4. Discussion

Ascidians are soft bodied and lack obvious physical defenses. Structural components of the tunic, i.e. leathery texture and calcareous spicules are not effective feeding deterrents (Lindquist *et al.*, 1992). Therefore, secondary metabolites have been implicated in defending these sessile marine invertebrates. Consequently, ascidians are well represented in the literature for producing many novel secondary metabolites; however, ascidians are different from other marine invertebrates because they may also be chemically defended by means other than secondary metabolites.

The presence of low pH environments and heavy metal accumulation in ascidians has long been recognized (Henze, 1911). Stoecker (1978), first hypothesized that inorganic acids and heavy metals, most notably vanadium, provided ascidians with effective anti-predatory and anti-fouling defenses. Parry (1984) disputed Stoecker's results. Arguments by Parry (1984) were correlative and based on the existence of other tunicate species in similar habitats that contained neither vanadium nor acid, yet remained uneaten or fouled. Parry (1984) did not consider the presence of secondary metabolites as defenses that might function in the absence of vanadium and sulfuric acid. Stoecker (1980a) conducted feeding assays with fish and crabs using the vanadium salts vanadyl sulfate and sodium vanadate. These salts putatively represented the oxidation state of vanadium when it was released from the ascidian during a predation event. The results of the feeding assays reported herein using the blue head wrasse, *Thalassoma bifasciatum*, were similar to those of Stoecker (1980a) that used the fishes: *Fundulus heteroclitus* (common killifish), *Abudefduf saxatilis* (sergeant majors), and *Haemulon sciurus* (blue striped grunt). However, salts of vanadium do not accurately depict vanadium as it is found within *P. nigra* tissues and blood; thus, the palatability of naturally occurring vanadium was not assessed. Furthermore, pH and vanadium accumulation and storage in adult ascidians are coupled together and therefore must be decoupled from one another to determine the chemical deterrence of the individual components.

In this study, the presence of vanadium did not always deter predation or inhibit microbial growth, whereas low pH was unpalatable to fish and exhibited anti-microbial activity. Crude extracts of *P. nigra* tunic and soft body that contained vanadium but were not acidic did not deter predation nor inhibit microbial growth. Vanadium species decoupled from acidity by chelation to DOPA/TOPA-type ligands did not deter predation or inhibit microbial growth; however, acidic aqua vanadium (+3 and +4) complexes were deterrent in both assay systems. This study is the first to attempt to decouple the chemical deterrence of vanadium from low pH in an adult ascidian.

Localization of chemical defenses to specific regions of an organism is exhibited in terrestrial plants and marine organisms (Zangerl and Rutledge, 1996; Dworjayn *et al.*, 1999; Van Alstyne *et al.*, 1999) and has been observed in some marine invertebrates such as mollusks (Pawlik *et al.*, 1988), gorgonians (Harvell and Fenical, 1989), sponges (Schupp *et al.*, 1999), and has also been suggested in tunicates (Pisut and Pawlik 2002). Surface associated defenses may enhance the survival of an organism by providing a mechanism to protect against predation, deter fouling organisms, and control epibiont populations (Wahl *et al.*, 1994). Acidity is important in deterring potential predators and is probably effective against most grazers and browsers (Thompson, 1960; Stoecker, 1980a). Davis and Wright (1989) hypothesized that surface acidity would be neutralized by the buffering capacity of seawater, but Pisut and Pawlik (2002) have demonstrated otherwise. Brief localized exposure to low pH may be enough to deter a potential predator (Hirose *et al.*, 2001). Pisut and Pawlik (2002) demonstrated that pieces of acidic *Ascidia interrupta* tunic were unpalatable to *T. bifasciatum* in feeding assays; crude organic extracts of *A. interrupta* tunic were palatable. Epibionts are rare on species that exhibit strong acid and/or concentrate heavy metals (Stoecker, 1980a). Some ascidians sequester acids and heavy metals to the tunic exterior (Goodbody, 1962; Stoecker, 1980a; Hirose, 1999). The allocation of chemical defenses to the tunic exterior would be effective against fouling organisms by preventing the surface of the tunic from being penetrated by stolons or other anchoring devices (Stoecker, 1978).

In the solitary tunicate *Phallusia nigra*, low pH and vanadium are associated with the tunic exterior. Acidic fluid is released from bladder cells localized near the exterior surface when the tunic is bruised or damaged (Hirose *et al.*, 2001; Pisut and Pawlik, 2002). Vanadium is not evenly distributed throughout *P. nigra* tissues (Fig. 3). The data presented here, confirms the results of Stoecker (1978) and Ciereszko *et al.* (1963) concerning the distribution of vanadium within the tissues investigated. Vanadium is stored mainly in blood cells called vanadocytes, but is also accumulated at the tunic surface (Bielig *et al.*, 1966; Carlson, 1975; Kustin *et al.*, 1976; Botte *et al.*, 1979; de Leo *et al.*, 1981; Brand *et al.*, 1989; Hirose, 1999; Hirose *et al.*, 2001; Frank *et al.*, 2001, 2002). Interestingly, vanadocytes are able to migrate from blood vessels located in the tunic and accumulate in the pigmentary layer located at the tunic surface (Anderson, 1971). However, the coordination environment of vanadium at the tunic surface has yet to fully clarified.

Acidic tissues are repellant to most fish (Thompson, 1960) and may partially explain why *P. nigra* is unpalatable. Tissues associated with low pH were unpalatable to *T. bifasciatum* however these same tissues that were frozen and then thawed did not exhibit low pH and were more palatable. Although this process would not affect the presence of vanadium in these tissues, the oxidation state and ligand coordination associated with vanadium may be affected due to changes in the storage environment and oxidative effects of cell lysis. Non-chelated vanadium (+3) ions are unstable in air or moisture and are rapidly oxidized at pH ≥ 2 (see review Rehder, 1995). For feeding assays with whole blood, freezing and thawing neutralized the low pH and increased palatability. Freezing and thawing did not change the pH of soft body samples, but did increase the palatability. Intact blood cells containing acidic fluids may have been present in the freshly dissected soft body and when mouthed by fish, released their acidic contents and were subsequently rejected. Crude organic extracts of whole *P. nigra* soft bodies were not deterrent to fish predation. However, Pisut and Pawlik (2002) suggested that the regions of the gonad and zooid within the soft body of *P. nigra* are defended by secondary metabolites because they were unpalatable to *T. bifasciatum*. In crude extracts of whole tissues (i.e. soft body) the effect of deterrent secondary metabolites localized to specific tissues (i.e. gonad or zooid only) may be altered by the inclusion of extracts from non-chemically defended tissues. The volume of the whole soft body includes tissue that dilutes the deterrent activity of the gonad or zooid that makes up only a small percentage of the entire soft body (Pisut and Pawlik, 2002). Interestingly, whole pieces of fresh *P. nigra* tunic tissue that was acidic and previously frozen tunic tissue that was not acidic were both unpalatable to *T. bifasciatum*, which is a result that cannot be readily explained. Stoecker (1980a) also noted this feeding behavior with fish during predation assays. Crude organic extracts of the tunic were palatable to *T. bifasciatum*. The results of feeding assays with crude organic extracts of *P. nigra* tunic were similar to those of Pisut and Pawlik (2002).

In standard disc diffusion assays, crude organic extracts of the tunic and soft body of *P. nigra* did not inhibit the growth of any of the bacteria assayed. Non-acidic vanadium (+3) compounds also did not inhibit microbial growth, whereas low pH and acidic aqua vanadium (+3 and +4) complexes did inhibit microbial growth. Although assayed at equivalent vanadium concentrations, the two vanadium salts have different effects on the panel of bacteria. Sodium vanadate (pH = 11) did not inhibit microbial growth, whereas vanadyl sulfate (pH 1.9 to 3.6) did produce clear zones of inhibition. Acidic fluids that are released from ruptured bladder cells may be able to kill or inactivate bacteria and/or other microorganisms that would otherwise gain access to tunic tissues.

Tunicates like *Phallusia nigra* are unusual among marine invertebrates because of their capacity to produce secondary metabolites, sequester acids, and accumulate heavy metals for use as chemical defenses. Non-acidic vanadium (+3) complexes did not deter fish feeding or inhibit microbial growth at any of the vanadium concentrations tested. Subsequently, acidic aqua vanadium complexes were unpalatable to *T. bifasciatum* and exhibited anti-microbial activity. The presence of vanadium in high concentrations is not enough to deter predation or inhibit microbial growth. The deterrence of vanadium may be affected by the chelating ligands and the coordination environment. The results of the laboratory assays of this report imply that for *P. nigra*, low pH including acidic vanadium complexes are more effective than non-acidic vanadium complexes as feeding and microbial growth deterrents. It is important to note that definitive conclusions are hindered by the fact that vanadium cannot be decoupled from low pH without altering the coordination environment and oxidation state, i.e. aqua vanadium (+3 and +4) versus DOPA/TOPA-like chelated vanadium (+3). Hence, the function of vanadium in *P. nigra* has yet to be fully clarified.