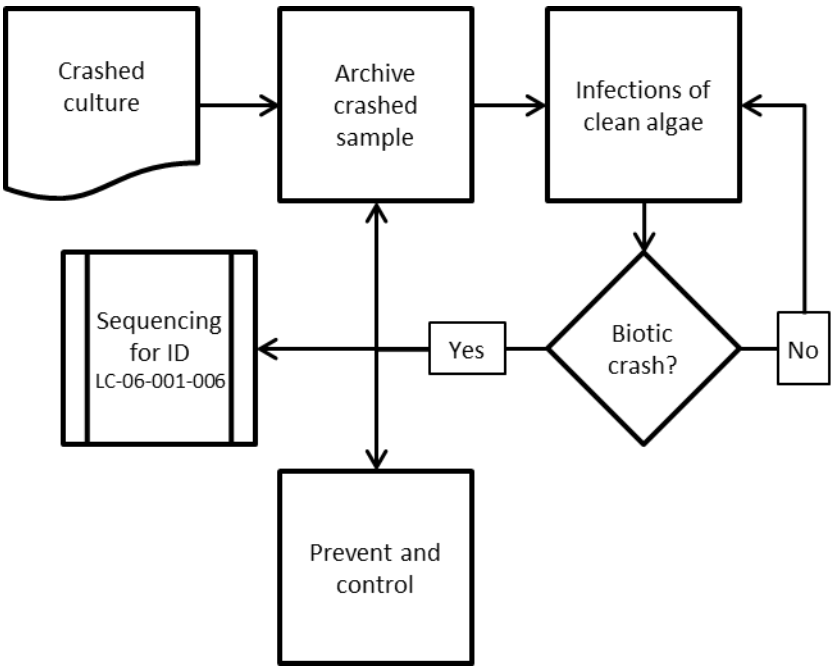
Biotic Review

This SOP describes the procedures that can be used to determine the cause of a culture crash. These activities form part of the after-action review (AAR) process which is typically under the purview of the lab. Experiments described herein should enable the user to determine if a culture crash was caused by a biotic factor. Data will inform experiments that may identify the detrimental organism, archive the infection source for further study, and identify measures that can be used to prevent future crashes caused by the same organism.

Flow Diagram



Process description

Crashed culture

Obtain a sample of the culture as soon as possible after a crash or process interruption has occurred, or, preferably, when observation suggests an interruption in the near future. It is far preferable that sampling occur as soon as a decline of culture health is documented in order to maximize the chances of isolating and studying the potential cause.

Archive crashed sample

Physical archive

1. Freeze multiple 1mL aliquots (at least 5 each) of culture at -80C under three conditions:
    a. 7% DMSO
    b. 25% Glycerol
    c. Untreated

2. Label vials and box appropriately and catalogue information in electronic or hard copy to include at least the following information:
    a. Crash Date/Time
    b. Freeze Date
    c. Source (e.g., pond, predose flask, lab crash etc.)
    d. Pond/culture ID
    e. Strain
    f. Media conditions
    g. Location in freezer
    h. Method of cryopreservation
    i. Suspected pest type or other phenotype observations
    j. Link to microscope pictures and other data
    k. Available/Used?

Data archive

As much information as possible should be recorded from the crash source. This may help determine what types of laboratory experiments will be run and what type of data should be taken, as well as starting to build an expected phenotype of the crash.

1. **Microscope observations**
   Save multiple pictures of infection source at all relevant magnifications
2. **Flowcam**
   Save 1000+ images via flowcam (if available)
3. **Photograph**
   Take photographs of samples. Some crash phenotypes can look quite different to others i.e., does the culture/media/biomass turn a different color? Does it clear out or remain opaque? How long does this take? Photographs of culture in small test tubes or falcon tubes with backlighting tend to give good pictures. Photos of crashing ponds may also prove useful.
4. **Cultivation data**
   Review Incident Report. Can anything be seen in the data that might help you determine the type of data to take in the laboratory experiments? E.g., Is a change observed in Fm but not in OD or fluorescence? Such observations could also aid early detection prior to pest ID and development of tracking tools.

Infections of clean algae

Infections in liquid media

The table below summarizes the different types of conditions that could be used to help determine the cause of a culture crash. Not all conditions would be used in every situation. The user should determine the best test conditions based on the information available at the time through data review and consultation with experienced employees observing the process interruption. This type of experimentation is often an iterative process; alternative conditions may be tested after gathering initial

data on a smaller set of conditions (e.g., one might first ask if a crash is even able to be propagated in the laboratory and then use this as an infection source for further studies).

Conditions	Testing for…
Uninfected	Control
v/v reinfection*	Is an agent present that can cause crash?
Treatments to infection source or infected cultures (typically in combination with 1% v/v infection):	
Boiled*	Should destroy an infectious (biotic) pest and prevent a crash
Filter*	Pest is smaller than pore size chosen , may preserve effects of toxins or allelopathic agents
Pesticides	Biotic? Potential treatments
Antibiotics	Biotic? Potential treatments, may give some information if prokaryotic
Transfer cells to fresh media	Toxicity or nutrient deficiency effects
Lab cells into source media	Abiotic (depending on treatment of media)
Add nutrient components	Nutritional status / treatment
Add nutrient mixes	Nutritional status / treatment
Add / drop-out nutrient components	Nutritional status / treatment
Environment conditions	Seasonality
Shaking	Pest propagation (some pests will not propagate under certain conditions)
Temperature	Pest propagation (some pests will not propagate under certain conditions)
Vessel type	Pest propagation (some pests will not propagate under certain conditions)
Light intensity/regime	Pest propagation (some pests will not propagate under certain conditions)

* Almost always included in the initial biotic review

Standard re-infections for Biotic review

The goals of the biotic review are:

1. Determine if the causative agent of the culture crash is likely biotic or not.
2. Gather preliminary data to guide next steps.
3. Establish if the phenotype observed in outdoor culture can be recapitulated in the lab.

A sample of unhealthy or crashed culture is diluted into clean laboratory culture and cultivated under standard conditions. Cultures are observed for appearance of the crash phenotype over 5-7 days. Typical lab infections use 1% v/v of infection source into clean laboratory culture of the host strain in the appropriate media. Higher levels of infection may be used and should result in a faster crash if the cause is biotic. A gradient of infection may be used (0.01, 0.1, 1.0, 10.0% v/v) to observe a gradient of crash speed. The maximum infection rate typically used is 10% v/v. At higher infection rates, using pond culture (especially unhealthy pond culture) can lead to large amounts of non-pest organisms obscuring the data (e.g. bacterial blooms).

The minimum conditions tested should include the following:

1. Control
2. 1% v/v infection
3. 1% v/v infection (boiled infection source)
4. 1% v/v infection (filtered infection source 0.22um)
5. 1% v/v infection (filtered infection source 0.45um)
6. [optional] 0.01% v/v infection
7. [optional] 0.1% v/v infection
8. [optional 10% v/v infection

Steps

1. Gather supplies need to complete the task.
2. Where gloves when handling pond samples
3. All steps with open culture containers must be done in a laminar flow hood or equivalent biosafety cabinet.
4. Transfer materials required to the flow hood / designated work area (See Laminar Flow Hood Use SOP LC-01-001-018 for further details on appropriate use of the hood).
5. Clean and set up your work area in such a way as to prevent the need to move hands or equipment above open containers.
6. Boil infection source (see separate procedure)
7. Filter infection source into clean vessel (see separate procedure)
8. Dilute laboratory culture to desired OD in appropriate media.
9. Aliquot culture into culture vessel, labeling or taking note of sample layout.
10. Add infection source to corresponding wells
11. Place culture in appropriate growth chamber to mix
12. Take initial data point
13. Take data at determined intervals. Minimal data collection is OD750nm and Fluorescence (ex480/em685) at ~24 hour intervals with frequent macro/microscopic phenotype examination (with saved images). Choice of additional data to collect will be determined by the expected crash phenotype based on field observations during original health trigger.

Typically, the density of lab culture used in re-infections is approximately 0.1-0.2 OD (750nm, measured in 200ul 96-well plate reader). The ideal culture density (and method of measuring) will depend on the strain being cultivated and the type of pest / cause of crash. Some pests are affected by culture density and a crash may be prevented if the wrong density is used. If no crash is observed, a gradient of starting densities should be used.

Some treatments will call for using the source culture itself without passaging as an infection source. This would typically happen when there is still algae present in the sample and the user suspects poor health, lack of nutrients, or some kind of abiotic toxicity, rather than when a sample has completely crashed and very little algae is left in the sample.

Typically, OD and fluorescence are measured on a daily basis during cultivation of infected cultures. PAM can also be useful (especially if the phenotype observed in source culture was observed in PAM data). The type of data collected, and culture conditions will be determined by the user and should match as closely as possible to the conditions where the problem was initially observed.

Microscope and photographic observations should also be made throughout experimentation, especially for any cultures demonstrating phenotype different from control.

On certain occasions, qPCR with the entire set of available primers will be used to determine if a known chytrid or closely related species is the cause, even if this particular host isn't thought to be susceptible. Usually this is inspired by scope observations (sporangia, zoospores or "chytrid death spot")

Treatments to infection source or infected cultures

Boiling

Boiling the sample for 10minutes should kill any pests or other biotic factors present in the sample and thus prevent an infection when added to healthy culture. Thus, if a reduction in health is observed after infection with a boiled sample it indicates that the causative agent may be chemical (abiotic) in nature.

Samples are typically "boiled" in PCR tubes using a 10minute cycle at 95C, or in Eppendorf tubes in a heat block.

Boiling samples with a PCR machine

1. Gather supplies required: Gloves, infected sample, sterile collection vessel (dependent on volume boiling but e.g., sterile microfuge), tube rack, pipette and tips, PCR plate, PCR plate seals, 70% Ethanol spray bottle, paper towels.
2. Refer to SOP LC-01-001-018 for proper set up and use of the hood.
3. Aliquot sample to be boiled by pipette into wells of a PCR plate. Add 100-200ul of sample per well. When working with unknown organisms it is advisable to use filter tips.
4. Seal PCR plate by removing backing from seal and placing adhesive surface over the wells. (If you plan to freeze the plate later, use a foil seal or replace seal with a foil seal prior to freezing as plastic seals are not suitable for freezing.)
5. Apply pressure with fingers or a soft rubber roller to complete contact with each well and the adhesive seal.
6. Place plate in PCR machine.
7. Start a program that will hold the sample at 95-98°C for 10 minutes, and that uses the heated lid setting.
8. Remove plate from PCR machine and transfer to the flow hood.
9. Label a sterile collection vessel of appropriate volume to contain the boiled sample.
10. Carefully remove seal from plate by gently peeling back the seal.
11. Collect boiled samples by pipette and transfer into collection vessel.
12. Close collection vessel and discard PCR plate in appropriate trash receptacle.
13. Clean work area for the next person.

Boiling samples with a Heat block

1. Gather supplies required: Gloves, infected sample, sterile microfuge tubes, tube rack, 70% Ethanol spray bottle, paper towels, pipette and tips, squirt water bottle, heat block with thermometer.
2. Refer to SOP LC-01-001-018 for proper set up and use of the hood.
3. Turn on the heat block and set to 100°C. Ensure a thermometer is in place.
4. Add water to wells using the squirt bottle. Fill the wells approximate half full so that when a tube is inserted the water surrounds the tube but does not spill over.
5. Also add water to the hole that the thermometer is in.
6. Aliquot sample to be boiled by pipette into microfuge tube(s). When working with unknown organisms it is advisable to use filter tips.
7. If the tubes do not have lids that prevent opening during incubation (e.g., safelock lids) then close lids with appropriate covers to prevent lids from opening during boiling step (e.g. Sherlock tube closures http://www.usascientific.com/Sherlock-tube-closures.aspx).
8. Ensure temperature of heat block is at >95°C.
9. Place tubes in heat block wells containing water.
10. Incubate for 10 minutes.
11. Carefully remove tubes from heat block and allow to cool before use. If tubes are not cooled, hoe liquids may spray out when opened.
12. Tubes may be spun briefly in a microcentrifuge to settle any condensation down the tubes.
13. Turn off heat block.
14. Clean work area for the next person.

Filtering

Infections sources can be filtered to help determine the size and/or nature of pests present. If a crash is observed from a filtered infection source, then a pest that is smaller than the pore size utilized is suspected as a causal agent.

Syringe filters of varying sizes can be used to filter the infection source prior to being used to infect culture. Typical filter sizes used are 0.22µm and 0.45µm. Care should be taken to note the material of the filter. Typically, PVDF membranes are used since pests may bind to cellulose-based filters.

Consider the size of the crop algae when determining the range of filter sizes to use.

Procedure for using a syringe filter to filter pest samples

1. Gather supplies required: Gloves, infected sample (in a suitable container that syringe will easily fit into to reach the sample), sterile syringe, sterile filter, sterile collection vessel (dependent on volume filtering but e.g. sterile microfuge tube, 15/50ml falcon tubes etc.), tube rack, 70% Ethanol spray bottle, paper towels.
2. Always wear gloves when working with environmental samples containing unknown organisms.
3. Clean work area. Refer to SOP LC-01-001-018 for proper use of the flow hood.
4. Label collection tubes.

5. Open syringe filter package by peeling back the paper seal. Leave filter in plastic packaging and place on work surface.
6. Open the syringe package and remove syringe.
7. Insert syringe into sample and aspirate sample into syringe by raising plunger.
8. Grasp filter from either side of the plastic packaging and firmly hold in place while attaching syringe to upper luer lock attachment point. Be careful not to depress the plunger while attaching the filter.
9. Remove filter from packaging by lifting the syringe and filter with one hand. Only do this when you are ready to start filtering. Work quickly to minimize the time that the collection tube is open. Avoid allowing the lower part of the filter unit (where filtrate comes out) to contact objects or surfaces.
10. With your other hand, open collection vessel. If vessel has a removable lid, hold in the fingers of the same hand. If you are unable to do this, it may be placed briefly on a clean work surface.
11. Move filter/syringe above collection vessel opening. Filter body may be rested on top of opening to add stability and cover opening.
12. Apply gentle pressure to the syringe plunger to push sample through filter and into collection vessel. Do not force plunger. If sample is difficult to filter, pre-filter through a larger pore size. If filtering becomes difficult before desired volume is filtered, replace filter, and repeat process.
13. Close collection tube.
14. Discard filter and syringe in appropriate trash receptacle and close sample collection vessel.
15. Clean up work area for the next person, including spraying and wiping with 70% ethanol.

Pesticides/Antibiotics

Treating the infected culture with pesticides or antibiotics (or other types of treatment available) can help determine the type of pest (e.g., based on mode of action of chemical treatment, or historical phenotypes) as well as suggesting potential preventative measures that can be taken before subsequent crashes.

Treatments are typically added to culture after infection but can also be used in “treating the infection source” experiments, where the treatment would be added to the infection source for an incubation period prior to infection. Choice of chemical, and the levels to use, will be dependent on the strain/media being used and relevant data concerning toxicity and efficacy. See appendix for typical concentrations of antibiotics and pesticides.

Transfer cells from crash source to fresh media

1. Gather supplies need to complete the task.
2. Where gloves when handling pond samples
3. All steps with open culture containers must be done in a laminar flow hood or equivalent biosafety cabinet.
4. Transfer materials required to the flow hood / designated work area (See Laminar Flow Hood Use SOP LC-01-001-018 for further details on appropriate use of the hood).
5. Clean and set up your work area in such a way as to prevent the need to move hands or equipment above open containers.

6. Label all containers, plates etc. appropriately with at least the date, initials, and an identifier.
7. Transfer sample to falcon tube or equivalent vessel suitable for centrifugation that is capable of holding the necessary volume. Multiple tubes may be required.
8. Take samples to the centrifuge.
9. Balance tubes in centrifuge (refer to centrifuge SOPLC-0x-00x-xxx).
10. Centrifuge at x,000g for 5 minutes. This step may vary with different algal species. If no pellet is formed after this step, repeat for 5-10 minutes longer.
11. Return samples to flow hood work area.
12. Pour off supernatant into a suitable waste container. If the supernatant is to be kept for use in other experiments, then pour into a sterile labeled container for later use.
13. Place tube in a rack or holder to allow remaining supernatant to settle to the bottom and then remove any further supernatant by pipette, being careful not to disturb the cell pellet with the pipette tip.
14. Add fresh media and re-suspend the cell pellet by pipetting, vortex, or shaking by hand. The volume of new media added will depend on the desired final OD of the resulting culture. If you use the same volume as the original starting sample the resulting OD will likely be lower than that of the starting sample since some loss will occur during the procedure. Add less media than you expect to need and dilute later to target OD with fresh media.
15. Aliquot re-suspended culture to new growth vessel (e.g., flask/s, plate/s etc.).
16. Place culture in appropriate growth chamber.
17. Clean up the work area.

This treatment can help determine if there is something in the media that is the cause of poor culture health/crash.

Cultivate lab culture in crash media

1. Follow steps in previous protocol through to and including centrifuge step.
2. Collect supernatant from centrifuge step
3. [Optional] Sterilize media. If using filtration, further centrifugation and filtration through large and then decreasing pore size filters is advisable since culture media contains many particles.
4. Inoculate lab culture into media to target OD. Do this either by diluting culture into media or concentrate culture by centrifugation and inoculating from concentrated biomass. Calculate inoculum by reading OD of culture and inoculum.
5. Place culture in appropriate growth chamber.
6. Clean up the work area.

This treatment can help determine if there is something in the media that is the cause of poor culture health/crash. Following the procedure with/without sterilization may also help determine cause but typically media used in this type of experiment is filter sterilized after centrifugation.

Adding nutrients to crash source or infected cultures

Adding nutrients to source sample and cultivating in the lab (see notes above in "infections" section) can help determine if the culture was deficient in any or all nutrients or may suggest the presence of pests competing for resources.

1. Dilute nutrient mixes / component stock solutions to 100-1000X final desired concentration (Where 1X = pond culture target).
2. Transfer culture to growth vessel as per previous procedure for biotic review.
3. Add nutrient components or mixes to 1(+)X levels.

Nutrient levels have also been shown to prevent crashes of certain pests (e.g., Phosphate level can prevent crashes caused by FD107 in SE00107 cultures). The level to use, if targeting higher than field target concentration should be determined based on known toxicity data for each component being tested.

Environmental conditions

Typically, constant temperature and light is used and will speed up time to crash as compared to outdoor conditions. Since pests are often seasonal, using the wrong temperature profile could prevent a crash. Ideally, reinfection experiments should be cultured in conditions as close to the original source culture, but this may not always be possible.

Shaking

Some pests can be sensitive to the type of mixing used as well as the cycling of this mixing. As much as possible conditions should match those used in the source culture. This may not be practical and so initial experimentation can be used to determine the necessity of altering this condition. Varying culture motivation is achieved by varying shaker speed and use of appropriate culture vessels at each chosen speed.

Vessel type

Typically, 5ml culture tubes/plates ("preview tubes") or flasks are used. Again, the culture condition can affect crashes so care should be taken in choosing or experimenting with different growth formats.

Light intensity/regime

Typically, constant light is used in laboratory experiments to speed up time to conclusion, but this may potentially prevent some pests from propagating.

Infections on solid media

Two major methods for pest isolation/determination can be used:

1. **Plaque plates**

Dilutions of source culture are mixed with clean culture and plated onto agar plates of appropriate media. After incubation areas of clearing (plaques) can be observed if certain pests are present.

Plaque plating procedure is detailed in SOP LC-xx-xxx-xx. The following steps are suggested for plaque plating an unknown source:

1. Dilute and plaque to at least 10-6
2. Plate multiple plates for replication and to place in different growth conditions (cultivate plates at different light levels, temperatures, and CO2)
3. Incorporate any information already gathered on the pest source e.g., antibiotic sensitivity, size to remove/prevent non-pest organisms from contaminating the plates and potentially masking the data.

2. Crawl plates (for motile pests and amoeba)

A suitable food source (algae/bacteria) is inoculated onto an agar plate of appropriate media and dilutions of source culture are spotted close to or on to the food source. Gradual clearing of food organism is observed when motile pests are propagated. The procedure for crawl plates is detailed in -. As per plaque plating, plate multiple dilutions and replicate for multiple conditions while incorporating any information on the pest organism that may aid in isolation.

A suspected biotic crash is observed

If a crash is observed and a biotic cause is suspected, proceed with the following steps:

1. Archive crashed samples as before. These samples should be used for future experimentation, preserving the original source for historical archiving and future back-up.
2. Submit sample for identification via sequencing
3. Use crash source for “prevent and control” experimentation to determine appropriate prevention measures that can be taken in the future.
4. Repeat algae infections to confirm and gather further information on phenotype

No crash is observed

No crash could be observed because the conditions used were not permissive for infection or no infective agent is present. Repeat infections using different culturing conditions as close to the original source culture as possible.

Prevent and Control

Once a crash is observed from a suspected pest, treatments that can be used in the field against such a pest in the future should be tested. Some or all of these may have been tested during the biotic review process, but all available treatments should be tested and/or confirmed for efficacy. Some typical concentrations for common chemical treatments are detailed in the appendix below.

1. Set up infections of laboratory culture as per biotic review, using 1% v/v infection or other as determined in previous experiments
2. Add treatments from stock solutions
3. Observe and compile data for 5-7 days
4. Collect data useful for confirming pest crash phenotype

5. Repeat successful treatments for confirmation while also refining concentrations for efficacy study
6. Test successful treatment in field setting before incorporating into standard crop protection schedule.

Appendix 1. Common biocides and concentrations.

Pesticide/ Antibiotic (active ingredient)	Typical concentration / range used
Omega 500F (fluazinam)	0.5-1 ppm AI
Bravo weather stik (chlorothalonil)	3-5 ppm AI
Defiant (thiram)	2-5 ppm AI
Dithane (mancozeb)	2-5 ppm AI
Spectrus CT1300 (benzalkonium)	0.5-5 ppm AI
Streptomycin	5-25 ug/ml
Tetracyclin	10 ug/ml
Ampicillin	100 ug/ml
Kanamycin	5-25 ug/ml
Penicillin	100 ug/ml
Chloramphenicol	5-25 ug/ml
Penicillin/Streptomycin/Neomycin cocktail (Sigma)	100-1000X dilution from manufacturers stock
GoalTender (oxyfluorfen)	10-50 ppb AI
Hyvar X-L (Bromacil)	0.5 ppm AI