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Identification of local pests for new strains

New pest organisms are observed every year for algae strains. The likelihood of finding new pests is increased when using new strains or media compositions since there may not be established populations of pests adapted to this environment already present. There are essentially two approaches to identifying the types of pests that will be a problem; test current pest models with the new media/strain, and capture new pests from the environment. Pests will appear when algae is cultivated in open ponds so proactive efforts were made to identify pests early in the process of scaling up of the new strains, as well as continuous pest capture efforts once large scale cultivation in raceways was established.

Proactive pest capture.

Two minipond experiments were designed for early pest capture. Laboratory experiments also attempted to capture pests from the local environment.

Exp_172 – "Pest Capture"

This experiment was designed to discover what pests would naturally invade outdoor cultivation ponds. Four "in house" strains of *Nannochloropsis*, that were available for immediate outdoor experiments, were inoculated in miniponds prior to other strains being cultivated outside. These cultures would then be the oldest outdoor cultures on site and the most likely to show pest pressure before other ponds. This provides an early indication of expected pest pressures in the local environment for *Nannochloropsis* generally. The experiment is summarized below.

- One minipond of each strain (00087, 00088, 60373, 60445) was cultivated in MASM based media using NaNO3 and KH2PO4 as the N and P sources.
- The experiment lasted 93 days from 2/23/15 to 5/26/15.
- No crashes were observed in any of the cultures used in this experiment.
- Two weed strains were identified – *Tetraselmis* sp. and *Amphora* sp. (diatom).

As noted above, weed algae appeared in all four cultures. *Tetraselmis* species were sequenced and identified as like strains that had been cultivated in the local environment recently, showing the possibility for cross contamination from nearby ponds when different strains are cultivated together or on sites where previous algae cultivation has occurred (e.g., Spirulina is still observed in CB ponds, years after cultivation ceased). The other weed strain identified was a diatom (*Amphora* sp.) which is commonly observed in algae ponds. The observation of both of these weed's strains led to research into potential treatments which will be described in more detail in further sections. The *Amphora* sp. diatoms responded to treatment by bleach and the *Tetraselmis* sp. algae responded to alteration of the paddle wheel speed and schedule. Thus, this experiment succeeded in the goal of early pest capture that led to research of potential crop protection solutions prior to similar problems occurring in larger cultures. *Tetraselmis* appeared after approximately one month of cultivation and the diatoms shortly after that. By mid-May Diatoms were at approximately 10-20% of the culture (as measured by OD750) highlighting the need for control and prevention for this pest especially during the spring growing season

Exp_0173 – "Weed/Pest Capture"

Once you have the ability to grow algae without culture failure due to infection/predation, the major class of pests that are likely to be observed is weeds. These organisms (typically algae or diatoms) compete for resources with the crop as well as potentially lowering the product quality. Experiment 0173 "Weed/Pest capture" aimed to capture and identify local algal strains and other organisms that could grow in the cultivation media. Such organisms should represent potential weed species.

Two ponds were used in this experiment with different media representing the potential range of media conditions that may be used at larger scale. One pond was started on 2/23/15 and the other on 3/10/15. Both ponds visually showed the presence of algae after approximately 40 days and were at cultivation pond densities after approximately 60 days. This highlights the speed at which contamination could propagate in a pond environment without any prevention methods.

Five different weed strains that were observed in significant numbers were isolated and sequenced (see the table below). Chlorella is potentially a problematic weed strain due to its similarity in shape and size to *Nannochloropsis*. Without diagnostic or tracking tools this weed could propagate undetected in the pond. When samples from these ponds were cultivated in the lab, the *Tetraselmis* species became dominant. Different algae weed species have appeared and propagated at different times of the year so whilst this experiment did successfully identify weed strains (that were in fact similar or identical to strains observed later in larger cultures), unique weeds may be identified if the same experiment was repeated at different times of the year.

Weed	Closest species similarity (ITS1&2 sequence comparison)	Notes	Observed before?
Tetraselmis sp.	Various *Tetraselmis sp.*, all similar to SE strains.	Likely cross contamination	Yes, cultivated *Tetraselmis* on site in recent time
Chlamydomonas sp.	*Chlamydomonas hedleyi*	Motile	No, although other *Chlamydomonas* species have been observed
Chlorella sp.	*Chlorella sp.*	Small algae, difficult to	Not sequenced/isolated

		distinguish from crop	but likely observed
SE61268-like	*Nephrochlamys / Scenedesmus sp.*	Small moon shaped	Observed as a weed strain in green algae ponds in recent years.
Razor diatom	Sequence failed	Lab cultivation failed	Yes, one of many diatoms often seen in cultures.

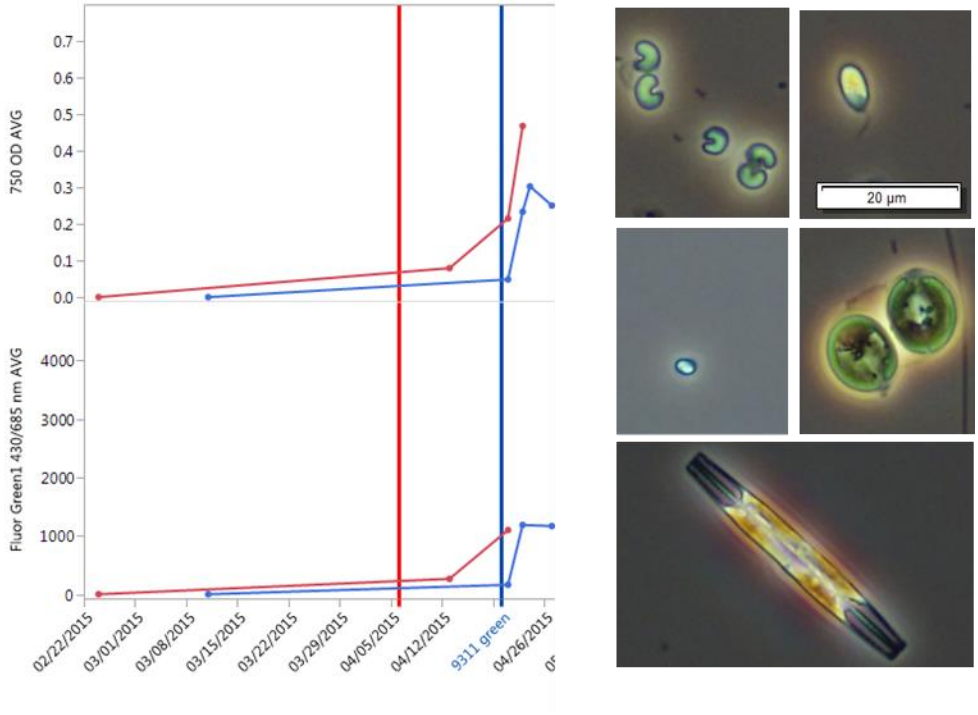

Figure 1. Natural inoculation of pest capture miniponds. Graphs show OD 750nm (top) and fluorescence (bottom) over time for ponds set up with media only. Data for pond 9311 (N1M1 media) is shown in blue and 9312 (NF-1) media) in red. Images to the right show examples of the organisms visualized in the ponds. From top left: 1. SE61268-like, 2. *Chlamydomonas hedleyi*, 3. *Chlorella* sp., 4. *Tetraselmis* sp., 5. "Razor" diatom.

Laboratory pest capture

Environmental samples from both the LC and CB sites were added to clean laboratory flask cultures of *Nannochloropsis* (SE00087) and observed macro- and microscopically for up to two weeks. Both solid (soil/dirt) and liquid samples (ponds, puddles, sumps, etc.) were used for inoculum and all samples were biologically active as shown by microscopic observations.

Many interesting organisms were observed during these experiments but no definitive pest organisms were identified since no culture crashes were observed. A number of organisms were observed consuming algae but were unable to decimate the culture and thus would not be considered pests (note: it is generally thought that certain organisms that are able to consume algae may be beneficial to the crop since they predominantly consume other organisms and help maintain the pond ecosystem as well as potentially reducing bona fide pest pressure by consumption). However, it is possible that these organisms could have catastrophic effects on the algae culture when present under alternative environmental conditions and thus these organisms are still relevant in the development of a yield protection strategy.

An expected major pest of *Nannochloropsis* is rotifers. A novel type of rotifer was observed in environmental samples used in laboratory pest capture efforts (see Fig. 2) however it was unable to survive in the *Nannochloropsis* culture media. This agrees with the literature available on these types of rotifers, that they are unable to tolerate high salinity environments, and the fact that they were observed in recently established rainwater puddles.

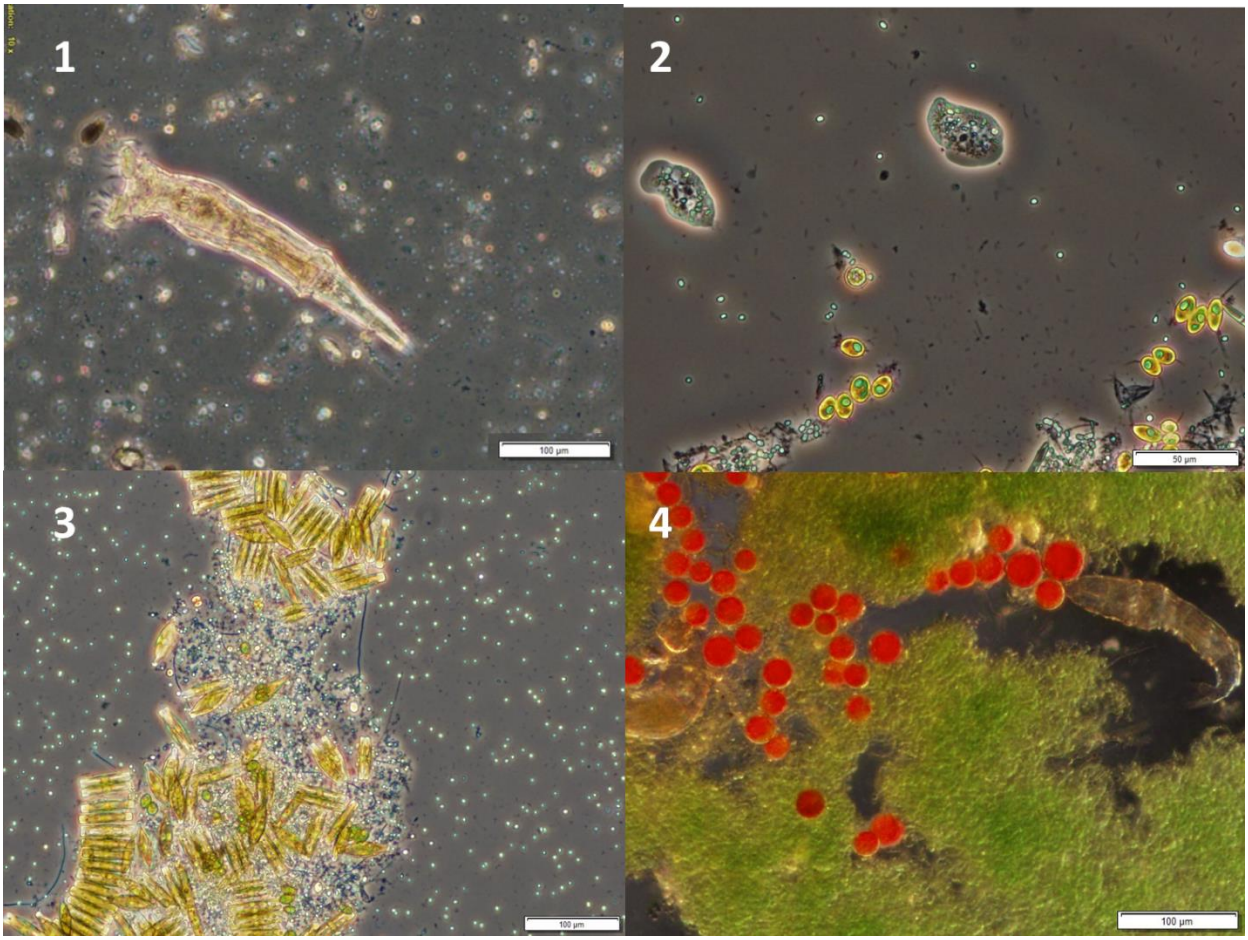

Figure 2. Examples of organisms observed in early pest capture efforts. 1. Rotifers – this type of rotifer was observed in natural standing water (rainwater) but failed to grow in algae media (though was successfully cultivated in laboratory conditions in other media). 2. Amoeba – this example is consuming *Nannochloropsis* but consumption rate and propagation were not significant enough for it to be considered a pest. 3. Diatoms – Various diatom species cultivated in the pest capture efforts both inside and outside suggesting that they are a potential pest organism. 4. Various – photosynthetic organisms such as cyanobacteria and red algae shown in this picture were observed in environmental samples but none out-competed or infected the crop strain.

Other laboratory work with archived pest models, preserved from infections of previous algae strains/cultures did not show successful infection in *Nannochloropsis* cultures highlighting the specificity of many pests and the likelihood of finding other pests once large-scale cultivation began.

Taken together with the minipond experiments described above, these efforts suggest that a pest population sufficient to quickly destroy a culture was not likely present in the local environment at this time, or if it were, conditions were not conducive to pathogenicity. This may change with environmental conditions and thus similar experiments may be beneficial at other times of the year. No further

dedicated pest capture pond experiments were used since by this time numerous ponds were active for other experiments, acting as de facto pest capture. It should be noted that numerous strains have been cultivated for short periods such as in the pest capture minipond experiment and resulted in culture crash, so it is understood that such an event is possible in such a short time. Diatom populations became established in many cultures highlighting Diatoms as a major class of likely weed organism.

Pest capture throughout the cultivation season.

Satellite culture

Satellite cultures are routinely used during cultivation to assess pest pressure (and assessment of other parameters), by taking samples of the pond and cultivating them under different conditions in the field or laboratory. Culture failure due to pest can be accelerated under laboratory conditions and thus this method is useful for pest capture. During routine observation and data collection in field trials, anomalies are often observed. Sometimes these anomalies are caused by pest organisms. Thus, pest capture efforts may be triggered periodically throughout outdoor experiments. The first step in such efforts is satellite culture, examples of which will be described later.

Rotifers

Rotifers are known to consume *Nannochloropsis* which is used as a food source in the commercial cultivation of rotifers. Rotifers have also been observed in many, if not all SEI algae cultivation ponds. As expected, rotifers were observed in *Nannochloropsis* ponds in 2015. All rotifers observed to propagate to significant population numbers were identified as *Brachionus* sp. (by phenotypic analysis) which is the expected type of rotifer based on past experience. Due to this, very little rotifer capture work was initiated and research in this area focused on treatment and monitoring strategies which will be detailed in other sections.

Isolation of pests from pond crashes

Two major pest events occurred in 2015, P9 (6/19) followed by P5 (6/25) resulting in pest capture efforts. The suspected pest in the P9 health decline at the time was rotifers. However, based the data and from previous experience it seemed likely that rotifers were not the only cause of culture loss. Work investigating the second pond crash identified a potential bacterial pest that was likely also a contributing factor in the earlier crash (in combination with a number of mechanical and environmental factors).

Bacterial pests FD111/2

After the rapid decline in health of the *Nannochloropsis* crop, a suspected biotic pest was identified as the causative agent by infection of lab culture with pond sample. Example data from this experiment is shown in Fig. 3. This and other similar experiments suggested that the pest was biotic and close to or smaller than 0.45um in at least one dimension, since it was able to move through a 0.45um syringe filter. A number of antibiotics were able to prevent the infection suggesting that the pest may be bacterial. Samples were archived for study and the pest named FD111.

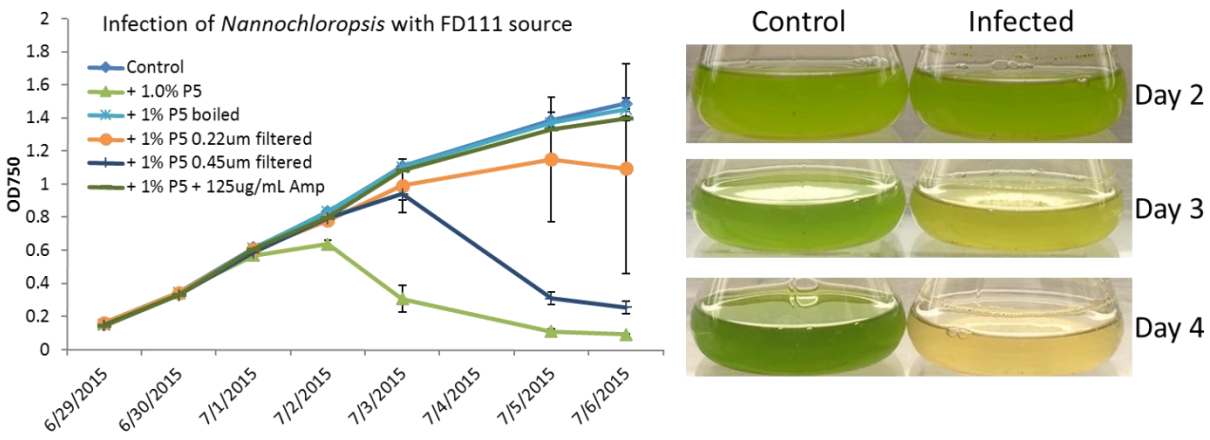

Figure 3. Infection of laboratory culture with crashed pond sample containing FD111. Graph shows OD (750nm) over time for *Nannochloropsis* cultured in 24-well blocks (5ml culture volume) with various treatments/infections. 1% v/v of crashed pond sample was added to laboratory culture after treatment (no treatment (+ 1.0% P5), boiling 10min (+1% P5 boiled), filtering 0.22 or 0.45µm (+1.0% P5 0.22/0.45µm filtered)). Also shown are uninfected control (Control) and infected culture treated with the addition of 125ug/ml Ampicillin (+1% P5 + 125ug/ml Amp). Infection is shown as a rapid decline in OD of the culture whereas healthy culture continues to increase in OD throughout the time period. Data shows that infection is prevented by boiling, filtering (≤0.22um), or the addition of antibiotics. Pictures to the right show appearance of infected culture in a similar experiment. Controls continue to get darker green throughout growth whereas infected culture loses green color over time showing a chlorotic appearance.

Microscope observation revealed the presence of both hook and rod-shaped bacteria attached to the surface of the algal cell. It is unclear if either one or both of these organisms are pests. Isolation efforts have so far been unsuccessful, however complete isolation is not necessary for study of treatments and may not be possible since many pathogenic organisms are host-dependent. Sequencing of 16S region of the bacterial genome identified a number of potential leads on the identification of the pest. The most commonly identified sequence, and the only one consistently identified in different sources was similar to *Pseudobacteriovorax antillogorgiicola* strain RKEM611 (Accession KJ685394), a bacterium that infects other bacteria with a life cycle that includes a stage called a bdelloplast which is an elongated cell found within the host that later divides into the daughter cells (1)(McCauley et al., 2015).

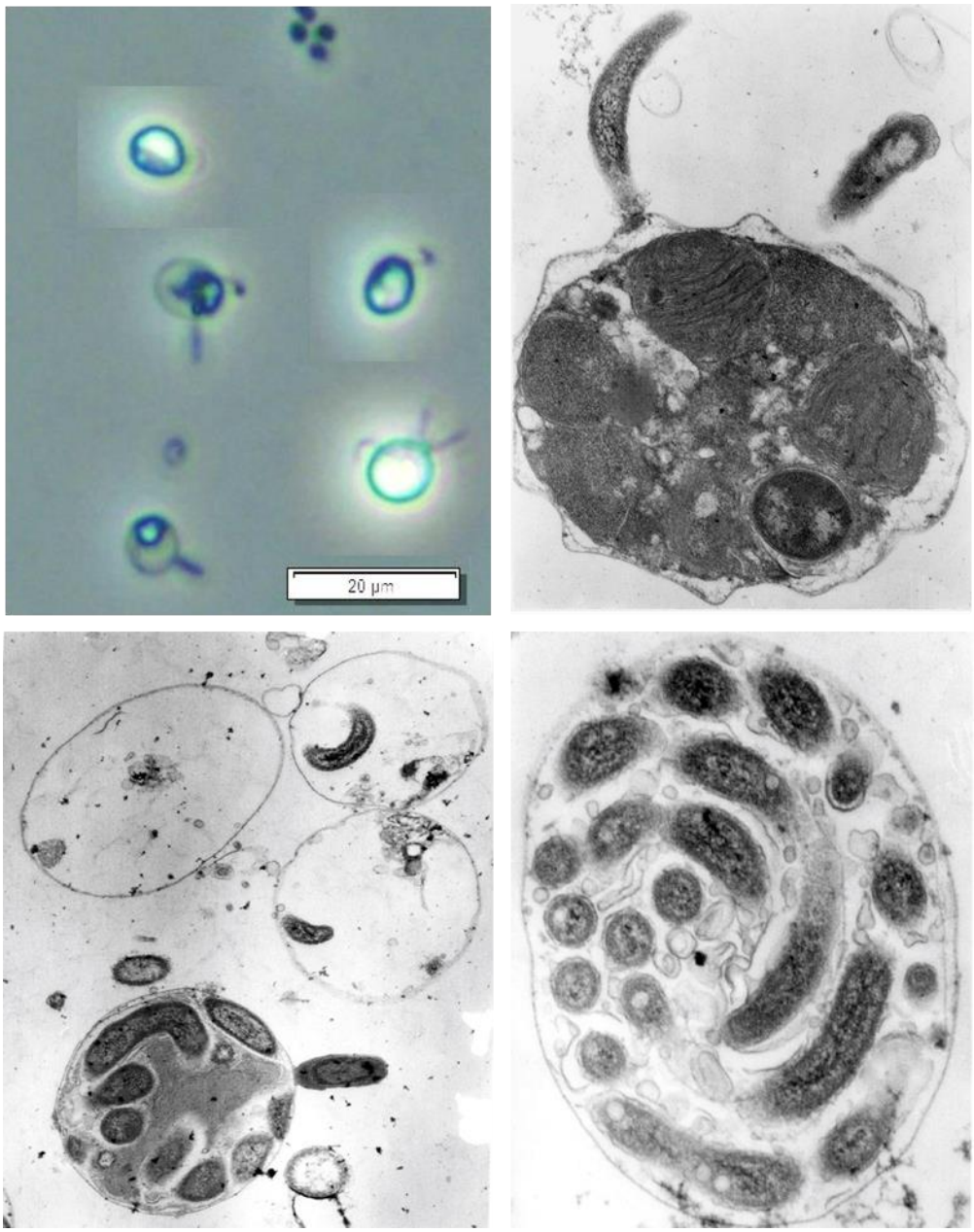

Figure 4. Microscopic observations of infection by FD111. Top left: Phase contrast image of *Nannochloropsis* cells at various stages of infection. Hooked and rod-shaped attachments can be seen on the cell surface as well as the receding pigmentation phenotype characteristic of this infection that ultimately leaves a hollow shell. Top right: Electron micrograph of an infected cell showing hooked bacteria attached to the cell surface. Lower left: bacterial cells within the host and empty host cells. A rod-shaped bacterial cell is associated with the algal host (bottom of image). Lower right: Propagation of the pest within the host forming a bdelloplast-like structure.

Samples of infections were sent to our academic collaborator Peter Letcher at the University of Alabama for observation by electron microscopy. This confirmed that the attached organisms, that were observed via light microscopy, were bacterial in nature. Bacteria were also observed propagating within the algal cell. Bdelloplast-like cells were also observed within the host and sections through such cells revealed that the bacteria were likely a continuous cell within the host. This observation is in alignment with previous sequencing data/observations mentioned above. Further isolation and identification

efforts are ongoing as well as the development of tracking tools in order to monitor for the presence of pest in the field.

A second presumed bacterial infection was also observed via plaque plating and named FD112. This infection has a similar macroscopic phenotype but differs microscopically in the appearance of the resulting dead cells. FD111 infection is characterized by the resulting hollow cells after pigment recession. Cells killed by FD112 infection appear darker (see Fig. 5.). Characterization and isolation of FD112 is ongoing.

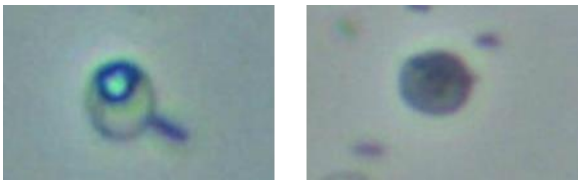

Figure 5. Comparison of cell death phenotypes from FD111 and FD112 killed cells. FD111 killed cells result in a hollow cell, often with attached bacteria (Left). FD112 killed cells result in dark dead cells (right).

References

1. *Description of Pseudobacteriovorax antillogorgiicola gen. nov., sp. nov., a bacterium isolated from the gorgonian octocoral Antillogorgia elisabethae, belonging to the family Pseudobacteriovoracaceae fam. nov., within the order Bdellovibrionales.* **McCauley, E P, Halti, B and Kerr, R G.** 2015, Int J Syst Evol Microbiol., Vol. 65(Pt2), pp. 522-30.