2. Materials and Methods

2.1. Specimen collection

Whole individuals of *P. nigra* were collected with substrate intact along sea walls and pilings in the Port Largo Canal, Key Largo, Fl, in October 2002 and January 2003. Individuals were collected by SCUBA at depths between 3 and 7 meters. Substrate attached to the basal portion of tunicates was carefully removed and each individual was immediately weighed and a volume determined by submerging the animal in a graduated cylinder and recording the amount of seawater displaced.

2.2. Specimen dissection and whole blood collection

Blood of *P. nigra* was isolated as described in Brand *et al.* (1989). In brief, blood was extracted by heart puncture with a sterile 3.0-ml syringe and an 18.5 gauge wide bore hypodermic needle. The blood collected from each individual was placed in a sterile 2.0-ml cryogenic vial. Extracted blood was flash frozen in an ethanol and dry ice slurry (-40 ºC) to limit oxidative effects. The tunic of each individual was then dissected from the soft body and the volume and mass of each were determined. After dissection both the tunic and soft body were rinsed with distilled water and frozen in separate containers.

Specimens of *P. nigra* were collected for the purpose of obtaining samples of the tunic bladder cell layer. This layer was separated from the rest of the tunic using a sterile scalpel (Stoecker, 1978; Hirose, 1999; Hirose *et al.*, 2001). The volume and weight of the tunic bladder cell layer were recorded. After dissection the bladder cells were flash frozen and stored as previously stated.

2.3. Organic extraction

Tunic and soft body tissues were chopped into small pieces and added to a 40-ml mixture of 1:1::dicholormethane:methanol (DCM:MeOH) in a 100-ml vessel. Capped vessels were agitated repeatedly on a bench-top shaker at room temperature for 10 to 16-hr. During this time, water within the sample combined with the methanol as the containers were shaken. After extraction, both phases were filtered through celite (Celatom, Eagle-Picher Minerals, Inc.). Both the DCM and the MeOH:H_2O extracts were evaporated to a residue using low heat (< 40 ºC) on a rotary evaporator and any remaining moisture removed by a Savant Speed Vac vacuum concentrator. The tissue sample was extracted a second time with methanol for 12-hr at room temperature and the extract filtered and evaporated as previously stated. The MeOH extract was evaporated to dryness, the extract was resuspended in 3-ml of solvent and then transferred to a 20-ml scintillation vial containing the other 2 extracts to form a whole crude organic extract. Any remaining residual solvents were evaporated by vacuum concentration (Pawlik *et al.*, 1995).

2.4. Chemical analysis

Frozen samples of tunic, tunic bladder cells, soft body, and blood were freeze dried until a constant mass was attained. Crude organic extracts were evaporated to dryness and like *P. nigra* tissue (whole tunic, whole soft body, and tunic bladder cell layer) and blood samples were digested in hot concentrated 2:1::nitric acid:sulfuric acid (HNO_3:H_2SO_4) (Sigma-Aldrich, trace metal grade) in a 25-ml teflon digestion vessel. The concentration of vanadium from each crude organic extract, tissue and blood sample was measured by flame AAS by the method of standard curve with a Perkin Elmer 3110 AAS and nitrous oxide (N_2O_2) (Kustin *et al.*, 1976; Stoecker, 1978).

2.5. Vanadium species and synthesis of vanadium complexes

A solution of vanadium chloride (VCl_3, Fisher Scientific) in 1M HCl was used to simulate the hexaaqua V (+3) component of *P. nigra* blood cell chemistry. The V (+3) fraction of the blood chelated by DOPA or TOPA-like ligand was simulated by V(acetylacetonate)$_3$ (Strem Chemicals Inc.) and K_3[V(catecholate)$_3$]·1.5 H_20 (Cooper et al. 1982), which was prepared as needed. The V (+4) fraction of the blood cells was reproduced as, $VOSO_4$·6 H_2O in 1M HCl (Frank *et al.*, 1999, 2003).

The techniques of infrared spectroscopy (IR) using a potassium bromide pellet (KBr) and ultraviolet spectroscopy (UV) were used to confirm the presence and coordination of vanadium in all synthesized vanadium compounds. Reaction products were purified by re-crystallization.

2.6. Preparation of stock solutions

Stock solutions of each vanadium-containing compound were made based on the molecular weight of the vanadium fraction in ratio to the molecular weight of the entire vanadium complex. Three stock solutions of each vanadium complex with vanadium concentrations of 1000-µg/ml, 2000-µg/ml, and 4000-µg/ml were prepared. These vanadium concentrations are relative to known vanadium concentrations in *P. niga* tissues. In instances where vanadium complexes were synthesized, stock solutions of the reaction precursors were also prepared. Stock solutions of the reaction precursors were made based on the molecular weight of the precursor fraction in ratio to the molecular weight of the entire vanadium complex. Three stock solutions of each reaction precursor were also prepared with concentrations

equivalent to the amount of the reaction precursor present in the vanadium complex minus the vanadium fraction.

The acidity of each stock solution was determined by pipetting 30-µl of solution onto analytical pH strips (EM-reagents 9580, 9581, and 9590). The pH of freshly dissected tunic and soft body tissues was also determined by analytical pH strip by pressing the test strip to the surface of the tissue with light pressure. The pH of tunic and soft body tissues that had been previously frozen and then thawed were analyzed in the same fashion. The pH of fresh and frozen blood samples was determined by pipetting 30-µl of blood onto the test strip. Each stock solution and tissue type was measured 3 times to determine a mean pH value; the pH values were consistent and did not vary with each reading.

2.7. Feeding assays

Feeding assays with the blue head wrasse, *Thalassoma bifasciatum*, a generalist Caribbean reef predator, were performed as described in Pawlik *et al.* (1995) in the wet laboratory located at the Center for Marine Science, Wilmington, NC. Anti-predation activity was assessed for all synthesized compounds and reaction precursors, as well as crude organic extracts of tunic and soft body tissues and blood lysates of *P. nigra* as described above. The vanadium complexes, reaction precursors, tissue and blood extracts were incorporated into a food matrix composed of freeze-dried squid mantle (5.0-g), alginic acid (Sigma-Aldrich, Na-salt form) (3.0-g), and distilled water (100-ml). The mixture was stirred until homogenous and then packed into a 3.0-ml syringe and extruded into a 0.25 M calcium chloride (CaCl) solution, which caused the squid matrix to polymerize. After a few minutes, the spaghetti-like strand was removed from the CaCl solution and rinsed with de-ionized water. The resulting string was cut

with a razor blade into 4.0-mm long pellets. Control pellets were made in the same manner with the addition of solvents, but without the addition of extracts. Some compounds reacted with the squid and alginic acid matrix and caused it to polymerize before being extruded into the CaCl solution. These compounds were instead incorporated into a food matrix composed of freeze-dried squid mantle (5.0-g), carageenan (3.0-g) (Sigma-Aldrich, type 1 commercial grade), and distilled water (100-ml). The mixture was stirred until homogenous and heated in a microwave until boiling. The mixture was then quickly remixed and poured onto a clean bench top and allowed to harden. Once the carageenan mixture had polymerized the resulting gel was cut with a razor blade into 4.0-mm long pellets. Control pellets were made in the same manner with the addition of solvents but without the addition of extracts.

Five 30-gallon aquaria were divided into 24 cells and groups of 2 to 4 yellow-phase *T. bifasciatum* were placed in each cell. Groups of fish were randomly chosen during feeding assays and offered a control food pellet, followed by a treatment pellet. If the fish rejected the treatment pellet, another control pellet was offered to determine whether the fish had stopped feeding; groups of fish that would not eat control pellets were not used in assays. A pellet was considered rejected if not eaten after a minimum of 3 attempts by one or more fish to take it into their mouth cavity, or if the pellet was approached and ignored after one such attempt. Fish were fed 10 to 30-min prior to assays to limit effects due to extreme hunger. Uneaten pellets were removed from tanks after each assay. The Fisher exact test was used to determine the significance of differences in the consumption of treated versus control pellets (Zar, 1984). For any single assay of 10 replicates, a treatment was considered significantly deterrent if 4 or more of the pellets were rejected ($p \leq 0.043$, 1-tailed test). Treatments were considered deterrent if the

mean number of pellets eaten in multiple samples of the same treatment was less than or equal to 6 (Pawlik *et al.*, 1995).

To assess the effect of basic pH on palatability, food pellets were made as before using carageenan with the addition of 1N sodium hydroxide (NaOH) that provided the desired pH, as indicated by an analytical test strip. Control pellets were made in the same manner, but did not include the addition of base. The mixture was stirred and microwaved for 30-sec. The heated mixture was poured onto a clean bench top and allowed to harden. Food pellets were cut from the hardened gel. Feeding assays were performed as before, however the pH of food pellets was determined with analytical test strips both prior to assay and after pellets were rejected to assure consistency in pellet pH values. The pH of successive assays was increased within a range starting at pH 7.0 to pH 14.0. Control pellets had a pH of 7.0.

2.8. Disc-diffusion anti-microbial assay

Anti-microbial activity was assessed for: vanadium-containing compounds and reaction pre-cursors, as well as crude organic extracts of *P. nigra* tunic and soft body tissues. Each compound and tissue extract was tested against an assembly of 4 marine bacteria that included: *Vibrio harveyi* (Microbial Identification, Inc., MIDI, Newark, Delaware), *Deleya marina* (American Type Culture Collection (ATCC) 35142), *Vibrio parahaemolyticus* (ATCC 27969), and *Leucothrix mucor* (ATCC 25906). The bacterium *V. harveyi* was isolated and identified as part of a previous study by Kelly *et al.* (2002 submitted) and has been recognized as an opportunistic pathogen of certain fish and marine invertebrates (Oakley and Owens, 2000 and references cited therein). The marine microbe *D. marina* is a common surface fouling bacterium (Shea *et al.*, 1985), and both *V. parahaemolyticus* and *L. mucor* are known marine pathogens that

can cause disease in marine invertebrates (Sparks, 1985). Standard disc-diffusion anti-microbial assays were performed by inoculating each bacterial test strain onto the surface of a YP agar medium plate: peptone (1.0-g·L^{-1}) and yeast extract (1.0-g·L^{-1}) in filtered seawater (32 to 36-‰) with the addition of agar (15-g·L^{-1}) (Berquist and Bedford, 1978; Jensen *et al.*, 1996; Newbold *et al.*, 1999). Culture concentrations were measured before each anti-microbial bioassay using a Milton Roy Spectronic 20 D at 500 nm. Only cultures that had an absorbance between 0.2 and 0.5 were used to inoculate bioassay media plates. A 100-µl aliquot of inoculated broth was spread with a sterile glass rod over the entire agar surface to form an evenly distributed film. Compounds were dissolved in solvent and tested by pipetting 30-µl onto a sterile circular paper disc (Becton Dickinson, 6.0-mm x 0.9-mm, disc volume = 30-µl), and allowing the solvent to evaporate. Crude organic extracts were resuspended volumetrically in methanol before transfer to paper disc. All solvents used to dissolve air-sensitive materials were degassed of oxygen prior to use, and all stock solutions were stored under inert atmosphere using purified nitrogen. After pipetting the stock solution onto a paper disc the solvent was evaporated under nitrogen. Stock solutions were monitored for color change that would indicate oxidation events. If oxidation occurred, the solutions were re-made before use in the assay. The discs were placed on the surface of the inoculated agar (up to 9 $plate^{-1}$). Pressing lightly on the disc with an analytical pH strip, the pH of each disc was checked once the discs had re-hydrated after being placed on the agar plate. All strains were incubated at room temperature (22 ºC) and a confluent film developed over the entire agar surface after 24-hr. Clear halos (zones) surrounding the discs denoted areas of inhibited microbial growth. The computer-imaging program Image J (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) was used to calculate area of inhibition zones by subtracting the area of the disc (28.27-mm^2) from the total area of clear zone.

Compounds that exhibited inhibition zones (total area of clear zone minus the area of the disc) of greater than or equal to 50-mm^2 were considered to possess anti-microbial activity. Statistical analysis was not used to assign significance between treatments as the disc diffusion assay is used to assess qualitative differences in antibacterial activity (Jensen *et al.*, 1996; Jenkins *et al.*, 1998; Newbold *et al.*, 1999).