AN ANALYSIS OF PLACOZOAN NUTRITION AND BIOMINERALIZATION

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

THESIS ADVISOR

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ABSTRACT

Trichoplex adhaerens, the only species in the phylum Placozoa, is the simplest and most primitive of all metazoans. Placozoans are found in the littoral zone of sub-tropical and tropical oceans, have a plate-like appearance and generally adhere to surfaces. These extremely simple organisms have no organs, muscular system or nervous system. Little information is known about their diet or behavior in the natural environment. In the laboratory placozoans feed via phagocytosis on algae, living protozoa, and detritus, and have been cultured using generic filter feeder food. Experiments were performed in order to compare the success of populations of placozoan strains who were fed a filter feeder medium versus a brine shrimp medium. Placing a single placozoan in a petri dish and allowing it to reproduce asexually in order to clone the organism resulted in different strains. There appeared to be no preference for either media shown between the strains. A significant distinction that was observed between strains was the presence of birefringent granules that exist in a ring around the perimeter of the animal. Optical analysis, combustion experiments, triammonium hydroxide tests, Scanning Electron Microscopy (SEM) and Raman spectroscopy analyses were performed in order to discover the structure and composition of the birefringent granules. Only certain strains were found to contain birefringent granules that remained present over multiple generations, which could imply there might be more than one species of Placozoa. The crystals were found to be inorganic by combustion. Birefringence interference tests indicate that it is improbable that the crystals are of calcium carbonate nature. SEM analysis uncovered crystals that likely originated from the organism that indicate the presence of calcium and oxygen.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. Introduction

1.1 *Trichoplax Adhaerens: Primitive Metazoan in its own phylum?*

The phylum Placozoa is comprised of only one described species, *Trichoplax adhaerens.* Trichoplax are the simplest and most primitive of all metazoans (animals) and are possibly closest to the common ancestor of all animals. F.E. Schulze discovered the organism in an aquarium in 1883, from material taken from the Adriatic Sea. There was little interest in the organism, however, because it was assumed Trichoplax was a modified planula larva. In the 1970's interest in the organism was sparked again once it was found that the organism could reproduce asexually, discarding the previous claim (Schierwater 2005). As of present, the relation of placozoans to other metazoans and their placement on the phylogenetic tree is uncertain (see Fig. 1).

Trichoplax adhaerens was named due to its plate-like appearance and its tendency to adhere to surfaces. *T. adhaerens* are comprised of only two epithelial layers and an internal mesenchyme (Pearse et al. 1994). The organism lacks any type of nervous system or muscular system. *Trichoplax adhaerens* move in two ways, by gliding on substrates and by buckling. The organism is able to glide by the use of cilia located on the ventral epithelium (Schierwater 2005). There is little known about the ecology of these organisms in their natural environment. *Trichoplax* is known to reproduce asexually, and recently Signorovitch et al. (2005) discovered molecular evidence for sexual reproduction in the phylum. Placozoans reproduce asexually either by binary fission or by the less commonly observed process of "budding" where an individual forms multiple buds around its perimeter that eventually separate from the individual and form new organisms.

As of present, *Trichoplax adhaerens* is the only recognized species in the phylum Placozoa and all observed specimens fit the common morphological description for *Trichoplax adhaerens*. Recently, however, Voigt et al. (2005) discovered widespread genetic variation in Placozoa that provides evidence against the idea that the phylum consists of only one species.

1.2 Trichoplax Adhaerens and Nutrition

Placozoans have been found to feed on different kinds of detritus, living protozoa, and certain types of algae, although the specifics are not yet known. When placed on a substrate, *Trichoplax* promptly adhere to the closest surface, termed a dorsoventral orientation reaction that is not yet completely understood (Pearse et al. 1994). They feed in the same manner, climbing atop their food and digesting the material through phagocytosis or simply by extracellular absorption (Schierwater 2005). One focus of this thesis is to explore the nutritional needs of placazoans. Typically, they are fed a cryptophyte algae. It is not known, however, what nutrients the organisms require and what they are digesting from the filter feeder food. Placozoans taken from their natural environment are noticeably larger in size than those that are cultivated on a filter feeder medium. This observation suggests that the filter feeder media is not supplying the optimum nutrition. One focus of this thesis was to compare the success of feeding strains of *Trichoplax* with a common filter feeder media versus a brine shrimp flake media. An additional focus was testing the hypothesis that if biomineralizing and non-biomineralizing strains are in fact different species, they could exhibit additional disparities such as a preference for a certain media.

1.3 Biomineralization in Trichoplax and other metazoans

A distinctive characteristic observed in certain isolates of placozoans by past scientists is the presence of birefringent granules located in the peripheral area of the organism (Pearse et al. 1994) (See Fig. 2). Pearse et al. (1994) found no geographic or seasonal explanation for the presence or absence of birefringent granules based on specimens taken from both the Atlantic and Indo-Pacific. The observation that only certain organisms in this phylum are practicing biomineralization, along with evidence for genetic variation, could offer considerable support to the proposal that the phylum Placozoa is significantly more diverse than previously thought.

The composition and structure of the biominerals produced by placozoans remain to be discovered. There are currently 64 known minerals that organisms produce; of these 64 minerals roughly 25% are amorphous and do not diffract x-rays (Weiner 1986). Amorphous solids are not birefringent, which leads to the conclusion that the granules found in placozoans are in fact crystalline due to their birefringent nature. It is possible to narrow down the potential biominerals produced by placozoans by taking into account defining characteristics such as crystalline nature and production by controlled or uncontrolled mineralization. It is also necessary to take into consideration biominerals produced by closely related phyla.

The following were considered as possible biominerals produced by placozoans. There are eight known crystalline polymorphs of calcium carbonate that include calcite, Mg-calcite, aragonite, vaterite, monohydrocalcite, protodolomite, and hydrocerussite. Another biomineral group to consider is sulfates, which include gypsum, bassanite,

barite, and celestite. A possible phosphate would include the commonly produced carbonated-hydoxylapatite, also known as dahllite. Additional minerals that should be included are pyrite, silica, magnetite, as well as crystalline organics. Table 1 summarizes the potential types of biominerals produced by placozoans.

The process by which biomineralization occurs can differ between species and phyla; however there are basic properties and necessary components of biomineralization held in common by most groups. See Weiner et al. (1986) for details. Biomineralization generally requires that there exist a localized zone that controls a set supersaturation. The zone must limit diffusion into or out of the system, and the fluid must be electroneutral in order for biomineral growth to occur. Ion supply into the compartment can occur either by active pumping or by passive diffusion gradients. The biomineralization process is classified into two groups; one that is “biologically induced” and the other “organic matrix-mediated”. Biologically induced biomineralization is the secondary precipitation of minerals that is a result of interactions between the environment and biological activity. The organism cannot control what type of mineral is deposited, and nucleation generally occurs directly on the cell wall. In organic matrix-mediated mineralization (also known as biologically controlled mineralization), the organism directs the nucleation, growth, morphology, and final location of the mineral. When examining the location of biominerals in placozoans, the crystals appear to be evenly placed around the perimeter of the organism (Gaidos, pers. comm.). This implies that they are practicing organic matrix-mediated biomineralization.

1.4 Objectives

The main objectives of this study were to identify the crystalline granules found in placozoans and determine whether they were a heritable trait of specific lineages. We applied chemical techniques, optical microscopy, combustion, scanning electron microscopy, and Raman spectroscopy. These techniques have helped narrow down the possible crystalline biominerals being produced by placozoans.

The goals of the nutritional aspect of this study were two-fold: to attempt to obtain a reliable medium that would supply sufficient nutrition to placozoan populations in the laboratory setting as well as determine if there are different nutritional preferences between different strains. To accomplish this, populations from each strain were cultivated and observed in two different media. Both the presence and absence of biomineralization and nutritional preference might serve as non-genetic markers in differentiating between possible strains and/or species of placozoans.

CHAPTER 2. Methods

2.1 Isolation and Cultivation of Placozoans

Placozoans were collected from Kewalo Basin Marine Research Facility by placing trays containing plain glass microscope slides in water tables for up to four weeks. The placozoans were then transferred via micro-pipette from the glass slide to glass petri dish. *Trichoplax adhaerens* were cultivated in petri dishes with media. Individual placozoans were placed in single petri dishes to found strains. Two particular strains, one biomineralizing (P13) and one non-biomineralizing (P8) were isolated in this way and used for further experiments. The petri dishes were placed adjacent to a window at room temperature. The medium in each petri dish was changed every two days.

2.2 Nutritional Media

Brine shrimp flakes (Sanfrancisco Bay Brand Inc., Newark, CA) were ground with a mortar and pestle and used to create a medium consisting of 0.01 grams of brine shrimp per liter of filtered seawater. The concentration of filter feeder food (Interpret Ltd, Surrey, England) used was 100 µl per liter of filtered seawater. Six petri dishes were prepared; three petri dishes contained 30 ml of brine shrimp medium and three dishes contained 30 ml of filter feeder medium. Three placozoans of a biomineralizing strain (P13) were transferred to petri dishes and cultivated for 18 days. The same procedure was repeated using a non-biomineralizing strain (P8). The petri dishes were placed on a counter adjacent to a window. The medium in each dish was changed every two days, and the number of placozoans in each dish was recorded for a total of 18 days.

2.3 Optical Microscopy

Individual placozoans were transferred via pipette onto a slide and allowed to adhere to the surface. The slide was then placed under an Olympus BX51 microscope fitted with crossed polarizers and rotated 360°. The birefringence interference colors transmitted by the crystals were recorded.

2.4 Combustion Method

Individual placozoans were transferred onto a glass slide in 20-30 µl of filtered seawater. The organisms were fixed by adding 10-15 µl of a para formaldehyde (PFA) 4% solution. De-ionized water was added to the slide and pipetted back out to remove as much salt as possible. The slide was left to dry and the location of the placozoans in reference to the slide was recorded. The slide was placed in a Lindberg 51748 oven at a temperature of 500°C for a period of 24 hours.

2.5 Triammonium hydroxide Test

An individual placozoan was deposited into the well of a concave slide in 30 µl of filtered seawater and allowed to settle. The presence of crystals located around the perimeter of the animal was verified under a polarizing microscope. A 0.5 molar aqueous solution of triammonium citrate ($C_6H_{17}N_3O_7$) was prepared by titration of diammonium citrate $(NH_4)\cdot 2HC_6\ H_5O_7$ with ammonium hydroxide (NH_4OH) to a pH of 8.1 +/-0.1. Approximately 10 µl of triammonium citrate was added to the well containing the placozoan. Photos were taken of the placozoan with an Olympus digital low-noise camera at one-minute intervals.

2.6 Scanning Electron Microscopy

Approximately 20 placozoans were transferred via pipette into the well of a concave slide containing 50 µl of filtered seawater. The placozoans were left to settle and adhere themselves to the bottom of the slide. 50 µl of sodium hypochlorite 5% was added to the well to digest the organic material and the slide was covered with a coverslip and left for 24 hours. The crystals were pippeted in 5 µl increments and deposited onto a vacuum filter fitted with a 0.2 µm GTTP Isopore membrane filter. The filter containing the crystals was dried, carbon coated, and placed into a JEOL JSM-5900L V Scanning Electron Microscope. Other preparations used 1x phosphate-buffered saline (PBS 1x**)** as a replacement for sodium hypochlorite. An individual placozoan was pipetted into a slide well in 40 µl of filtered seawater. The placozoan was allowed to settle onto the slide and examined under a polarizing microscope. An estimate of the number of crystals present in the organism was recorded, and 50 µl of PBS 1x was added to the well and left for one hour. The crystals were then pipetted via 5 µl pipette and vacuum filtered. The crystals were rinsed three times with de-ionized water on the filter, dried, carbon coated, and placed into the SEM.

2.7 Raman Spectroscopy

Approximately 15-20 placozoans were placed in a petri dish in 30 ml of filtered seawater. 30 ml of PFA solution was added to the petri dish and placed in a 4°C refrigerator for 12 hours. The placozoans were rinsed in a de-ionized water bath and returned to the slide. Photos were taken with of the fixed organisms under a polarizing microscope for reference. Placozoans were pipetted individually onto a slide covered with aluminum tape, allowed to dry out, and placed into a Raman Microprobe (Kaiser Optical Systems, Inc.)

CHAPTER 3. RESULTS

3.1 Nutrition

The numbers of placozoans in each petri dish were recorded over an 18-day period. Table 2 displays population counts for strain 13 (a biomineralizing strain) and Table 3 displays population counts for strain 8 (a non-biomineralizing strain). Figures 3 and 4 represent averages of these population counts.

3.2 Persistence and Heritability of Biomineralization in Placozoan Strains

It was observed over a 10 month time period that the presence or absence of biomineralization is persistent. Biomineralizing strains continued to biomineralize over multiple generations and non-biomineralizing strains also remained non-biomineralizing over multiple generations.

3.3 Identification of the Biominerals

Under cross polarizers, the crystals did not transmit a broad color range of birefringence interference. Although rather difficult to judge, the observed colors ranged from a pale yellow to shades of pale green and gray. Combustion of the animals was repeated various times over 24 and 48-hour time intervals. The location of the crystals could be verified by using previously made measurements as a guide. The crystals remained completely intact and birefringent (Figs. 5, 6). The triammonium hydroxide completely dissolved the biominerals in less than five minutes. Photos were taken once every minute and arranged in a time-series (Fig. 7).

Filter samples that had been placed in the SEM contained numerous round and plate-like crystals, as well as spicules (Fig 8). In an un-rinsed sample that had been exposed to sodium hypochlorite, crystals were observed that ranged in size from 4 to 20 microns (Fig. 9). An area was selected that appeared to contain various crystals, and element maps for calcium (Ca), magnesium (Mg), sodium (Na), chlorine (Cl), oxygen (O), silicon (Si), iron (Fe), potassium (K), sulfur (S), and phosphorous (P) were produced (Fig. 10). The element maps confirmed the presence of Na, Mg, and Cl from precipitated seawater. There was also the presence of Ca and O found in small, round crystals three to four microns is size. The remaining elements examined (Si, Fe, K, S, and P) were not found to be present in the sample area.

The second set of filter samples that had been exposed to the PFA buffer appeared to have less contamination from organic material, dust, and salts. The sample that had been rinsed in de-ionized water did not have any Na or Cl peaks. Roughly 10 to 15 crystals were present in the sample. Two types of crystals could be identified: those with a darker complexion that had very strong Ca and O peaks and those with a very bright luminescence that produced strong Mg and P peaks. The crystals containing Ca and O ranged from three to five microns in size and were located within or very close to the remaining organic matter. Those that contained P and Mg were slightly larger in size, ranging from five to fifteen microns in size.

The Raman spectrometer, when focused on areas thought to contain crystals along the perimeter of the animal, produced peaks at 1009, 984, 438, 625, 1342, and 1447 ± 5 cm^{-1} (see Fig. 12). A reading for the PFA solution was also taken which produced peaks at 544, 916, 1043, and 1491 ± 5 cm^{-1} (Fig. 11).

CHAPTER 4. DISCUSSION

4.1 Different Properties between Strains

It can be concluded that the ability to biomineralize is a heritable and persistent trait that is observed only in certain strains of placozoans. As far as nutritional preference, there did not appear to be any significant difference in media preference between the strains, although there does appear to be a preference for the filter feeder media over the brine shrimp media observed in both strains. It is still not clear what the placozoans are digesting in either media or what nutrients they require.

4.2 What are the Biominerals?

The fact that the crystals remained visible, completely intact and birefringent after combustion leads to the conclusion that the crystals are inorganic. The triammonium hydroxide test was used to separate calcite from apatite. As seen in Figure 7, the crystals dissolved in less than five minutes when exposed to the solution, which indicates that the crystals are not composed of apatite. The test for birefringence interference colors is based on the concept that anisotropic minerals that are placed under a cross-polarizing microscope transmit visible colors. When a mineral is rotated between the crossed polarizers (the crossed polarizers are at an angle of 90°), a spectrum of "interference colors" is produced that goes through a repeating color sequence (Nesse 1991). Interference colors are produced when light is split into two different rays at right angles to one another as it passes through the mineral (Nesse 1991). The birefringence value of a mineral represents the difference between the fast and slow wave when the light is split. When the birefringence value and the thickness of a mineral is known, an interference

color chart (Fig. 13) can be used to determine what interference colors should be observed.

There are significant differences between the interference color spectra of calcium carbonate minerals and sulfate minerals. Calcium carbonate minerals transmit a very broad, vibrant range of colors (even at a thickness of <10 μm) that was not observed in crystals found in placozoans. The interference colors observed in crystals found in placozoans were pale yellow, beige, and shades of pale gray/green. These results closely correlate to the spectra produced by gypsum, followed by barite, celestite, and dahllite. It would be difficult to identify the biominerals based solely on birefringence interference; however it is possible to at least eliminate certain biominerals. It is feasible to conclude that the biominerals produced by placozoans do not produce the interference color spectra that would be expected of calcite, aragonite, vaterite, or monohydrocalcite, subject to the assumption that optical path changes by at least a few microns.

The SEM was able to acquire fairly detailed images of all the crystals in the sample. The phosphate-containing crystals could have been a precipitate of the PBS buffer; it is unclear whether the magnesium crystals were contamination or could have come from the organism. The size range and abundance of the calcium- and oxygen-containing crystals in the sample closely matches that of what was previously observed in the live animal. The same distinct crystals were found in the second unwashed sample, as well as sodium chloride crystals, which can be expected, as they are a precipitate of seawater. Again, the quantity of calcium- and oxygen-containing crystals found in the second sample was very close to the quantity of crystals found in the original placozoan. Therefore it is very probable that calcium is an element present in the targeted

biominerals. Unfortunately element maps could not be completed for either of these samples due to time restrictions.

The results obtained from the Raman spectrometer readings were difficult to interpret due to the interference from other materials and the possibility that the crystals are not large enough in size to produce significant spectrum peaks. It is not possible to view the birefringence of the crystals when the placozoan is placed on the aluminum tape to be put into the Raman spectrometer; therefore it is difficult to determine whether or not the laser is focused on an area containing a crystal. Multiple spectrum readings were taken at various locations in an attempt to obtain spectra of the crystals as well as the animal body where no crystals were present. The spectra readings strongly correlated to gypsum; however salt crystals precipitated from seawater contain many of the same spectrum peaks as does gypsum, which makes it very hard to differentiate between the two.

A table was constructed to summarize the test results and narrow down the possible list of biominerals produced by placozoans (See Table 4). The areas marked in black indicate with certainty that the candidate biomineral has been eliminated through the respective test. Areas marked in gray indicate that the biomineral is unlikely but cannot be eliminated, and white areas indicate that no statement can be made.

The results obtained from all of the filtered SEM samples suggest that the crystals being produced by the organism at least contain calcium and oxygen. Although silica is biomineralized by Porifera, a closely related phylum of placozoans, the element maps produced by the SEM showed no evidence of silica in any of the samples. Taking this into account, the remaining possible biominerals include all of the carbonates (minus

hydrocerrusite), gypsum, and fluorite. When interference color spectra are considered, calcite, aragonite, vaterite, bassanite, and monohydrocalcite all have a large spectrum of colors. This is not what is observed when the crystals are rotated 360° under a polarizing microscope. It is also noteworthy to point out that the Raman spectrometer did not produce any readings that correlated at all to calcite, Mg-calcite, aragonite, or vaterite. Gypsum was not eliminated by any test, however the occurrence of gypsum as a biomineral in marine organisms (specifically cnidaria) has conflicting research. A recent study claims that the stratoliths produced by certain cnidaria are bassanite ($CaSO_4 \cdot 0.5H_2O$), as opposed to gypsum ($CaSO_4 \cdot 0.5H_2O$) (Tiemann 2006). Bassanite, however, forms needle-like crystal structures which is not the crystalline structure observed in biomineralizing placozoans. It was not possible to explicitly determine the identity of the biominerals in this study; however the results point towards the elimination of calcium carbonates as potential biominerals as well as determine that the biominerals contain at least calcium and oxygen.

CHAPTER 5. Concluding Remarks

Based on genetic evidence as well as non-genetic indicators, it appears that the phylum Placozoa is more diverse than previously thought. Although not enough information has been collected to determine the exact biomineral being produced by placozoans, this study has determined that the target biominerals contain both calcium and oxygen and are not of calcium carbonate nature. Future prospects include more proficient SEM analyses as well as the use of a Confocal Raman Microprobe.

TABLES

Table 1. Candidate crystalline biominerals produced by placozoans

Name	*Formula*
Carbonates	
Calcite	$CaCO_3$
Mg-calcite	$(Mg_xCa_{1-x})CO_3$
Aragonite	$CaCO_3$
Vaterite	$CaCO_3$
Monohydrocalcite	$CaCO_3 \cdot H_2O$
Protodolomite	$CaMg(CO_3)_2$
Hydrocerussite	$Pb_3(CO_3)_2(OH)_2$
Phosphates	
Dahllite	$Ca_5(PO_4,CO_3)_3(OH)$
Apatite	$Ca_5(PO_4)_3(OH,F,Cl)$
Sulfates	
Gypsum	$CaSO_4 \cdot 2H_2O$
Bassanite	$CaSO_4 \cdot 0.5H_2O$
Barite	$BaSO_4$
Celestite	$SrSO_4$
Other	
Pyrite	FeS_2
Silica	SiO_2
Magnetite	Fe_3O_4
Crystalline Organics	

Table 2. Strain 13 Population Counts
Filter Feeder Medium (F) vs. Brine Shrimp Medium (B)

	F 1	F 2	F 3	B 1	B 2	B 3
Day 2	5	6	10	7	4	5
Day 4	8	11	11	2	4	7
Day 7	13	5	9	3	4	3
Day 11	22	16	17	7	6	6
Day 14	24	19	25	8	6	11
Day 18	43	26	18	3	12	10

Table 3. Strain 8 Population Counts
Filter Feeder Medium (F) vs. Brine Shrimp Medium (B)

	F 1	F 2	F 3	B 1	B 2	B 3
Day 2	4	5	3	4	0	1
Day 4	4	4	3	5	2	2
Day 7	7	6	5	5	2	3
Day 11	10	27	0	3	2	0
Day 14	0	60	0	9	5	0
Day 18	0	118	0	12	6	0

Table 4. Summary of Test Results

Biomineral	Combustion	Birefringence Interference	Triammonium Citrate	Raman Spectroscopy	SEM
Calcite					
Mg-calcite					
Aragonite					
Vaterite					
Monohydrocalcite					
Protodolomite					
Hydrocerussite					
Dahllite					
Apatite					
Gypsum					
Bassanite					
Barite					
Celestite					
Pyrite					
Silica					
Magnetite					
Crystalline Organics					

FIGURES

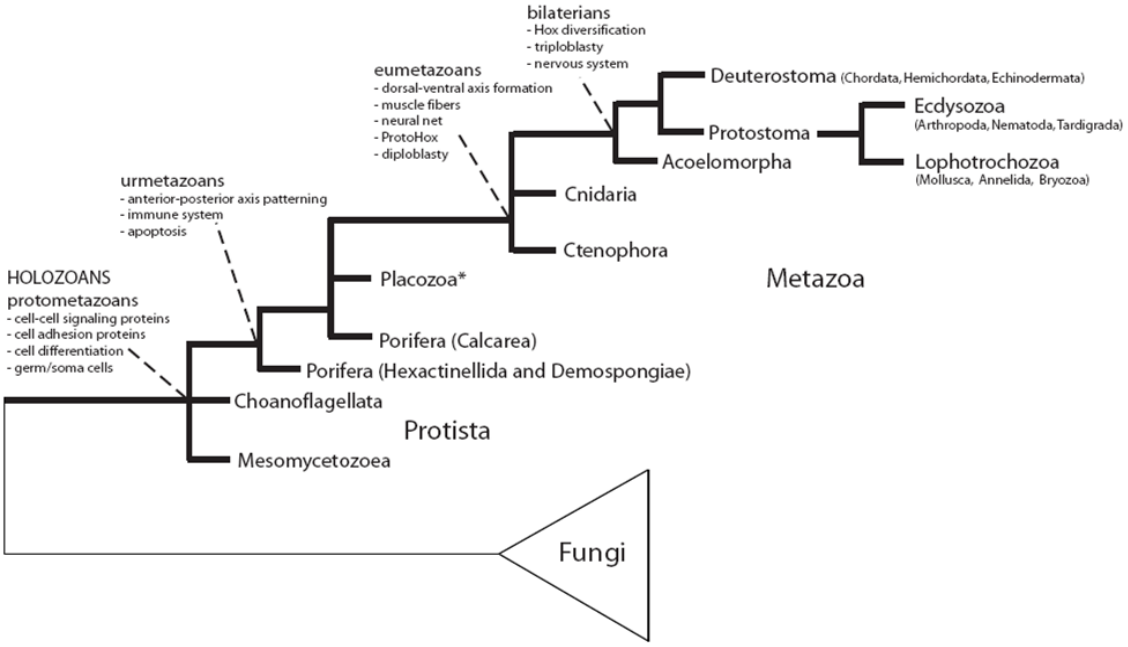


Figure 1. Hypothesized placement of Placozoa on phylogenetic tree

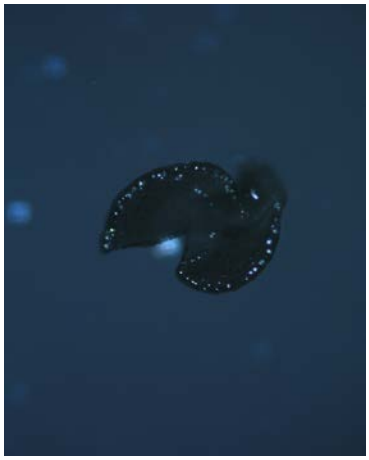


Figure 2. Image of a biomineralizing placozoan

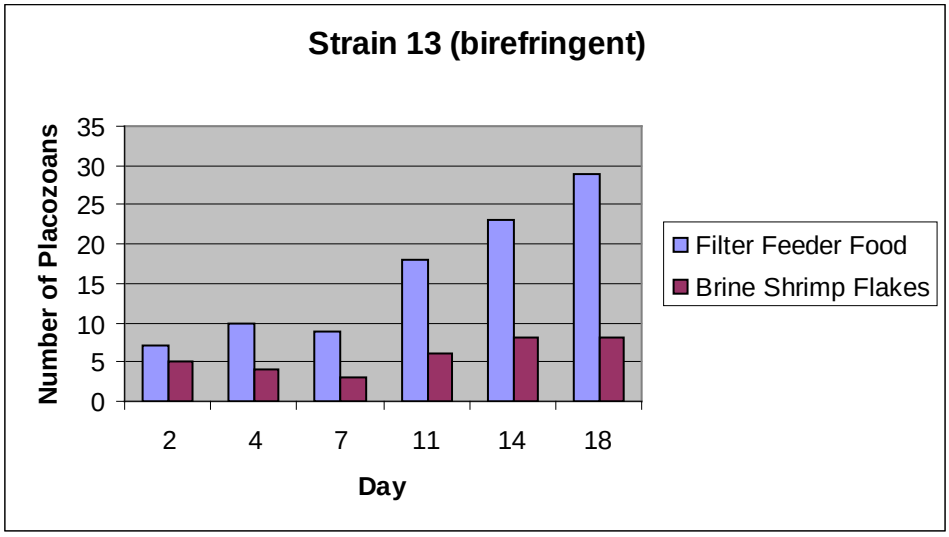


Figure 3. Strain 13 daily population counts in two different media

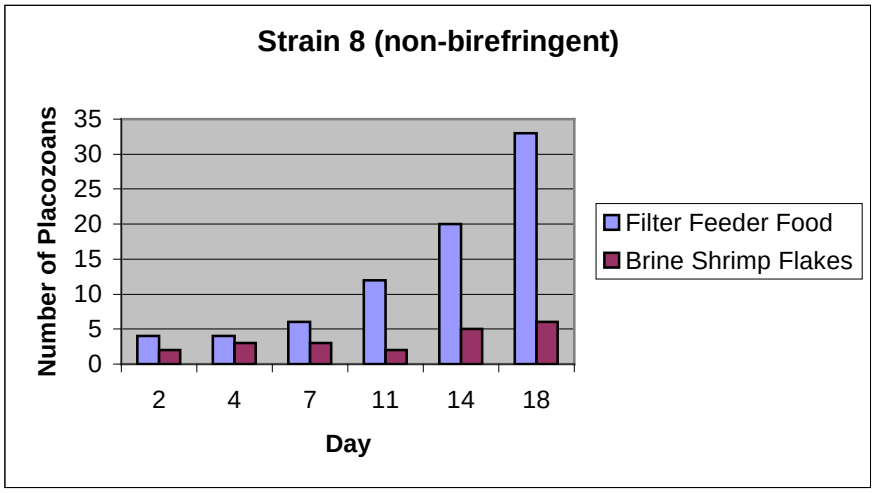


Figure 4. Strain 8 daily population counts in two different media

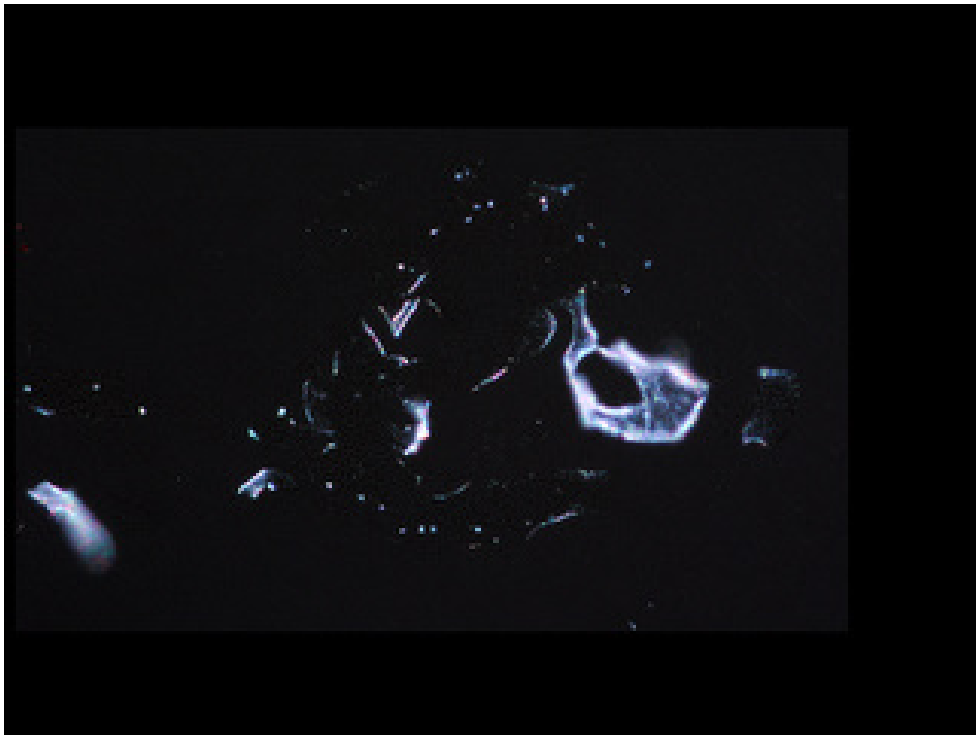


Figure 5. Placozoan crystals after combustion

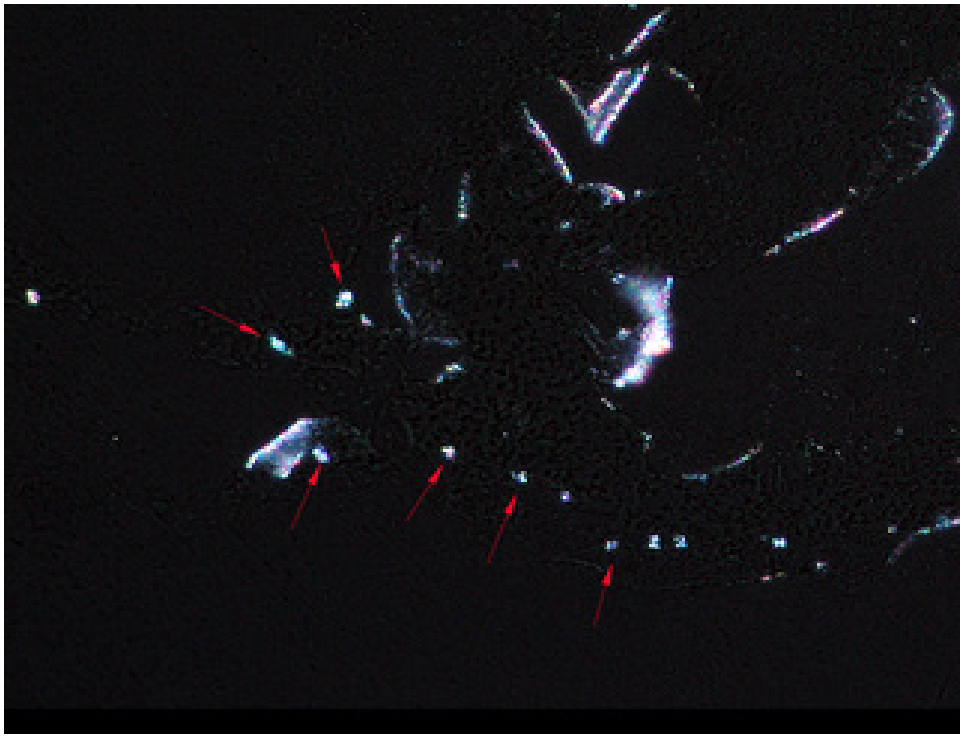


Figure 6. Close up of placozoan crystals

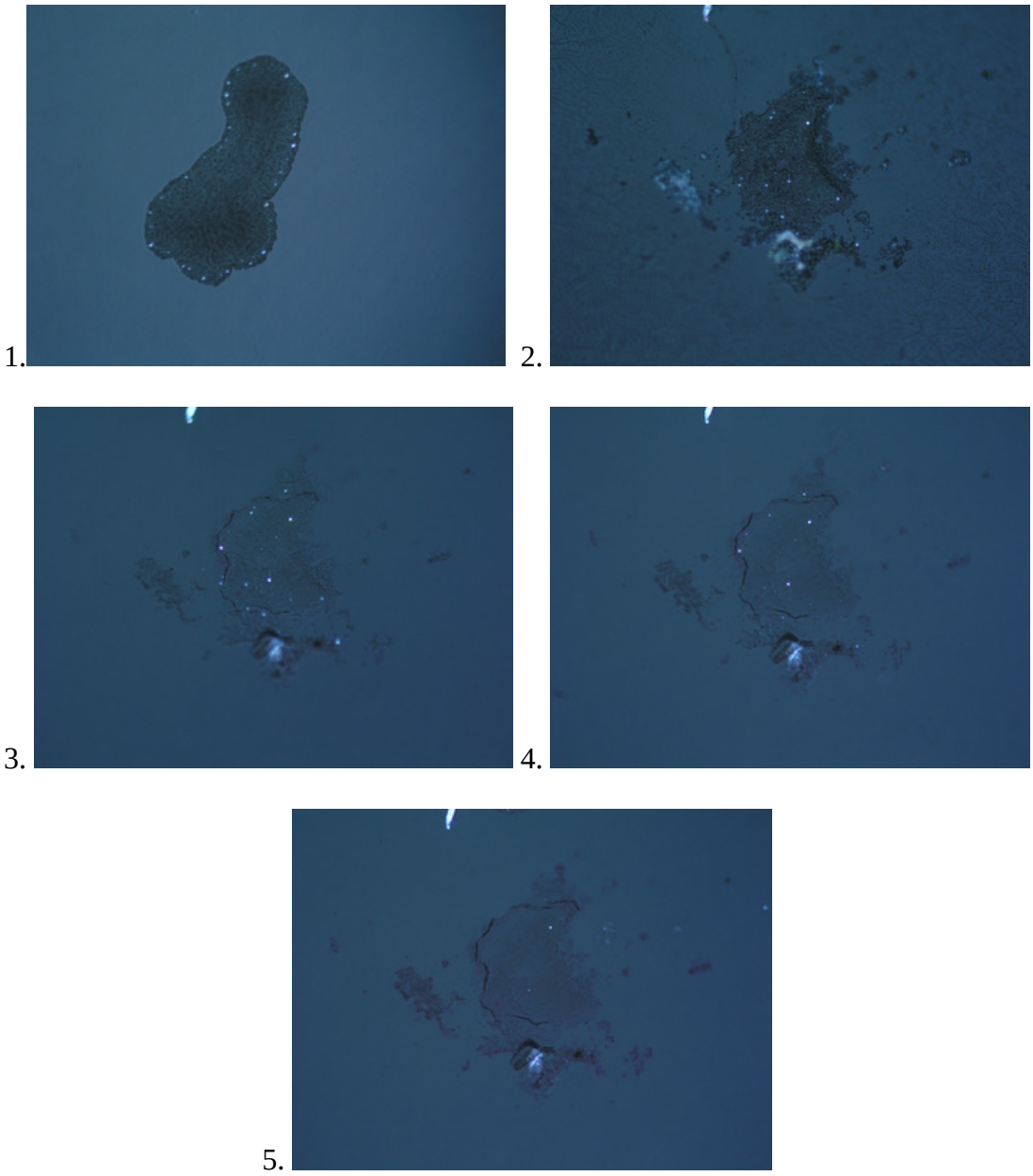


Figure 7. Digital Photos were taken once every minute after the application of 10 µl of Triammonium Citrate

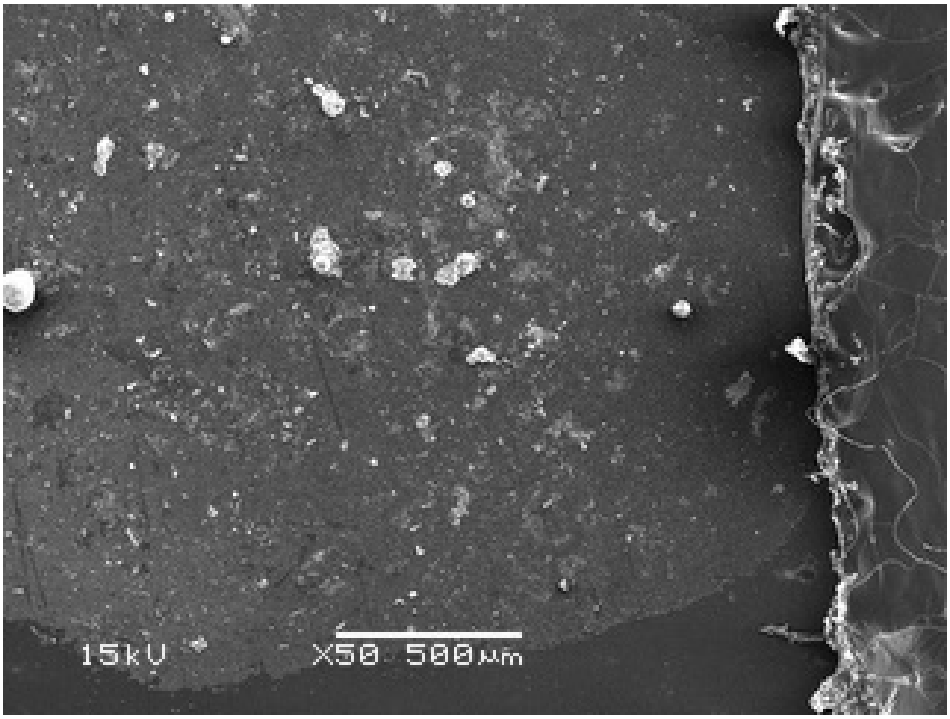


Figure 8. SEM Image of sample exposed to bleach

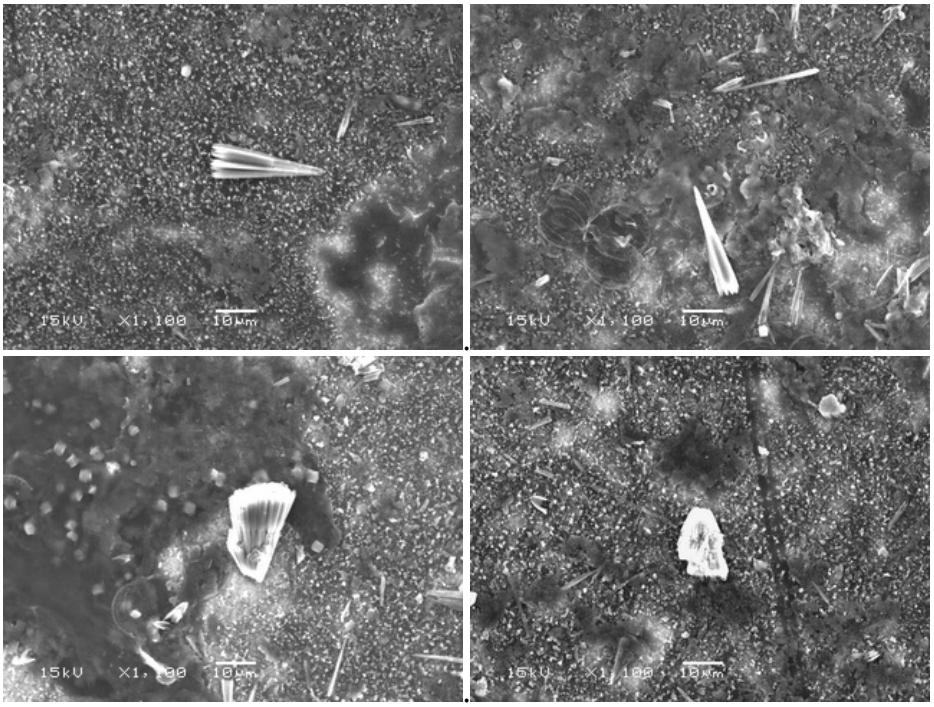


Figure 9. Close up of various crystals

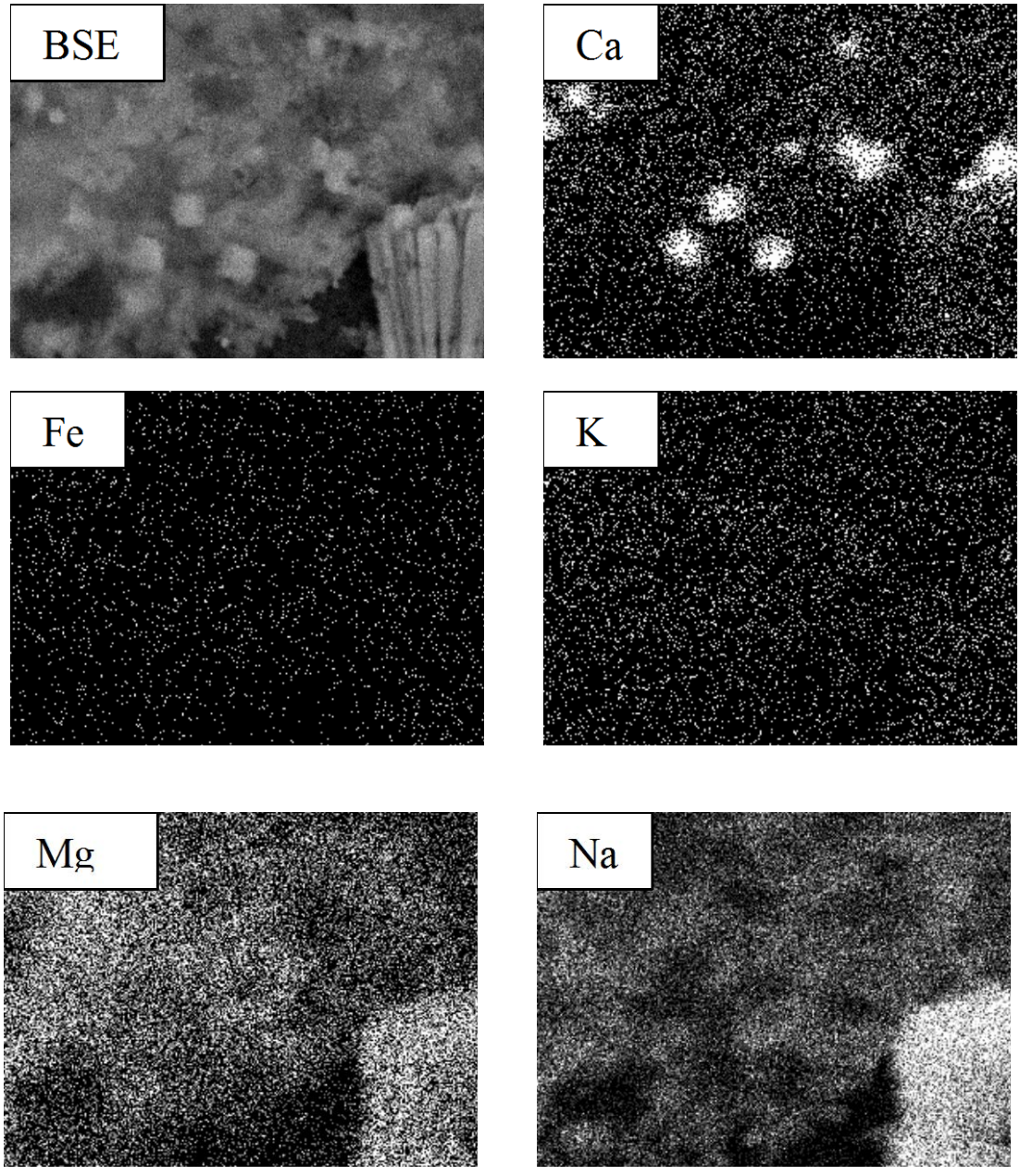


Figure 10. Element Maps of Rinsed Filter Sample

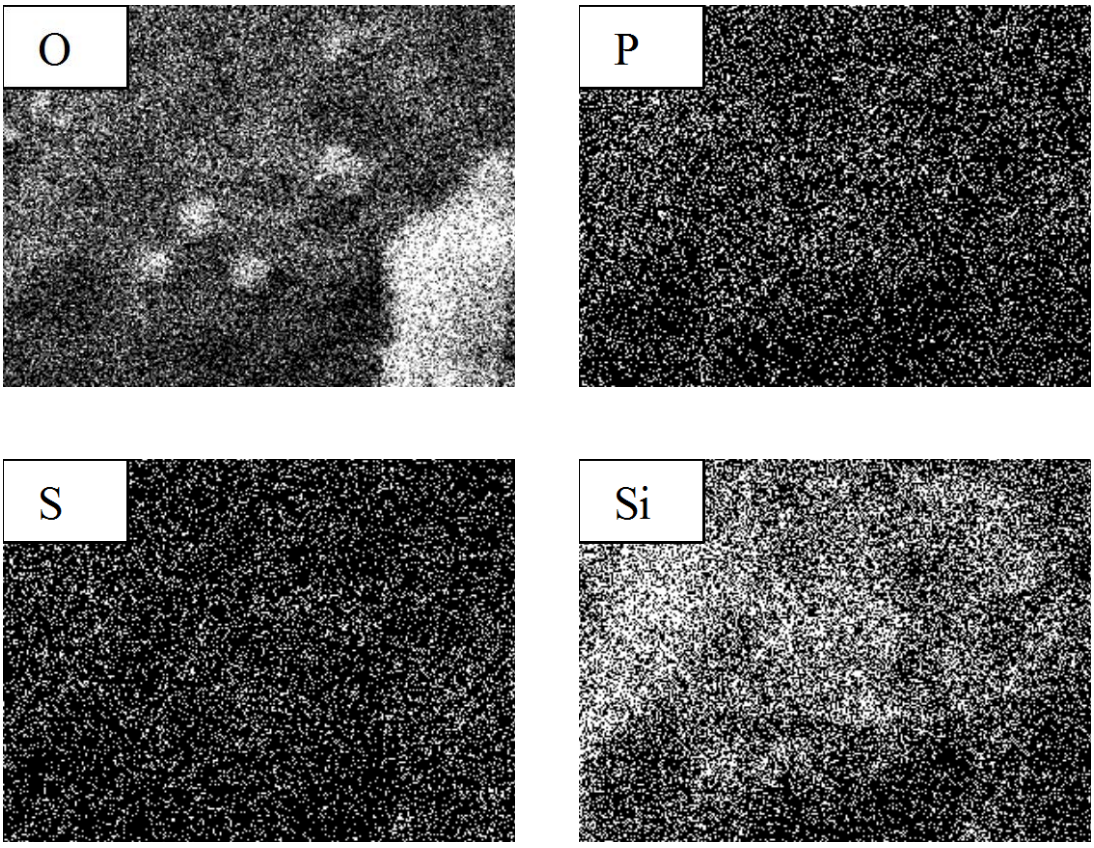
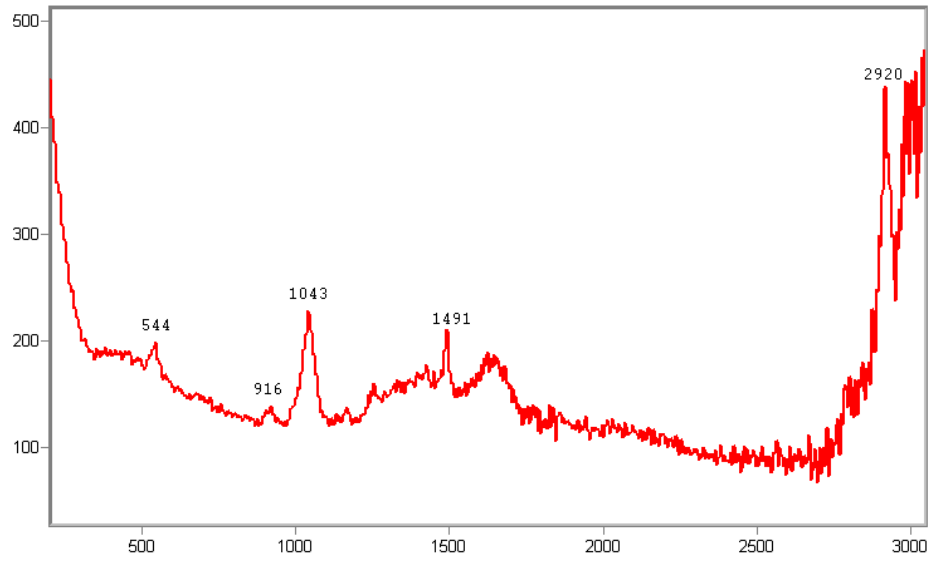
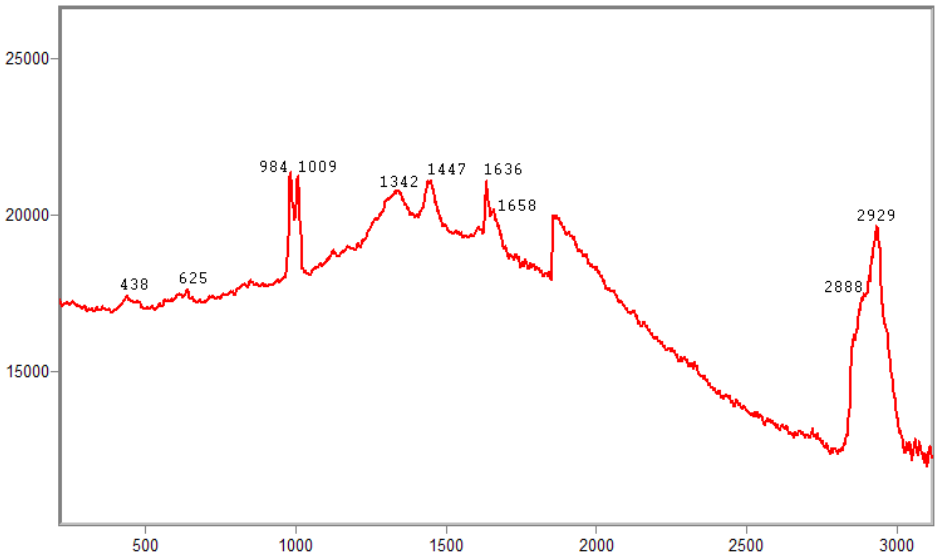


Figure 10. Element Maps of Rinsed Filter Sample



File # 1 : 4 PFA SOLUTION 5S 11MW-M

Figure 11. Raman spectrum of 4% PFA solution



File # 1 = DRIED OUT ANIMAL 6

Figure 12. Raman spectrum of probable crystal

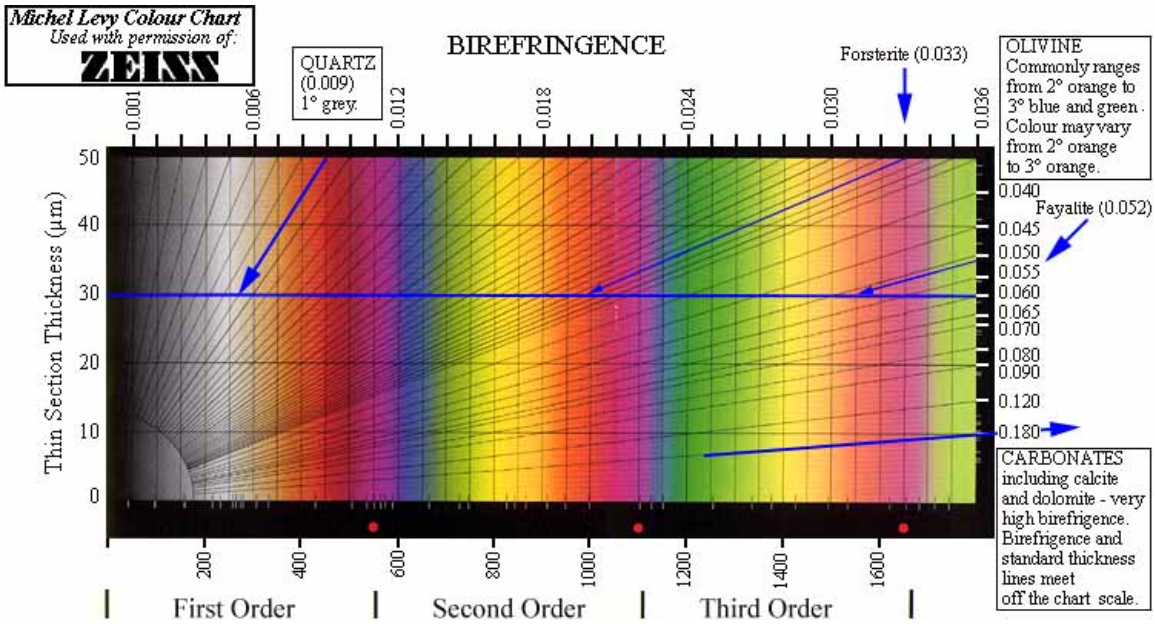


Figure 13. Interference Color Chart (Zeiss 2005)

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