

GUT CONTENT AND PIGMENT ANALYSIS IN THE MARINE ISOPOD
PENTIDOTEA RESECATA

by

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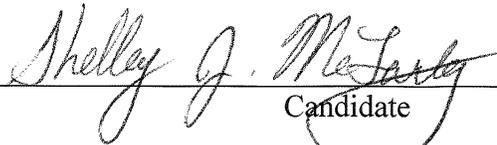
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ABSTRACT

The marine isopod *Pentidotea resecata* lives on eelgrass (*Zostera marina*) along the west coast of North America. These green animals closely match their eelgrass substrate in color, suggesting that these isopods may obtain their green color at least partly from the eelgrass. This suggestion is further supported by evidence that the isopods feed on eelgrass, including the presence of large sections of whole eelgrass cells within the lumen of the isopod hindgut. These eelgrass cells contain chloroplasts and at least some functional chlorophyll, as indicated by chlorophyll fluorescence. Chlorophyll pigments can also be extracted from isopod tissue; however, most of the pigment present is actually pheophytin, a degradation product of chlorophyll. Diatoms are also frequently found within the contents of the gut, and most likely represent the source of chlorophyll *c* found within the isopod. Thus these isopods contain photosynthetic machinery within the lumen of their hindgut in the form of whole eelgrass cells and diatoms.

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INTRODUCTION

Photosymbiotic Associations

A variety of organisms form photosymbiotic associations in which a non-photosynthetic organism houses a photosynthetic organism (Clark 1992). A classic example of such can be found in coral reef communities where dinoflagellates (unicellular algae often referred to as zooxanthellae) live within the gastrodermis of the coral animal. This sustained physical association permits the transfer of metabolites from the alga to the animal and vice versa, and may help sustain coral reefs in nutrient-poor waters (Muscatine & Porter 1977). These dinoflagellates are members of the genus *Symbiodinium* and have been found in a variety of organisms other than corals including anemones, coronate jellyfish, hydrozoans, sponges, bivalves, and gastropods (Baker 2003).

Sacoglossan Model

Perhaps the best-known photosymbiotic association in a complex (triploblastic) animal has been observed in mollusks. In a phenomenon termed “kleptoplasty”, some marine, opisthobranch gastropods from the order Sacoglossa obtain chloroplasts from their algal diet and sequester the plastids within the epithelium lining their digestive system (Clark 1992). The chloroplasts continue to photosynthesize for some time within the slug and thereby appear to contribute more than just green camouflage to the animal host. In fact, the kleptoplastic sacoglossan *Elysia chlorotica* can survive up to nine months in the absence of food if provided with light and CO₂ (Green et al. 2000). *Elysia*

timida, another kleptoplastic species, exhibits greater survival and less decrease in size if starved while on a 12 h light-dark cycle than if maintained in continuous darkness without food (Casalduero & Muniain 2008). Given that the chloroplasts are sequestered within epithelial cells of the slug's digestive system, this long survival time most likely indicates that the slug is benefitting metabolically from the photosynthate produced by the chloroplasts. Under optimal lighting, the slugs spread out their parapodia or cerata, increasing chloroplast exposure to light and further demonstrating the animals' dependence on light (Clark 1992).

One of the most astounding features of this association is the duration over which the captured chloroplasts retain their functionality, *i.e.* continue to photosynthesize. In *E. chlorotica*, the thylakoid membranes retain their capacity for photosynthetic electron transport (PET) for more than six months inside the slug (Green et al. 2000). The high-energy process of photosynthesis damages chloroplasts, and so for the thylakoids to remain functional, these damaged proteins must be repaired or replaced. Green and colleagues (Green et al. 2000) found that several thylakoid polypeptides, including a Rubisco peptide, are actively translated inside the slug, indicating *de novo* protein synthesis in the animal. Most of the chloroplast proteins are actually encoded by the algal nuclear genome, not the plastid genome. This should make plastid repair very difficult, if not impossible for the slug. *E. chlorotica* appears to have overcome that challenge due to horizontal gene transfer of the necessary genes from algal nuclear DNA to the slug chromosomes (Pierce et al. 1996, Hanten & Pierce 2001, Rumpho et al. 2008, Schwartz et al. 2014, Pierce et al. 2015).

Crustacean Models

Not all photosymbiotic associations are as intimate as that between the sea slug and its algal prey, in which the chloroplast is sequestered within animal cells. In some cases, the association could be as simple as the presence of intact cells or chloroplasts within the lumen of the animal's digestive system. However, even such a simple scenario would bring the chloroplast into close enough association with the animal that the animal might benefit metabolically if the chloroplast continued to photosynthesize during its stay in the animal's gut.

In a pattern similar to kleptoplasty in sacoglossans, *Daphnia obtusa* accumulates plastids of two morphologies, referred to as dark and light, within the endocytes of its midgut. The plastids are generally larger than its mitochondria and contain stacks of thylakoid membranes. Both types of plastids are bounded by a single membrane; but the dark plastids are more prevalent, enclose an electron-dense stroma, and occasionally include a pyrenoid. These features suggest either a cyanobacterial or green algal origin for the plastids, since cyanobacterial chloroplasts are bound by a single membrane, but lack a pyrenoid body whereas the chloroplasts in green algae are bound by a double membrane and can contain pyrenoid bodies. The lighter plastids may simply be senescent dark plastids (Chang & Jenkins 2000).

Another species of *Daphnia*, *D. magna*, feeds on the colonial green alga *Sphaerocystis Schroeteri*. However, in contrast to the above example, *D. magna* species does not sequester plastids within cells of its digestive system. Instead, 90% of the ingested algae remain whole and viable following their passage through the animal's

digestive system. The algae absorb nutrients such as phosphorus that are present in the animal's gut. These nutrients appear to fuel an increase in carbon fixation by the algae. The net result is that grazing by *D. magna* actually increases algal growth. This relationship allows *S. Schroeteri* to bloom when most other phytoplankton are in decline due to low nutrient availability (Porter 1976).

In the previous example, it appeared that the *Daphnia* gained comparatively little from the phytoplankton, given how much of the algae passed through the gut without any apparent digestion occurring. Porter (Porter 1976) suggested that perhaps the gelatinous sheath encasing the algal cells provides at least limited nutritive value to the crustacean (Porter 1976). However, greater metabolic benefit has been suggested for another phytoplankton-consuming crustacean, the copepod *Acanthocyclops vernalis*. Respirometry experiments indicate that the phytoplankton continues to produce oxygen for at least 24 h after being ingested by the copepod. As in *Daphnia magna*, much of the phytoplankton is not digested during its stay in the copepod's gut and appears intact and viable upon its exit. It is possible that the copepod benefits from gases and/or photosynthate produced by the algae. This association has been referred to as a nonobligatory mutualistic symbiosis, with the phytoplankton likely benefiting from nutrients present in the animal's gut and the animal gaining an additional source of oxygen while having carbon dioxide removed from its tissues (Epp & Lewis 1981).

Potential Photosynthesis in *Pentidotea resecata*

Pentidotea resecata (Stimpson 1857) (Isopoda: Valvifera) (Figure I) is found along the west coast of North America from Baja California to southeast Alaska (Jones 1971). Though abundant in the summer, their populations disappear over the course of the fall and winter for unknown reasons only to reappear the following spring and summer. This isopod is dorsoventrally flattened with seven free pereonites and can be identified based on its concave pleotelson. Like other valviverans, its uropods form a ventral valve over the pleopods. The pleotelson consists of two free pleonites and one partially-free pleonite attached to the telson. The palp of the maxilliped is composed of five articles (Kozloff 1996). These isopods can grow up to 64 mm long from the front edge of the cephalon to the tip of the telson, but most of the adult isopods collected from eelgrass beds in Padilla Bay, WA from June through August of 2014 were between 30-50 mm long (Cowles 2015).

P. resecata lives and feeds on either eelgrass (*Zostera marina*) or brown kelp such as *Macrocystis pyrifera*. Isopods found on eelgrass are green, whereas those found on kelp are brown. The coloration of the isopod is influenced by the degree of chromatophore expansion, cuticle color, and general body color (Lee & Gilchrist 1972). Lee and Gilchrist (Lee & Gilchrist 1972) stated that brown isopods get their color from a dark yellow body surrounded by a green endocuticle and red exocuticle, whereas the green isopods have a light yellow body covered by a bright green endo- and exocuticle. They also noted a visible difference in chromatophore appearance between the green and



Figure 1. Two eelgrass isopods (*Pentidotea resecata*) collected from eelgrass beds in Padilla Bay, WA. The concave telson with a sharp point on each side is one of the easiest ways to identify this species of isopod.

brown animals. The differences in appearance, diet, and habitat, along with a lack of evidence for the ability of this isopod to change color, suggests that exchange of individuals between green and brown populations is highly unlikely to occur (Lee & Gilchrist 1972).

These observations suggest that the isopod's coloration may depend upon its diet. This is further supported by a small study in which green isopods taken from an eelgrass bed progressively lost their green coloration when starved for 17 days (Cowles et al. 2011). The same study obtained absorption spectra characteristic of chlorophyll *a*, *b*, and *c* from isopod tissue, and respirometry work indicated that the isopods were consuming less oxygen in the light than in the dark. Taken together these findings suggest the possibility of photosynthesis occurring within the isopod. Lee and Gilchrist (Lee & Gilchrist 1972) observed that the gut contents of *Pentidotea resecata* often contained undigested plant material. These undigested plant cells may receive enough light to photosynthesize within the isopod's gut.

The isopod digestive system consists of a mouth, esophagus, stomach, digestive glands (which form the hepatopancreas or midgut), hindgut, and anus (Figure 2). The isopod stomach is divided into two regions (dorsal and ventral) by the presence of a set of filters. The dorsal region of the stomach connects to the hindgut, whereas the ventral region containing the filters directs the fluid component to the digestive glands of the hepatopancreas (Wägele 1992).

Isopods feed by a combination of mandible activity and suction generated by muscle lining the esophagus and stomach. Large particulate matter travels through the

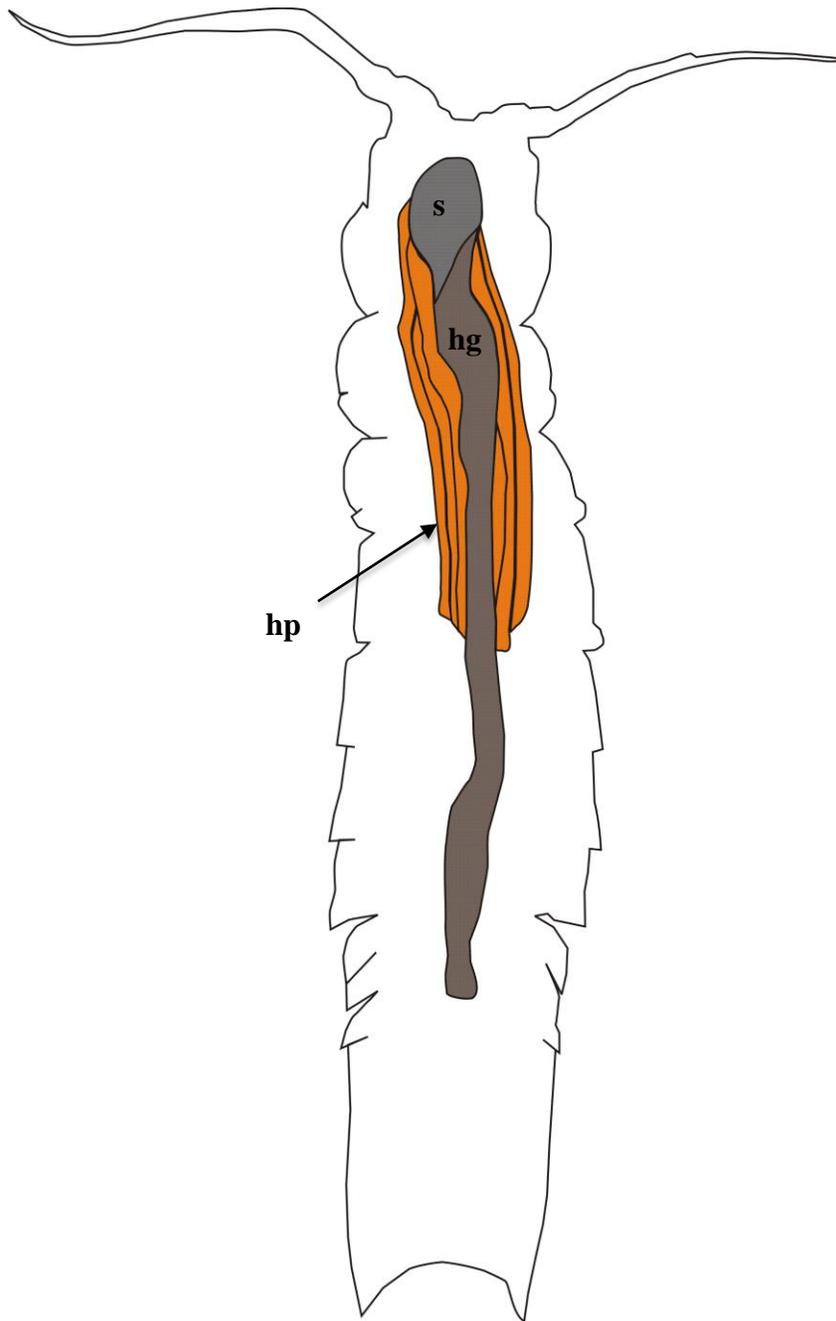


Figure 2. Diagram of the digestive system of *Pentidotea resecata* created as a composite of a trace of a photograph and observations of multiple isopods. The various parts are indicated using the following abbreviations: stomach (**s**), digestive glands of the hepatopancreas (**hp**), hindgut (**hg**).

dorsal stomach and to the anterior hindgut for storage. Ingested liquids and very fine particles are passed through two filters in the ventral stomach to the digestive glands of the hepatopancreas. Some of the digestive enzymes secreted by the hepatopancreas also pass through a valve into the stomach and hindgut to facilitate further digestion of the chyme there. Material from the dorsal stomach and hindgut can also be moved back into the ventral stomach and pressed through the filters into the digestive glands. Particulate matter that is too large to pass through the filters is returned to the hindgut, enclosed by a peritrophic membrane, and expelled as fecal pellets (Holdich & Ratcliffe 1970, Wägele 1992).

The entire length of the digestive system, with the exception of the digestive glands, is lined by chitinized cuticle (intima). Since chitin is relatively impermeable, it has generally been assumed that most nutrient absorption occurs within the digestive glands, whereas the hindgut has been assigned osmoregulatory and storage roles. However, electron microscope studies of the hindgut have revealed the presence of electron dense bodies within the intima and the development of infoldings in the apical plasma membrane following feeding on carbohydrates and proteins. These changes suggest that the hindgut cuticle in certain species is actually permeable to some macromolecules, at least following a period of food deprivation (Hryniewiecka-Szyfter & Storch 1986). If *P. resecata* contains photosynthesizing chloroplasts within its digestive system, a similar increase in the permeability of its gut epithelium could enable the isopod to absorb photosynthate produced by the chloroplasts.

Photosynthesis depends upon the presence of light-absorbing pigments, most notably, chlorophylls. Tissue from *P. resecata* has previously been examined for the presence of carotenoid pigments, including several carotenes and xanthophylls (Lee & Gilchrist 1972). All of the isolated pigments were present in the cuticle of green isopods, but only β -carotene and xanthophylls were found in the gut diverticula. The major xanthophyll present was lutein. Lee and Gilchrist (Lee & Gilchrist 1972) also noted the presence of a green, water-soluble carotenoprotein pigment with strong absorption at 400-500 nm, but they lacked sufficient material to determine the pigment's identity other than that it contained canthaxanthin and lutein. They did not isolate chlorophyll over the course of their pigment analysis, but neither did they look for it specifically. Moreover, the majority of their samples were taken from brown isopods.

The bright color of green *P. resecata* closely matches that of its eelgrass substrate. Observations that the isopods gradually lose their bright green color when removed from eelgrass (Cowles et al. 2011) suggest that the source of the eelgrass isopod's bright green color is the eelgrass itself. As a plant, eelgrass contains both chlorophyll and carotenoid pigments within the thylakoid membrane of its chloroplasts. Both types of pigments are non-covalently linked to proteins and absorb the light energy that fuels photosynthesis. The carotenoids have an additional function of photoprotection in which they help dissipate excess light energy that could otherwise cause oxidative damage to the chloroplasts (Young 1991). Most of the chlorophyll in plants, such as eelgrass, consists of chlorophyll *a*, which is present both in the reaction center complexes and in conjunction

with chlorophyll *b* as part of light-harvesting antennae to the reaction centers (Green & Durnford 1996).

The goal of this study was to further clarify the source of the green isopod's coloration by verifying the presence of eelgrass cells, whole chloroplasts, and functional chlorophyll pigments and identifying their location within *P. resecata*. The presence of functional copies of such photosynthetic elements would suggest that this isopod contains the machinery needed to perform photosynthesis. The location of each of these photosynthetic elements would provide information regarding the potential metabolic benefit to the animal of harboring photosynthetic material. Since the animal would obtain these elements through its diet, the results from this study include a detailed description of the digestive system of *Pentidotea resecata*.

MATERIALS AND METHODS

Sample Collection Site

All samples of eelgrass (*Zostera marina*) and isopods (*Pentidotea resecata*) were collected from near the boat launch at March Point, in southern Padilla Bay, WA (Figure 3; 48° 29' 50" N, 122° 33' 23" W).

Experiments with Eelgrass

Eelgrass Collection and Maintenance

Samples of eelgrass (*Zostera marina*) collected from Padilla Bay were transported in buckets of seawater taken from the bay to Rosario Beach Marine Laboratory for study. At the laboratory, the eelgrass was maintained in indoor, running seawater tanks, exposed only to fluorescent ceiling lights.

Eelgrass Microscopy

I began by acquainting myself with the appearance of eelgrass cells and chloroplasts. Using a Nikon Eclipse E200[®] compound light microscope, I examined 14 blades of fresh eelgrass for chloroplasts and recorded their size and appearance under 400x and 1000x magnification. I used an ocular micrometer to measure the dimensions of the cells. The ocular micrometer was calibrated using a stage micrometer.

I also looked for chlorophyll fluorescence in eelgrass using two different dichroic edge filters. I secured a short-pass filter (Edmund Optics 47-288) to a white LED incident-light illuminator and a long-pass filter (Edmund Optics 64-701) to the objective



Figure 3. Isopod (*Pentidotea resecata*) and eelgrass (*Zostera marina*) samples were collected in Padilla Bay, WA near March Point. The collection site is indicated as well as the location of Rosario Beach Marine Laboratory (RBML) where the actual study was conducted. This map is a composite of six topographical maps taken from the U.S. Geological Survey, 2011.

lens of a Jenco GL7-280 dissection microscope. The short-pass filter allowed wavelengths of light up to 550 nm through, which included the optimum excitation wavelength for chlorophyll of 430 nm. Based on the specifications charts, this filter blocked 99% of the light above 575 nm. To prevent longer-wavelength light from escaping out the side of the light source, two layers of blue cellophane were taped over the short-pass filter and the room lights were turned off. This created an almost pure light source of wavelengths shorter than 550 nm. The long-pass filter on the microscope objective allowed wavelengths greater than 600 nm through. Maximum chlorophyll fluorescence has been observed at an emission wavelength of 685 nm (Gitelson et al. 1999). Thus, the filters functioned such that blue light would pass from the light source through the filter, strike the eelgrass sample, excite chlorophyll molecules, and cause them to fluoresce. The filter over the objectives allowed light of the fluorescence (emission) wavelength for chlorophyll through the objective lenses, but only very little of wavelengths shorter than 600 nm. I used this edge-filter system to look for chlorophyll fluorescence in eelgrass samples and photographed the results using a Nikon D70[®] digital camera attached to one of the microscope's ocular lenses. The fluorescence was detected by the camera with slow shutter speeds ranging from 8-30 sec.

Chlorophyll Extraction from Eelgrass

Following the standard protocol in Rice et al. (2012), I extracted chlorophyll from eelgrass samples using a Wheaton 7-ml glass tissue grinder and 90 parts acetone + 10 parts magnesium carbonate solution. The magnesium carbonate helped to minimize

chlorophyll degradation. This procedure was performed in the dark using a headlamp with a red filter to minimize degradation of the chlorophyll. First, I ground a 4 cm-long, pre-weighed strip of eelgrass in a tissue grinder with 2 mL of acetone solution. The extract was transferred to an acetone-resistant 15 mL screw-cap centrifuge tube. I then rinsed the tissue grinder twice with 1 mL acetone solution and added each 1-mL rinse to the centrifuge tube. Lastly, I adjusted the final extract volume to 5 mL by adding acetone solution. The centrifuge tube was kept on ice in the dark for at least 2 h to allow for thorough extraction of the chlorophyll. I then centrifuged the tube for 20 min at 3,000 rpm and 4°C. After centrifuging, I poured the supernatant containing the extracted chlorophyll into a clean 15-mL screw-cap centrifuge tube. The samples were tested immediately for the presence of chlorophyll, according to the procedures described below. They were maintained on ice and in the dark when not in use.

Spectrophotometric Procedure for Chlorophyll Determination

I tested for the presence of chlorophyll by measuring the absorbance of 2 mL aliquots of extract in either quartz or UV BRAND (acetone-resistant) cuvettes using a Beckman DU 520 UV/Vis spectrophotometer. I obtained a complete absorbance spectrum from 400-760 nm for seven samples. For chlorophyll determination, I employed two different protocols and then compared the results between the two. Both methods were conducted on 18 samples of chlorophyll extract from fresh eelgrass blades.

The trichromatic method (Rice et al., 2012) determined the relative amounts of chlorophyll *a*, *b*, and *c* based on the absorbance at 664, 647, and 630 nm, respectively.

Each value was first corrected for turbidity by subtracting the absorbance at 750 nm. I calculated the amount of each pigment in each sample ($\mu\text{g pigment/g sample}$) using standard equations (Rice et al. 2012); however I substituted the mass of the sample for the sample volume.

The acidification protocol was used to distinguish between chlorophyll *a* and pheophytin *a*, an inactive degradation product of chlorophyll (Rice et al. 2012).

Chlorophyll *a* can be converted to pheophytin *a* through the addition of acid.

Acidification removes the magnesium ion from the middle of chlorophyll's porphyrin ring, replacing it with two protons and in the process slightly altering the absorption spectrum. The effect of acidification on a given extract's absorption spectrum can be used to estimate the relative amount of chlorophyll that was present in the original sample, versus that which had already degraded to pheophytin. This is done by calculating an acidification ratio based on the absorbance at 664 nm before the addition of acid and at 665 nm after the addition of hydrochloric acid. Pure chlorophyll *a* has an acidification ratio of 1.70 when using 90% acetone. Pure pheophytin *a* has an acidification ratio of 1.0 (Rice et al. 2012).

I carried out this protocol in the dark using a headlamp with a red filter to minimize unintentional degradation of chlorophyll *a* to pheophytin *a*. First I transferred 2 mL of extract to a cuvette. I recorded the absorbance at 750 and 664 nm. Then I acidified the extract in the cuvette with 66.6 μL of 0.1M HCl. I gently tapped the cuvette for 90 s and then recorded the absorbance at 750 and 665 nm. The absorbance at 750 nm was used to correct for any background turbidity. I used the absorbance at 664 and 665 nm to

calculate the acidification ratio for each sample. The absorbance values from the acidification method were also used to calculate the relative amounts of chlorophyll and pheophytin in each eelgrass sample ($\mu\text{g pigment/g sample}$) according to a standard equation (Rice et al. 2012) in which I substituted the sample mass for sample volume.

Finally, I used the proportion of pheophytin *a* to chlorophyll *a* in the acidified samples to estimate how much of the chlorophyll *a* as calculated in the trichromatic method was actually functional chlorophyll *a* and how much was probably degraded to pheophytin *a*.

Experiments with Isopods

Animal Collection and Care

Green *Pentidotea resecata* were collected from March Point (Figure 3) during low tide by dragging a 6-foot seine over the eelgrass beds and scooping up the disturbed isopods. The forty-nine individuals used in this study were collected on four different dates: June 25, July 14, August 7, and August 11, 2014. The mass and length from the front of the cephalon to the tip of the telson was measured and recorded for each isopod. The isopods were maintained in either outdoor seawater tanks exposed to ambient sunlight or in indoor seawater tanks exposed only to artificial, fluorescent light. Eelgrass was kept in both tanks with the isopods. Some isopods were kept in individual containers, and others were kept as groups in the tanks. The animals' exoskeleton was gently brushed every 2-3 days with a small, soft toothbrush to minimize diatom growth. The isopods were anesthetized using carbonated saltwater prior to dissection or tissue extraction. The

carbonated saltwater was made by adding 29.5 g table salt to 1 L seltzer water. Isopods placed into this carbonated saltwater became inactive and unresponsive after 30-60 min.

Isopod Microscopic Analysis

I dissected 25 adult isopods by cutting transversely across the posterior end of the dorsal pleon, then making two long cuts dorsally along each side of the exoskeleton from the telson to the head. I then used forceps to carefully remove the dorsal exoskeleton and expose the isopod digestive system (Figure 4). I examined and photographed the digestive system using a Nikon D70® digital camera attached to the ocular lens of a Jenco GL7-280® dissecting microscope. I then carefully removed the entire digestive system onto a slide for examination under either a Nikon Eclipse E100 or Nikon Eclipse E200 compound microscope at 40-1000x. I used the compound microscope to check for the presence of green plant cells and chloroplasts within each section of the digestive system (stomach, hepatopancreas, and hindgut, Figure 2). I also checked for fluorescence on the isopod's cuticle, in its digestive system, and in gut contents using the edge filter system described above for eelgrass.

Isopod Tissue Sample Preparation

The isopod tissue extraction and spectrophotometry were performed in the dark using a headlamp with a red filter. I generated whole-body isopod extracts by grinding an entire isopod with 2 mL acetone solution in a tissue grinder. The extract was transferred

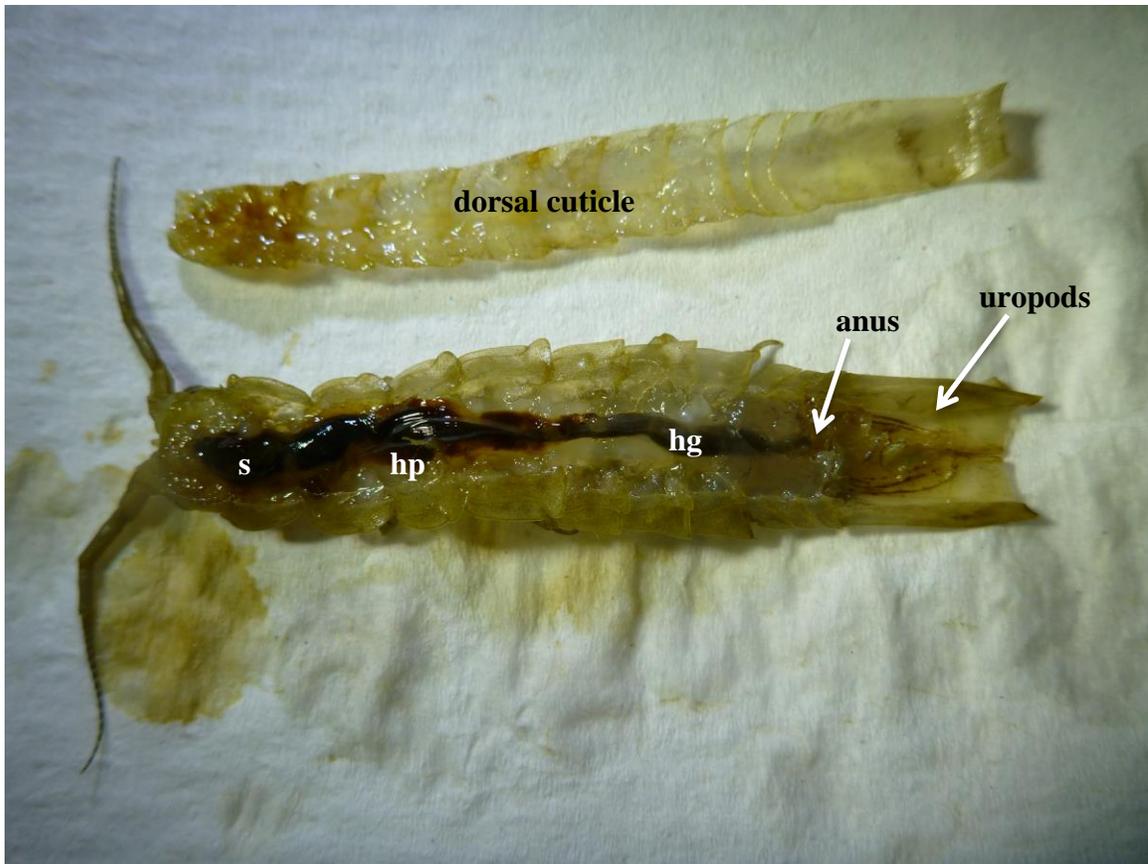


Figure 4. Dorsal view of the isopod digestive system after the removal of the dorsal exoskeleton (**s** = stomach, **hp** = digestive gland of the hepatopancreas, **hg** = hindgut). Note that the hindgut forms a long tube attached directly to the stomach. The digestive glands of the hepatopancreas are tan-colored and most visible on either side of the hindgut. This isopod measured 51 mm long.

to an acetone-resistant 15 mL screw-cap centrifuge tube. I then rinsed the tissue grinder twice with 1 mL acetone solution and added each rinse to the centrifuge tube. Lastly, I adjusted the final extract volume to 5 mL by adding acetone solution. The centrifuge tube was kept on ice in the dark for at least 2 h to allow for thorough extraction of the chlorophyll. I then centrifuged the tube for 20 min at 3,000 rpm and 4°C. After centrifuging, I poured the supernatant into a clean 15-mL screw-cap centrifuge tube.

Isopod Pigment Analysis

I followed the same procedures for analyzing the isopod tissue extracts as I used to look for chlorophyll in the eelgrass extracts. For five isopod samples I obtained a complete absorption spectrum from 400-760 nm. The trichromatic method (Rice et al. 2012) was used to estimate relative amounts of chlorophyll *a*, *b*, and *c*, and the acidification method was used to estimate the how much of the chlorophyll present in the animal extract was already degraded to pheophytin. For the acidification method of chlorophyll determination (Rice et al. 2012), I acidified 2 mL of tissue extract with 66.6 μ L of 0.1M HCl. The same two sets of equations were used to calculate μ g pigment/g sample from both the acidification and trichromatic absorption data, just as had been done for the eelgrass extracts. These data were collected from 16 isopod samples.

Statistical Analysis of Pigment Data

I compared the acidification ratios from the isopod extracts to those from the eelgrass using a two-tailed t-test for unequal variance, since a Bartlett's test indicated

unequal variance. I calculated the percent of total extracted chlorophyll for each pigment type in the both trichromatic and acidification methods separately for each sample. The percentages were then transformed using an arcsine transformation. I tested the data for equal variance and normal distribution using a Bartlett's test and Shapiro-Wilkes test, respectively, in R studio. I then ranked the data and performed a two-way Analysis of Variance (ANOVA) on the ranked data, followed by a Tukey Honest Significant Differences post-hoc test. The ANOVAs were used to compare the mean amount of each pigment type extracted from eelgrass and isopod samples in both the trichromatic and acidification methods and to compare the relative amount of each pigment type based on the ranked percentage data. The results were four separate ANOVAs. Since two of the ANOVAs were based on the same data set, I did a Bonferonii correction by dividing the critical p-value (α) by two. Thus the p-value for each test needed to be smaller than 0.025 for the test result to be considered statistically significant.

RESULTS

Experiments with Eelgrass

Eelgrass Microscopy

Individual cells were clearly visible when viewing eelgrass blades under the light microscope (Figure 5A). The eelgrass cells ranged in diameter from 15-28 μm . The cells were filled with green disc-shaped organelles that were 2-5 μm in diameter. Individual thylakoid stacks were not visible within the organelles, yet their size, color, and location supported their identification as chloroplasts.

All the living eelgrass blades examined appeared pink when photographed in the dark using the light filters meant to detect pink chlorophyll fluorescence. Portions of the eelgrass blades that appeared dead (brown) exhibited less color when viewed under the filters (Figure 6).

Eelgrass Pigment Analysis

The chlorophyll extracts from eelgrass blades exhibited absorption spectra characteristic of chlorophyll *a* with two strong peaks, one between 430-460 nm and the other around 664 nm (Figure 7). The amounts of pigment extracted from eelgrass can be seen in Table 1A and Figure 8. The trichromatic method showed that most of the chlorophyll present in the eelgrass extracts was chlorophyll *a* (73%) and *b* (25%), with chlorophyll *c* making up less than 2% of the extracted chlorophylls. Denaturation by acidification of eelgrass chlorophyll extracts with 0.1M HCl caused a decrease in absorbance overall and a shift in the peaks that resulted in acidification ratios between 1.1

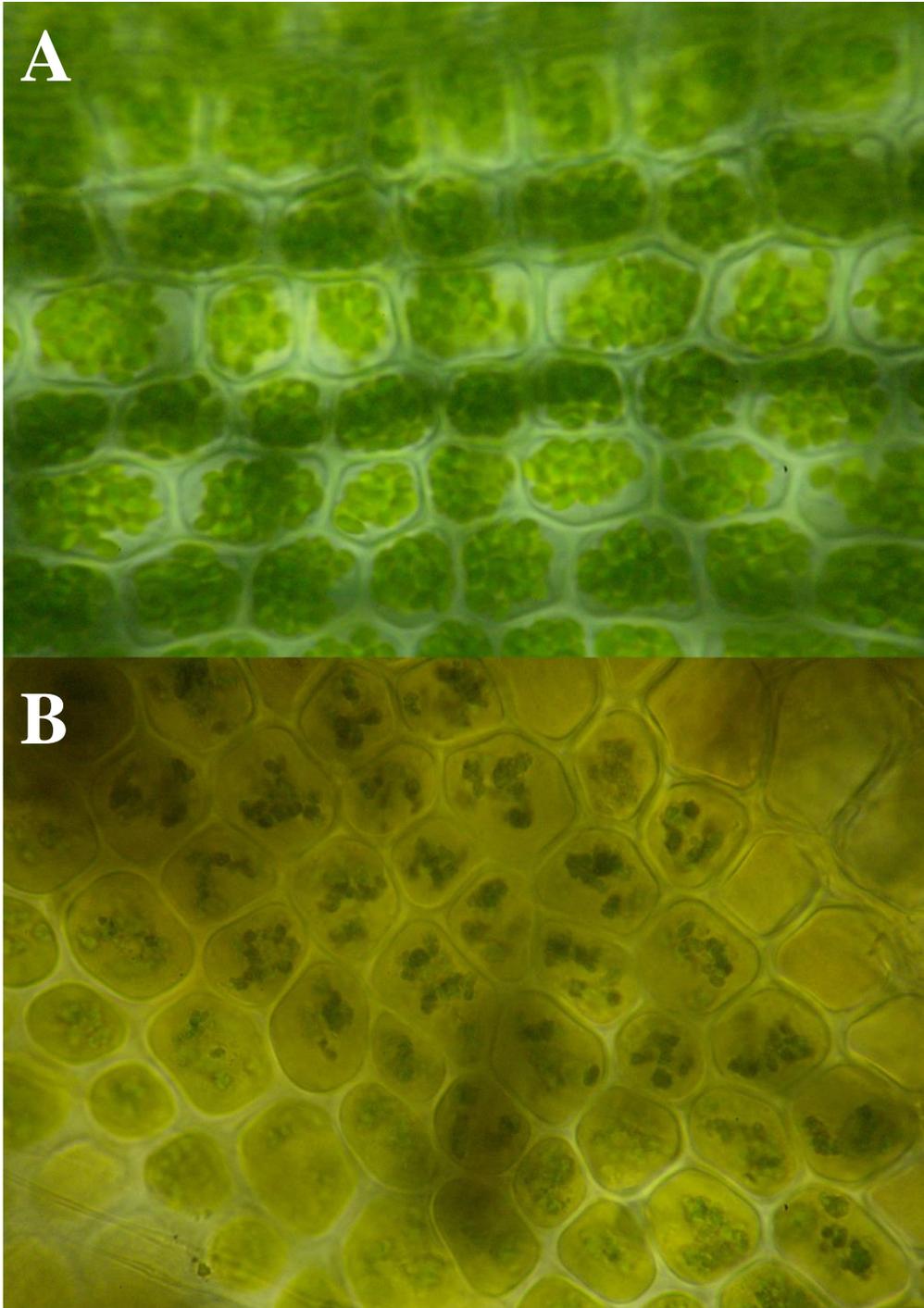


Figure 5. Eelgrass cells with chloroplasts in a blade of fresh eelgrass (**A**) and in the hindgut of an isopod (**B**). Photographed at 1000x.

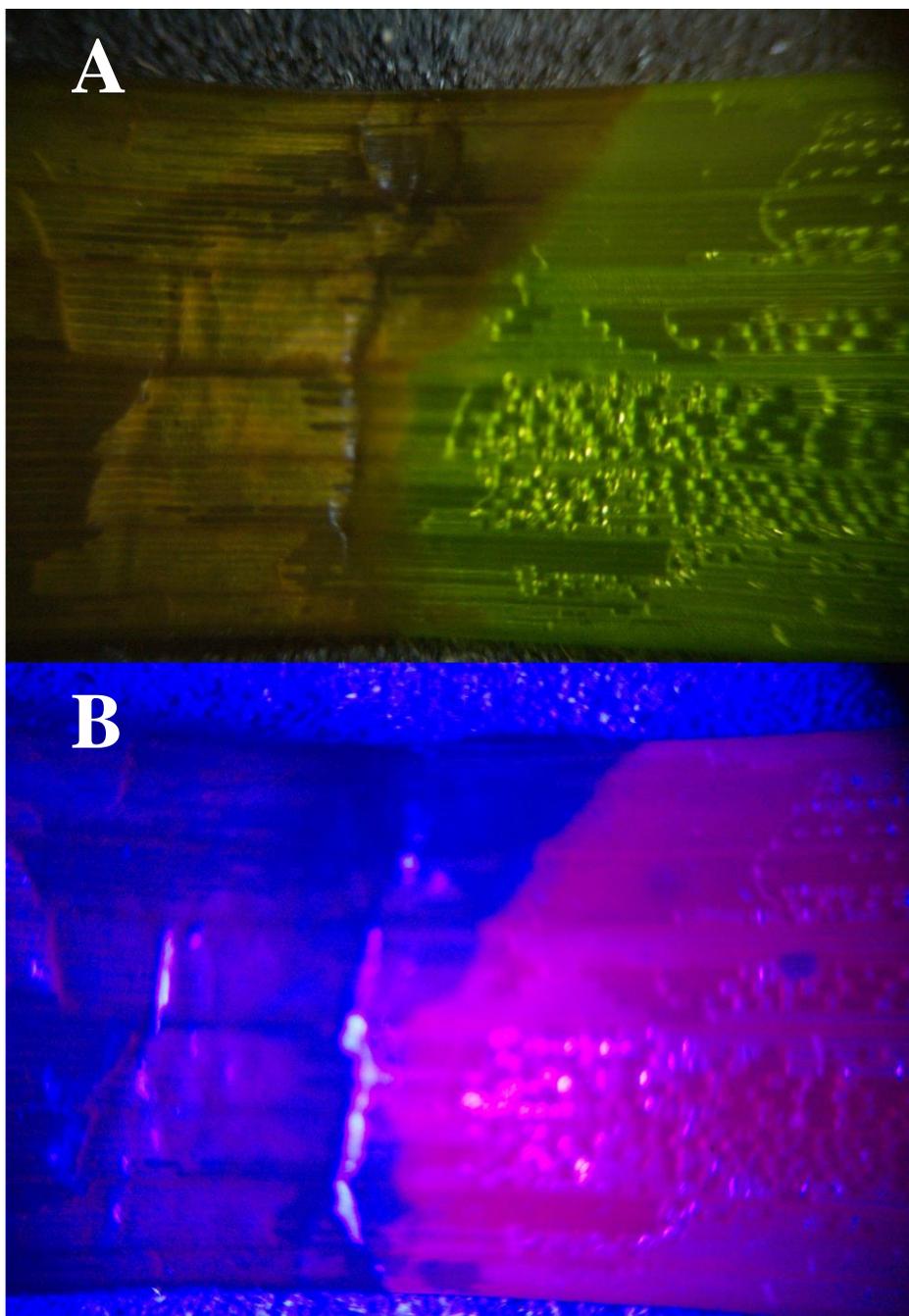


Figure 6. An eelgrass blade under normal lighting (**A**) and under filters meant to detect pink chlorophyll *a* fluorescence (**B**). The portion of the blade in the left side of the photo appeared unhealthy as indicated by the brown color and contrasted strongly with the rest of the blade, which appeared healthy. This contrast was apparent both under normal lighting and when checking for chlorophyll fluorescence using the edge filter setup. Photographed at 10x.

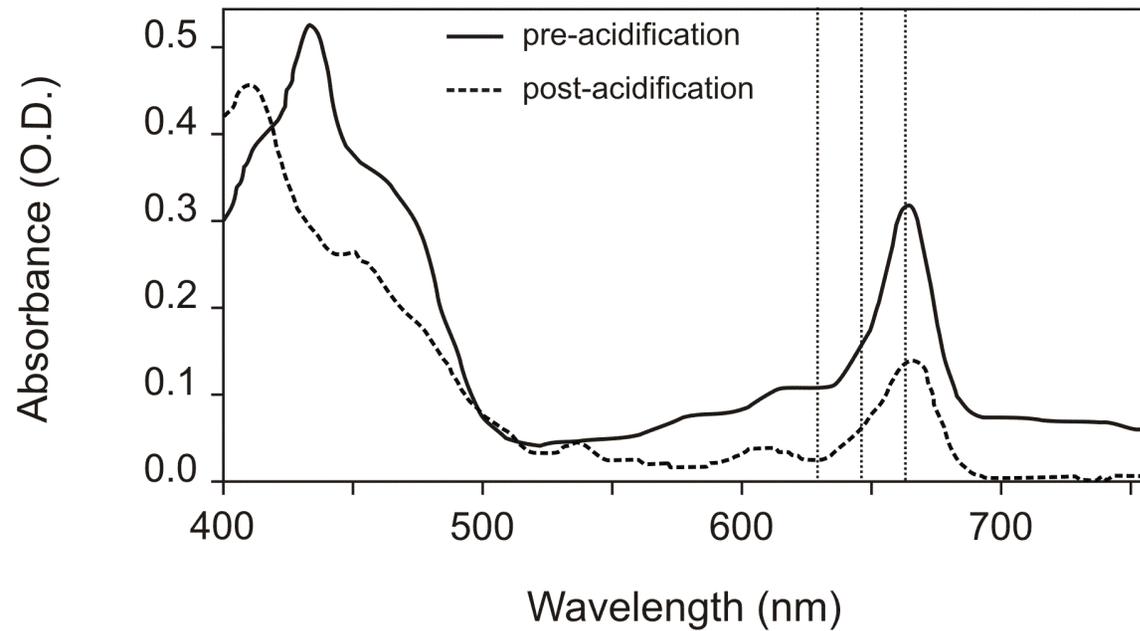


Figure 7. Absorption spectrum of fresh eelgrass before and after acidification with 0.1M HCl. The pre-acidification extract had a peak at 663 nm. This peak shifted to the right (665 nm) and exhibited a decrease in absorbance following acidification. The three vertical lines indicate the wavelengths tested in the trichromatic method (630, 647, and 664 nm). The acidification protocol compared absorbance at 664 and 665 nm.

Table 1. A comparison of the amount of chlorophyll pigments extracted from eelgrass and isopod tissue. The trichromatic method was used to determine relative amounts of chlorophyll *a*, *b*, and *c* based on the absorbance at 664, 647, and 630 nm, respectively. The acidification method was used to determine how much of the extracted chlorophyll *a* was actually pheophytin *a*, a degradation product of chlorophyll. The percentage of pheophytin obtained from the acidification method was used to correct the amount of chlorophyll *a* as determined by the trichromatic method to a more realistic estimate of the amount of actual chlorophyll *a* present. The results were obtained from 18 blades of eelgrass and 16 isopod samples.

| A. EELGRASS SAMPLES | Trichromatic Method | | | Acidification Method | |
|--|----------------------------|----------------------|----------------------|-----------------------------|---------------------|
| | Chlorophyll <i>a</i> | Chlorophyll <i>b</i> | Chlorophyll <i>c</i> | Chlorophyll <i>a</i> | Pheophytin <i>a</i> |
| Mean Extracted Pigment (μg pigment/g blade) | 611 | 214 | 16 | 352 | 437 |
| Percent of Total Extracted Pigments | 72.6 | 25.5 | 1.9 | 44.6 | 55.4 |
| Actual Chlorophyll <i>a</i> (μg pigment/g blade) | 273 | | | | |

| B. ISOPOD SAMPLES | Trichromatic Method | | | Acidification Method | |
|---|----------------------------|----------------------|----------------------|-----------------------------|---------------------|
| | Chlorophyll <i>a</i> | Chlorophyll <i>b</i> | Chlorophyll <i>c</i> | Chlorophyll <i>a</i> | Pheophytin <i>a</i> |
| Mean Extracted Pigment (μg pigment/g isopod tissue) | 12.8 | 0.7 | 2.8 | 1.3 | 15.4 |
| Percent of Total Extracted Pigments | 78.4 | 4.3 | 17.3 | 7.5 | 92.5 |
| Corrected Chlorophyll <i>a</i> (μg pigment/g isopod tissue) | 0.96 | | | | |

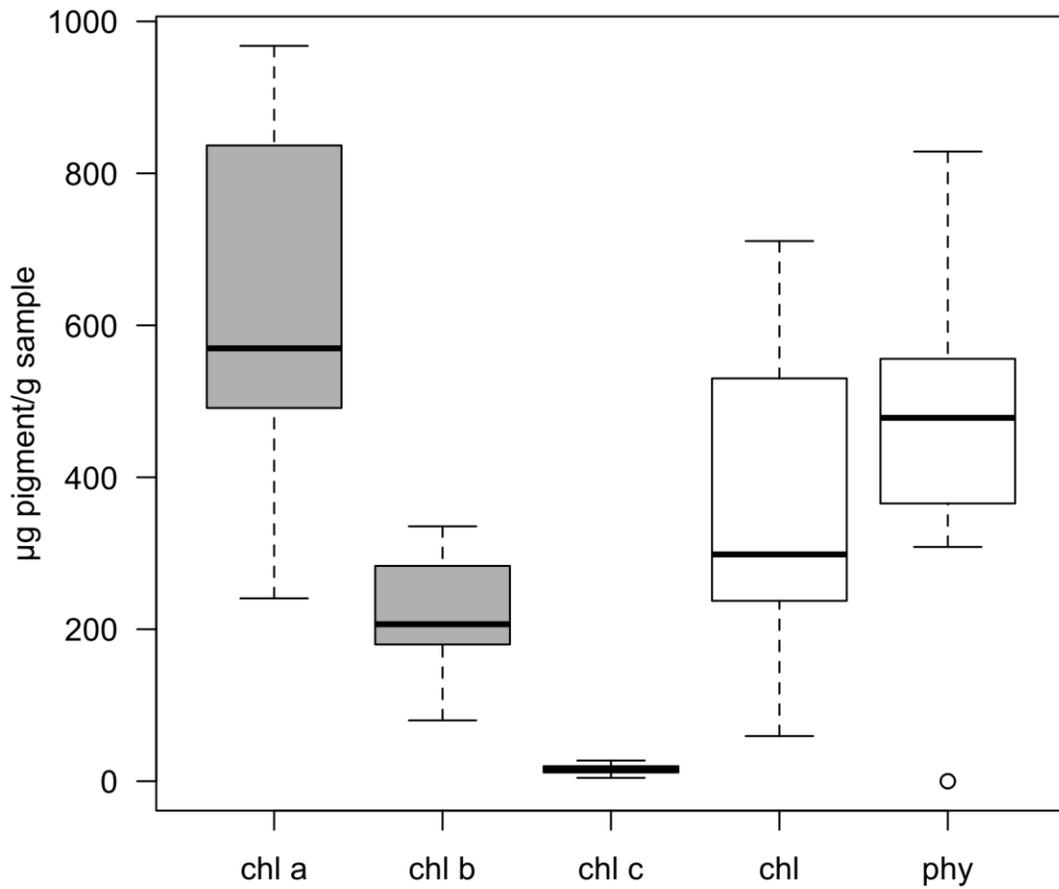


Figure 8. Boxplot of estimated amount of photosynthetic pigment extracted from eelgrass blades based on corrected absorption data (Rice et al. 2012; chl = chlorophyll, phy = pheophytin). The shaded boxes represent data gathered using the trichromatic method. The empty boxes represent data from the acidification method. The crossbar in each box represents the median of 18 samples. Box height represents the interquartile range, and the error bars represent the data that fall within 1.5x the interquartile range. Data that lie outside that range are shown by the small circles.

and 1.7, with a mean acidification ratio of 1.3. These acidification results suggested that roughly 55% (range 36-85%) of the eelgrass extracts consisted of pheophytin *a*. Thus, while the trichromatic method indicated that 1 g of eelgrass contained 611 µg of chlorophyll *a*, the low acidification ratio suggested that in actuality, functional chlorophyll *a* only accounted for 273 µg of the extracted eelgrass pigment after the extraction procedure. The remaining 338 µg most likely consisted of chlorophyll *a* which had already degraded to pheophytin *a*.

Experiments with Isopods

Isopod Digestive System Anatomy

The complete digestive system of *Pentidotea resecata* can be seen in Figure 4. Near the junction of the stomach, hepatopancreas, and hindgut lay a filter apparatus (Figure 9), which filters materials moving from the stomach to the digestive glands of the hepatopancreas (Wägele 1992). The hepatopancreas consisted of up to six digestive glands surrounding the hindgut. These glands varied in length relative to the hindgut in different animals and often bulged with brownish fluid (Figure 10). The hindgut, which is chitinized in crustaceans, formed a long tube with a striated appearance under the light microscope (Figure 11) and exited ventrally into the valve formed by the uropods.

Isopod Hindgut Contents

The hindgut of the isopods frequently contained large fragments of eelgrass blades containing whole plant cells. These cells contained dark oval organelles ~2 µm in

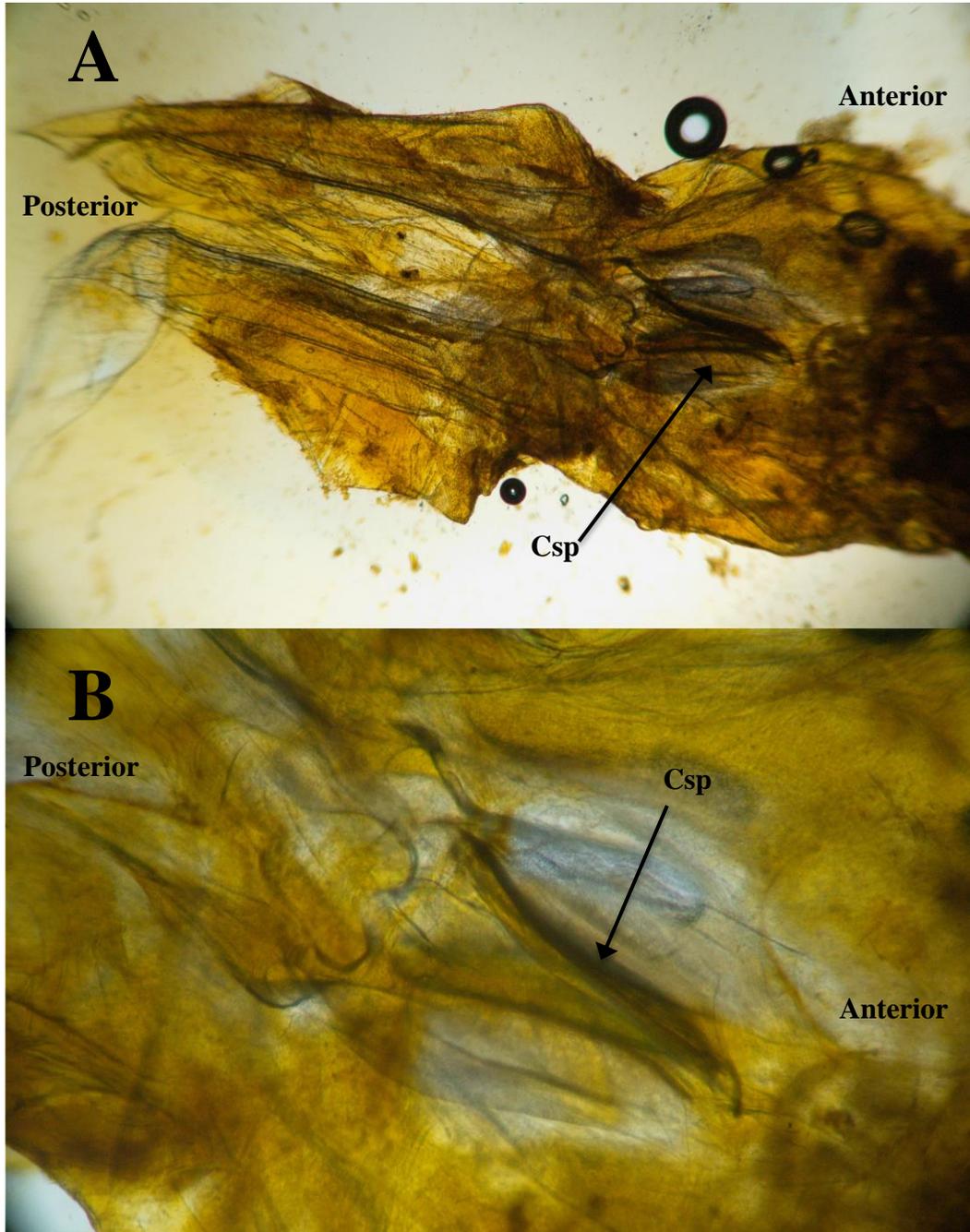


Figure 9. Filter apparatus within the stomach, shown at 40x (A) and 100x (B). This secondary filter, the clatri setarum posteriores (Csp), is visible as the dark “V” in the photograph. It screens the entrance to the digestive glands of the hepatopancreas so that only fine particles can pass through.



Figure 10. Comparison of two digestive glands from one animal's hepatopancreas. One digestive gland is empty and the other bulges with brownish liquid. Photographed at 100x.



Figure 11. Comparison of the glandular appearance of a digestive gland of the hepatopancreas with the striated appearance of the hindgut. Photographed at 40x.

diameter (Figure 5B). The isopods also contained a variety of diatoms within their gut. A long, pennate diatom can be faintly seen in the bottom left of Figure 5B and a brown, spherical diatom is shown in Figure 12. The gut contents also occasionally contained parts of other isopods, such as part of a pereopod or the palp of a maxilliped.

Fluorescence Microscopy of Isopod Tissue and Gut Content

Isopod tissue appeared pink under the microscope filter setup meant to detect pink chlorophyll fluorescence. When viewing the gut, more color was visible in sections of hindgut containing ingested matter than in sections that were empty (Figure 13).

Isopod Pigment Analysis

The absorption spectra of isopod tissue extracts exhibited two rounded peaks near 430 nm and 650 nm (Figure 14). The trichromatic method indicated that most of the extracted chlorophyll pigments were chlorophyll *a* (78%) and *c* (17%), with chlorophyll *b* constituting only 4% of the extracted chlorophylls (Table 1B). Overall, the isopod tissue contained much less pigment, as was evident both in appearance (the extract was much lighter in color) and as indicated by the absorbance data. On average, 1 g of eelgrass contained 51 times as much extractable pigment (chlorophyll and pheophytin pigments combined) compared to isopod tissue based on the yields calculated from the trichromatic method.

Acidification of the isopod extracts with 0.1M HCl resulted in acidification ratios of 1.0-1.1. These acidification ratios are significantly different from those obtained from

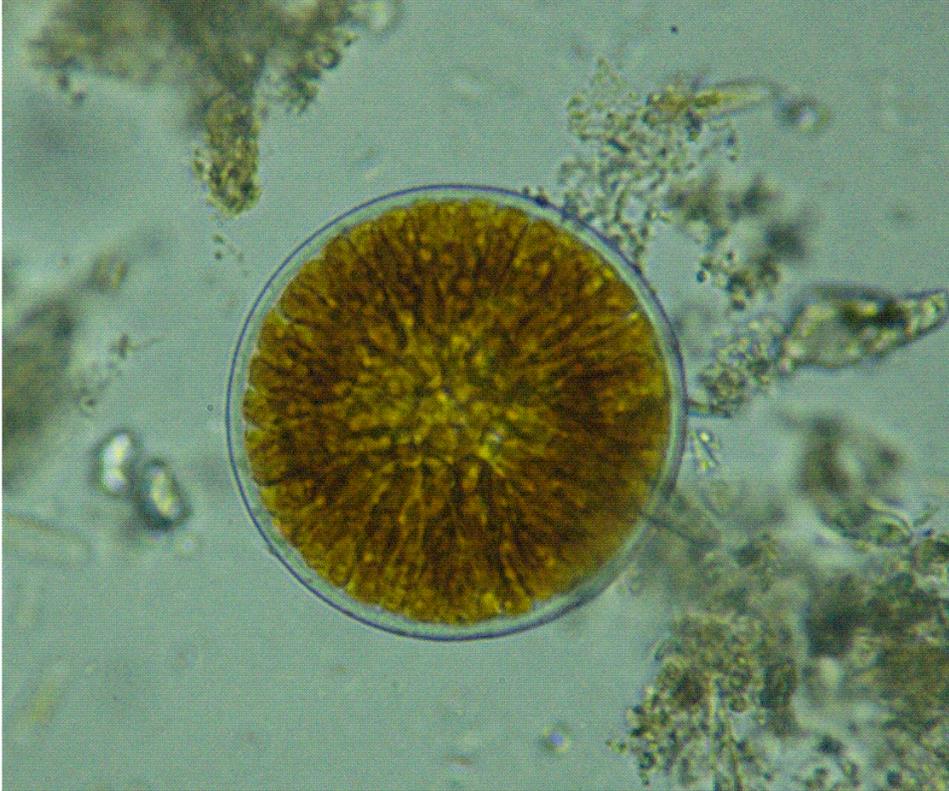


Figure 12. An example of a diatom found within the hindgut of *Pentidotea resecata*. This diatom measured 28 μm in diameter and was photographed at 400x.

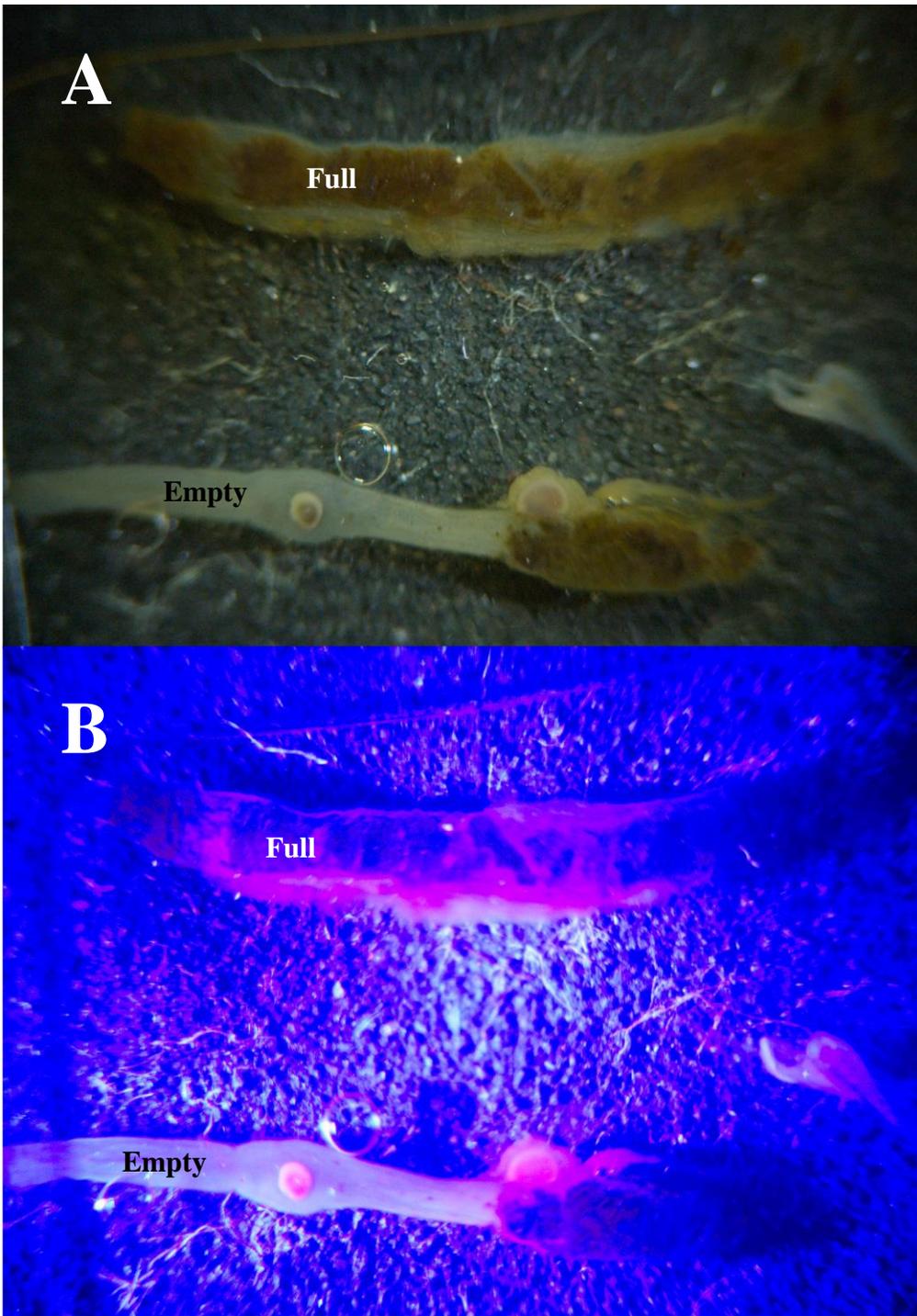


Figure 13. Isopod hindgut under normal lighting (**A**) and under filters meant to detect chlorophyll *a* fluorescence (**B**). The portions of the hindgut containing more ingested material exhibited greater fluorescence as indicated by the pink color.

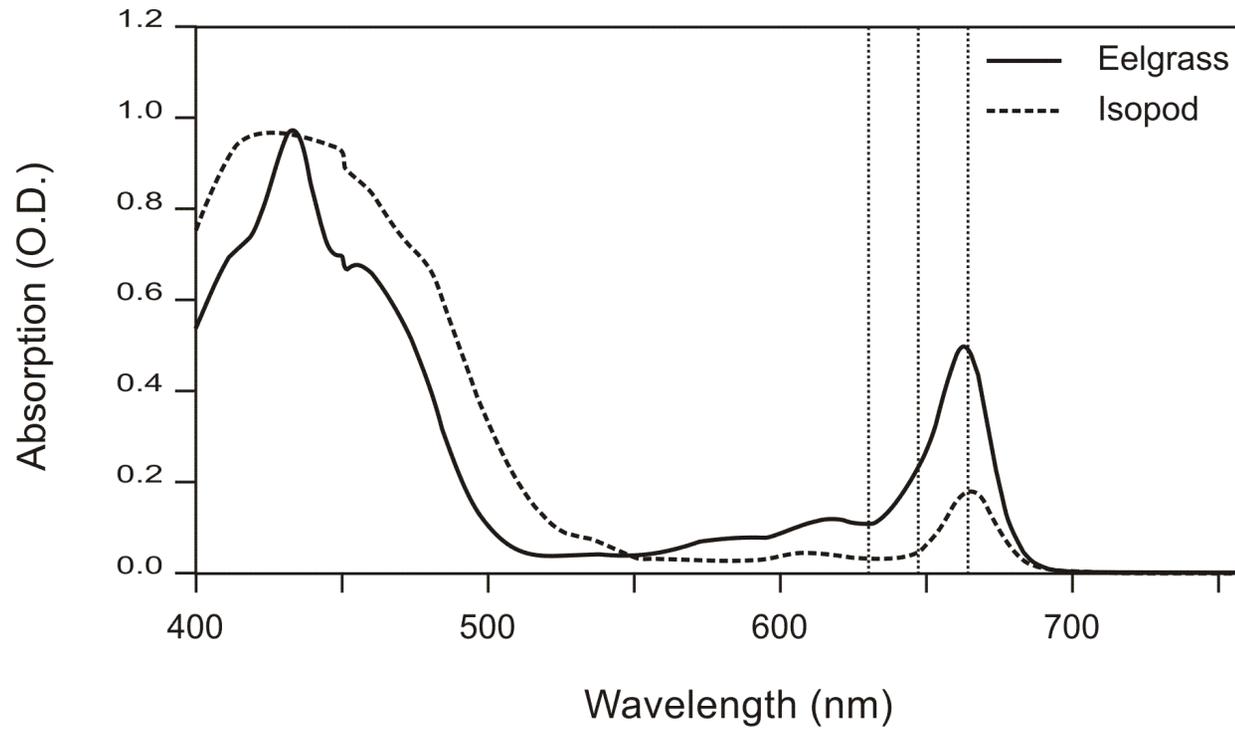


Figure 14. Absorption spectra of fresh eelgrass (solid line) and isopod tissue (dashed line) extracts between 400-760 nm. The three dotted lines indicate the wavelengths tested using the trichromatic method (630, 647, and 664 nm).

eelgrass chlorophyll extracts ($p=6.65E-06$, d.f. =32). The trichromatic method indicated that 12.8 μg of chlorophyll *a* could be extracted from 1 g isopod tissue (Figure 15). However, comparison with the acidification results suggested that 92% of the extracted chlorophyll *a* was actually already degraded to pheophytin *a*. Thus, less than 1 μg functional chlorophyll *a* was extracted from 1 g of isopod tissue (Table 1B).

Statistical Analysis of Pigment Data

The four Ranked Analysis of Variance (ANOVA) tests performed on the pigment data yielded significant p-values for both differences between samples (eelgrass and isopod) and differences between relative amounts of pigment (chlorophyll a, b, c and pheophytin a) in a given sample (Table 2). There was one exception in that the ANOVA did not indicate a significant difference between eelgrass and isopod pigment percentages from the acidification method. Post-hoc tests also revealed highly significant differences between nearly all of the variables compared (Tables 3 and 4).

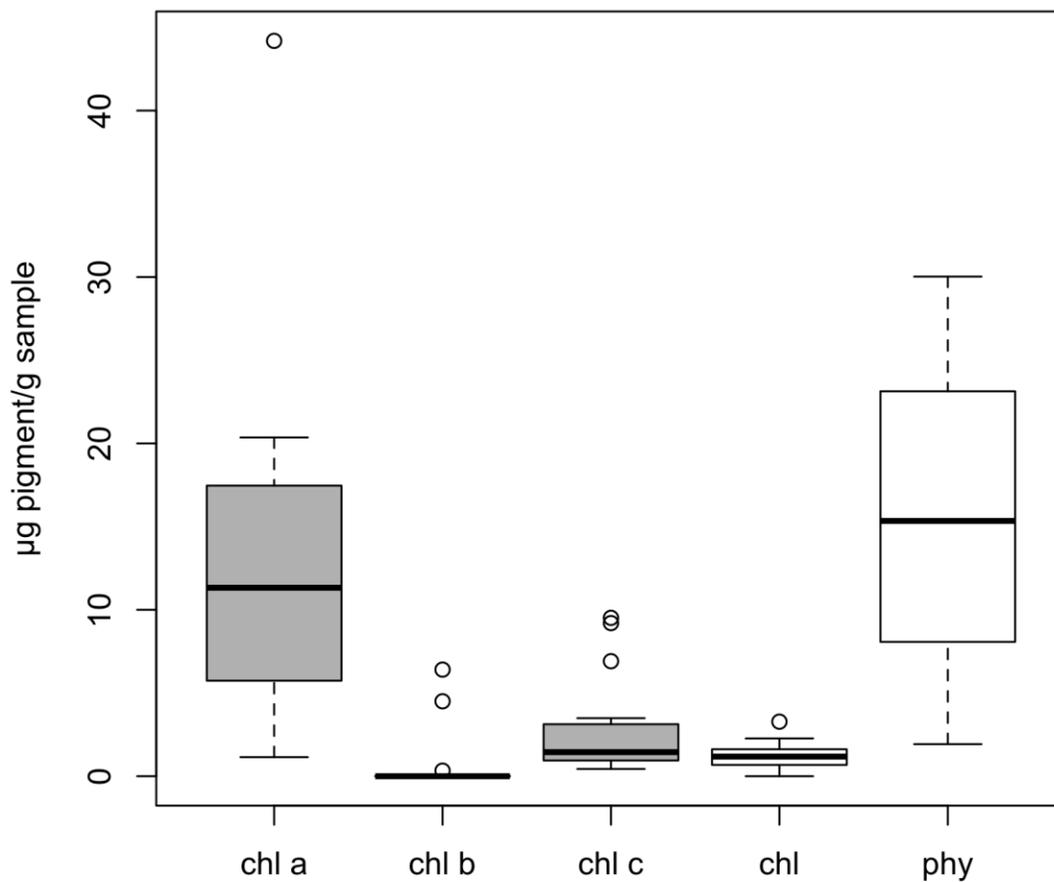


Figure 15. Relative amounts of photosynthetic pigments extracted from isopod tissue extracts. Amounts were estimated based on corrected absorption data. Shaded boxes represent data from the trichromatic method, while empty boxes represent data gathered following the acidification protocol (chl = chlorophyll, phy = pheophytin). The crossbar in each box represents the median of 16 samples. Box height represents the interquartile range, and the error bars represent the data points that fall within 1.5x the interquartile range. Outliers are represented by small circles.

Table 2. The results from four Ranked Analysis of Variance tests performed on pigment data using R. For each data collection method (trichromatic and acidification), a test was done on both the raw pigment data (untransformed) and on the percentage data, which were transformed using an arcsine transformation (percentage). Due to a Bonferroni correction, p-values must be < 0.025 to be considered statistically significant. Sample refers to comparisons between eelgrass and isopods. Pigment refers to comparisons between chlorophyll a, b, c and pheophytin a within the same organism.

| Ranked ANOVA | Trichromatic Method | | Acidification Method | |
|-----------------------|----------------------------|-------------------|-----------------------------|-------------------|
| | Untransformed | Percentage | Untransformed | Percentage |
| d.f. (sample) | 1 | 1 | 1 | 1 |
| d.f. (pigment) | 2 | 2 | 1 | 1 |
| p-value (sample) | 2.00E-16 | 0.00998 | 6.32E-16 | 1 |
| p-value (pigment) | 2.00E-16 | <2E-16 | 0.00358 | 2.48E-13 |
| p-value (interaction) | 2.99E-13 | <2E-16 | 0.01963 | 4.02E-12 |

Table 3. Summary of the results from a Tukey Honest Significant Difference post-hoc test on the Ranked Analysis of Variance performed on pigment data generated using the trichromatic method. Part A shows the results using the untransformed pigment data (TRI). Part B shows the results from transformed percentage data (TRIP). Due to a Bonferroni correction, p-values must be < 0.025 before being considered statistically significant. Significant p-values are indicated by bolded font. Key to label abbreviations: eel = eelgrass, iso = isopod, chl = chlorophyll.

| A. TRI TukeyHSD | eel chl a | eel chl b | eel chl c | iso chl a | iso chl b | iso chl c |
|------------------------|------------------|-----------------|-----------------|------------------|------------------|-----------|
| eel chl a | | | | | | |
| eel chl b | 0.0000017 | | | | | |
| eel chl c | <1E-7 | <E-7 | | | | |
| iso chl a | <1E-7 | <1E-7 | 0.2923706 | | | |
| iso chl b | <1E-7 | <1E-7 | <1E-7 | <1E-7 | | |
| iso chl c | <1E-7 | <1E-7 | <1E-7 | 0.0000002 | 0.0002966 | |

| B. TRIP TukeyHSD | eel chl a | eel chl b | eel chl c | iso chl a | iso chl b | iso chl c |
|-------------------------|-----------------|------------------|------------------|-----------------|-----------------|-----------|
| eel chl a | | | | | | |
| eel chl b | <1E-7 | | | | | |
| eel chl c | <1E-7 | <1E-7 | | | | |
| iso chl a | 0.0774679 | <1E-7 | <1E-7 | | | |
| iso chl b | <1E-7 | <1E-7 | 0.0112135 | <1E-7 | | |
| iso chl c | <1E-7 | 0.0027535 | 0.0057557 | <1E-7 | <1E-7 | |

Table 4. Summary of the results from a Tukey Honest Significant Difference post-hoc test on the Ranked Analysis of Variance performed on pigment data generated using the acidification method. Part A shows the results using the untransformed pigment data (ACID). Part B shows the results from transformed percentage data (ACIDP). Due to a Bonferroni correction, p-values must be < 0.025 before being considered statistically significant. Significant p-values are indicated by bolded font. Key to label abbreviations: eel = eelgrass, iso = isopod, chl = chlorophyll, phy = pheophytin.

| A. ACID TukeyHSD | eel chl a | eel phy a | iso chl a | iso phy a |
|-------------------------|------------------|------------------|------------------|-----------|
| eel chl a | | | | |
| eel phy a | 0.9437181 | | | |
| iso chl a | <1E-7 | <1E-7 | | |
| iso phy a | 0.0000075 | 0.0000009 | 0.0017105 | |

| B. ACIDP TukeyHSD | eel chl a | eel phy a | iso chl a | iso phy a |
|--------------------------|------------------|------------------|-----------------|-----------|
| eel chl a | | | | |
| eel phy a | 0.8264386 | | | |
| iso chl a | 0.0000006 | <1E-7 | | |
| iso phy a | <1E-7 | 0.0000006 | <1E-7 | |

DISCUSSION

The purpose of this study was to establish whether the eelgrass isopod, *Pentidotea resecata*, contains the machinery needed to perform photosynthesis, *i.e.* intact chloroplasts and functional chlorophyll, in a location and condition that may potentially benefit the animal. Since the isopod would presumably obtain such elements from its diet, I examined the digestive system of *P. resecata* for whole plant cells with intact chloroplasts and tested for chlorophyll in animal tissue extracts.

Digestive System of *Pentidotea resecata*

The isopod digestive system consists of a mouth, esophagus, stomach, hepatopancreas, hindgut, and anus. The mouth, esophagus, and stomach are located within the cephalothorax of *Pentidotea resecata* and may extend into the first pereonite. The isopod stomach is divided into two regions by the presence of a set of filters (Wägele 1992). The dorsal region of the stomach connects to the hindgut, whereas the ventral region containing the filters directs the fluid component to the digestive glands of the hepatopancreas. Both filters consist of setae that overlay channels formed by invaginations in the wall of the stomach. The primary filter is located more anteriorly and its setae cover the dorsal entrance to the filter channels. The secondary filter, shown in Figure 9, consists of finer setae that cover the ducts leading to the digestive glands. This filter is the clatri setarum posteriores. These filters restrict the size of ingested material that is allowed to pass into the digestive glands.

The digestive glands that make up the hepatopancreas are the only parts of the isopod digestive system that are not lined with chitinized cuticle (Hryniewiecka-Szyfter & Storch 1986), and thus bring isopod digestive epithelium into direct contact with the gut contents. Over the course of my dissections, I counted three pairs (six total) of digestive glands in each animal. Most isopod species have two to three pairs, but some species, including some Valviferans, are known to have up to four pairs (Wägele 1992).

There was great variation in the length and structural integrity of the glands. In some animals I was easily able to find and remove six separate glands. In others, the glands appeared to be already partially disintegrated within the animal, and it was nearly impossible to separate them from each other without tearing them. These differences in length and structural integrity may be related to the nutritional status of the individual. A study of an Antarctic marine isopod (*Natatolana obtusata*) found that the digestive glands increased in mass and length after intensive feeding (Storch et al. 2002). However, this species is a scavenger, and so it befits it to feed voraciously when food is present so that it may have energy stores in times of scarcity. In contrast, *P. resecata* feeds on the very substrate on which it is found, and so does not appear to risk starvation as long as the eelgrass beds are in good condition. Thus, a more detailed study would be required for any correlation to be drawn between the condition of the digestive glands and the nutritional status of individual *P. resecata*.

Another possibility for the variability in the structure of the digestive glands could be that they deteriorate as the animal ages. Most of the dissections were conducted near the end of the summer. This species seems to disappear in the fall (Lee & Gilchrist 1972)

as indicated by failed attempts to collect animals at times other than during the summer months. This “disappearance” of the species could be due to the adult generation dying off after breeding. The small, newly released isopods are quite small, and would be very difficult to collect without the use of a plankton net. Thus, it could be that adult animals cease feeding at the end of the summer, and as a result their digestive glands begin to deteriorate.

The digestive glands are surrounded by a network of myocytes (Wägele 1992), and the cross-sectional, radial arrangement of microtubules in the myocytes may contribute to the “constrictions” seen in the empty gland of Figure 10. Other glands within the same animal bulged with brownish fluid. The gland epithelium consists of two alternating cell types: large alpha cells and smaller beta cells. The alpha cells are believed to function in absorption and secretion, whereas the beta cells appear to serve as storage sites for lipid droplets and glycogen (Storch & Lehnert-Mortitz 1980, Wägele 1992). I did not find any plant cells or chloroplasts within the digestive glands. Plant cells and chloroplasts may have been too large to pass through the filters in the ventral stomach and into the digestive glands. The digestive glands of one animal did contain a large number of what appeared to be yellow lipid droplets (Figures 10 and 16).

The hindgut of the isopod forms a long tube extending from the stomach to the anus, which exits ventrally into the valve formed by the uropods (Figure 4). The anterior portion of the hindgut may be enlarged following feeding as it often serves as a storage chamber for food. Like the stomach, the inner surface of the hindgut is lined with chitinized cuticle. It is surrounded by an inner layer of circular muscles and outer layer of

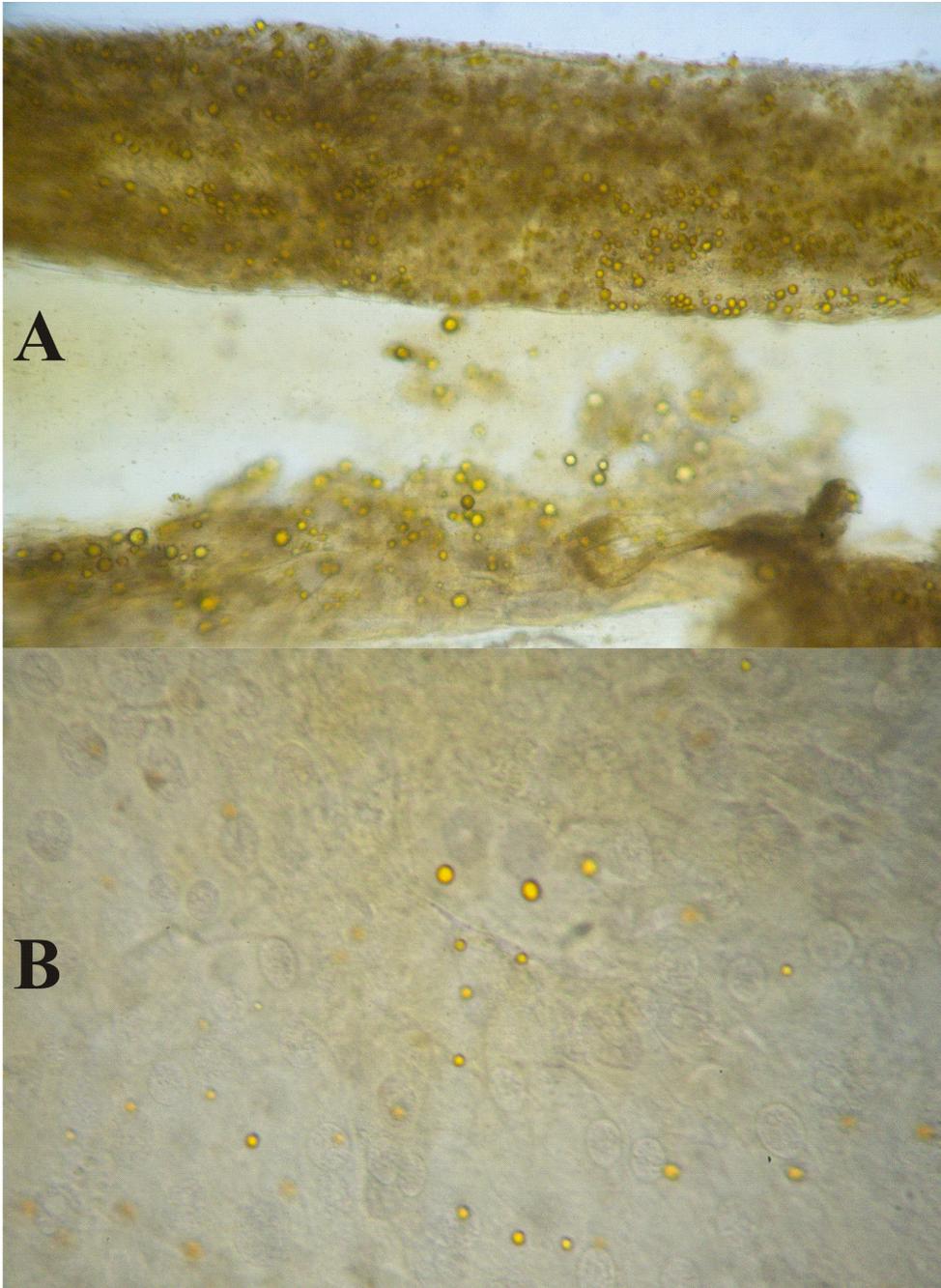


Figure 16. Digestive gland tissue from two different isopods filled with many yellow droplets. Photographed at 100x (A) and 400x (B).

longitudinal muscles which work together to move material up or down the gut by peristalsis (Wägele 1992). Under the light microscope, the hindgut had a striated appearance, perhaps due to the presence of these outer, longitudinal muscles (Figure 11). In addition to food storage and passage, the hindgut may also play a role in osmoregulation (Holdich & Ratcliffe 1970) and the absorption of some macromolecules (Hryniewiecka-Szyfter & Storch 1986).

Photosynthetic Gut Contents

Dissection of the digestive system of *Pentidotea resecata* frequently revealed the presence of large chunks of whole plant cells containing chloroplasts within the lumen of the hindgut. These cells were easily identified based on their similarity in size and appearance to the eelgrass cells (Figure 5). The large size of these cells likely prevented them from passing through the filters into the digestive glands. If these plant cells were able to photosynthesize within the hindgut of the isopod, then the cells would likely release oxygen into the lumen and could potentially also release some of the photosynthate. More work would need to be conducted to determine whether this was occurring and if it was occurring, whether the isopod could actually absorb the photosynthetic products through the cuticle layer lining the hindgut. I did not find evidence of isolated chloroplasts within the digestive glands or in any other isopod gut tissue. The filters within the isopod stomach likely restrict the passage of material into the digestive glands to particles smaller than the size of whole chloroplasts. However,

regurgitation of hindgut contents into the stomach could facilitate animal uptake of photosynthate, which would likely pass through the filters into the digestive glands.

At least some of the photosynthetic material within the isopod's hindgut contained un-degraded chlorophyll as indicated by the fluorescence under the light filters (Figure 13). Portions of the hindgut containing ingested material exhibited stronger pink color than did empty sections of the hindgut. While some of the color could be due to light reflecting off the subject, comparison with the eelgrass results support the interpretation that the pink color actually represented chlorophyll fluorescence. The strong difference in the amount of color visible through the light filters when viewing brown, decaying portions of eelgrass compared to greener portions of an eelgrass blade indicated that the edge-filter setup was sensitive enough to detect chlorophyll fluorescence (Figure 6).

The pink color was not limited to just the digestive system of the isopod. The isopod's cuticle and hemolymph also appeared pink when viewed under the edge filters. The pink color of the cuticle could be partly the result of light reflecting off the animal's shiny surface, but it was more likely due to the presence of diatoms growing on the animal's exoskeleton. The animals were brushed with a small toothbrush to try to prevent diatom growth on their exoskeleton, but this method was unsuccessful in completely eliminating diatoms from the exoskeleton. They were especially difficult to remove from the small spaces between joints/hinges in the exoskeleton, such as between the edges of the pereonites.

Diatoms photosynthesize using chlorophyll *a* and *c* (Green & Durnford 1996). Thus, some of the apparent chlorophyll fluorescence could be due to the presence of diatoms growing on the animal's surface, as also attested to by the detection of chlorophyll *c* by the trichromatic method (Figure 15/Table 1B). Since the isopods frequently contained diatoms within their gut as well, some of the fluorescence found in the animal's gut could also be due to the presence of diatoms. The diatom *Fragilariopsis kerguelensis* has been noted to survive passage through the digestive system of several copepods (Jansen & Balthmann 2007). Thus the presence of viable diatoms within the gut of *P. resecata* could contribute fluorescing chlorophyll. Nonetheless, the relative abundance of eelgrass material within the hindgut, compared to that of diatoms, would implicate the eelgrass cells as a strong contributor to the apparent chlorophyll fluorescence.

Photosynthetic Pigments within *Pentidotea resecata*

I used spectrophotometry to test for the presence of functional chlorophyll pigments in isopod tissue extracts. The two rounded absorption peaks near 430 and 665 nm seen in these extracts (Figure 14) overlap the ranges of the peaks seen in the absorption spectrum of fresh eelgrass extract containing chlorophyll, but the peaks from the isopod tissue extract were much broader. Furthermore, the second peak (at 665 nm) was much shorter than the corresponding eelgrass peak and was shifted slightly to the right. A chlorophyll extract from fresh eelgrass showed a peak at 663 nm, whereas the isopod extract exhibited a peak at 665 nm. This shift of only a couple nm was notable

since it is used to distinguish between chlorophyll *a* and one of its degradation products, pheophytin *a*. Chlorophyll can be degraded to pheophytin by the addition of acid.

Differences between the peaks in the eelgrass and isopod absorption spectra paralleled the changes in the eelgrass chlorophyll absorption spectrum that were generated by acidification. This comparison demonstrates that while the isopod may contain some chlorophyll pigments, greater than 90% of the chlorophyll within the isopod has already been degraded to pheophytin. These data were strongly supported by the significant difference in the acidification ratios generated from eelgrass and isopod extracts. Some of the ratios generated from eelgrass extracts were as high as 1.7, indicative of pure chlorophyll *a*. In contrast, all of the acidification ratios from isopod extracts were between 1.0-1.1, indicating the presence of primarily pheophytin rather than chlorophyll. These results were further supported by the statistically significant difference between the percentages of chlorophyll and pheophytin from the two organisms (Table 4B).

The lower absorbance of the isopod tissue extracts at 664 nm compared to that of eelgrass (Figure 14) indicated that the pigments were also less concentrated in the isopod extract than in the eelgrass blade. A blade of eelgrass contained 241-968 μg pigment/g plant, whereas isopod extracts contained only 1-20 μg pigment/g isopod tissue (Table 1). These results were to be expected since as a photoautotroph, eelgrass depends upon the presence of photosynthetic pigments for its ability to generate energy from sunlight. In contrast, the heterotrophic isopod does not produce such photosynthetic pigments and so would obtain them only through its diet. Thus one could expect the pigments to be less concentrated within isopod tissue. This was further supported by the ANOVAs on both

the trichromatic (TRI) and acidification (ACID) data, which showed that the two samples differed significantly in the amount of extractable chlorophyll pigments (Table 2, Table 3A, and Table 4A).

The composition of the pigments also differed between the isopod and eelgrass extracts. In addition to the lower proportion of chlorophyll *a*, the isopod extracts also had a significantly lower proportion of chlorophyll *b* and a significantly higher proportion of chlorophyll *c* compared to those found in the eelgrass extracts (Table 3B). The two primary chlorophyll molecules in higher plants are chlorophyll *a* and *b* (Green & Durnford 1996). Thus, as expected, the eelgrass extract contained more chlorophyll *b* than chlorophyll *c*. However, the opposite was true in the case of the isopod tissue extracts. The fact that the proportions of chlorophyll molecules in the isopod extract were different from those in the eelgrass extract suggested that the isopod obtained a substantial portion of its chlorophyll molecules from a source other than eelgrass. Diatoms contain chlorophyll *a* and *c* (Green & Durnford 1996), and constitute the most likely source of elevated chlorophyll *c* levels in isopod tissue extracts. This explanation is further supported by the presence of diatoms both on the exoskeleton and within the hindgut of the isopods.

One of the motivating factors for this study was that the presence of functional photosynthetic machinery could suggest the possibility of photosynthesis occurring within the isopod, and potentially benefitting the isopod metabolically. While I found whole plant cells containing chloroplasts within the lumen of the isopod's hindgut, I did not find plant cells or chloroplasts within the digestive glands of the hepatopancreas. If

the plant cells were photosynthesizing within the hindgut of the isopod, such photosynthesis would be unlikely to contribute much to the metabolic demands of the animal, except perhaps as an extra oxygen source. Most of the chlorophyll pigment present in the isopod was degraded by the end of the extraction procedure; however, isopod tissue exhibited fluorescence characteristic of chlorophyll *a*. Thus, while it is possible and probable that the isopod contains some functional chlorophyll due to its eelgrass diet, it is unlikely that significant amounts of photosynthesis are occurring within the animal.

The isopod's eelgrass diet may benefit it in another way. During dissections, I noticed that the hemolymph was bright green and even exhibited small amounts of fluorescence when viewed under the edge filters. Furthermore, the cuticle of green *P. resecata* appeared rather transparent when removed from the animal. Lee and Gilchrist (Lee & Gilchrist 1972) ascribed the difference in color between green and brown isopods to differences in cuticle composition, noting that the green isopods had a yellow body covered by a green endo- and exocuticle. However, my observations indicated that the bright green hemolymph may be the primary difference responsible for the isopod's green coloration. Loss of hemolymph during dissection resulted in an immediate loss of coloration, even when the body was still covered by cuticle. Since the isopod's coloration closely matches that of its eelgrass substrate, concentrating chlorophyll, or, more likely, degraded chlorophyll (*i.e.* pheophytin) within its hemolymph may serve an adaptive function of camouflage for these isopods.

Summary

The marine isopod *Pentidotea resecata* can be found living on eelgrass along the west coast of North America. The green coloration of these individuals closely matches that of their eelgrass substrate. These isopods feed on eelgrass, and large chunks of whole eelgrass cells containing chloroplasts are frequently found within the lumen of their hindgut. Chlorophyll pigments can be extracted from isopod tissue, but only in very small concentrations. Some of the chlorophyll appears functional, as evidenced by fluorescence characteristic of chlorophyll *a*. However, greater than 90% of the extracted chlorophyll pigments appear to be rapidly degraded to pheophytin.

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APPENDIX

Observations on the Natural History and Behavior of *Pentidotea resecata*

An extensive literature search on *Pentidotea resecata* yields scarce information regarding the animal's natural history and behavior. Much of what is known comes from anecdotal remarks in the few studies that have been conducted on the species. In addition to the results discussed above, this project afforded me the opportunity to add to the body of general knowledge regarding this species of isopod. Thus, the purpose of this appendix is to enhance what little is known about this species by giving a written account of my observations regarding the natural history of *Pentidotea resecata*. Many of these observations were made while working with another graduate student, Joanna Cowles. Further information on some of the included topics can be found in her master's thesis (Cowles 2015).

Reproduction and Development

This study was conducted between June 25 and September 2, 2014. The animals were breeding during this time. It was not uncommon for us to find males clasping females, both in the collection bucket and in the field. The male clasped the female from above, such that he was on top of her dorsal surface (Figure A1). This behavior made it much easier to collect the isopods because once the male clasped a female, he would not let go and she could not swim away as quickly as she could without him holding her.

The female carries her eggs in a marsupium under her venter (Figure A2) where they develop into juveniles. The juveniles are released as miniature adults, with a few



Figure A1. A larger male isopod claspng a smaller female isopod on the underside of a blade of eelgrass. The isopods usually cling to the underside of eelgrass blades rather than sitting on top of the blades.

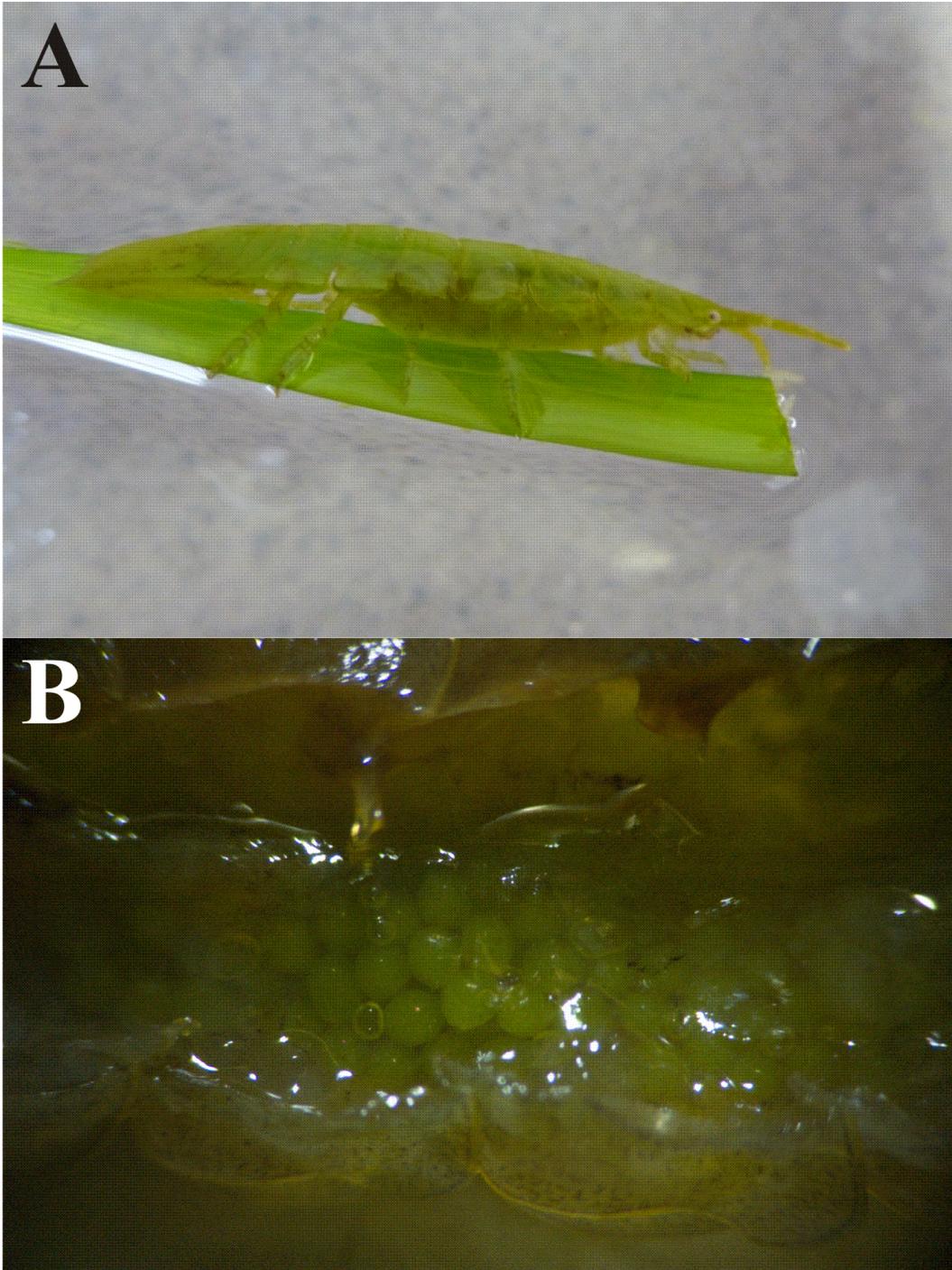


Figure A2. **A)** A female isopod with a bulging marsupium on a blade of eelgrass. **B)** A closer look at the marsupium revealing bright green eggs inside. These eggs were photographed at 20x.

important morphological differences. These differences were noted in a previous study by Menzies and Waidzunas (Menzies & Waidzunas 1948), and so this is not new information. Nonetheless, I will describe these anatomical differences here for the benefit of a reader who might be naïve regarding this species' development. The smallest juveniles that we found in our tanks measured ~ 3 mm long from the tip of the telson to the front of the cephalon (Figure A3). If the length of the antennae were included, then it would measure 4 mm long. The juveniles had 6 clearly identifiable pereonites with 6 well-developed pereopods. It was difficult to tell if an additional segment between belonged to the pereon or the pleon. The telson was very odd-shaped (Figure A3). Unlike that of adult *P. resecata*, the telson was not concave. It had one point on either side and a third protuberance or bump in the middle, between the two points. There was a white band of tissue between the two points. The eyes of the juveniles were decidedly lateral in position, and the palp of the second maxilliped consisted of four articles (not five as in adult *P. resecata*). In addition to these observations, Menzies and Waidzunas (Menzies & Waidzunas 1948) also noted that the length of the flagellum of the second antenna increased in length due to the addition of articles as the animal grew. Knowledge of these differences in appearance between juvenile and adult *P. resecata* is critical for correct identification of juvenile *P. resecata* since several of these features could result in them keying out to a different species.

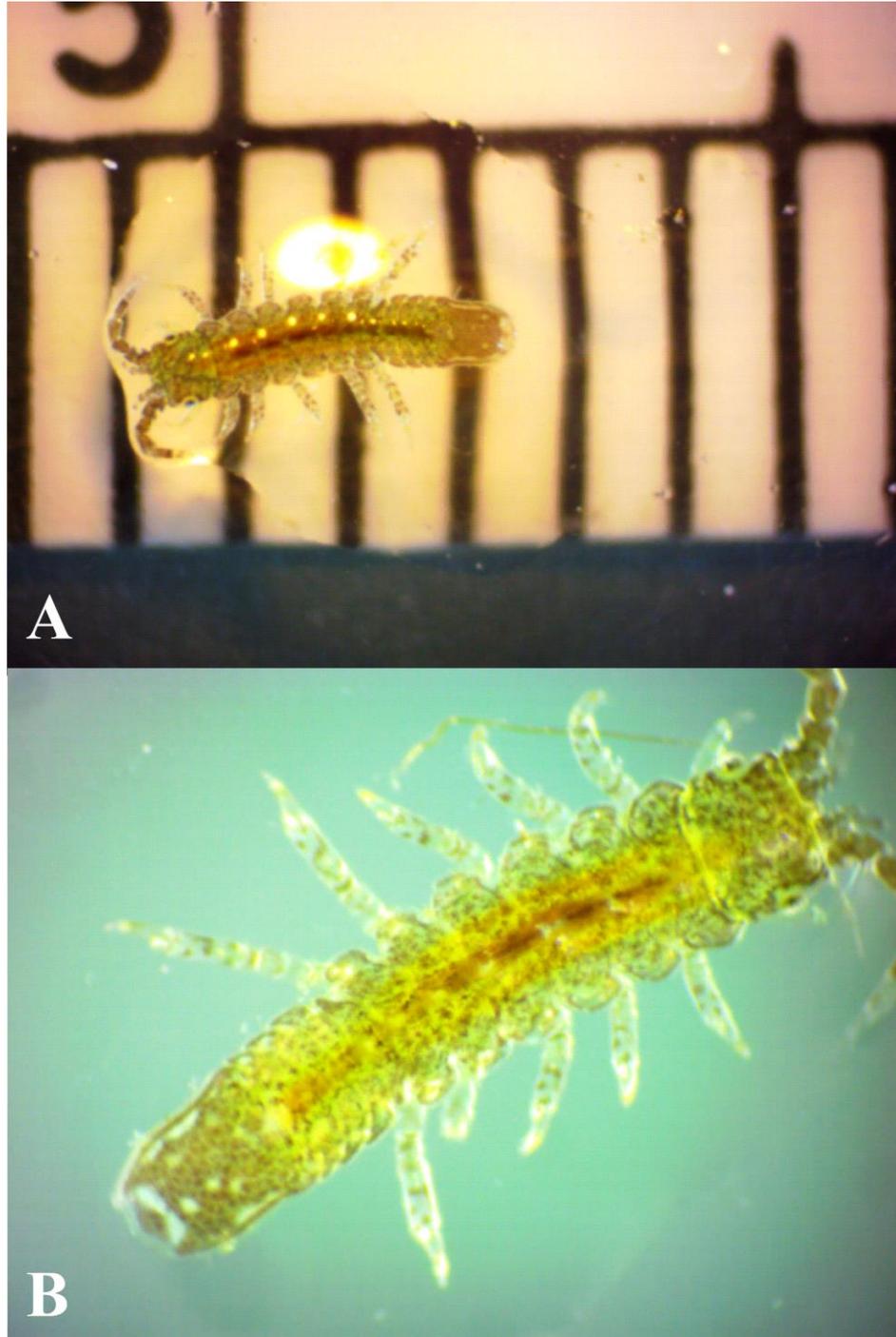


Figure A3. Juvenile *Pentidotea resecata* measuring ~ 3 mm long (A). A closer view of another juvenile *P. resecata* (B). Note the convex rather than concave telson with the white bar of tissue in the middle.

Size and Molting Pattern

It has been reported that this species grows up to four cm long (Kozloff 1993). However, over the course of our collections, Joanna and I found that these isopods quite commonly exceed four and even five cm. In fact, we collected one individual who measured 64.5 mm (Figure A4A).

Another large individual, shown in Figure A4B, provides a good example of biphasic molting in these isopods. They first molt the posterior portion of their exoskeleton, and then they molt the anterior portion. Between the molting of the two halves, the older (anterior) part of the exoskeleton is browner in color than the freshly molted posterior part. This difference in color can largely be attributed to the colonization of the exterior of the isopod by diatoms.

Cannibalism and Parasitism

In addition to the photosynthetic material such as eelgrass cells, the hindgut of some animals also contained parts of other isopods, such as the palp of a maxilliped or a pereopod. The presence of these body parts within another animal's gut would suggest either scavenging behavior by the isopods, cannibalism, or both. While it could be possible that isopod had consumed its own molt, a behavior known to occur in some crustaceans (Jones 1971), old molts were frequently recovered from the containers of isopods kept individually with eelgrass, suggesting that this species does not habitually consume its molted exoskeleton. Behavioral observations of the isopods indicated that they do consume the remains of other dead isopods, and will even begin eating another



Figure A4. Two large *Pentidotea resecata*. Specimen **A** measured 64.5 mm, and was the largest individual that we found over the course of our collections. Specimen **B** was also quite large, measuring 56.5 mm, and exhibits the biphasic molting pattern characteristic of these isopods. It has recently molted the posterior portion of its exoskeleton, which is why the rear half of the isopod is brighter green. It has yet to molt the anterior portion of its exoskeleton, which appears brown due to the presence of diatoms on its surface.

isopod that is not fully dead. However, this behavior may have resulted from the animals being kept in too small of a tank and/or with inadequate eelgrass substrate. I did observe one large juvenile eat a smaller juvenile when attempting to sedate both of them in the same fingerbowl. Similar observations regarding cannibalism in this species were also reported by Jones (Jones 1971).

On two occasions I found what looked to be some sort of nematode inside the digestive system of an isopod, once in a juvenile and once in an adult (Figure A5).

Variation in Prevalence and Appearance of Adults

While I did not conduct a specific study of changes in the size of the Padilla Bay population of *Pentidotea resecata*, the isopods seemed to become scarcer towards the end of the summer. The last collection was made on August 11, 2014, and we had a difficult time finding enough isopods within our desired size bracket of 4-5 cm long.

We also noticed that the inside of several isopods was filled with a white substance (Figure A6). This substance was visible through the cuticle and gave the isopods an odd appearance. It also appeared to increase in the amount to which it filled the isopods over time. When it first appeared in the isopods, we thought that it might indicate an animal that was in the process of dying, since their tissues do turn whitish following death. However, some animals lived for several weeks with the white substance inside them, and only died as result of being anesthetized and dissected.

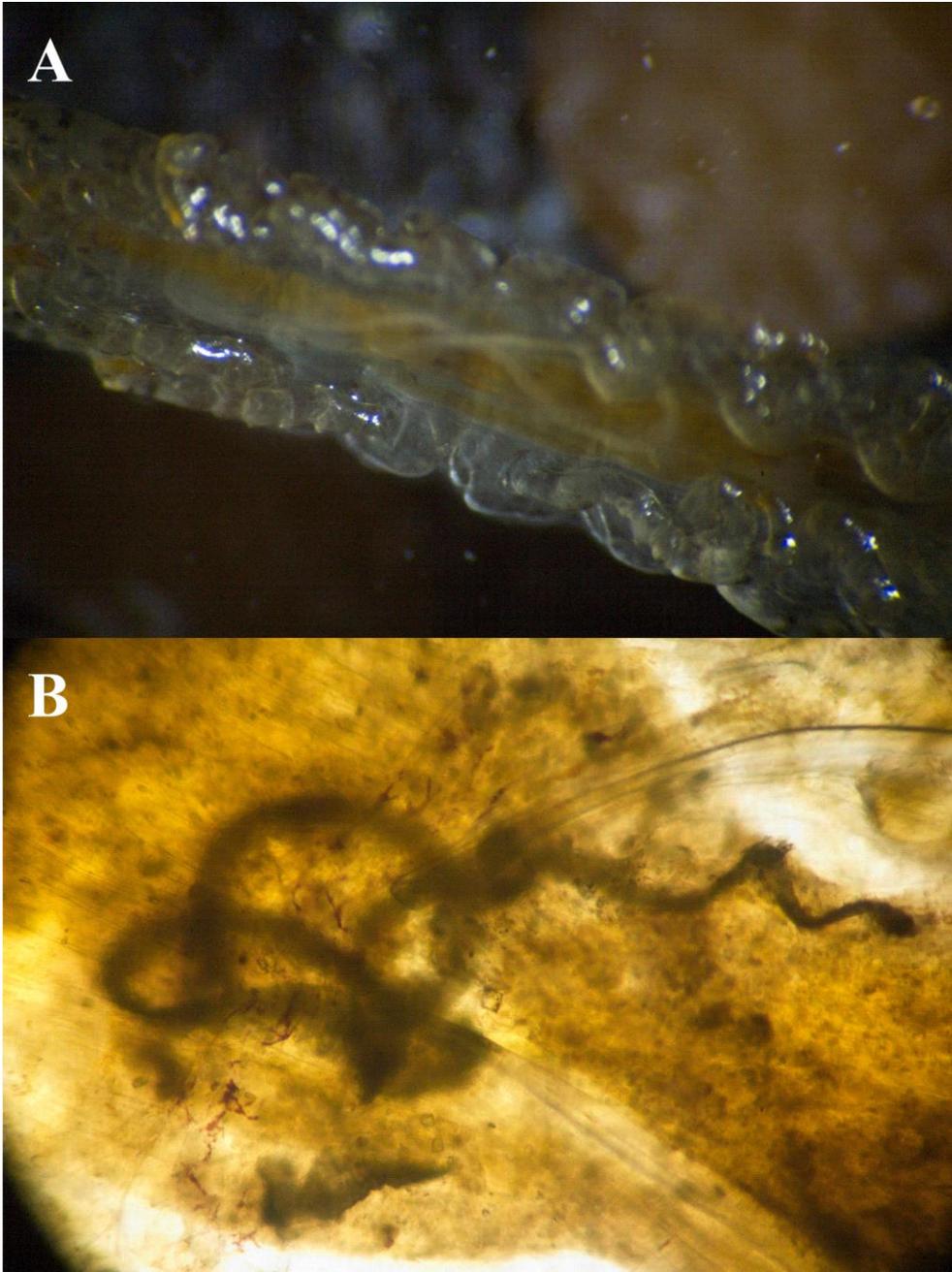


Figure A5. Two examples of what could be a parasitic worm inside *Pentidotea resecata*. Example **A** is from a juvenile isopod, photographed at 45x. Example **B** is from an adult isopod, photographed at 100x.



Figure A6. Two examples of the white substance inside several different isopods over the course of the summer. The top photograph shows a ventral view of the isopod at 6.5x, and the bottom photograph is of the dorsal surface of a different isopod at 15x.

Dietary Fidelity

In addition to feeding isopods eelgrass, 10 isopods were kept in containers with blades of *Macrocystis* instead of eelgrass for a period of 23 days. The *Macrocystis* blades disintegrated more rapidly than eelgrass blades, making it difficult to determine whether the loss of the blade was caused by the isopod or not. However, isopods maintained on *Macrocystis* did grow over the trial period, as indicated by what appeared to be small increases in length and mass. Just as was reported by Lee and Gilchrist (Lee & Gilchrist 1972), I did not observe any change in color from green to brown in the isopods that were fed *Macrocystis*.