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Investigation of Anatomy of Zooplankton by DAPI, Nile Red and Phalloidin Staining

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ABSTRACT

The behavior of phototaxis is essential for the living of benthic and biofouling organisms, and it is also related closely with factors such as locomotion, sense of light and ecological niche. This experiment sought to find the correlation by analyzing and comparing body structure of some zooplanktons, these include eye structure, muscle, lipid, and fat distribution. In this experiment, samples of bryozoan (*Bugula neritina*) larvae, amphipod (*Niphargus* sp.), isopod (*Cirolanidae* sp.), and copepod (*Harpacticoid* sp.) were treated with three types of fluorescent stain, DAPI, Nile Red and iFour™ 448 Phalloidin. The result of the experiment showed that the advance eye structure in Amphipod (*Niphargus* sp.) and Isopod (*Cirolanidae* sp.) may allow them to identify color, shape and react to change in light condition faster than bryozoan (*Bugula neritina*) and copepod (*Harpacticoid* sp.) which bears simple eye structures. It was observed that more muscle fibers in of Amphipod (*Niphargus* sp.) and copepod (*Harpacticoid* sp.) may contribute to faster locomotion comparing to bryozoan (*Bugula neritina*) larvae. Beside these results, Nile Red signals also suggested the presence of microplastics inside some zooplankton specimens, indicating an environmental issue that can further influence the ecosystem and Human.

Introduction

This experiment is an attempt to prove the relationship between the anatomy of organisms and their phototaxis behavior. The experiment used both normal microscopic imaging as well as fluorescence microscopic imaging of pigmented samples, to compare structure of types of planktons and benthic organisms. The experiment targets include larvae of bryozoan (*Bugula neritina*), barnacles (*Balanus* sp.), Amphipod (*Niphargus* sp.), Isopod (*Cirolanidae* sp.), and copepod (*Harpacticoid* sp.).

Bryozoans are small, diverse invertebrates that live in groups of thousands. The coronate larvae released from the parental bryozoan zooid wander freely in water and later settle and forms biofouling colonies, in which they transform into adult that feed on plankton and bacteria by sweeping the surrounding water with their lophophore. The sample used in this experiment belongs to the species *Bugula neritina*. *Bugula neritina* larvae are positively phototactic on emergence from the brood chamber (Wendt et al. 1999). This, however, changes after 4 or 5 hours into negative phototaxis. The sample used in the experiment is freshly released bryozoan larvae.

Copepod is one of the most common zooplankton found in both freshwater and seawater. They are tiny crustaceans that exist in a diverse niche from biofouling communities, benthic communities to open waters. Like barnacle, copepods have nauplius larvae that grew into juvenile stage and finally reach maturity. Beside distribution, copepod is also famous for its locomotion, which could happen in fraction of second similar to jumping. This gave copepod high mobility compared to other plankton. The sample used in this experiment likely belongs to the nauplius larvae and adult of order Harpacticoid. Phototactic behavior of the marine harpacticoid copepod *Tigriopus japonicus* is related to developmental stages and light intensity (Danielson 1976).

Amphipods are marine and freshwater shrimp-like crustaceans that often makes up an important part of benthic communities. They have eight pairs of leg and they use first five of them do walk, and three to thrust and swim. Amphipods vary a lot in size and shape, as some of them can grow up to 30 cm while others are as small as grains. They usually feed on organic matters but don't mind hunting for plankton and creatures smaller than them. The sample used in this experiment belongs to the genus *Niphargus*. In the previous experiment (NOAA Ocean Exploration, 2017), the *Niphargus* sp. can sense and respond to lasers with wavelengths of 532nm, 515nm and 445nm with negative phototaxis and do no response to wavelength of above 635. Their locomotion rate was also fast given their size and locomotion method.

Cirolanidae is a family that belongs to the order of Isopod. Like amphipod, marine isopods vary a lot in size, but are crustaceans that have seven body segments and seven pairs of legs. The short abdominal section contains six segments, called "pleons," and one or more of these segments is fused into a tail section. They also occupied many environments and can also be parasite of fish (Thorp et al. 2010) The phototaxis behavior of isopod is considered negative. Isopods are adapted to the red and blue wavelengths prior to exposure to the white light (Greater Atlantic Regional Fisheries Office 2017). The samples collected for this experiment likely belong to the family *Cirolanidae*.

Barnacles are marine sessile crustaceans and compose a large portion of the organisms in the biofouling community. Their life cycle includes two major free-moving larval stages: the nauplius stages and the subsequent cyprid stage during which the larvae search for a place to attach and metamorphose into juvenile barnacles. Unable to move and covered with a calcified shell, they feed on zooplankton by obtaining them with their fans-like limb. The nauplius larval sample used in the experiment belongs to the genus *Balanus*, which was previously examined for phototaxis behavior (NOAA Ocean Exploration, 2017) of barnacle larvae. The nauplius larvae showed positive phototaxis behavior towards blue and green light but have no response to red light.

Materials and Methods

The samples were collected from the biofouling communities in Dapeng at around 3 pm 2022/12/21, The GPS coordinate is 22°34'04.2"N 114°31'52.0"E. At the sampling site, pH and salinity were tested. In total samples of barnacle communities and *Bugula neritina* communities were collected and stored in boxes with aerated seawater. Both samples have other biofouling organisms attach on them. Samples of plankton was obtained using plankton net at the same location.

The samples return to the lab at 6pm. The barnacle samples were picked out and amphipods and isopod specimens were separated out. The barnacle samples were then cleaned and washed with fresh water to remove other biofouling organisms. The barnacles were left overnight in aerated seawater in dark container. The *Bugula neritina* adult colonies were left overnight in aerated seawater in dark container. The plankton samples were stored in 4oC refrigerator. Meanwhile the amphipods and isopod specimens were each transferred to a 50ml centrifuge tube with 50 ml of sea water. Relaxation solution (7% Magnesium Chloride solution) was then added to the centrifuge tube. After 10 min the samples were transferred into two 2ml centrifuge tubes and the supernatant were extracted with a pipette. 1mL of 4% paraformaldehyde dissolved in 1X PBS were added and left overnight for sample fixation.

On the second day, barnacle adults kept in the dark were transferred to a transparent container with seawater. Strong white light illumination was applied to the barnacle adult to trigger the release of nauplius larvae. After 2 hours the barnacle larvae released and concentrated at the light source was transferred to a 2ml centrifuge tube. The same process was done to the *Bugula neritina* adult colonies to obtain its larvae. The fixed plankton samples were separated into four 50ml centrifuge tubes and condensed. The plankton specimens were then transferred into another four 2ml centrifuge tubes. Up to this point, there were one tube of amphipod specimen, one tube of isopod specimen, three tubes of *Bugula neritina* larvae, four tubes of plankton samples and one tube of barnacle larvae. 1 mL of PFA fixation solution was then added to each tube and left for 10 min and then exchange for new PFA for 3 times. The samples were left overnight on shaker.

On the third day, each sample tube except the B. neritina larval sample was exchanged with 1 mL of freshly

prepared 1 x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4). The samples were exchanged with new PBS for three time. After this the PBS in each sample were removed and 10 ml of PBST, which was 10% Triton X-100 solution in PBS, was added and the 10 samples were left on the shaker spare. Meanwhile the staining solution was prepared. The staining solution was PBST containing 2% of DAPI (100%), 0.1% of Nile red (1mg/ml) and 0.1% of iFlour™ 488 Phalloidin. All three pigments were stored at 20 oC. This staining solution was freshly made and was applied to the samples for two days before it was replaced with new washing solution. The *Bugula neritina* larvae sample was stained for 90 min.

After the staining step, the samples were washed with 800 microliter of PBS solution for three times. The final sample is transferred to a fluorescence microscope and microscopic image is taken under the emission wavelength of each pigment. Image acquisition was carried out with the Soft-Top fluorescent microscope (company info) and a CCD colour (company info). The light source for fluorescence excitation was generated by an camera name (company info). Fluorescence filters were applied to observe fluorescence staining of different histological dyes.

Results

Fluorescent images were collected successfully for bryozoan larvae, copepod nauplius larvae and adult, Isopod and amphipod. The sample of nauplius larvae of barnacle was not observable in the final sample.

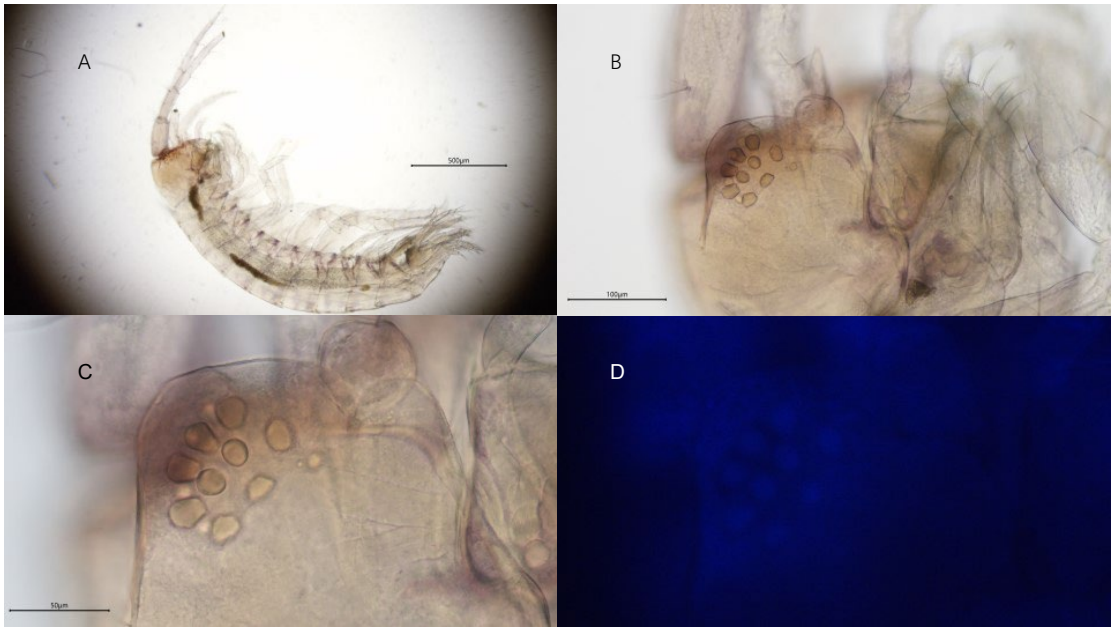

Figure 1. The Images of amphipod sample 1. 1A is the image of the whole body under white light; 1B and C are the eye structure under white light; 1D is the eye structure under DAPI emission wavelength. Notice that the scale bar for 1D is same with 1C.

The amphipod specimen, with length of about 2000 micrometers (Figure 1A) (excluding antenna) have no florescence signal for Phalloidin and Nile red staining but showed weak signal of DAPI in the eye structure. Under 40x amplification (Figure 1C), the eye structure of amphipod shows a compound eye with diameter of about 50 micrometers. The compound eye is located on both side of the cephalothorax segment and consist of 10-15 cones of crystal, likely structures of Ommatidia. Under fluorescent microscope, blur detail indicates signal from cells and the cone crystals. (Figure 1D)

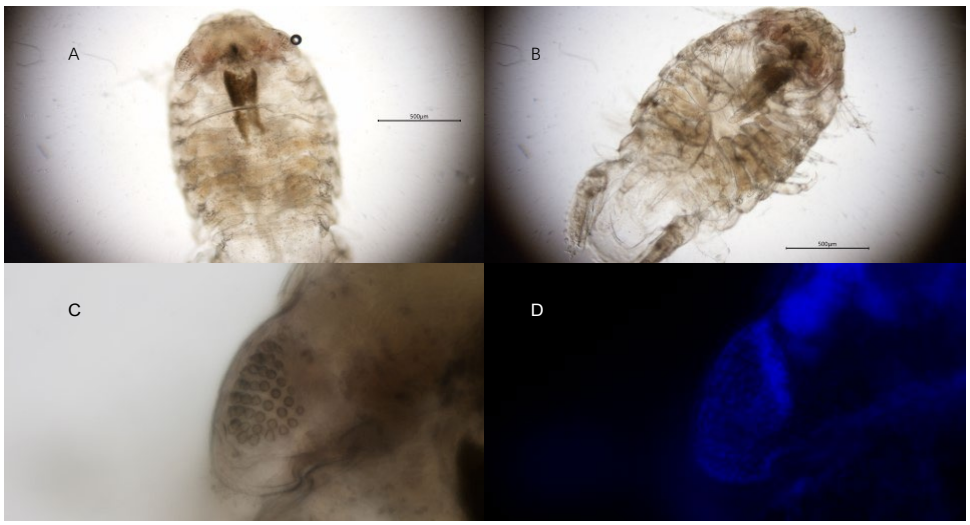

Figure 2. Images of Isopod sample 1. 2A and B are the whole body of sample under white light; 2C is the overlap of 16 eye structure of sample under white light ; 2D is the overlap of 16 image of eye structure of sample under DAPI emission wavelength. Notice the scale bar for 2C and D is same with 1b.

The isopod specimen, with length of about 2000 micrometers (Figure 2A) also have no fluorescence signal for Phalloidin and Nile red staining, but showed weak signal of DAPI pigment of eye structure. A set of pictures with 16 different foci were taken for the eye detail under 20x amplification. The stack of DAPI and bright-field images shows complex eye structure with more than 50 cone crystals each side of the head (Figure 2C). Under fluorescent microscope, detail indicates significant signal from cells and the cone crystals. (Figure 2C)

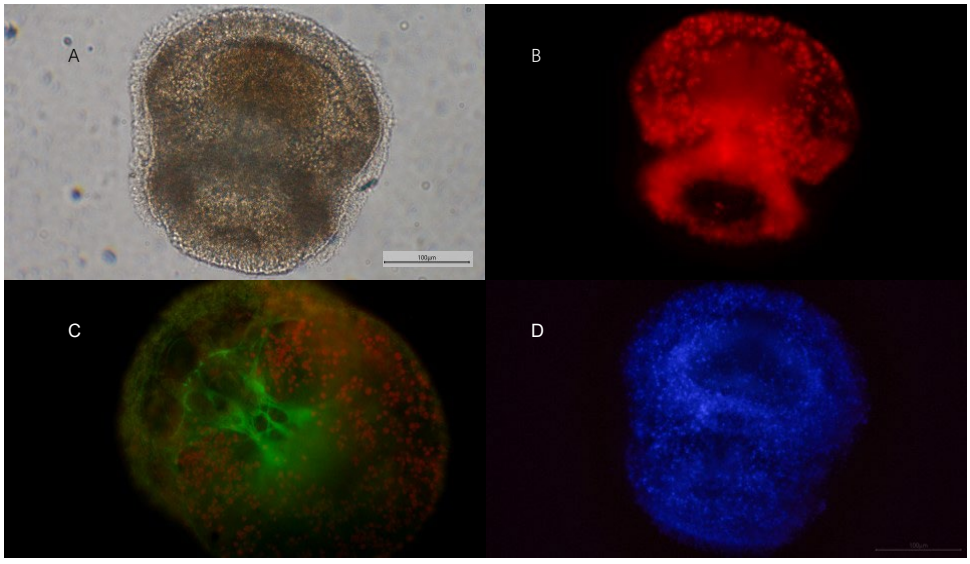

Figure 3. Images of Bugula neritina coronate larvae sample 1 (3C) and 5 (3A,B,D). A is the image of sample under white light; B is the overlap of 16 image of sample under Nile red emission wavelength; C is an overlapped image of sample under Nile red and Phalloidin emission wavelength; D is the overlap of 40 image of sample under DAPI emission wavelength. Notice that the scale bar of 3B and 3D is the same as 3A, and the scale bar of 3C is the same as 1C.

The bryozoan (*Bugula neritina*) larval sample, with a diameter of around 300 micrometers (Figure 3A) showed strong signal of DAPI, Phalloidin as well as Nile Red stain. Strong DAPI signal can be observed throughout its tissues, forming a donut-shape body (Figure 3D). The Nile Red signal is more concentrated on the surface of the specimen (Figure 3B). Besides, indication of Phalloidin signal shows significant bands of actin filament located in the center of the body, indicating existence of a well-organized muscle fibers system (Figure3C). The eye structure is clearly observed in the bright-field image when the sample was alive. Image shows single eye on both side of the body, both surrounded by black tissues (Figure 4).

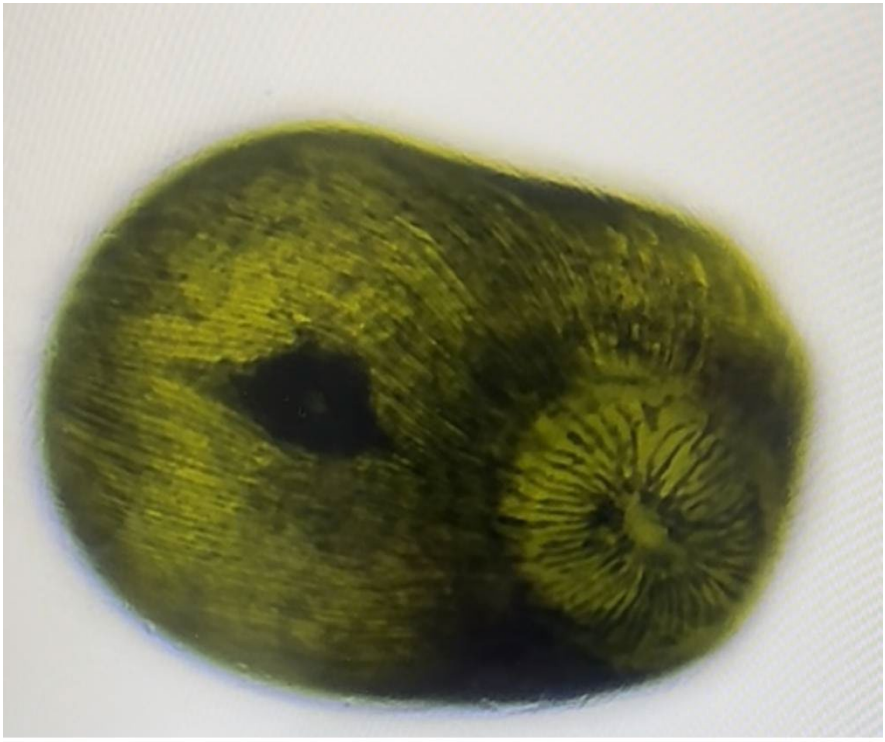

Figure 4. Image of one of the living samples of the bryozoan *Bugula neritina* coronate larvae used in this experiment. Notice the single eye structure of the sample that is surrounded by a dot of dark tissue. This eye structure exists on both side of 1 sample.

Two copepod adult specimens sample 2 (Figure 5) and 3 (Figure 6), each with a length of around 600 micrometer (Figure 5) and 350 micrometer (Figure 6) (excluding antenna and tail tip), shows signals of Nile Red and Phalloidin but weak signal of DAPI (Figure 6D & 5D). Nile Red staining images shows lipids droplets in the body of both samples (Figure 5B & 6B). Phalloidin signals shows muscle fibers located in the legs in both samples, but in sample 3 (Figure 6c) strong also signal appear from body all the way to the tail, indicating well developed muscle throughout the body .

Three copepod nauplius larval specimen, sample 1, 3 and 5, each with a length of around 200 (Figure 7), 230 (figure 8) and 250 micrometer (Figure 9) (excluding antenna and tail tip), shows weak signal of DAPI and Nile red (except sample 1, which DAPI signal is unidentifiable), but significantly strong signal of Phalloidin. Sample 1 shows almost identical Phalloidin signal, with muscle fibers locating at the appendages, the digestive system, the antennae, the base of seta and skeletal muscle that covers the side of the specimen (Figure 7B&C and 8B&C). Sample 5 shows bright band of Phalloidin signals in the appendages.

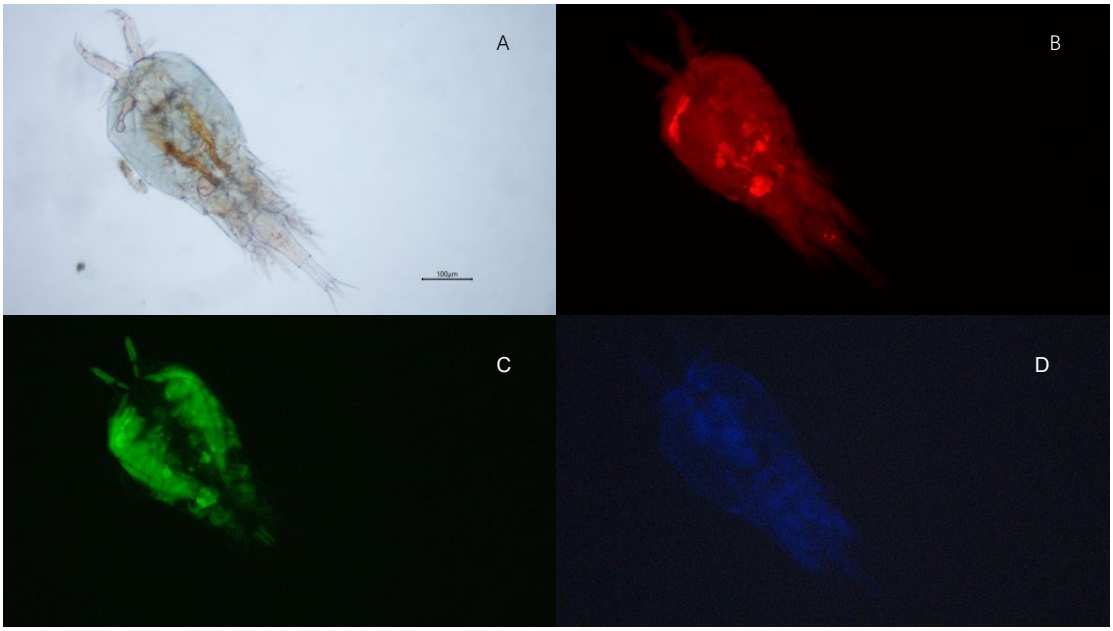

Figure 5. The image of Copepod adult sample 2. A is the sample under white light; B is the sample under Nile red emission wavelength. C is the sample under phalloidin emission wavelength. D is the sample under DAPI emission wavelength. Notice that 5B, 5C and 5D share the same scale bar as 5A.

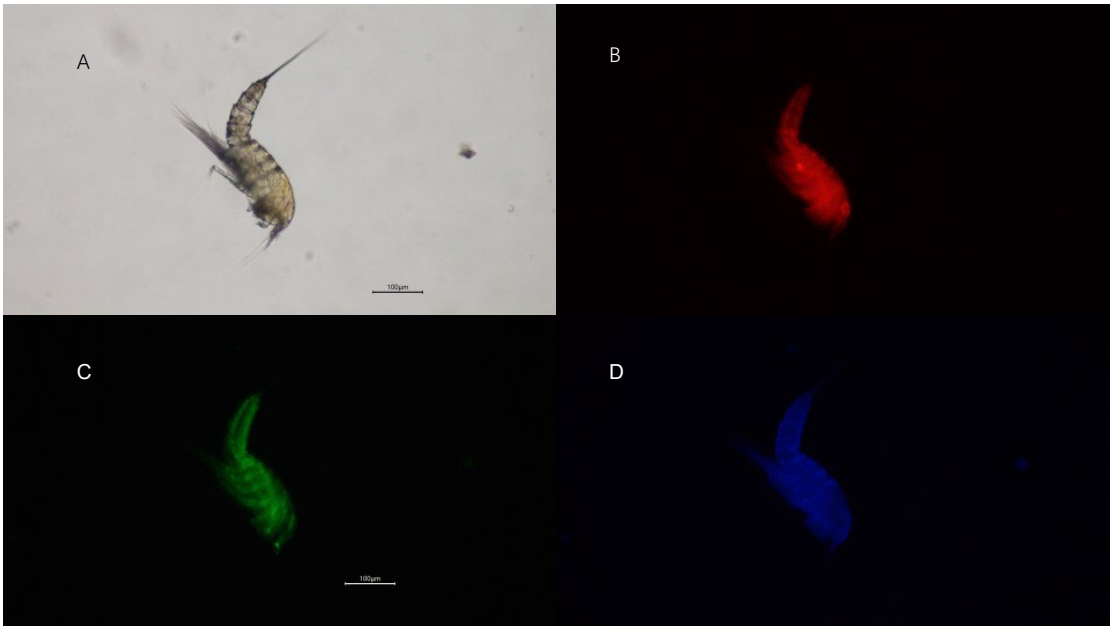

Figure 6. The image of copepod adult sample 3. A is the sample under white light; B is the sample under Nile red emission wavelength. C is the sample under phalloidin emission wavelength. D is the sample under DAPI emission wavelength. Notice that 6B and 6D share the same scale bar as 6A.

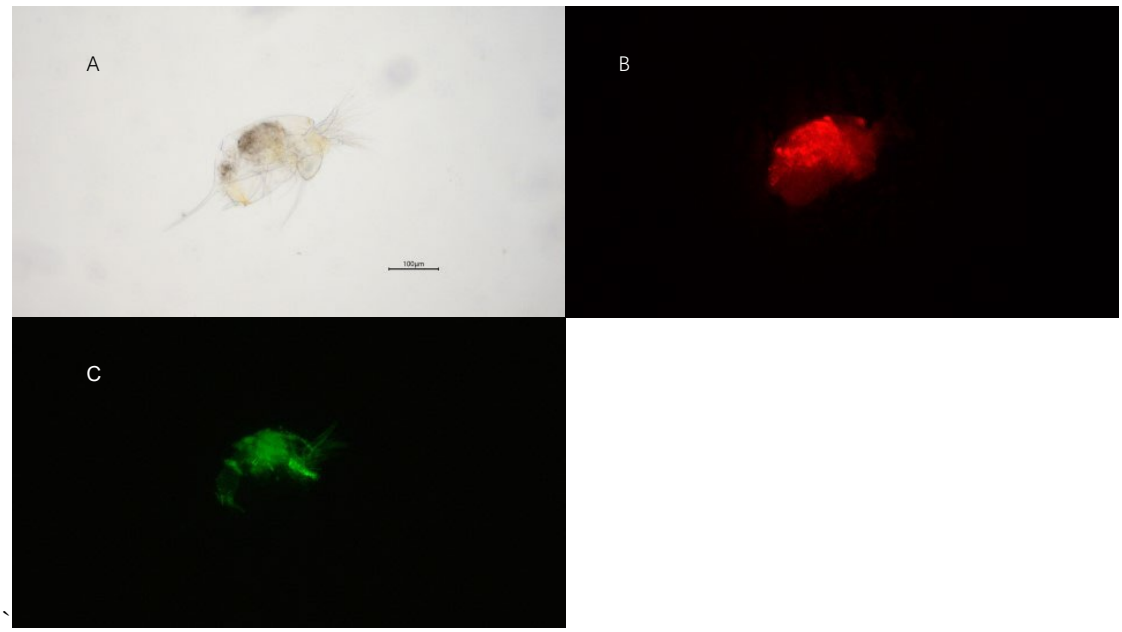

Figure 7. The image of copepod larvae sample 1. A is the sample under white light; B is the sample under Nile red emission wavelength. C is the sample under phalloidin emission wavelength. Notice that 7B and 7C share the same scale bar as 7A.

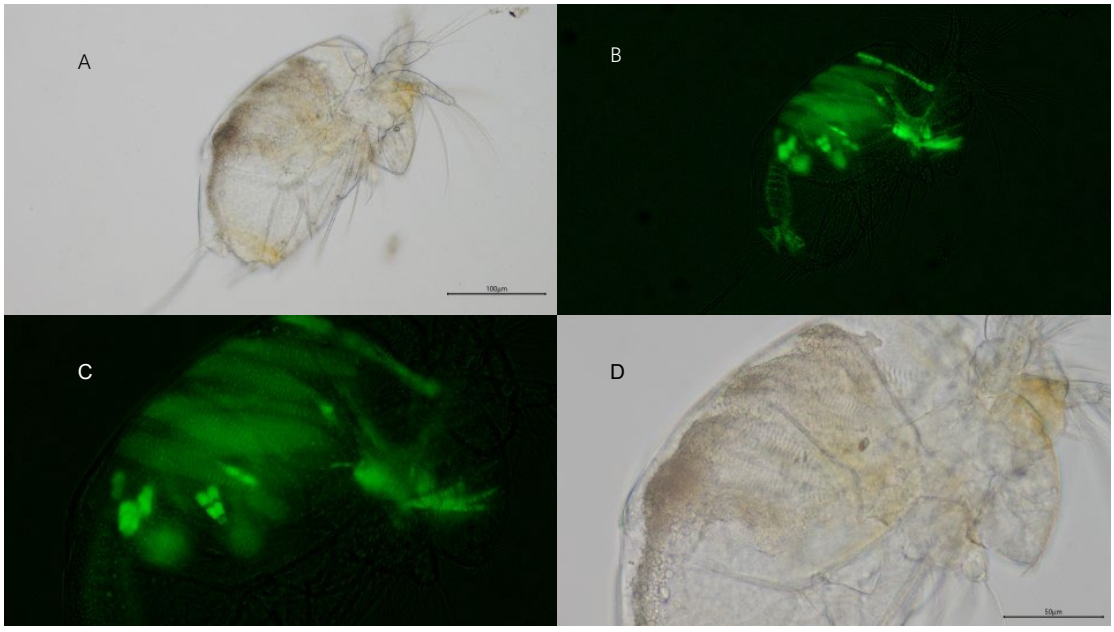

Figure 8. The image of copepod larvae sample 3. A and D is the sample under white light; B and C is the sample under phalloidin emission wavelength. 8B has the same scalebar as 8A and 8C has the same scalebar as 8D

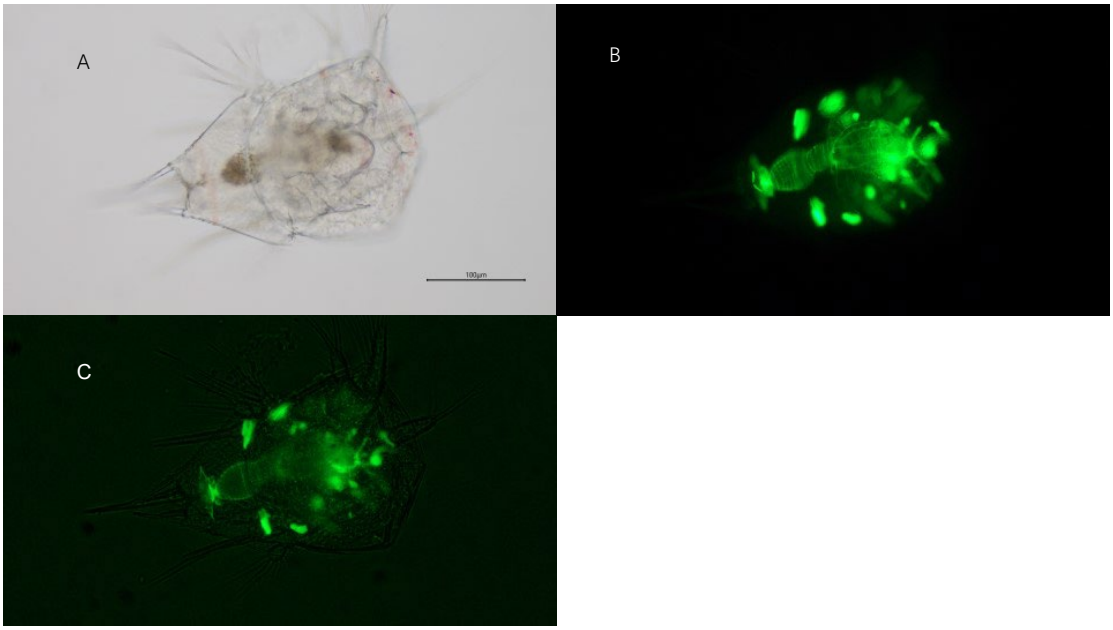

Figure 9. The image of copepod larvae sample 5. A is the sample under white light; B and C is the sample under phalloidin emission wavelength. Notice that 9B and 9C share the same scale bar as 9A.

Discussion

Fluorescence staining is one of the common ways to indicate structures of organisms, organs, or cells. Fluorescent dye works by absorbing photons under certain wavelengths of light and emitting them in a region of wavelength visible to human eyes. For different fluorescent dyes, they favor bonding with different molecules. In this experiment, three types of pigment are used: DAPI, that bind with deoxyribose nucleic acid which indicate cell units; iFour™ 448 Phalloidin, that bind with and stabilize filamentous actin; Nile red, while indicating lipid, can also be associated to micro-plastic particulates. For all the three pigments to work properly at the same time, samples needed to have their cells permeabilized and/or fixed to allow pigments to enter. When the staining molecules failed to enter the sample due to the presence of exoskeletons for example, staining might become unsuccessful, as in Phalloidin staining in some of the copepod and amphipod samples.

Eye structure

Among all the eye structures of samples, *Cirolanidae* sp. (Isopod) and *Niphargus* sp. (Amphipod) have the most advanced eye structure, composing of multiple crystal cones. While the signal of DAPI is weak in the specimen of *Niphargus* sp., the signal was stronger in the eye structure of *Cirolanidae* sp., showing a larger and more developed (presumably specialized) compound eye structure, suggesting the structure may be able to sense brightness and distinguish color. Compound eye also provide a wide range of view and allow fast detection of movements and change in brightness, resulting in a quick responsive phototaxis behavior. As both species specimens were recovered from a heavily fouled underwater structure, the compound eye structure may also contribute to the role of the two species in benthic and biofouling communities as generalist, since they both scavenge organic matter and hunt for organisms.

Compared to the *Cirolanidae* sp. and *Niphargus* sp., *Bugula neritina* (Bryozoan) larvae has a relatively simple eye structure, with two eyespots locate on the side of the body (Figure 4). Such eye structure is only capable of identifying basic changes in brightness. The black tissues around the eye structures likely function to block the light from

going into the photoreceptor cells to increase sensitivity towards environmental light source. This sense of brightness can assist their role in this free-roaming larval stage (coronate larvae) to sense their position relative to the substrate during searching for surface to attach to.

The eye structure of both adult and larvae samples of the copepod *Harpacticoid* sp. was not found, it is suspected that damage of eye structure occurred during the process of treatment of samples. However, according to literature, in most copepod species the (nauplius) eyes are small, immobile, and located in a central cephalic position (Porter et al. 2017). Such an eyespot can only identify general changes in light intensity.

Muscle structure

Phalloidin staining of the amphipod *Niphargus* sp. was not successful in this experiment (likely because the pigment did not penetrate through the exoskeleton), yet their muscle structure is likely the most developed among all the samples. The living sample moved in a drag-based type of mechanism (Boudrias 2002). These structures may contribute to the fast movement of locomotion when performing phototaxis, as observed in the previous experiment done. It also corresponds to the sample's role in benthic and biofouling communities as generalist that both scavenge and hunt.

Phalloidin staining of the isopod *Cirolanidae* sp. was also not successful, likely due to the same reason as the *Niphargus* sp. samples. Their muscle structures are similar to that in amphipod, but unlike amphipod, the living sample of isopod performs locomotion in a slow-rate crawling pattern. When disturbed, they can curl into sphere-shape and either sank quickly or roll away. This results in the slow-moving pattern in the phototaxis experiment, as well as matching its niche as a generalist that favor mostly organic matter, plant and algae.

The muscle structure of the coronate larvae of *Bugula neritina* can be observed within the body. There are significant fiber structures in the body, and thinner, shorter fibers located at the surface of body. The major muscle structure contributes to the sample's deform/stretch and shrinkage of body. Larval movement was performed with the cilia located all over its surface coronate cell layer. They enable the *Bugula neritina* coronate larvae to find suitable biofouling substrate or benthic surface to attach and settle.

Phalloidin staining of both adult and nauplius larvae of copepod were successful. Skeletal muscle can be found in the body and legs, along with networks of fibers in its digestive system. The highly developed muscle structure despite their small body size allows them to perform locomotion with a movement pattern of swim-and-jump and demonstrated fast phototactic responses.

Fat/oil droplets and Nile Red staining pattern

The samples of *Bugula neritina* had shown clear oil droplets located at the surface of their body. This indicates storage of nutrition and energy that is essential for the coronate larvae of *Bugula neritina*. Since *B. neritina* larvae does not eat, they will have to sustain the whole swimming and subsequent metamorphosis process based on the nutrition inherited from the parent.

While some Nile red signals in other samples also show the presence of lipid or fat, Nile Red staining might also reveal something else. Nile Red is known as a stain for indicating the presence of microplastics, which are plastic particles (<5 mm) in the environment [8]. In samples like the copepod *Harpacticoid* sp., abnormal concentration of DAPI signal is indicated in the body, with irregular shape and size that might indicated microplastic. The presence of microplastic in these planktons and organisms in lower trophic level has a serious influence. Through the process of bioaccumulation, the concentration of microplastic will increase as it goes up the trophic level, which eventually results in edible fish containing a high concentration of microplastic. This increase in microplastic consumption can put negative influence to organisms throughout the ecosystem including humans.

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