3. Results

3.1. Flame absorption spectroscopy

Whole tunic, tunic bladder cell layer, soft body, and blood of *P. nigra* were analyzed for vanadium content by flame atomic absorption spectroscopy with N_2O_2 by the method of standard additions. Results of the analysis are displayed in Table 1.

The soft body contains 2280 ± 822-ppm (mean vanadium concentration ± S.D., dry weight) of vanadium, the blood contains 1886 ± 567-ppm, and the tunic contains 259 ± 157-ppm. Further investigation of the vanadium distribution within the tunic revealed that the tunic bladder cell layer contains 871 ± 390-ppm. The distribution of vanadium concentration between the *P. nigra* tissues was tested with a one-way ANOVA, after which the tissues were grouped into similar subsets using the Ryan-Einot-Gabriel-Welsch Multiple Range test. The distribution of vanadium within the soft body and blood are similar but are significantly different than that of the tunic bladder cell layer and of the whole tunic.

3.2. $K_3[V(catecholate)_3]$ synthesis and complex confirmation

The vanadium (+3) tris(catecholate) complex, $K_3[V(catecholate)_3]$ (molecular weight = 519.55), was synthesized using the methods described by Cooper *et al.* (1982). All operations were carried out using standard Schlenk techniques under nitrogen (N_2) atmosphere. The synthesized product was analyzed by infrared spectroscopy by KBr pellet. Vanadium (+4) catecholate and vanadium (+3) catecholate complexes can be distinguished from one another by the presence of a V═O stretch at 977 cm^{-1} within the IR spectra of V (+4) tris(catecholate) and the absence of this band in corresponding V (+3) tris(catecholate) complexes (Selbin, 1966).

Table 1. Flame atomic absorbance spectroscopy results of the vanadium content (ppm ± SD) of freeze dried *P. nigra* tissues, blood samples, and crude organic extracts of whole tunic and soft body. Wet weight vanadium concentrations were converted from dry weight. Results of one-way Analysis of Variance to test for spatial differences in the distribution of vanadium in *P. nigra* tissues and blood samples are followed by Ryans-Einot-Gabriel-Welsch Multiple Range Test for comparison between tissue and blood samples (homogeneous subsets at p = 0.05). SB = Whole Soft body; B = Blood; TE = Tunic Bladder Cell Layer; T = Whole Tunic.

Sample	**n**	**Vanadium Content**	
		Dry weight	**Wet weight**
Whole Tunic	6	259 ± 157	5 ± 3
Tunic Bladder Cell Layer	7	871 ± 390	18 ± 8
Whole Soft Body	5	2280 ± 822	63 ± 23
Blood	7	1886 ± 567	38 ± 12
Crude Extract: Soft Body	4	750 ± 289	31 ± 12
Crude Extract: Tunic	4	225 ± 126	7 ± 4

	One-way Analysis of Variance				Ryans-Einot-Gabriel-Welsch subsets			
Vanadium Concentration (ppm)	n	F	*P*	R^2				
Dry Weight	3	41.02	0.0001	0.7688	SB	B	TE	T

The results of the IR spectra by KBr pellet of synthesized $K_3[V(catecholate)_3]$ are shown in Figure 1. Comparisons of the observed peak values from the IR spectra to the expected peak values from the crystallographic data are summarized in Table 2. The observed peak values from the $K_3[V(catecholate)_3]$ spectra are characteristic of catecholate complexes (Cooper *et al.* 1982).

3.3. Feeding assay

Dissected whole tissue and blood samples, crude organic extracts of tunic and soft body tissues, vanadium complexes, and vanadium salts were assessed for anti-predation activity in feeding assays with a common generalist reef fish, the bluehead wrasse, *Thalassoma bifasciatum*. The results of the feeding assay are presented in Figure 2.

Assays with freshly dissected whole tunic tissue (pH ~ 2.0) and previously frozen tunic (pH = 7) were not palatable to *T. bifasciatum*. In both cases, treatment pellets were cut from the whole tunic. The low pH associated with fresh tunic tissue was neutralized after being frozen and thawed however this did not affect the palatability of the food pellet. In all cases, fishes approached and mouthed the pieces of the tunic but it was not eaten. Food pellets that were made with finely chopped tunic tissue, fresh and frozen, were also mouthed and the squid matrix consumed; however, the bulk of the tunic tissue was not consumed. Freshly dissected soft body tissue (pH = 7) was unpalatable to *T. bifasciatum*. Freezing and thawing the tissue did not change the pH of the tissue, but did increase palatability. Fishes rejected freshly collected *P. nigra* blood (pH ~ 2) incorporated into food pellets, whereas neutralized blood was palatable.

Figure 1. Infrared spectra by KBr pellet of the non-acidic vanadium (+3) catecholate compound, $K_3[V(catecholate)_3]$.

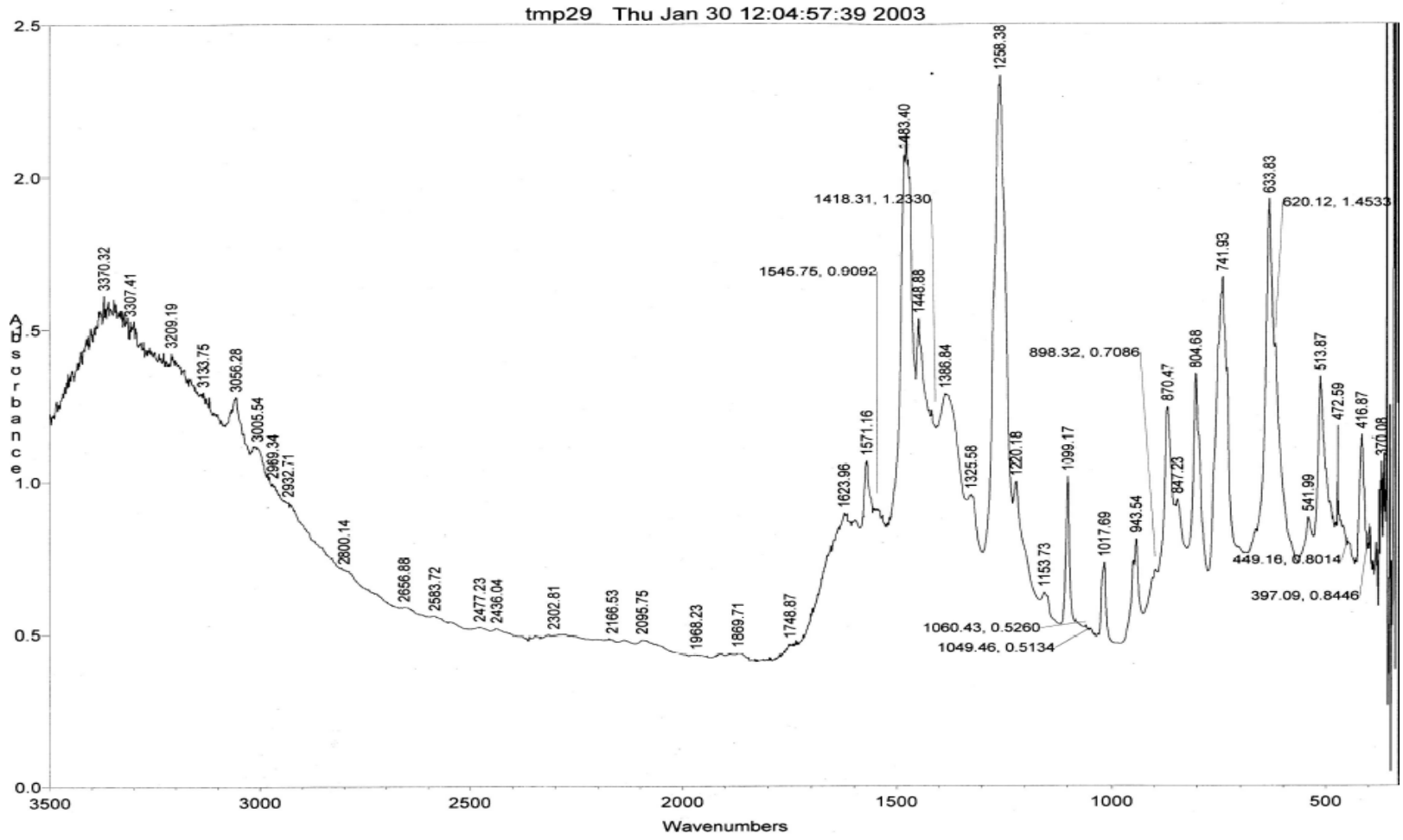

tmp29 Thu Jan 30 12:04:57:39 2003
Absorbance
Wavenumbers
3500
3000
2500
2000
1500
1000
500
0.0
0.5
1.0
1.5
2.0
2.5
3370.32
3307.41
3209.19
3133.75
3056.28
3005.54
2969.34
2932.71
2800.14
2656.88
2583.72
2477.23
2436.04
2302.81
2166.53
2095.75
1968.23
1869.71
1748.87
1623.96
1571.16
1545.75, 0.9092
1483.40
1448.88
1418.31, 1.2330
1386.84
1325.58
1258.38
1220.18
1153.73
1099.17
1060.43, 0.5260
1049.46, 0.5134
1017.69
943.54
898.32, 0.7086
870.47
847.23
804.68
741.93
633.83
620.12, 1.4533
541.99
513.87
472.59
449.16, 0.8014
416.87
397.09, 0.8446

Table 2. Infrared Spectroscopy of $K_3[V(catecholate)_3]$ by KBr pellet. Comparison of the expected peak values from crystallographic data reported by Cooper *et al.* (1982), for $(Et_3NH)_2V(catecholate)_3 \cdot CH_3CN$ versus observed peak values of $K_3[V(catecholate)_3]$. Spectra are characteristic of catecholate complexes. Notable changes in peak strength or size are highlighted in **bold**.

Expected Peak Values (cm-1)	Peak Strength	Observed Peak Values (cm-1)	Peak Strength
3420	b	**3370**	b
3050	m	3056	m
3000	m	3005	**m,b**
2975	m	2969	**b**
2940	m	2933	**b**
2660	b	2657	b
2480	b	2477	b
1960	w	1968	w,b
1630	w,b	1624	w,b
1569	m	1571	m
1465	s	**1483**	s
1450	s	1448	s
1400	m	**1418**	w
1365	w	**1386**	w
1318	w	1326	w
1300	w	**no peak**	
1260	s	1258	s
1223	m	1220	**w**
1140	w	**1154**	w

Expected Peak Values (cm-1)	Peak Strength	Observed Peak Values (cm-1)	Peak Strength
1095	m	1099	m
1064	w	1060	w
1030	m	**1049**	w
1021	m	1018	m
1013	m	**no peak**	
935	w	944	**w, d**
895	w,d	898	w,d
870	m	870	m
840	w	847	w
801	s	805	s
732	s	741	s
620	s,d	**634**	s,d
552	w	542	w
532	w	**no peak**	
500	s	**514**	s
442	w	449	w
413	s	417	s
364	m	370	m
310	w	**no peak**	

Legend

s =	strong
m =	medium
w =	weak
b =	broad
d =	doublet

Freezing and thawing neutralizes low pH of tissues but does not affect the vanadium content.

Organic crude extracts of *P. nigra* tunic and soft body tissues were palatable to *T. bifasciatum* in laboratory feeding assays.

The non-acidic vanadium (+3) compounds bound to DOPA/TOPA-like chelation environments were also tested. The compounds V(acetylacetonate)$_3$ and K_3[V(catecholate)$_3$] were palatable to *T. bifasciatum* at all of the concentrations assayed.

Food pellets with vanadyl sulfate were palatable to *T. bifasciatum* at the lowest vanadium concentration (pH = 2.2). However, increasing the vanadium content of the treatment pellets reduced the pH of the pellets and subsequently decreased palatability. Food pellets made with the vanadium salt sodium vanadate at all concentrations (pH = 11) were not palatable to *T. bifasciatum*. Results of this assay with the vanadium salts, sodium vanadate and vanadyl sulfate, confirmed earlier results reported by Stoecker (1980a).

Food pellets with a pH ≥ 13.0 deterred feeding by *T. bifasciatum* (Figure 3). The pH of food pellets was the same immediately after being mouthed and rejected by *T. bifasciatum*.

3.4. Disc-diffusion anti-microbial assay

Crude organic extracts of tunic and soft body tissues, vanadium complexes and vanadium salts were assessed for anti-microbial activity against a panel of 4 marine bacteria known to infect marine invertebrates. The results of the standard disc-diffusion anti-microbial assay are presented in Table 3.

Figure 2. Consumption by the blue head wrasse, *T. bifasciatum*, in laboratory assays of *P. nigra* tissues and blood as well as food pellets of vanadium complexes and vanadium salts. Compounds were considered deterrent if the mean number of pellets (n = 5) eaten for any individual assay were less than or equal to 6 ($p \leq 0.043$, Fisher exact test, 1-tailed), as indicated by the dotted line on the graph.

Treatment	Vanadium Concentration µg/ml	Food Pellet pH
Whole Tunic (fresh)	5 ± 3	2.0
Whole Tunic (frozen)	5 ± 3	7.0
Soft Body (fresh)	63 ± 23	7.0
Soft Body (frozen)	63 ± 23	7.0
Blood (fresh)	38 ± 12	2.0
Blood (frozen)	38 ± 12	7.0
Crude Extract: Tunic	7 ± 4	5.0
Crude Extract: Soft Body	31 ± 12	5.0
$V(acetylacetonate)_3$	1000	6.5
$V(acetylacetonate)_3$	2000	6.5
$V(acetylacetonate)_3$	4000	6.5
$K_3[V(catecholate)_3]$	1000	11.0
$K_3[V(catecholate)_3]$	2000	11.0
$K_3[V(catecholate)_3]$	4000	11.0
VCl_3 in 1M HCl	1000	0.0
VCl_3 in 1M HCl	2000	0.0
VCl_3 in 1M HCl	4000	0.0
$VOSO_4$ in 1M HCl	1000	0.0
$VOSO_4$ in 1M HCl	2000	0.0
$VOSO_4$ in 1M HCl	4000	0.0
$VOSO_4$	1000	2.2
$VOSO_4$	2000	1.9
$VOSO_4$	4000	1.6
Na_3VO_4	1000	11.0
Na_3VO_4	2000	11.0
Na_3VO_4	4000	11.0

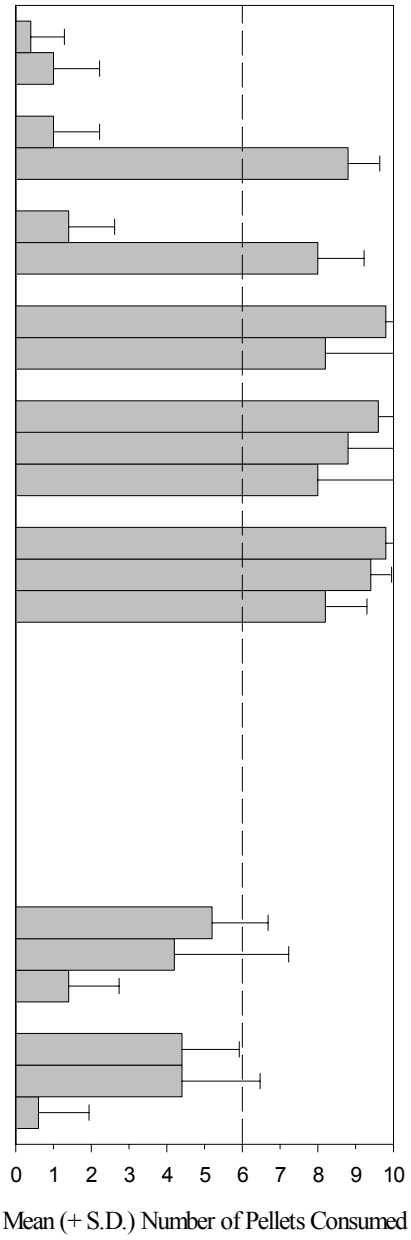
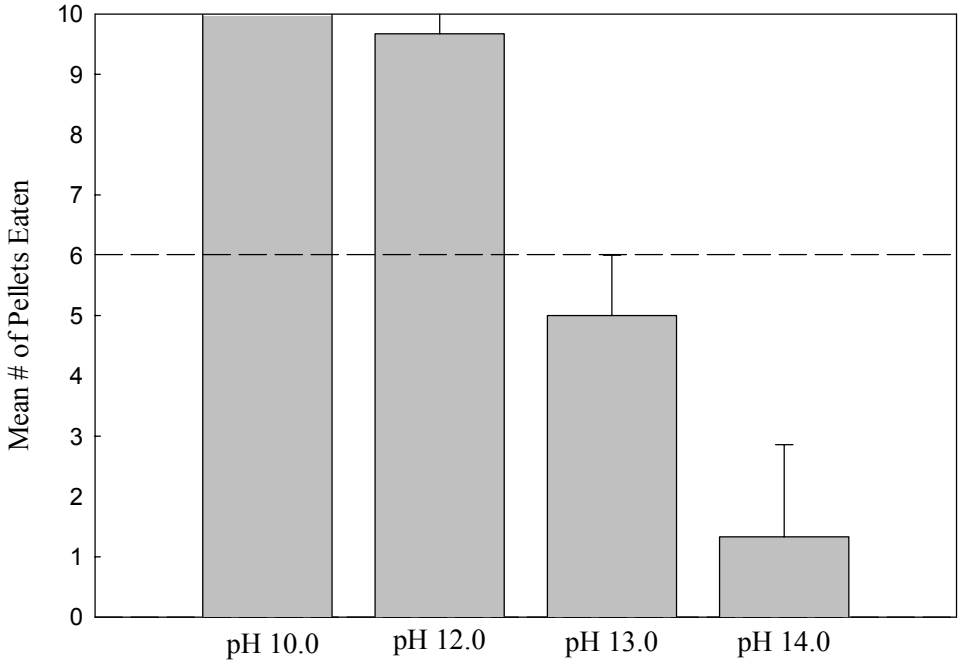


Figure 3. Consumption by *Thalassoma bifasciatum* of food pellets (mean + S.D., n = 3) buffered in sodium hydroxide. Fish consumed 10 control pellets in all cases. Treatments were considered deterrent if the number of pellets eaten was less than or equal to 6 ($p \leq 0.043$, Fisher exact test, 1-tailed), as indicated by the dotted line on the graph.



Organic crude extracts of dissected tunic and soft body tissues (n=3 for each tissue type versus each bacteria) of 4 individual tunicates did not exhibit any anti-microbial activity against the panel of marine bacterial lines tested.

The non-acidic vanadium (+3) complexes, V(acetylacetonate)$_3$ and K_3[V(catecholate)$_3$] did not exhibit any anti-microbial activity against the entire panel of marine bacterium. However, the acidic aqua vanadium (+3 and +4) complexes inhibited microbial growth. The aqua vanadium (+3) complex inhibited growth against the entire panel of marine bacteria at all concentrations tested, but only exhibited significant anti-microbial activity when assayed at the highest vanadium concentration. The results of the acidic aqua vanadium (+4) complex were similar to those of the acidic aqua vanadium (+3) complex. However, the aqua vanadium (+4) complex exhibited significant anti-microbial activity against 3 of the 4 bacteria lines tested at only the highest vanadium concentration.

Although assayed at equivalent vanadium concentrations the two vanadium salts had different effects on the panel of bacteria. Sodium vanadate at all concentrations (pH = 11) did not inhibit microbial growth, whereas vanadyl sulfate (pH 1.9 to 3.6) did produce clear zones of inhibition. Vanadyl sulfate showed significant inhibition effects at the highest vanadium concentration for 3 of the 4 marine bacteria.

Table 3. Anti-microbial activity (mean area of inhibition (standard deviation), mm^2) of vanadium compounds and vanadium salts versus bacterial test strains using standard disc diffusion assay. Mean areas (n = 8 for all treatments) that produced clear zones of inhibition greater than or equal to 50 mm^2 denote significant anti-microbial activity. A (0) denotes no activity, a (+) denotes activity, and a (+++) denotes significant activity.

Compound	Vanadium Concentration (μg/ml)	Solution pH	Disc pH	*Vibrio parahaemolyticus* Mean Area of Inhibition	*Deleya marina* Mean Area of Inhibition	*Leucothirx mucor* Mean Area of Inhibition	*Vibrio harveyi* Mean Area of Inhibition
$V(acetylacetonate)_3$	4000	7.0	4.0	0	0	0	0
$V(acetylacetonate)_3$	2000	7.0	5.0	0	0	0	0
$V(acetylacetonate)_3$	1000	7.0	6.0	0	0	0	0
$K_3[V(catechol)_3]$	4000	11.0	10.0	0	0	0	0
$K_3[V(catechol)_3]$	2000	11.0	10.0	0	0	0	0
$K_3[V(catechol)_3]$	1000	11.0	9.0	0	0	0	0
VCl_3 in 1M HCl	4000	0.0	2.5	+++	+++	+++	+++
VCl_3 in 1M HCl	2000	0.0	3.3	+	+	+	+
VCl_3 in 1M HCl	1000	0.0	3.6	+	+	+	+
$VOSO_4$ in 1M HCl	4000	0.0	1.6	+	+++	+++	+++
$VOSO_4$ in 1M HCl	2000	0.0	1.9	+	+	+	+
$VOSO_4$ in 1M HCl	1000	0.0	2.2	+	+	+	+
$VOSO_4$	4000	1.6	1.9	+	+++	+++	+++
$VOSO_4$	2000	1.9	2.5	+	+	+	+
$VOSO_4$	1000	2.2	3.6	+	+	+	+
Na_3VO_4	4000	11.0	10.0	0	0	0	0
Na_3VO_4	2000	11.0	9.0	0	0	0	0
Na_3VO_4	1000	11.0	8.0	0	0	0	0