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Short Communication

Title: The effects of several common anthropogenic contaminants on proliferation of the parasitic oyster pathogen *Perkinsus marinus*.

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Abstract:

Estuarine contaminants have varying effects on estuarine inhabitants and host-parasite interactions. Some field collected contaminant mixtures have been shown to increase oyster susceptibility to parasitism by *Perkinsus marinus*, but little is known about contaminant effects on the parasite itself. This study examined the effects of ammonium, nitrate, phosphate, fluoranthene, phenanthrene and a common herbicide mixture (Weed-B-Gone®) on in vitro

proliferation of P. marinus. Only the herbicide had a significant effect, but not at or below the

25 manufacuture's recommended application rate (7.81 µl mL⁻¹). The herbicide's active ingredients (3.1% 2,4-dichlorophenoxyacetic acid, 10.6% mecoprop and 1.3% dicamba) mimic growth hormones of broadleaf plants; over stimulation of growth results in death. The mode of action of these compounds on *P. marinus* warrants further investigation which may provide insight towards the identification of biocides to control *P. marinus*.

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Key words: shellfish, disease, dermo, protozoan, estuary, pollution

During the past century, populations of the Eastern oyster *Crassostrea virginica* have
declined dramatically in mid-Atlantic bays along the coast of North America (e.g., Rothschild et
al. 1994). Concurrently, extensive anthropogenic alterations have changed many facets of the
estuaries where the oysters once thrived. One noteworthy alteration has been the increase in
chemical contaminants, several of which have been shown to negatively impact oysters and
increase their susceptibility to dermo disease (Chu and Hale 1994, Anderson et al. 1996, 1998,
Fisher et al. 1999, Chu et al. 2002). Several parasitic pathogens plague the eastern oyster (Ford
and Tripp 1996), but the protozoan parasite *Perkinsus marinus*, which causes dermo disease, has
been a primary source of oyster mortality for decades (Burreson and Calvo 1996) and is a major
impediment to the recovery of eastern oyster populations (Brown et al. 2005). Few studies,
however, have examined the impact that estuarine contaminants have on *P. marinus*.

Lafferty (1997) described how the effects of anthropogenic inputs could be positive, 45 negative, or neutral with respect to parasites and disease. In general, pesticides, metals and PAHs have negative impacts on most biota, although some microbes can use these compounds to

their advantage (Heitkamp et al. 1988). If *P. marinus* can utilize a contaminant that is detrimental to oysters, the negative impact of that particular contaminant on the disease status of the oysters may be exacerbated. On the other hand, some contaminants may harm the parasite

without affecting the host, resulting in a net benefit to the host. Indeed, herbicides, pesticides and antibiotics are explicitly designed and applied for just this purpose. Once such compounds move beyond their target populations, in runoff flowing into estuaries for example, they become contaminants. The interacting forces of different contaminants may confound the interpretation of the impact that a particular contaminant or class of contaminants has on any particular
organism. The present study examined the effect of six common anthropogenic chemical contaminants (Table 1) on in vitro proliferation of *P. marinus*.

Parasites. *Perkinsus marinus* strains (ATCC 50509, 50775 and 50763) that represent three distinct genotypes (Reece et al. 2001) were grown in ATCC 1886 medium (www.atcc.org) with minor modifications described in Bushek et al. (2000). Genetically distinct strains derived from different geographic locales were used as replicates to ensure that results were not specific to the sensitivity of any particular genotype. Parasites were harvested for chemical challenge experiments from cultures undergoing log-phase growth. Cell density was determined by repeated (n = 3) hemocytometer counts (La Peyre and Chu 1994) and 1-mL aliquots containing equivalent numbers of parasites used to seed wells in a 24-well culture plate. Seeding densities varied among chemicals from 0.5 to 2.0 x 10^6 parasites mL⁻¹, but not among treatments within chemicals. These seeding densities yield rapidly proliferating populations within a few days (Gauthier and Vasta 1995).

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Chemicals and Treatments. Table 1 identifies the chemicals and concentrations tested. Reagents were dissolved in deionized water or acetone, depending on solubility, then in fresh

- 70 culture medium to create 10x working stocks relative to test concentrations. Final dilutions were performed with culture medium that contained *P. marinus* in log-phase growth. Ammonium chloride, sodium nitrate, and sodium phosphate were used as sources of ammonium, nitrate and phosphate with test concentrations selected to include and exceed the range of these chemicals observed in estuarine waters at the North Inlet-Winyah Bay National Estuarine Research
- Reserve, South Carolina, US (data available at http://cdmo.baruch.sc.edu/). The herbicide
 Weed-B-Gon®, which contained a mixture of 2,4-dichlorophenoxyacetic acid (= 2,4-D, CAS# 2008-39-1); 2-(2-Methyl-4-chlorophenoxy)propionic acid, dimethylamine salt (= mecoprop, CAS# 32351-70-5); and 2,5-Dichloro-6-methoxybenzoic acid (= dicamba, CAS# 1918-00-9)
 (Table 1), was initially tested at concentrations of 0.781, 7.81 and 78.1 µg mL⁻¹ based on the
- 80 recommended application concentration of two tablespoons per gallon (= $7.81 \,\mu L \,m L^{-1}$). A subsequent trial tested concentrations of 7.81, 15.6 and 39.1 78.1 $\mu g \,m L^{-1}$ to increase resolution across the observed effective range. PAH test concentrations were based upon levels reported from Murrells Inlet, South Carolina, US (Fortner et al. 1997). Increases in PAH toxicity due to photoactivation following exposure to ultraviolet light (Lyons et al. 2002) was also examined.
- 85 Ultraviolet A and B light levels measured in streams and tributaries of coastal South Carolina, US indicated that 200 µWs cm⁻² would provide a realistic exposure level for testing photoactivated toxicity (J. Weinstein, The Citadel, pers. comm.). Because acetone was used as a solvent for PAHs and also in the herbicide concentrate, its effect was tested at concentrations spanning those used in the treatments.
- Each isolate was challenged in separate 24-well plates with only one chemical at a time to avoid cross-contamination among chemicals or isolates. All experiments were performed at 25°C and controls in fresh culture medium were run for each chemical tested. All treatments

were prepared in triplicate (3 wells x 3 concentrations + three control wells = 12 wells) for each isolate. One well from each isolate (n = 3) was sacrificed a 2, 48 and 144 hr to determine cell proliferation via replicated hemocytometer counts (LaPeyre and Chu 1994). Counts were repeated until multiple counts were within about 10% of each other.

Two-way analysis of variance (ANOVA $\alpha = 0.05$) indicated no interactions between time and chemical concentration for any treatments (Table 2), enabling the effects of time and concentration to be interpreted independently. Time was significant in all experiments as a result

100 of cell proliferation, but only the herbicide Weed-B-Gon® showed a significant effect of concentration (Table 2). In trial 1, cells were killed within 2 hr at 78.1 μ g mL⁻¹, but there was no difference from the control at 7.81 and 0.781 μ g mL⁻¹ (data not shown). The second trial indicated a dose response with reductions in proliferation at concentrations of 15.6, and 39.2 μ g mL⁻¹ that became more pronounced over time (Figure 1).

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Shellfish that inhabit estuaries can be affected directly by contaminants through adsorption and ingestion or indirectly through the food web (Karlander et al. 1983, Croonenberghs 2002). In addition, shellfish might be indirectly affected when contaminants affect their parasites. Many anthropogenic chemical contaminants are introduced daily into estuarine systems from point and non-point sources. Fertilizers, pesticides and herbicides enter estuaries in runoff from farmlands, golf courses, and residential areas (Knutzen 1987, Howarth et al. 2002). Polycyclic aromatic hydrocarbons (PAHs) are by-products of fossil fuel combustion

that enter estuaries as runoff from roads and via atmospheric deposition (Knutzen 1987). The chemical contaminants used in the present study were selected because previous studies

115 documented their presence in estuarine waters of the southeastern United States (Vernberg et al.

1997) were *P. marinus* commonly infects oysters (Bobo et al. 1996). Four of the contaminants are commonly used on golf courses, farms and residential lawns as fertilizers (ammonium, nitrate and phosphate) or herbicides (Weed-B-Gon®). The active ingredients of Weed-B-Gon® (2,4-D, mecoprop and dicamba) are general broadleaf herbicides common to both industrial (e.g., agriculture, golf courses) and residential (lawn and garden) use (Burnside 1996). Concentrations of 2,4-D, mecoprop and dicamba in estuarine waters were not available, although they are listed among the anthropogenic chemicals found in southeastern US estuaries (Vernberg et al. 1997). The other two compounds (fluoranthene and phenanthrene) are polycyclic aromatic hydrocarbon (PAH) by-products of combustion engines.

- 125 The lack of effect by PAHs is noteworthy due to the effects that these chemicals can have on oysters. For example, PAHs are known to increase hemocyte activity, cause a thinning of the digestive epithelial tissue, disrupt larval development, and prevent embryos from entering the Dshell larval stage (Weinstein 1997, 2002, Oliver et al. 2001, Lyons et al. 2002). Some of these effects may decrease the oyster's ability to fight infection. Because oysters filter large volumes
- 130 of water and are known to concentrate contaminants, even relatively low concentrations of chemical contaminants may have significant effects on their health and vitality (Croonenberghs 2000). Moreover, by concentrating contaminants, parasites within the oysters might be exposed to higher contaminant concentrations than exist in the surrounding waters. *Perkinsus marinus* infection intensities and PAH distribution within estuaries have also been correlated suggesting
- 135 that either both have identical responses to environmental changes or there is a link between the two (Wilson et al. 1990, Wilson et al. 1992). Chu et al. (2002) demonstrated that oysters exposed to field contaminated sediments that contained several PAHs, metals and other organic compounds were more susceptible to *P. marinus* infection and/or intensification of extant

infections. Results reported herein demonstrate that individual exposure to two common PAH

140 compounds, flouranthene and phenanthrene, at levels comparable to those used by Chu et al.(2002) did not affect in vitro proliferation of *P. marinus*.

The effects of increased nutrient loading are a concern in estuaries throughout the world. Nitrogen and phosphorus derived from various point and non-point sources have increased globally over the past 40 years by two to three times (Howarth 2002). The effects of nutrient

145 loading on *P. marinus* had not been previously examined, but results from this study suggest there is no direct effect. These nutrients encourage algal blooms, often leading to eutrophication that may increase abundance and biomass of benthic fauna like oysters (Nixon and Buckley 2002). In contrast, harmful algal blooms, which have also been linked to eutrophication, may produce toxins that kill or decrease the overall health and spawning of oysters (Pelley 1998,

Weed-B-Gon® significantly reduced proliferation of *P. marinus*, but only at concentrations greater than the recommended application rate. The herbicides contained in Weed-B-Gon® target broadleaf plants and mimic plant growth hormones or auxins. Both 2,4-D and mecoprop are phenoxy acid compounds whereas dicamba is a benzoic acid (PAN Pesticide
Database: www.pesticideinfo.org). In small doses they promote growth in broadleaf plants and research on its actions continues to reveal new insights (e.g., Hensel et al. 2002). At higher concentrations over stimulation interferes with fundamental processes eventually killing the plant (Munro et al. 1992). The effect on in vitro proliferation of *P. marinus* was also dose dependant, but stimulation at the lower dosages tested was not observed. Because *P. marinus* is not a plant, the mechanism of action is quite likely to be different.

Several studies have examined the toxic effects of 2,4-D on cells from other organisms. For example, in human hepatoma cells, 2,4-D induced apoptosis by disrupting mitochondrial membrane potential in a dose dependent manner (Tuschl and Schwab 2003). Aria (2002) examined genotoxic effects of 2,4-D on developing chick embryos and found a dose dependent

- 165 response of increasing sister chromatid exchange rates as well as an inhibition of the cell cycle. Mecoprop may have similar effects at high dosages while dicamba appears to be more benign (ECOTOXNET, <u>http://extoxnet.orst.edu</u>). Recently, Lund et al. (2005) found that the antimicrobial compound triclosan inhibited *P. marinus* proliferation but effective concentrations did not harm oyster hemocytes. Triclosan is chemically related to 2,4-D. As an antimicrobial,
- 170 triclosan interferes with fatty acid synthesis, but the concentration needed to affect *P. marinus* proliferation was less than half that needed to affect fatty acid synthesis of *P. marinus*. Working with *P. olseni*, a closely related molluscan pathogen, Elandalloussi et al. (2005) found that some antimalarial compounds designed to target folate synthesis inhibited *P. olseni* proliferation. These studies provide potential mechanisms to explain the dose response observed in the
- herbicide treatment from the present study and warrant further investigation. Determining the mechanism(s) of action of the herbicide 2,4-D on *P. marinus* may identify metabolic pathways to target in the development of chemotherapeutants that may be useful under certain conditions (e.g., protecting valuable brood stock). The ability to target multiple pathways with different chemicals should help minimize the development of *P. marinus* populations resistant to any
 particular treatment.

In the environment, parasites and their hosts are likely to be exposed to a suite of contaminants (e.g., Chu et al. 2002). Because the effect of each contaminant on the host and the parasite may differ and be further complicated by the interaction of various compounds,

interpreting the effect of any single contaminant is specific to the entire suite of contaminants

185 present. In the current study, contaminant exposures were performed *in vitro* and most showed little effect. This is perhaps not surprising as the in vitro culture medium was designed to promote proliferation of *P. marinus*. Free-living stages or parasites within a host oyster are under conditions less likely to favor proliferation and may react differently to the contaminants employed here.

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	Compound	Chemical formula	Concentrations tested			
	Weed-B-Gon [®]		0.781, 7.81, 15.6, 39.2 and 78.1 $\mu L \ m L^{-1}$			
	2,4-D (3.05%)	$C_{10}H_{13}Cl_2NO_3$				
	Mecoprop (10.6%)	C ₁₂ H ₁₈ ClNO ₃				
315	Dicamba (1.3%)	$C_8H_6Cl_2O_3$				
	Ammonium	NH ₄	0.18, 1.8 and 18 μM			
	Nitrate	NO ₃	0.036, 0.36, and 3.6 µM			
	Phosphate	PO ₄	0.0055, 0.055, 0.09 and 0.9 μM			
	Fluoranthene	$C_{20}H_{12}$	0.236, 2.36, and 23.6 μg/L			
320	Phenanthrene	$C_{14}H_{10}$	0.45, 2.3 and 4.5 μg/L			
	Acetone	C ₃ H ₆ O	0.1, 1.0 and 10% v/v			

310 Table 1. Chemicals and concentrations tested.

Table 2. Probability values from two factor ANOVAs of chemical challenge experiments.

Headings represent sources of variation. Significant values are in bold.

	<u>Chemical</u>	Time*Treatment			<u>Fime</u>	<u>Concentration</u>			
	Ammonium		0.999	().000	0.906			
	Nitrate		0.999	().000	0.845	4		
	Phosphate		0.999	().000	1.000	Contraction of the second seco)	
330	Weed-B-Gon® (trial 1)		0.406	().003	0.006	6	<i>v</i>	
	Weed-B-Gon® (trial 2)		0.353	().000	0.009			
	Fluoranthene		0.995	().000	0.852	<i>v</i>		
	Fluoranthene UV-activat	ted	0.552	().000	0.128			
	Phenanthrene		1.000).064	0.978			
335	Phenanthrene UV-activa	ted	0.943	().000	0.409			
	Acetone	4	0.814	().000	0.829			

Figure 1. Effect of Weed-B-Gon® herbicide on in vitro proliferation of *P. marinus*. Data are means with standard errors of the three replicate parasite strains.

