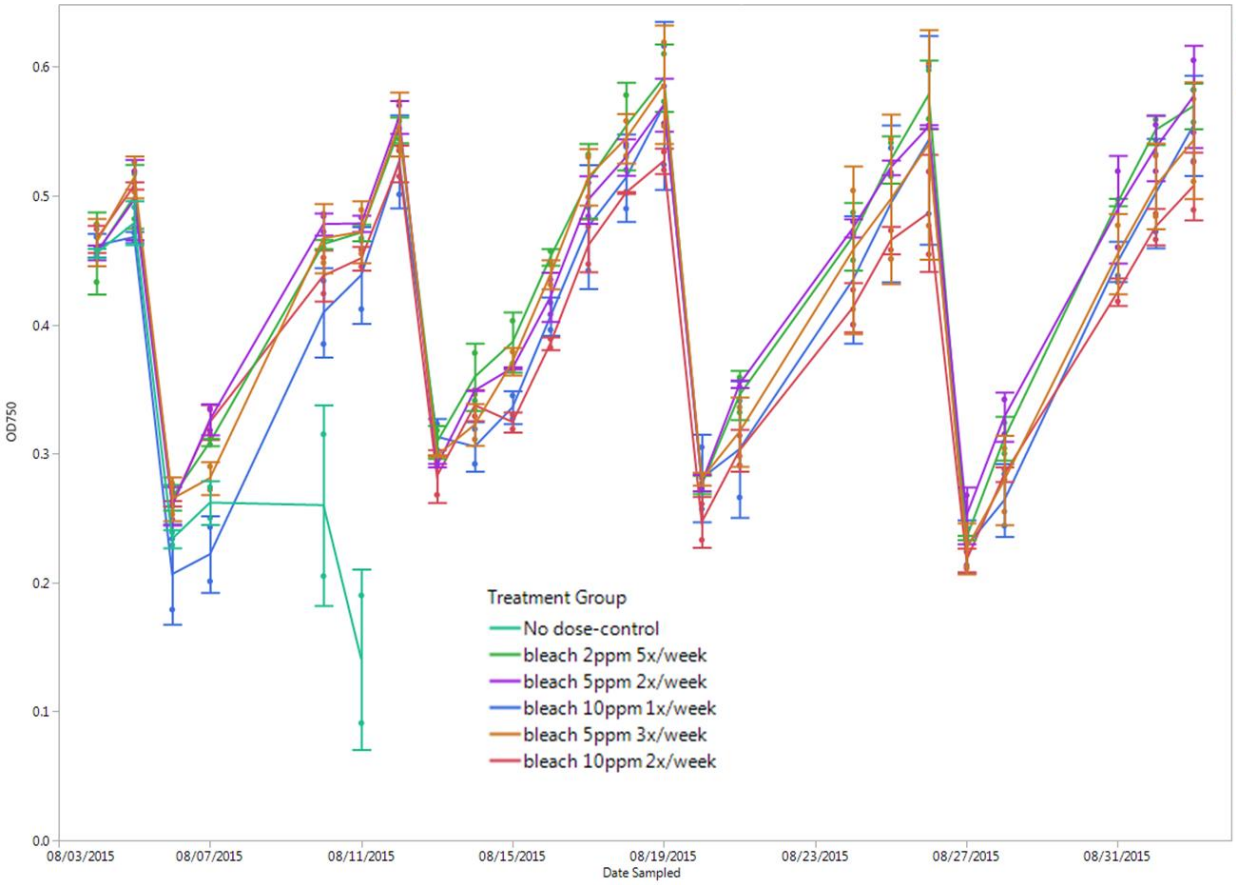

Figure 10. Scheduled dosing of bleach for crop protection of *Nannochloropsis*. Graph shows OD over time for minipond cultures of *Nannochloropsis* inoculated from raceway culture and dosed with bleach at the concentration and frequency shown. The no dose control died within the first week and data collection ceased (green line).

After treatment for a particular pest it is typical for the pest to eventually return. This could be due to the dose merely reducing the pest population below detection thresholds rather than killing all pests, or due to the presence of pest life stages that are resistant to treatment, for example cysts or spores. A second scheduled dosing experiment was used to assess options for removing rotifers and then preventing their return. This experiment was divided into multiple phases. In phase one, miniponds infected with rotifers were treated with chlorine via either bleach or calcium hypochlorite until rotifer numbers were reduced below detection (<1/10ml). In phase two the ponds were treated with chlorine again on a schedule and the return of rotifer population was monitored. As shown in Figure 11, rotifers returned faster in ponds treated with bleach as compared to calcium hypochlorite (compare bleach, no schedule to calcium hypochlorite, no schedule). Rotifers were not observed in ponds treated with a schedule of calcium hypochlorite. A difference in rotifer return was observed between schedules for bleach where rotifer numbers increased the most in ponds dosed on schedule 2ppm 5x/week. This experiment highlights the need for more detailed understanding of pest life cycles, sensitive pest monitoring in the ponds, and further experimentation on improving best practices for use of crop protection methods. It again also highlights the need for long-term, large-scale experiments to answer these questions (i.e., how long would it take for rotifers to return in the calcium hypochlorite ponds or would they remain rotifer free?).

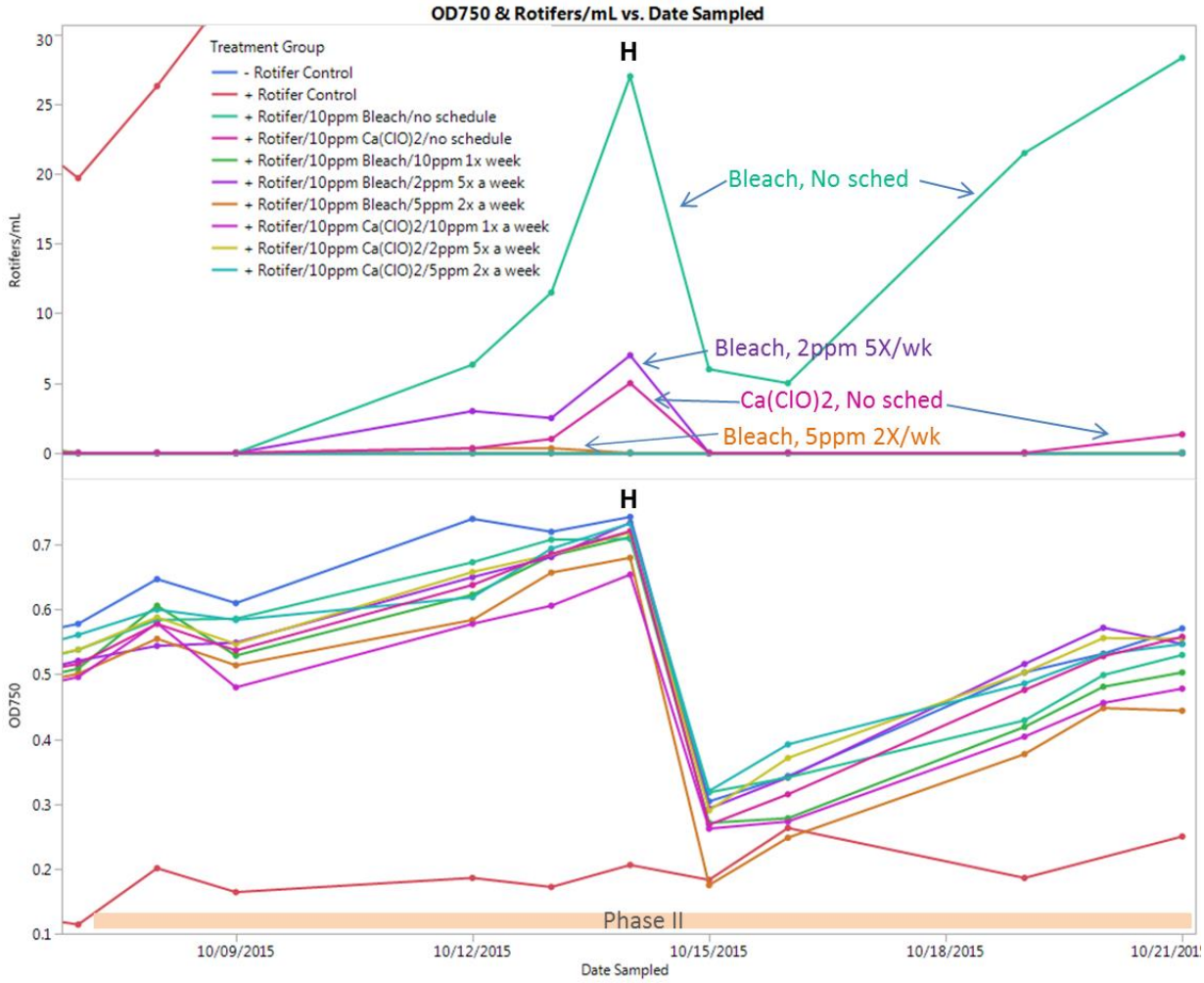

Figure 11. Effect of scheduled dosing on the return of pests after treatment. Miniponds were infected with rotifers and allowed to get to high levels (>100/ml) before initiating treatment with either 10ppm bleach or calcium hypochlorite until rotifers were below detection (<1/10ml). After this, scheduled dosing of either chemical was applied (none, 2ppm 5x/wk, 5ppm 2x/wk, 10ppm 1x/wk). Rotifers/ml over time is shown in upper graph (scale adjusted to highlight schedule dosed ponds). Growth of culture is shown by OD750nm over time in lower graph.

Density effects

In previous years it was noted that efficacy (and toxicity) of certain chemicals was affected by the density of the culture. One likely reason for this is the presence of increased organic and inorganic matter to react with or inhibit the chemical at higher densities. This was again observed this year, in particular with the oxidizing agents. An example of this is shown below. An example of how this observation might be applied in future experiments to refine use, may be to only apply crop protection when the culture density is low e.g. after harvest, or using culture density as a factor in calculating the applied dose. This could result in better efficacy and a lower use of chemicals over time, thus also reducing cultivation costs. Experimentation to develop these approaches is planned as part of work towards future milestones. This will also integrate with other sections of the proposed work for the grant e.g. agronomic practices.

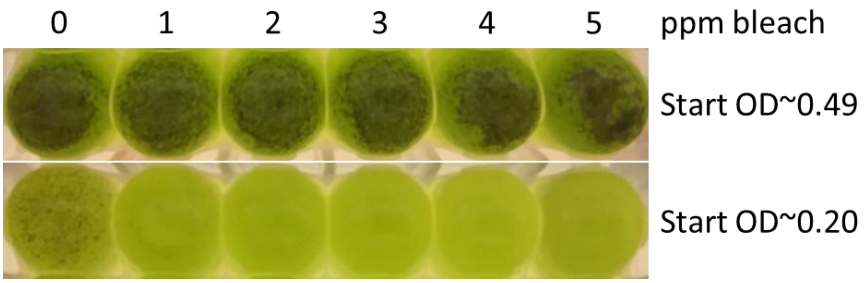

Figure 12. Effect of culture density on efficacy of bleach for crop protection. A culture containing *Nannochloropsis* and diatoms was treated with different levels of bleach at two different starting culture densities as shown. Presence of diatoms is shown by dark particles at the bottom of the wells. Bleach was ineffective at preventing diatom growth at the higher starting density whereas little to no diatoms are observed in any of the treatments started at the lower density.