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Antibody Production in Spot (*Leiostomus xanthurus* Lacepede): A Model to Test the Impact of Elizabeth River Sediments on the Humoral Immune System of Fish

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ANTIBODY PRODUCTION IN SPOT (Leiostomus xanthurus Lacepede): A MODEL
TO TEST THE IMPACT OF ELIZABETH RIVER SEDIMENTS ON THE HUMORAL IMMUNE
SYSTEM OF FISH.

A thesis

presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
of the Requirement for the Degree of
Master of Arts

by

Catheriné N. Pourreau

1984

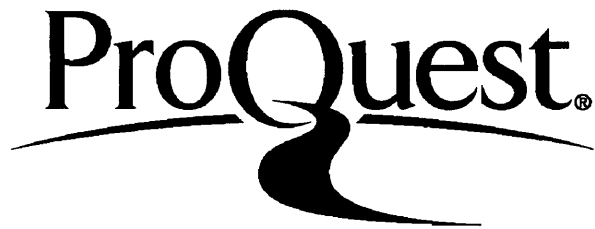
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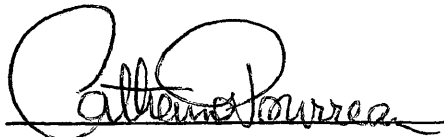
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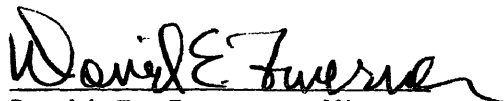
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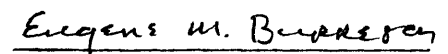

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A mio caro amico Maurizio Castelli.

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ABSTRACT

A single intraperitoneal injection of sheep red blood cells in spot, Leiostomus xanthurus Lacepede, elicited significant serum antibody production within 25 days at a temperature range of 25-27 C and 17ppt salinity. Serum antibodies were first detected 10 days post-injection using the microtiter agglutination test. The rate of antibody production was dose-dependent. Similar maximum titers were obtained after 25 days with all antigen concentrations injected. Natural hemagglutinins were very low. Spot did not appear adversely affected by captivity, or experimental manipulation.

This humoral immune test model was used to evaluate the impact of polyaromatic hydrocarbon contaminated sediments from the Elizabeth River on the competence of spot to produce antibodies. Fish exposed continuously to contaminated sediments showed significantly higher antibody levels 25 days post-injection than those exposed to reference sediments. Overall antibody levels were low. Pollutant-exposed fish also showed altered feeding behavior, hyperactivity, emaciation, and gross damage to gills, fins, skin, and liver. Fish exposed to reference sediments exhibited few pathological signs.

ANTIBODY PRODUCTION IN SPOT (LEIOSTOMUS XANTHURUS LACEPEDE): A MODEL
TO TEST THE IMPACT OF ELIZABETH RIVER SEDIMENTS ON THE HUMORAL IMMUNE
SYSTEM OF FISH.

INTRODUCTION

Water quality in the Elizabeth River

The Elizabeth River, a southern tributary of the James River, opens into Hampton Roads in the lower Chesapeake Bay. It consists of a main stem starting at Sewell's point and Craney Island and divides to the south into four subtributary arms: the Lafayette river and the Eastern, Western, and Southern Branches (Figure 1). This river system is a major water quality problem in the lower Chesapeake Bay (Gilinski and Roland, 1983a). Since the founding of Norfolk in the late 1900's, it has been the site of increasing waterborn transportation, docking facilities, naval, municipal, and commercial wastewater discharges, and urban stormwater runoff (Neilson and Sturm, 1978). In 1980, 13,166 av.lb./day of biological oxygen demand, and 18,050 av.lb./day of organic pollutants were discharged into the river (Commonwealth of Virginia, State Water Control Board, 1981). Currently 48 industrial and 15 domestic point sources along the river, as well as numerous non-point sources of pollution exist within the 300 square miles of drainage area (Gilinski and Roland, 1983a).

The Elizabeth River has poor flushing characteristics, due to slight topographic relief, weak salinity gradient, and limited gravitational circulation (Neilson and Sturm, 1978), causing increased residence time of pollutants and sediments. The limited source of

freshwater is the Great Dismal Swamp drainage, regulated by the Corps of Engineers, and stormwater runoff. (Neilson and Sturm, 1978). Whereas any noxious materials discharged near the mouth of the river are removed rapidly from the system by tidal exchange, pollutants released further upriver (eg. the Southern Branch) may take several days to reach the mouth (Neilson and Sturm, 1978). Construction of the Craney Island dredge spoil disposal area near the mouth has further reduced flushing and trapped pollutants within the system.

Poor water quality, particularly in the Southern Branch is attributed to low dissolved oxygen (D.O.), particularly during the summer months, high total phosphate and nitrogen values, high temperatures from cooling water discharges, high heavy metal concentrations (eg. arsenic, cadmium, chromium, copper, lead, mercury, nickel, and zinc) in the water column and in sediments, and very high concentrations of aromatic hydrocarbons (10 to 200 ppm) in sediments (Cronin et al., 1977; Bieri et al., 1982; Lu, 1982; Gilinski and Roland, 1983). As a reference, the York River sediments contain aromatic hydrocarbon concentrations of 7 to 10 ppm.

Impact of pollution in the Elizabeth River on biological communities

Studies of benthic populations show that they are dominated by pollution tolerant organisms such as polychaetes, and oligochaetes (Boesch, 1971). Schaffner and Diaz (1982) found low diversity in benthic populations indicative of pollution stress. American oyster and hard shell clam, commercially important invertebrates, are present in very low numbers in the Elizabeth River (Hampton Roads Energy

Company, 1977). Water quality degradation particularly from wastewater discharges has made portions of the river unsuitable for commercial shellfish purposes since 1914 (Smith, 1950) and in 1982, the river was completely closed for harvesting of shellfish (Commonwealth of Virginia, State Water Control Board, 1981). High levels of zinc, cadmium, and copper are found in oyster tissues (Gilinski and Roland, 1983b).

Little data exist on finfish species present, their abundance, and migration patterns in the Elizabeth River. The Hampton Roads Energy Commission report for 1977 states that the most common fish species in the river are spot (Leiostomus xanthurus), Atlantic menhaden (Brevoortia tyrannus), bay anchovy (Anchoa mitchilli), Atlantic silverside (Menidia menidia), and hogchoker (Trinectes maculatus) with seasonal variations in their abundance. Fish populations in the Elizabeth River are below commercial fisheries levels (Department of the Army, 1974).

Studies conducted at the Virginia Institute of Marine Science show that fishes from the Southern Branch of the Elizabeth River exhibit chronic, idiopathic signs of disease (eg. gill damage, pancreatic necrosis, and impaired cellular immunity as characterized by reduced phagocytic activity of macrophages in comparison to fishes from the more pristine Ware River (Weeks and Warriner, 1984; Hargis et al., in press). Laboratory experiments show that exposure to water associated with PAH-contaminated Elizabeth River sediments (3.9 million ppb of low and high molecular weight PAHs) kills spot in 8 to 28 days. Chronic toxicity tests show that spot exposed to Elizabeth River sediments develop skin lesions, fin erosion, ocular opacity, and

gill and liver abnormalities within three weeks. They also become anemic and stop feeding (Hargis et al., in press).

Spot

Spot, Leiostomus xanthurus Lacepede, a member of the Family Sciaenidae, is a marine migrant, mesopolyhaline, bottom feeding fish important to commercial and sport Fisheries in the Chesapeake Bay. Spot have broad temperature and salinity ranges which permit wide distribution in the Bay (Pacheco, 1957; Parker, 1971). They are abundant in "clean" and heavily polluted waters including the Elizabeth River. Their feeding habits and life history within the Bay make them particularly susceptible to chronic exposure to sediment-associated pollution. Spot feed non-selectively on epi- and infauna by agitating the sediment surface and sorting out benthic phytoplankton, zooplankton, microcrustaceans, and burrowing invertebrates from bottom particles using its gill rakers. They are often found in calm, shallow waters which provide a greater food supply. Adults, up to four years old, migrate out of the Bay when water temperatures decrease below 10^o C, spawn offshore, and return with the post-larvae in May-June, where they remain until the next fall. The young of the year overwinter and remain in the channels for about one year.

Testing biological effects of pollution on fish

Interpreting the results of field studies on the impact of pollution is difficult because of the dynamic interaction of

environmental and biological variables. Establishing direct cause and effect relationships is particularly problematic in aquatic toxicology where signs of poor health in organisms may be due to one or many physical, chemical, or biological factors. Tissue residue concentrations are relative to contaminant exposure.

Laboratory studies allow for better control of environmental factors so that the effects of pollution on organisms can be estimated with greater certainty than that obtained from field studies. However, responses obtained in laboratory tests can differ significantly from field results owing to differences in bioavailability of pollutants, feeding habits, avoidance reactions, or presence of other stressors. Previous studies show that chemical pollutants released in an aquatic environment may affect fishes by:

1. A direct toxic effect on cellular, neural, biochemical, or physiological functions of fishes (Gardner, 1975; Sastry and Miller, 1981; Meyers and Hendricks, 1982) with possible development of external or internal lesions particularly with repeated contact with the chemical.
2. A depletion of food supplies with a potential increase in inter- and intra-species competition.
3. An indirect toxic effect from ingestion of contaminated food.
4. An indirect effect caused by lowering animals' resistance to disease. In particular, opportunistic pathogens normally present in the water may become a threat to stressed individuals.

Using the fish humoral immune system to monitor pollution impact

I decided to use the immune system of fish to monitor pollution impact because:

1. Immunological tests give both qualitative and quantitative indications of the health status of fishes.
2. The immune system of fishes is, as in higher vertebrates, a very sensitive system that responds quickly to poor environmental conditions (natural stress or man-made pollution).
3. A deleterious impact on the immune system may have major ecological consequences at the organismic, species, and even population levels.

Fish immune processes include: non-specific cellular responses (phagocytosis by leucocytes or macrophages, lytic activity, granuloma or fibrosis formation), acquired cell-mediated immunity (delayed hypersensitivity), humoral non-specific immunity (natural hemolysin, hemagglutination, iso- and hetero-agglutination, lysozyme, complement, transferin, and interferon activity), and specific humoral antibody activity (agglutination via the immunoglobulin IgM, analogous to that in mammals). (Anderson, 1974; Corbell, 1974; Heartwell, 1975; Rijkers, 1982).

Although humoral immune mechanisms are generally similar in all fishes studied, different phylogenetic groups vary appreciably in their response to different antigens. Higher teleosts produce

antibodies to a wider variety of antigens and at different rates and intensities than fishes in the lower phylogenetic groups (Rowlands, 1969). Also, each species of fish has its own inherent responsiveness to antigens, kinetics of antibody synthesis, memory formation, and clearance time of antigens from circulation. These criteria are themselves dependent on temperature (Paterson and Fryer, 1974a; Rijkers et al., 1980a, 1980b, 1981), antigen type (soluble or particulate, combined or not with a carrier, live or inactivated), nature (whole cells, fragments of cells, or extracellular products), dose (Paterson and Fryer, 1974a; Rijkers et al., 1980a), and route of administration (parenteral, oral, or bath).

Fishes exhibit a humoral immune response characterized by a latent period, after the initial introduction of the antigen, when antigen uptake by macrophages, mobilization and stimulation of antibody-producing cells are thought to occur (Hodgins et al., 1967; Anderson, 1974, Ingram, 1980; Anderson et al. 1983a). Specific antibodies then appear in the serum after twenty days or more depending on the fish species, individual physiological state of the animal, stressors, and other environmental conditions such as temperature. Antibody levels increase and remain at a constant plateau while the antigen is present in the tissues or blood of the fish, and decrease gradually to a stable baseline level until the system is challenged again by the same antigen (Anderson, 1974; Lamers and Polarczyk, 1982). After the initial immune response to a "new" antigen, referred to as the primary response, subsequent exposure to the same antigen elicits an enhanced and more rapid response referred to as secondary, anamnestic, or memory response. In vitro tests show that once synthesized, antibodies

combine specifically with antigens forming antigen-antibody complexes that facilitate phagocytic attack, and lead to lysis of foreign cells or pathogens, and inactivation of their toxic extracellular products (Anderson, 1974). Hemolytic plaque assays show that once the plaque forming cells (PFCs) are activated, antibody production will continue even if the environmental conditions later become unfavorable (Anderson, pers. commun.).

The hemopoietic organs involved in the immune system of fish include: the anterior kidney with many blast cells undergoing differentiation, the spleen, believed to be the main processing and storing organ, and the thymus involved in lymphocyte stimulation and antibody production (Chiller et al., 1969; Anderson, 1974). It is reasonable to expect that damage to any of these organs or excessive blood loss would seriously impair a fish's immune response to invasion by pathogens and lead to disease or mortality. Such pathological changes have been reported due to chemical pollutants in the water (Meyers and Hendricks, 1982). This has not been specifically examined in fishes from the Elizabeth River.

Certain chemicals and/or physical conditions are known to activate or inhibit the immune system. These include: Freund's adjuvant, irradiation (Strand, 1975; Zeeman and Brindley, 1981), antibiotics such as tetracycline (Rijkers et al., 1981; Grondel and Boeston, 1982), and chloramphenicol (Zeeman and Brindley, 1981), corticosteroids, adrenocortical hormones, and alkylating agents such as cyclophosphamides (Bisset, 1948; Sneidermann and Wilson, 1975; Anderson et al., 1982, 1983b), introduction of antibody into the fish prior to the antigen challenge (Anderson, 1974), low temperatures

(Bisset, 1948; Avtalion, 1969; Cushing, 1970; Anderson, 1974; Paterson and Fryer, 1974; Rijkers et al., 1981; Sypek and Burreson, 1983), antigen tolerance in young fish and thymectomy in adults (Zeeman and Brinsley, 1981), captivity for more than four weeks (Miller and Tripp, 1981), or toxicants in the water such as phenol (Goncharov and Mikryakov, 1970; Anderson et al., 1983a), copper (Hetrick et al., 1979), zinc (McLeay, 1975; Sarot and Perlmutter, 1976), and cadmium (Robohm and Nitkowski, 1974). Another application of immunology in aquatic toxicology is to detect differences in antibody levels against specific bacteria in marine fish from "clean" versus polluted coastal waters, particularly high antibody levels against human pathogens (Jansen and Meyers, 1968; Stolen et al., 1981).

Study objectives

The objectives of this study were twofold. The first experiment (phase 1) was designed to test if antibody production could be induced in spot using sheep red blood cells (sRBCs) as antigen, and if so, to select an optimal injection procedure for antibody synthesis. The protocol developed in the Phase 1 experiment would then allow me to determine in a second experiment (Phase 2), the impact of environmental stressors such as pollutants in Elizabeth River sediments on the humoral immune competence of spot under laboratory conditions.

MATERIALS AND METHODS

Fish

Spot were selected for this study because they are commercially important to Chesapeake Bay Fisheries, are abundant in the lower Bay including the Elizabeth River from May until the end of September, are suspected to be exposed chronically to pollution in the Elizabeth River, and are physiologically affected by these environmental conditions (Bender et al., 1977; Bellanca and Bailey, 1977; Hargis et al., in press; Stehlik, 1982). As experimental animals, they tolerate transportation, can be maintained in confinement for several months, will feed on prepared commercial diets, and are resistant to stressors often encountered in the laboratory (eg. high organic load, handling, crowding, etc...).

Antigen

Criteria for selection of the antigen are that it should be readily available, easily stored, and be known to induce antibody production when introduced in fish under laboratory conditions. Also the baseline serum antibody titers against the antigen (due to previous exposure, natural antibody levels, and/or cross-reactivity with antigens present in the field) should be very low or non-existent. Alsever's sheep red blood cells (sRBCs) were a good

candidate antigen since they have been proven to elicit a significant primary immune response two to three weeks after intramuscular (i.m.) or intraperitoneal (i.p.) injections in rainbow trout (Salmo gairdneri) (Kitao et al., 1981), carp (Cyprinus carpio) (Kitao et al., 1981; Rijkers et al., 1981), yellowtail (Seriola quinqueradiata) (Kitao et al., 1981), and goldfish (Carassius carassius) (Azzolina et al., 1981).

PHASE 1

Experimental plan

One year old spot, 140 to 200mm in fork length, returning from sea in May, were collected by 16 foot balloon trawl in the lower York River, Chesapeake Bay, 10 to 20 kilometers from the mouth, then immediately brought back to the laboratory and placed in 400 liter flow-through aquaria. A randomly selected group of 60 fish were examined for baseline information. The sera from these fish were obtained and stored frozen for future titration to determine natural antibody levels against sRBCs using the microtiter agglutination test (Stavitsky, 1974). The health status of these fish was determined immediately by macroscopic and microscopic examination of the skin, eyes, fins, gills, hemopoietic organs, and gut, for lesions or extremely heavy parasite burdens. Samples of kidney tissue (organ routinely used for diagnosis of bacterial infections in fishes) were removed aseptically, streaked on TSA plates, and checked after 72 hours to detect subclinical bacterial infections.

Two hundred experiment fish (10 fish x 3 antigen concentrations and 1 control x 5 time intervals) were acclimated to the laboratory over a period of 48 hours and inspected grossly for signs of poor health (ie. lesions, failure to feed, abnormal respiration rate, or erratic swimming behavior, etc...). Unhealthy fish were not used for the experiments.

SRBC-antigen preparation

Sheep red blood cells (Gibco whole blood in equal parts of Alsever's solution) were washed three times by centrifugation (300g for 15 minutes at 4⁰C) in 0.85% sterile phosphate buffered saline (PBS) at pH 7.2 immediately before use and resuspended in PBS at the desired cell concentrations for the microtiter assay (1.0%) and for the injections (20%, 35%, and 50%).

Injection protocol for antibody production

Experimental fish (50 randomly selected fish per group) were anesthetized with 300 mg of MS-222/1 (Tricaine methanesulfonate) and injected i.p. with a 0.15 ml solution of 20%, 35%, or 50% sRBC in sterile PBS. Controls were injected with 0.15 ml of PBS. Twenty five and thirty five percent sRBC-injected fish were kept in separate 400 liter flow-through tanks, fifty percent sRBC-injected (fin-clipped) and control fish were kept together in a 750 liter flow-through tank. The water flow in the tanks was 14 l/min. During the experiments the fish were fed Zeigler Brothers Trout Chow (No. 3 starter pellets

containing no antibiotics) daily except for the 24 hours prior to injection or bleeding to avoid excess fat in the sera. Water temperature, salinity, D.O., pH, and any mortalities in each tank were recorded daily.

Blood from ten fish in each treatment group was collected every five days and the sera were frozen for future serum antibody tests. This time schedule for monitoring antibody production was based on the works of Rijkers et al. (1980b, 1981) in carp, and Kitao et al. (1981) in yellowtail and trout using sRBCs as antigen. After collecting the blood each fish was immediately measured, weighed, sexed, and a determination of its general health made.

Blood collection and processing for serum antibody tests

Fish were anesthetized. Blood was drawn from the caudal vessel with a 3cc sterile, non-heparinized syringe and 25g 7/8 in. needle. The blood was allowed to clot for two hours at room temperature, and then overnight at 4°C. The serum was then separated from the clot, and frozen without antibiotics in 1.0 ml polystyrene tubes for future testing.

Preliminary microtiter agglutination test

Optimum conditions for antibody-antigen agglutination in the microtiter assay were determined by titrating fish sera collected 15 days post-injection against various concentrations of sRBCs in PBS (1%, 2%, 5%, 10%). Sera from injected fish tested against PBS alone, and

uninjected-fish-sera tested against sRBCs served as controls. Experimental sera were subsequently tested for antibody-antigen agglutination using this optimal cell concentration for the assays.

Sample processing

Serum samples were kept frozen until tested and processed within five days after bleeding to avoid bacterial contamination and/or denaturing of the sera. Serum antibody levels were determined using the microtiter agglutination test (Stavitsky, 1974). This assay was chosen because it is simple, sensitive, inexpensive, and gives a rapid, reproduceable, quantitative indication of the presence of antibodies in a serum. Two-fold dilutions (0.05 ml) of sera from injected fish were made from 1:2 to 1:4096 with PBS (12 wells). An equal volume (0.05 ml) of the 1% washed sRBC-PBS suspension was added to each well. Microtiter plates were covered, allowed to incubate overnight at room temperature, and read after 18 and 24 hours. Maximum well number showing positive agglutination was recorded for each test serum. Maximum well numbers were used in statistical tests and graphs. Antibody titers represented the reciprocal of the end point dilution showing positive agglutination.

Data analysis

Fork lengths and weights of the experimental fish were recorded and used to calculate an average condition factor ($K = \frac{W}{L^3} \times 10$) (Lagler,

1952) for each sRBC concentration and time interval. Mean sizes and condition factors were tested for significant differences at the 95% confidence level using Student's t test. Mean, standard deviation, and variance were determined for groups of ten fish in the serological tests. Group values were then tested for significant differences at the 95% confidence level using the least significant difference of means test (Li, 1964).

PHASE 2

Sediments

Contaminated sediments were collected with a Smith-McIntyre grab from the Southern Branch of the Elizabeth River near a defunct creosote plant (Figure 1). Sediments at this site have been determined to contain high concentrations of PAHs and have been used as a source of contaminated sediments for several toxicological experiments. Reference (or control) sediments were collected from the lower York River (Figure 1), which is known to be relatively pollution free. Experimental sediments consisted of 10%-contaminated Elizabeth River sediments mixed with 90% York River sediments (volume to volume) to produce a controlled chronic exposure for spot (Roberts, unpublished data). Reference sediments consisted of 100% York River sediments. Experimental and control tanks were similar flow-through systems (flow 9 l/min). Sediments in both tanks were 3cm deep and were covered with a plastic mesh (pore size 6mm^2) that permitted free exchange of sediment-associated chemicals with the water above. The mesh sediment

cover reduced resuspension of particles caused by natural turbulence and foraging activities of the animals, and most probably reduced the B.O.D. in the water and prevented anoxic conditions. Several sediment cores were taken from each tank at the beginning and at the end of the experiment and frozen. The cores were analyzed for selected polynuclear aromatic hydrocarbons (PAHs). Samples were freeze-dried and extracted with methylene chloride. The extracts were fractionated with gel permeation chromatography and high-performance liquid chromatography, then analyzed by glass capillary gas chromatography using a FID detector.

Use of the humoral immune test model in assessing pollution impact in spot

Spot were collected from the York River, acclimated to the laboratory for seven days, and checked for obvious poor health as in Phase 1. Twenty five fish were culled out to serve as baseline samples. Sera from these fish were tested to confirm low natural antibody levels against sRBCs as with the baseline fish from Phase 1. The baseline fish were necropsied and checked macroscopically and microscopically for disease as in Phase 1.

Two hundred experimental fish were randomly divided into two groups. Half were exposed to contaminated sediments, and the others to reference sediments. Only healthy fish were used for the experiment. Sample size for each fish group (25 fish/group) was considered adequate to account for the variability in serum titers as seen in phase 1. The experiment was set up in the following manner:

- Fish kept over reference sediments for seven days, injected sRBCs, maintained over reference sediments for 25 days, and bled to collect the sera.

- Control fish (fin-clipped) kept over reference sediments for seven days, injected PBS only, maintained over reference sediments for 25 days with sRBC-injected fish, and bled to collect the sera.

- Fish kept over contaminated sediments for seven days, injected sRBCs, maintained over contaminated sediments for 25 days, and bled to collect the sera.

- Control fish (fin-clipped) kept over contaminated sediments for seven days, injected PBS only (controls), maintained over contaminated sediments with sRBC-injected fish, and bled to collect the sera.

The antigen dose, and time post-injection for antibody production as determined in Phase 1 was used. The microtiter test described in Phase 1 was used here also to determine antibody levels for each group tested. Statistics and data analysis were as in phase 1.

RESULTS

PHASE 1.

SEROLOGY

Preliminary microtiter agglutination test

Agglutination in microtiter wells was readily visible using 1% sRBCs in PBS with sera from sRBC-injected fish for the assays. Higher concentrations of sRBCs gave less obvious results. No agglutination was seen in control treatments. Therefore, a one percent solution of sRBC was used for subsequent microtiter tests.

Natural hemagglutinins

Baseline fish sera indicated that spot have negligible natural antibody levels against sRBCs (mean titer 2.36 ± 2.86).

Kinetics of antibody production against sRBCs in spot

Microtiter tests revealed an increase in antibody levels, significant at the 95% confidence level, that was dose and time dependent (Figure 2). The rise in antibody levels started on day 10

post-injection but significant differences between the controls (PBS-injected) and the test groups were not apparent until day 15. At that time 20, 35, and 50% sRBC-injected fish showed similar mean antibody titers of 2.60, 2.66, and 3.00. By day 20 post-injection differences between 20, 35, and 50% sRBC-injected fish were most obvious with antibody titers being proportional to antigen concentration injected. Antibody level continued to increase throughout the experiment in all SRBC-injected fish. A slight increase in mean titer was also noted in PBS-injected fish. By day 25 post-injection mean maximum antibody levels of 41.28, 42.56, and 32.00 were recorded for 20, 35, and 50% sRBC injected fish. Although these titers were not significantly different at the 95% confidence level, the 50% sRBC-injected fish showed slightly lower maximum titers than 20 or 35% sRBC-injected fish. The experiment was ended after 25 days when all fish had been sampled. See Appendix 1 for serological raw data for this portion.

Based on data above, 35% sRBCs in PBS was selected as the concentration of immunogen to inject into spot and 25 days post-injection was chosen for maximum antibody production in Phase 2.

SPOT AS A TEST ANIMAL

Capture, transport, and acclimation of 300 fish to laboratory conditions was accomplished with a low mortality rate (less than 3%). Fish appeared to recover from handling within 12-15 hours with no morphological or behavioral signs of trauma.

Baseline fish

A random subsample of sixty fish was used to estimate average size (fork length= 163.39mm, weight= 51.8g), condition factor (K=1.19) and general health (Table 2) of the population of fish collected. All fish were immature. The absence of gross external or internal signs of pathology (Table 3), or of bacterial infection, indicated that the fish were healthy. They had little or no food in the gut. Parasite load in 97% of the fish was moderate (48.7% had Trichodina on the gills, 21.7% had digenean trematodes and 5.4% had nematodes in the gut) (Table 4).

Test fish

The experimental fish seemed to recover immediately from the injections and started feeding within 24-48 hours. Total mortalities from the injections and during the rest of the experiment was 2.0%. The fish were all immature. Sex ratio was 47.55% females and 52.45% males. Size, condition factor, and general health are presented in Tables 1a and b, 2, and 3. Only lengths were recorded for the fish in the first sample so the condition factors were not calculated for day 5.

Mesurements of size (fork length and weight) showed no significant differences between the groups or with time at the 95% confidence level.

Differences in condition factors between the various concentration groups were not significant at the 95% confidence level throughout the experiment. Mean condition factors of the experimental fish were generally high as compared to baseline fish ($K=1.19$). On day 20 post-injection all treatment groups showed lower mean condition factors than at any other sampling time. The mean K value on day 25 was 1.34.

Necropsies (Table 3) revealed no external or internal signs of pathology in fish until day 10 post-injection. Afterwards all sRBC-injected fish exhibited pale gills and pale livers. Slight emaciation was noted in 2/10 PBS-injected and 3/10 50%-injected fish 20 days post-injection. On the last sampling day only 50%-injected fish showed pale gills and slight emaciation. Parasite loads and percent of the fish affected fluctuated in all groups throughout the experiment. Ectoparasite infestations were more common in experimental fish than in baseline fish except in those sampled on day 15. Nematode infestations greatly increased in experimental fish. Trematodes were common in all groups and comparable in prevalence to baseline fish.

WATER QUALITY IN THE TANKS

Water quality data are presented in figures 3,4,5, and 6. Dissolved oxygen, salinity, pH, and temperature were similar in all tanks. Mean values recorded for the first three parameters were 7.3 mg/l, 15.9 ppt, and 7.22 respectively. Temperature increased throughout the experiment from 19°C in May to 27°C in late June (Figure 6). Dissolved oxygen and pH values rose from an initial low to

a stable maximum within five days. There was a slight drop in these two parameters at day 22 which coincided with a loss in water pressure. The oxygen supplied to the tanks was adjusted periodically to maintain a D.O. level of about 7.0 mg/l in the tanks.

PHASE 2.

SEROLOGY

Natural hemagglutinins

Serological tests confirmed very low natural antibody levels in spot against sRBCs (mean titer 2.42 \pm 2.94).

Effects of Elizabeth River sediments on the humoral immune response of spot against sRBCs

The results show low overall antibody levels (mean titers of less than 8) after 25 days (Figure 7). SRBC-injected fish in the contaminated tank gave significantly higher anti-sRBC antibodies (mean titer 14.52) than the ones in the reference tank (mean titer 2.40). Antibody titers of control fish in both tanks (mean titer 2.50) were not significantly different from each other at the 95% confidence level, but were significantly lower than titers obtained in sRBC-injected fish from both tanks. Serum antibody levels between male and female fish in reference (mean titer 5.50 \pm 3.42 for females and 4.60 \pm 4.32 for males) and contaminated tanks (mean titer 14.68 \pm 3.78 for

females and 14.20 ± 4.42 for males) were not significantly different at the 95% confidence level. See Appendix 2 for serological raw data.

SPOT AS A TEST ANIMAL

Baseline fish

I intended to examine 60 fish for baseline tests for Phase 2 but severe losses most probably due to capture and acclimation stresses (16% mortality) forced me to reduce the group to 25. All fish were immature. Forty percent were females and 60% males. Their mean length was 165.7mm, weight was 56.8g, and condition factor was calculated to be 1.25. The fish had no food in the gut but were not emaciated. Ten percent of them had pale livers. No gross signs of pathology were noted except for some fish with focal scale loss, presumably due to capture. The fish used for the experiment were therefore checked carefully for absence of scale loss.

Test fish

Mortalities from handling stress and/or trauma from injections was 0% in PBS-injected and 8.7% in sRBC-injected fish in the reference tank, and 32% in PBS-injected and 4% in sRBC-injected fish in the contaminated tank.

Fish began to eat prepared food after 5 days in the laboratory. When transferred to the tanks with sediments, fish in the reference tank started to feed actively three days post-injection, while those

in the contaminated tank did not actively feed until 20 days post-injection, but fed voraciously (twice the initial ration or more) thereafter. Fish in the contaminated tank were also hyperactive for about a week.

The experimental fish were all immature, 43.82% were females and 56.18% males. Their mean size, condition factor, and general health are presented in Tables 4,5, and 6. Although there was no significant difference in size (at the 95% confidence level) between controls and sRBC-injected fish from the two tanks, control fish from the reference tank seemed to decrease in weight and length during the experiment whereas 35% sRBC-injected fish in the same tank seemed to increase in length and particularly weight. This difference between test groups was less marked in the contaminated tank where both controls and sRBC-injected fish gained in length and weight.

Although mean condition factors of fish sampled on day 0 and day 25 post-injection showed no significant differences at the 95% confidence level, control fish in the reference and contaminated tanks seemed to have higher mean condition factors than sRBC-injected fish in these tanks. Also, mean condition factors of fish in the contaminated tank seemed to be generally higher than those from fish in the reference tank.

Necropsies of the fish revealed differences between fish from reference and contaminated tanks (Table 6). Fish from the contaminated tank exhibited the following gross signs of pathology: pale, congested gills, fin hyperemia, skin discoloration with loss of scales, and blisters, emaciation, hepatomegaly, and loss of liver firmness. These signs were rarely found in fish from the reference tank. Fish from the

contaminated tank often had full gallbladders. This was also noted in control fish and a few sRBC-injected fish from the reference tank. Ocular opacity was seen in some control fish from the reference tank and sRBC-injected fish from the contaminated tank.

Ectoparasites (Trichodina sp.) were significantly reduced in both tanks as compared to baseline fish (Table 6.). Antigen-injected fish, particularly those in the contaminated tank, showed a higher prevalence of Trichodina infestations than did control fish. Prevalence of nematode infestations were generally comparable to fish from treatment and baseline groups except that no nematodes were found in control fish from the contaminated tank. Acanthocephalans and trematodes were more frequently found in baseline than in experimental fish (less than 4% prevalence).

WATER QUALITY IN THE TANKS

Water quality parameters, D.O., salinity, and pH are represented in Figures 8,9,10, and 11. Means recorded were 5.65mg/l, 18.57ppt, and 7.59 respectively. Salinity, and pH were similar in both tanks throughout the experiment. Dissolved oxygen levels were maintained sometimes with difficulty, particularly in the contaminated tank, at 5.0mg/l or above. Oxygen levels in both tanks decreased gradually from 6.0-6.5mg/l initially to about 5.0mg/l at the end of the experiment. A drop in dissolved oxygen on day 27 was recorded, coincidentally with a dinoflagellate bloom maximum in the river. Salinity levels increased with time from 16.3 ppt to 21.0 ppt. The pH levels were fairly stable with a slight increase with time, except for a drop at day 31.

Temperatures were high throughout the experiment and ranged from 25.6 to 28.6°C with minor daily fluctuations (Figure 11).

CHEMICAL ANALYSIS OF THE EXPERIMENTAL SEDIMENTS

Cores of control and experimental sediments showed total PAH concentrations of 7×10^3 ppb for the initial sample and 1×10^4 ppb for the final sample in the reference tank, and 6×10^5 ppb for the initial sample and 7×10^5 ppb for the final sample in the contaminated tank. The difference in PAH concentrations between the two tanks was greater than one hundred-fold. Higher concentrations of PAHs in the final samples, as compared to the initial values were noted.

Among the twenty PAHs selectively analyzed for in the sediments (Table 7), the most concentrated were fluorene, phenanthrene, fluoranthene, and pyrene (concentrations above 3×10^4 ppb) in the contaminated tank. Presence of low molecular weight hydrocarbons could not be determined because they were generally lost during processing (Huggett, pers. commun.).

DISCUSSION

PHASE I

Serology

Spot produced significant serum antibody titers against a single i.p. injection of sRBCs in PBS at 20-25⁰C ambient river water temperature. The kinetics of antibody production in spot were similar to that reported in other fish challenged with sheep, horse, or human red blood cells, bovine serum albumin, bacterial, or viral antigens (McGlamery et al., 1971; Paterson and Fryer, 1974b; Robohm and Nitkowski, 1974; Khalifa and Post, 1976; Serero and Avtalion, 1978; Anderson et al., 1979a, 1982; Stolen et al., 1981).

Antibody levels rose throughout the experiment. It is possible that they may have continued to increase for several days before reaching a maximum.

Higher concentrations of antigen have been shown to give more rapid and more intense responses than lower concentrations (Rijkers et al., 1980a). In this study, the rate of antibody production was dose-dependent, but not the intensity of the response since similar mean titers were obtained after 25 days. The dose-dependent effect was particularly evident 20 days post-injection and less so after 5, 10, and 25 days post-injection. Therefore, unless antibody production was monitored throughout the experiment, differences in antibody levels at

various antigen concentrations injected may not have been detected. Since maximum titers reached by all test groups were similar after 25 days post-injection, that length of time was subsequently used in Phase 2.

Antibody levels against sRBCs obtained 25 days post-injection in spot were comparable to those reported by Robohm and Nitkowski (1974) in cunner, Tautogolabrus adspersus, (maximum titer 32 after 14 days at 23⁰C) and were higher than those reported by Stolen et al. (1981) in three species of flatfish (maximum titer 8-16 after 21 to 28 days at 17⁰C). Higher antibody levels might have been obtained in flatfish had they been challenged at a temperature closer to their normal maximum.

The slightly lower mean titer obtained in 50% sRBC-injected fish after 25 days may have reflected a physiological stress from the high concentration of antigen injected or most probably represented a leveling off of antibody levels often seen after maximum titers are reached (Anderson et al., 1979a). This would have indicated that maximum antibody levels were reached within 25 days in 50% SRBC-injected fish and probably also within a couple of days in 20 and 35% sRBC-injected fish.

In fishes studied, bovine serum albumin (BSA), and chicken and sheep red blood cells are considered fair to poor antigens as compared to bacterial antigens: maximum titers of 256 were obtained in rainbow trout, Salmo gairdneri, at 16.5⁰C against Aeromonas liquefaciens in 21 days (Khalifa and Post, 1976), and Yersinia ruckeri O-antigen at 11⁰C in 14 days (Anderson et al., 1979a, 1982), and in salmon, Oncorhynchus kisutch, at 12⁰C against Aeromonas salmonicida in 4 weeks (Paterson and Fryer, 1974b). Vesicular stomatitis virus (VSV) induces antibody

titers of 16 in catfish, Ictalurus punctatus, in 4 weeks (McGlamery et al., 1971). Bovine serum albumin gives a good response in carp with maximum titers of 128 at 25⁰C (Serero and Avtalion, 1978) but elicits little or no antibody production in catfish (McGlamery et al., 1971).

Bacterial or viral antigens could not, however, safely be used to study antibody synthesis in feral marine or estuarine fishes because of possible previous exposure in nature or cross reactivity with similar antigenic determinants. Among the "non-natural" antigens mentioned in the literature horse and human red blood cells may be better antigens than sRBCs since they elicit higher antibody responses in flatfish (Stolen et al., 1981). This would have to be confirmed in other fish species including spot.

Low levels of natural hemagglutinins (mean titer 4) were noted in baseline (uninjected) and control (injected PBS) fish. Natural hemagglutinins against various antigens are also found in hagfish (Linthicum and Hildemann, 1970), elasmobranchs (Voss et al., 1971) and other teleosts (Boyden, 1966; Sindermann and Krantz, 1968; Evelyn, 1971; Robohm and Nitkowski, 1974; Ingram and Alexander, 1977). Boyden (1966) states that these probably arise against bacteria and react with red blood cells due to the presence of shared antigenic determinants. Reports show that natural hemagglutinins are low in cunner (titer 64) as reported by Robohm and Nitkowski (1974) and non-existent in trout (Ingram and Alexander, 1977). Ingram and Alexander (1977) also reports a 59% prevalence of natural hemolysins in brown trout against sheep, horse, and human red blood cells. The amount of natural bacterial agglutinins is known to vary with season and temperature in salmonids (Fujihara and Hungate, 1972) and

elasmobranchs (Sindermann and Honey, 1964). It can be expected that natural hemagglutinins in spot may also vary with these parameters.

Spot as a test animal

Spot were easy to capture in sufficient numbers at the mouth of the York River in May as healthy juveniles, could be transported to the laboratory with minimum physiological stress, acclimated to captivity in less than a week, and rapidly started feeding on artificial trout pellets.

Parasites were common in the fish but not more than that expected in spot from the Chesapeake Bay (Burreson, pers. commun.). Trichodina, digenetic trematodes, acanthocephalans, and nematodes were frequently seen in the fish. The prevalence and intensity of Trichodina on the gills of experimental fish increased relative to baseline fish. This result was not surprising since intensity of parasites with direct development can be stimulated by high density of fish hosts and low water flow in the confinement of holding tanks (Bauer et al., 1973). An increase in prevalence of nematodes in experimental fish was surprising since these parasites have an indirect life cycle.

Weights of Phase 1 spot fluctuated but not significantly. It is not uncommon to see fish experiencing no weight gain, or even weight loss, when they are brought from the field into the laboratory. Up to four percent weight loss is reported by McCain et al. (1977) in flatfish within 1-2 months after capture. These fish regained weight by the fourth month in captivity.

Assuming that the condition factor (K) is an accurate indicator of nutritional status (Lagler, 1952), increased K values in experimental fish as compared to the baseline fish seemed to indicate that the fish were eating and assimilating the commercial trout pellets. The decrease in K values between days 15 and 20 post-injection in all test groups may have reflected utilization of energy reserves for processing the injected foreign material (PBS and/or sRBC) or for the activation of antibody producing cells in SRBC-injected fish. Studies show that 10 to 20 days post-injection corresponds to multiplication and differentiation of lymphocytes into antibody producing plasma cells (PFCs) which are stored in the spleen (McGlamery et al., 1971; Paterson and Fryer, 1974; Khalifa and Post, 1976; Serero and Avtalion, 1978; Anderson et al., 1979b, 1982). Antibody levels then begin to rise in the serum to reach a peak concentration five to ten days later (Anderson et al., 1979a). Fish injected with 50% sRBCs (highest concentration) showed a drop in K values earlier than 20% and 35% sRBC-injected fish. A possible correlation between antibody production and change in condition factor would have to be confirmed by further tests.

The necropsy data also showed an increase in prevalence of pathological signs (pale gills, slight emaciation, pale liver) in fish starting on the 10th day post-injection. Infestation of both Trichodina and nematodes increased 20 days post-injection. Rapid proliferation of Trichodina on possibly weakened hosts was not surprising.

PHASE 2

Serology

This study clearly showed that Elizabeth River sediments affected the ability of spot to produce antibodies. Higher antibody levels were obtained in spot exposed to contaminated sediments than those exposed to the reference York River sediments, 25 days after a single injection of sRBCs at 25-27⁰C. Test fish from both tanks showed higher antibody titers than control fish.

The antibody levels obtained in Phase 2 were significantly lower in all test fish than those from Phase 1. Temperature is known to have a major influence on the rate and possibly the intensity of antibody production (Muroga and Egusa, 1969; Avtalion et al., 1973; Paterson and Fryer, 1974; O'Leary et al., 1978; Rijkers et al., 1980b; Avtalion and Clem, 1981). Low temperatures have been shown to inhibit fishes' humoral immune system leading to unresponsiveness or delayed reactions to antigens. No studies have been published on the effect of higher than normal temperatures on antibody production in fishes. In Phase 1, it was unlikely that water temperature had any deleterious effect on the build up of serum antibodies since it remained within the fish's normal range (10 to 26⁰C). However, it is possible that long exposure to temperatures of 27⁰C or more as seen in Phase 2, and common during the summer months in the Chesapeake Bay waters, could have adversely affected the immune competence of spot. The two experiments were run at a month and a half interval from each other and with different populations of spot. It was therefore not possible to say if this

difference in antibody production was, in fact, due to differences in genetics, temperature, time of the year, age, maturity, physiological or nutritional state of the fish, presence of sediments at the bottom of the tanks with associated differences in dissolved oxygen, or presence of hydrocarbons or other compounds in reference and contaminated sediments. It is important to note, however, that it was possible in both Phase 1 and 2 to elicit significant antibody production in less than a month with a single challenge of sRBCs. Obviously, high antibody titers to sRBCs does not infer protection against naturally occurring pathogens unless they cross-react.

The increased levels of antibodies in fish exposed to contaminated sediments as compared to fish exposed to reference sediments could be explained in several ways:

1. Pollutants from Elizabeth River sediments at low concentrations in the water may have acted as general irritants. Damage to the skin, fins, and gills in this study, and also reported by DiMichele and Taylor (1977), Heansley et al. (1982), Meyers and Hendricks (1982), and Solangi and Overstreet (1982), also suggests that hydrocarbons act as general irritants. As such they may have non-specifically activated the defense system. This phenomenon is described by Selyes (1950) as the "general adaption syndrome", by Smyth (1967) as "sufficient challenge", and by Sastry and Miller (1981) as "physiological compensation".

Selyes' general adaption syndrome develops in three stages: the alarm reaction where the animal senses an unfavorable change in environmental conditions, the stage of resistance where metabolic and

physiological activity is increased to restore homeostasis, and a stage of exhaustion where the animal becomes overwhelmed by the stressing agent. In most cases the animal adapts to or escapes unfavorable conditions. However, if these conditions are extreme or persist for a long period of time exhaustion may lead to permanent tissue damage or death.

Selye (1950) theorizes that during the stage of resistance, endocrine and nervous systems are activated causing the hypothalamus-hypophysis system to gear the body for defense. Secretions of FSH, LH, prolactin, and tyrotropin are depressed, while secretion of ACTH is enhanced. The latter induces the adrenal cortex to produce glucorticoids that affect the blood count, thymolympathic tissues, gluco-genesis, and cause the reticuloendothelial system to increase phagocytosis and antibody production. Increased energy demand noted in the fish exposed to contaminated sediments as greater food consumption or emaciation in this study may have been due to increased glucocorticoid production since these hormones also enhance catabolism of proteins and lead to extensive loss of body protein, fat, and carbohydrate (Selye, 1950).

Examples of apparent benefit from small doses of toxic chemicals when large doses injure are not uncommon non-specific responses measured in toxicity studies (Smyth, 1967). Eldridge et al. (1977) reports beneficial effects of exposure of fish to low levels of aromatic hydrocarbons that probably result from a temporary stimulation of metabolic functions under chronic conditions.

The enhanced response seen in spot exposed to contaminated sediments in this study may have involved an "overshoot" of metabolic

functions in response to the environmental stress. Sastry and Miller (1981) points out that such initial overcompensations are generally followed by acclimation after several days or weeks if the environmental challenge persists. The most common pattern of overcompensation is observed in studies dealing with temperature change. Different classes of enzymes also show compensatory "overshooting" (Sastry and Miller, 1981). It is possible in this case that the same phenomenon may have occurred with antibody production in spot exposed to low concentrations of sediment-associated pollutants.

I would expect, however, that with continued exposure to low concentration of pollutants some negative feedback eventually would have occurred when the adaptation energy of the organism was gradually lost. Stress hormones (adrenergic substances, ACTH, corticoids, and RPS) in higher vertebrates are beneficial only for short periods and can eventually cause complications if they persist (Selye, 1950). The same should be true with fishes since they respond similarly to stress (Mazeaud et al., 1977; Donaldson, 1981; Schreck, 1981).

2. Antibody levels in fish exposed to reference sediments may have, in fact, been higher than those in the fish exposed to the contaminated sediments before the 25 days post-injection sample was taken and may have begun to taper off, as is suggested in several studies on kinetics of antibody production (Paterson and Fryer, 1974; Sailendri and Muthukkaruppan, 1975; Ingram and Alexander, 1977; Anderson et al., 1979a). The assumed lower immune response may have actually been an enhanced response on the decline. Repeated serum sampling of impacted and control fish would clarify this point.

3. Organisms or other proteinaceous materials in contaminated sediments but not in reference sediments may have stimulated the immune system and indirectly enhanced antibody production against SRBCs. These organisms would not have cross-reacted with sRBCs since control fish did not show antibody production against the antigen.

4. Finally, hydrocarbons in Elizabeth River sediments may have been immune potentiators. This is unlikely since carcinogens are frequently immune suppressants (Prehn, 1963; Cerilli and Hattan, 1974; Harris, 1976), and these compounds are similar to those known to inhibit the immune system in humans (Mavraganis and Beek, 1980), mice (Hodgins et al., 1977), and other fish species (Weeks and Warriner, 1984). Sjernsward (1966, 1967, 1969) established that a single injection of low level tumor inducing dose of 3-methylcholanthrene (3-MCA) or diben(a)anthracene (DMBA) into mice prior to administration of antigen results in depressed levels of antibody cells. This seems to suggest an effect of the hydrocarbons at the time of initiation of antibody production. It is possible, but unlikely, that spot may not have been exposed long enough before challenge with the immunogen for early toxic effects on antibody synthesis to occur. Other PAHs such as 9,10 dimethyl-12-benzanthracene and 3,4 benzpyrene produce a markedly diminished capacity for rejection of tumors or foreign tissue grafts in mice whereas non-carcinogenic hydrocarbons produce little or no effect. Other hydrocarbons showing immunosuppressive activity are p-dimethylaminoazobenzene and mineral oil. Compounds containing heavy metals such as cadmium or lead which may be associated with petroleum are also known to be immunosuppressive (Hodgins et al., 1977). Weeks and Warriner (1984) find depressed phagocytic activity in spot, and

hogchoker from the Elizabeth River. To my knowledge there are no published reports on the effect of PAHs on the humoral immune system of fish.

Phenols found in creosote and as byproducts of hydrocarbon degradation in the environment, and also produced during detoxification of hydrocarbons in fish liver (Whipple et al., 1981) are known to inhibit immune responses in fishes. Anderson et al. (1983a) suggest that phenol immunosuppresses by preventing antigen uptake and recognition by specific cells in the gills. It may be that immunosuppression can not occur if the antigen is introduced into the fish parentally thus bypassing uptake via the gills. Goncharov and Mikryakov (1970), however, held injection-immunized carp for long periods in low concentrations of phenol and also demonstrated lower humoral antibody titers. Anderson et al. (1983a) finds that phenol immune suppression is dependent on antigen dose and time of exposure. High doses or prolonged treatment with the challenge antigen abrogates the phenol induced inhibition. Challenge with the antigen and subsequent exposure to a continuous phenol bath results in a normal immune response. Fish seem to have the ability to adapt to phenol exposure.

HEALTH OF THE FISH

It was more difficult to catch, transport, and handle spot in July than it was in May, without overly stressing them because of high temperatures. They also exhibited more pathological signs and carried higher numbers of parasites.

The experiment showed that Elizabeth River sediments had an impact on the general health and behavior of spot after 25 days of continuous exposure. Lethal effects occurred only within the first week. Restlessness was also noted for less than a week. The fish did not feed for ten days. Parasites decreased in reference and contaminated tanks during the experiment. This was not seen in Phase 1, and may be due to the presence of sediments (unfavorable environmental conditions for the parasites) or fish were better able to control parasite burdens. Higher prevalence of Trichodina and acanthocephalans were more frequently seen in fish from the contaminated tank than in the reference tank.

Gross damage to gills, eyes, fins, skin, and liver were detected in fish from the contaminated tank. Many fish were emaciated. Some of these pathological signs were also detected in fish from the reference tank but at a much lower prevalence. Evidence based on numerous histological studies on fish exposed to aromatic hydrocarbons show frequent responses associated with the vascular system (damage to the heart, pseudobranch, blood vessels, gills, and eyes), the neurosensory system (damage to taste buds, the lateral line, olfactory mucosa), and the liver (Meyers and Hendricks, 1982). These anomalies are generally detected within 1 to 2 weeks of exposure to 200 ppm or less of water soluble fractions of oil (DiMichele and Taylor, 1977; Payne et al., 1977; Eurell and Heansley, 1981; Malins, 1982). My results agree with the literature. It is possible that the damage observed in spot exposed to contaminated sediments could have been detected earlier (1 to 2 weeks as seen in most studies) had the fish been sampled before the 25th day. Detection of damage to the neurosensory organs, the

pseudobranch, and blood vessels requires histological evaluation. It was therefore not possible to say whether or not these organs had been affected. Fin erosion as seen in some impacted fish in this experiment is also reported in a study by Hargis et al. (in press). These lesions and also the small blisters observed on the ventral surface or near the mouth of some of the fish were probably due to exposure to higher concentrations of pollutants near the sediment surface.

The presence of discolored patches on the body of fish in reference and particularly in contaminated tanks has not been reported previously in hydrocarbon toxicity studies. This pathological sign was probably not due to a direct toxic action of the pollutants but may have been indirect evidence of toxicity. The lesions resembled a pathological sign known as "sunburn". The actual skin burn is generally due to the effect of direct sunlight on fish in shallow water or as they frequently come up to the surface to avoid unfavorable or toxic conditions in the water (Goede, Fish Health Inspector, pers. commun.). "Sunburn" can also be caused by UV light (Bullock and Roberts, 1981). Even though the tanks were covered by a dark colored plastic mesh to prevent the fish from escaping, the fish may have developed "sunburn" from the incident light. The higher prevalence of "sunburn", and associated scale loss, in fish exposed to Elizabeth River sediments may have again confirmed the toxicity of the sediments.

Damage to gills and livers of fish exposed to Elizabeth River sediments was expected from knowledge of the mechanism of hydrocarbon uptake, accumulation, and excretion by fish. Fish, like many other aquatic organisms, readily accumulate hydrocarbons present even at low

concentrations in the ambient water (Anderson et al., 1974; Neff et al., 1976a and b, Neff, 1978; Anderson, 1979). Since hydrocarbons are highly hydrophobic and lipophilic lipid/water partition coefficients favor their rapid transfer from aqueous solution into lipophilic compartments such as biological membranes, macromolecules, and depot lipid stores in organisms (Lee et al., 1972; Neely et al., 1974). Bioaccumulation increases as the molecular weight of the soluble aromatic hydrocarbons increases (Neff et al., 1976b). Gill lamellae where the water-blood barrier is only one cell thick are good target tissues for rapid hydrocarbon uptake. Gills are in fact thought to be the main route of hydrocarbon uptake by fish (Whipple et al., 1981). The delicate sensory organs on the surface of fishes are also perfect tissues for absorption and possibly damage by chemical compounds. Polyaromatic hydrocarbons ingested with the food can be detected in the liver, gallbladder, and bile, within 72 hours after ingestion (Korn et al., 1976; Neff et al., 1976a and b; Roubal et al., 1977; Neff, 1978). Recent findings show, however, that hydrocarbon absorption in the gut is less than that via the gills (Whipple et al., 1981).

Aromatic hydrocarbons then rapidly accumulate in the liver, the main organ of detoxification of foreign compounds in fish (Korn et al., 1976; Bend et al., 1977; Roubal et al., 1977), and are excreted as unmetabolized products by the gills, or are metabolized and slowly released by the bile. Lipid rich mesenteric fat and the brain also accumulate hydrocarbons (Korn et al., 1976). McCain et al. (1977) exposed English sole to hydrocarbons from experimentally oiled sediments (700 ppm dry weight) for four months. The fish had

accumulated maximum levels of hydrocarbons in tissues by 11 days. After 27 days only livers contained significant concentrations of hydrocarbons, suggesting that the fish detoxified the toxic chemicals. Concentrations of aromatic hydrocarbons originally found in tissues of sole were of the same order of magnitude as hydrocarbon concentrations in contaminated sediments. This observation is also reported for other fish species (McCain et al., 1977, McCain and Malins, 1982; Neff et al., 1978).

Accumulation of hydrocarbons from oiled sediments in experimental tanks is generally considered less significant than from oil introduced directly in the water, but may be better suited for long term exposure since hydrocarbons are leached out of sediments for several weeks (Anderson et al., 1974; Vendermeulen and Penrose, 1978). Oil in water (not associated with sediments) often becomes non-toxic to organisms within 48 hours due to weathering processes (microbial degradation, chemical oxidation, and evaporation) (Eisler, 1975; Malins, 1975; Templeton et al., 1975; Kallio, 1976; McCain et al., 1977; Rice and Karinen, 1977).

In this experiment, it was not possible to identify all the compounds present in Elizabeth River sediments since they were contaminated with thousands of different substituted and unsubstituted hydrocarbons (chiefly aromatic hydrocarbons from creosote and manufacturing byproducts, but also alkanes and aliphatic compounds). Twenty aromatic hydrocarbons were therefore selectively analyzed for on the basis of ease of detection, and known toxicity to aquatic organisms including fish (Moore and Dwyer, 1974; Rice et al., 1976; Roubal et al., 1977; Neff, 1978; McCain and Malins, 1982). Chemical

analysis of reference York River and experimental Elizabeth River sediments showed that both contained aromatic hydrocarbons. Concentrations of hydrocarbons in experimental sediments were greater than one hundred times that in reference sediments. Higher concentrations in samples taken at the end of Phase 2, as compared to initial samples, was probably due to uneven distribution of aromatic hydrocarbons as seen in a previous study by Hargis et al. (in press). As expected contaminated sediments contained high levels of di- and polyaromatic hydrocarbons characteristic of creosote and its byproducts (Black et al., 1980). Fluorene, phenanthrene, fluoranthene, and pyrene were particularly abundant in experimental sediments.

Toxicity of petroleum to aquatic organisms is directly correlated to the content of soluble aromatic derivatives (Neff et al., 1976b). Concentrations of compounds in the water were not measured because of the difficulty in analyzing water extracts. However, water soluble fractions of aromatic hydrocarbons are generally found to be four orders of magnitude lower than that detected in sediments (Anderson et al., 1974). Based on concentrations of selected chemical species in my test sediments, soluble aromatic hydrocarbons in the experimental tanks could have ranged from 0.1 to 15 ppb in the contaminated tank, and 1 to 2×10^{-6} ppb in the reference tank.

Data from bioassays report that lethal effects from soluble fractions of petroleum (LD_{50} at 96 hours) can be expected in the 1 to 500 ppm range for adult aquatic organisms (Hyland and Schneider, 1976). For the more sensitive larval and juvenile stages lethal effects from oil usually occur at 0.1 to 10 ppm. Sublethal toxic effects are detected at 0.1 to 1.0 ppm. Chemical communication which

plays a crucial role in feeding, reproduction, and social behavior in fish, can be disrupted by soluble aromatic hydrocarbons at concentrations of 1 to 10 ppb. (Gardner, 1978). Only phenanthrene and fluoranthene were at concentrations near 0.1 ppb in this experiment (the lowest value reported in the literature for lethal effects in juveniles). All other hydrocarbons were less abundant. Mortalities recorded in oil exposed fish at such low concentrations of soluble oil fractions may have indicated that spot were unusually sensitive to hydrocarbon toxicity or that other physical (eg. high temperatures, low dissolved oxygen, or confinement) or biological factors accounted for the high susceptibility. Accumulation, toxicity, and release of hydrocarbons varies tremendously between fish species (Moore and Dwyer, 1974; Devries, 1976; Neff et al., 1976b; Rice et al., 1976; Anderson, 1979; Molés et al., 1979; Stegeman et al., 1983), and within a species varies with age (Rice et al., 1975a and b; Neff et al., 1976a), sex and fat content (Foureman et al., 1983), metabolic activity and nutritional state (Fingerman et al., 1983). It is also possible that other chemical compounds (eg. heavy metals) may have acted synergistically with hydrocarbons and increased the toxicity of the sediments.

Mortalities noted in impacted fish were probably due to volatile, low boiling point hydrocarbons particularly mono-, di-, and tri-aromatic hydrocarbons. Creosote contains aromatic hydrocarbons lighter than phenanthrene that are very soluble in seawater (Sirota and Uthe, 1980). Early toxic effects (after less than a week of exposure) suggested that the more soluble hydrocarbons, possibly phenanthrene or fluorethene, were responsible for mortalities. These findings would

agree with the literature that shows that most of the toxicity of petroleum to fish is attributed to the soluble di- and tri-aromatic hydrocarbons (naphtalene, fluorene, phenanthrene, and anthracene) (Neff et al., 1976a and b; Neff, 1978). Aliphatic compounds and alkanes are not known to produce significant toxicity in fish. Monoalkanes are generally toxic via alteration of cell membrane permeability (Morrow et al., 1975). Polycyclic hydrocarbons although present in high concentration in experimental sediments, are usually found at very low levels in water soluble fractions of oil because of their low solubility in water (McAuliffe, 1965; McAuliffe et al., 1975). They are usually not acutely toxic to fish (Neff et al., 1978). Long term bioassays show substantial chronic toxicity of polyaromatic hydrocarbons if these become metabolized into carcinogens in impacted organisms that have an efficient mixed function oxidase enzyme system in the liver (Grover and Sims, 1968; Stegeman and Sabo, 1976; Bender et al., 1977; Gruger et al., 1977; Stegemann, 1977; Staham et al., 1978; Fu et al., 1980; Ahokas and Pelkonen, 1983; Fingerman et al., 1983; Stegeman and Woodin, 1983, Stegeman et al., in press). Tumors and cancers are reported in feral fish inhabiting PAH-contaminated waters in the New York Bight, Southern California, Puget Sound in Washington, the Great Lakes, and Florida (Harshbarger, 1977). Tumors are also induced in fish in the laboratory after exposures to carcinogens for 6 months or more (Couch and Coutney, Hawkins et al., and Hinton, in press). However, it is highly unlikely that such changes would have occurred in the oil-exposed spot due to the short time of exposure. The absence of tumors in fish exposed to Elizabeth River sediments was, therefore, not surprising.

SUMMARY

1) Spot proved to be a good experimental marine animal for humoral immunology studies. They also were easy to capture, transport, acclimate to the laboratory, and could be maintained on commercial diet for six weeks. Spot caught in May and June were all immature, healthy fish, which carried moderate parasite burdens.

2) A single i.p. injection of 20,35, and 50% SRBCs in PBS could elicit a significant production of serum antibodies in spot within 25 days at 25-27⁰C and 17 ppt salinity. Serum antibodies were first detected 10 days post-injection and rose continuously until the experiment was ended. The rate of antibody production was dose dependent, but similar maximum titers were obtained with all antigen concentrations injected 25 days post-injection. Natural hemagglutinin levels in spot were low.

3) Sera of spot inoculated with SRBCs, and exposed continuously to PAH-contaminated Elizabeth River sediments, showed significantly higher antibody titers 25 days post-injection than those exposed to reference sediments. Overall antibody levels were low.

4) Pollutant exposed fish showed altered feeding behavior, hyperactivity, emaciation, and gross damage to gills, fins, skin,

and liver. Fish exposed to reference sediments exhibited low prevalence or none of these pathological signs.

6) The potential for compensatory responses at low doses must not be overlooked in toxicity studies. These may influence the magnitude and temporal course of responses to stressors. Each fish species considered may respond differently.

TABLE 1a.
Fork length of the fish injected 0,20,35, and 50% SRBCs with time.

Percent immuno- gen injected	Days post-injection				
	5	10	15	20	25
	----- (Mean \pm S.D. fork length)				
0		154.6 (\pm 10.78)	159.8 (\pm 11.9)	162.4 (\pm 13.16)	161.6 (\pm 12.5)
20		161.3 (\pm 7.6)	155.7 (\pm 14.3)	162.5 (\pm 14.1)	168.4 (\pm 12.3)
35		167.5 (\pm 13.6)	162.3 (\pm 13.7)	164.5 (\pm 11.9)	157.7 (\pm 14.5)
50		150.5 (\pm 9.9)	161.3 (\pm 16.3)	156.7 (\pm 7.3)	154.4 (\pm 18.4)

Table 1b.
Weight of the fish injected 0,20,35, and 50% SRBCs with time.

Percent immuno- gen injected	Days post-injection				
	5	10	15	20	25
	----- (Mean \pm S.D. weight)				
0		51.5 (\pm 14.5)	48.8 (\pm 12.4)	47.5 (\pm 9.4)	62.6 (\pm 9.6)
20		61.3 (\pm 14.0)	55.6 (\pm 8.7)	57.4 (\pm 15.6)	61.3 (\pm 14.5)
35		52.1 (\pm 16.9)	69.7 (\pm 17.0)	57.5 (\pm 13.3)	47.6 (\pm 9.5)
50		44.6 (\pm 15.0)	43.6 (\pm 7.3)	47.0 (\pm 7.6)	44.6 (\pm 15.0)

TABLE 2.

Mean Condition Factor ($K = \frac{W}{L^3} \times 10^7$) of the fish at different immunogen concentrations with time (Phase 1).

Percent immunogen injected	Days post-injection			
	10	15	20	25
	(Mean \pm S.D. condition factor)			
0	1.38 (\pm 0.30)	1.24 (\pm 0.41)	1.13 (\pm 0.25)	1.44 (\pm 0.67)
20	1.48 (\pm 0.45)	1.54 (\pm 0.46)	1.21 (\pm 0.32)	1.29 (\pm 0.41)
35	1.40 (\pm 0.65)	1.51 (\pm 0.75)	1.27 (\pm 0.08)	1.30 (\pm 0.39)
50	1.41 (\pm 0.66)	1.09 (\pm 0.34)	1.22 (\pm 0.12)	1.31 (\pm 0.60)

TABLE 3.

FISH NECROPSIES - External and internal signs of pathology (Phase 1).

	Baseline fish	Days post-injection				
		5	10	15	20	25
GROSS EXTERNAL SIGNS:						
1. PALE GILLS:	0.0					
0% SRBC		0.0	0.0	10.0	20.0	0.0
20% SRBC		0.0	10.0	40.0	30.0	0.0
35% SRBC		0.0	30.0	30.0	30.0	0.0
50% SRBC		0.0	30.0	20.0	20.0	10.0
2. EMACIATION (slight)	0.0					
0% SRBC		0.0	0.0	0.0	10.0	0.0
20% SRBC		0.0	0.0	0.0	0.0	0.0
35% SRBC		0.0	0.0	0.0	0.0	0.0
50% SRBC		0.0	0.0	0.0	30.0	20.0
3. PARASITES:						
ciliated Protozoa (Trichodina)	48.7	64.0	63.0	45.0	70.0	58.0
GROSS INTERNAL SIGNS:						
1. PALE LIVER:	0.0					
0% SRBC		0.0	20.0	10.0	0.0	20.0
20% SRBC		0.0	10.0	30.0	0.0	12.0
35% SRBC		0.0	20.0	30.0	10.0	10.0

(table 3. continued)

50% SRBC	0.0	50.0	20.0	0.0	10.0	
2. PARASITES:						
Nematoda	5.4	64.0	63.0	45.0	80.0	58.0
Platyhelminthes (Digenean Trematodes)	21.7	26.0	22.0	35.0	25.0	20.0

TABLE 4.

Sizes of the fish at the time of injection (Day 0) and bleeding (Day 25) in reference (TR) and contaminated (TC) tanks.

Tank	Percent SRBCs	Day 0		Day 25		L	W
		Ave. Length (mm.)	Ave. Weight (grs.)	Ave. Length (mm.)	Ave. Weight (grs.)		
TR	0	180.8 (+ 9.75)	88.0 (+ 16.2)	178.2 (+ 9.09)	95.2 (+ 14.3)	-2.59	-7.14
TR	35	181.9 (+ 9.64)	91.0 (+ 16.2)	182.0 (+ 8.14)	100.8 (+ 13.3)	0.09	9.83
TC	0	184.5 (+ 11.2)	92.5 (+ 15.5)	185.6 (+ 10.7)	92.8 (+ 16.1)	1.68	0.23
TC	35	176.6 (+ 12.6)	83.2 (+ 18.5)	177.0 (+ 12.1)	86.6 (+ 22.3)	0.42	3.32

TABLE 5.

Mean Condition Factor (K) of control and SRBC-injected fish, at injection time (day 0) and bleeding (day 25), in reference (TR) and contaminated (TC) tanks.

Tank	Percent immuno- gen injected	Days post-injection		K
		0	25	
		----- (Mean \pm S.D. condition factor)		
TR	0	1.36 (\pm 0.38)	1.50 (\pm 0.26)	0.14
TR	35	1.44 (\pm 0.31)	1.53 (\pm 0.45)	0.09
TC	0	1.20 (\pm 0.55)	1.51 (\pm 0.09)	0.31
TC	35	1.40 (\pm 0.31)	1.52 (\pm 0.16)	0.12

TABLE 6.

FISH NECROPSIES - External and internal signs of pathology (Phase 2).

	Baseline (n=25)	Reference tank		Contaminated tank	
		Control (n=25)	SRBC (n=23)	Control (n=17)	SRBC (n=24)
(percent fish affected)					
GROSS EXTERNAL SIGNS:					
1. GILLS:					
pale (anemic)	0.0	4.0	0.0	11.8	4.8
congested	0.0	4.0	4.3	29.4	12.5
2. EYES:					
opacity	0.0	4.0	0.0	0.0	4.2
3. FINS:					
erosion	10.0	0.0	0.0	5.9	0.0
hyperemia	0.0	4.0	0.0	11.8	8.3
4. SKIN:					
Discolored patches	0.0	0.0	0.0	29.4	8.3
loss of scales	11.7	0.0	0.0	5.4	8.3
blisters	0.0	0.0	0.0	5.9	12.5
5. EMACIATION:					
	0.0	5.3	0.0	7.2	16.8
6. PARASITES:					
(moderate to low numbers)					
ciliated Protozoa (Tri- chodina)	90.0	0.0	4.3	5.9	20.8

(table 6. continued)

7. FUNGAL INFECTION: (mild on gills)	0.0	0.0	0.0	13.4	0.0
GROSS INTERNAL SIGNS:					
1. BILE:					
full (Bilirubin)	5.0	12.0	6.0	17.6	29.2
2. LIVER:					
hepatomegaly	0.0	16.0	8.7	58.8	87.5
pale color	10.0	4.0	0.0	0.0	0.0
loss of firmness	0.0	4.0	0.0	35.3	83.3
3. PARASITES:					
Nematoda	16.7	28.0	17.4	0.0	20.8
Acanthocephala	10.0	4.0	0.0	0.0	0.0
Platyhelminthes (Digenean Trematodes)	40.0	4.0	0.0	0.0	0.0

TABLE 7.
Initial and final concentrations (in ppb dry weight sediment)
of selected PAHs of increasing molecular weight in
reference and contaminated tanks.

COMPOUND	Reference		Contaminated	
	conc.	conc.	conc.	conc.
Naphtalene	26	36	3878	2156
Benzothiopene	10	43	108	0
2-Methylnaphtalene	31	24	5330	1751
1-Methylnaphtalene	53	71	4327	6130
Biphenyl	16	92	5226	5338
Fluorene	25	98	30039	37889
Dibenzothiopene	0	36	9821	12369
Phenanthrene	118	249	120487	151505
Anthracene	0	11	10193	9740
Fluoranthene	151	310	71345	92936
Pyrene	130	238	40246	52680
Benzo(a)fluorene	0	23	9036	11774
Benzo(b)fluorene	0	54	8338	11450
Benzo(a)anthracene	48	73	9667	15364
Chrysene	105	150	9659	15006
Benzo(j,b,k)fluoranthenes	0	100	6643	11003
Benzo(e)pyrene	57	38	1867	3497
Benzo(a)pyrene	26	26	2199	4177
Perylene	118	92	626	1698
Indeno(cd-2,3)pyrene	56	65	0	1428
Benzo(ghi)perylene	<u>28</u>	<u>35</u>	<u>0</u>	<u>0</u>
TOTAL	998	1864	349035	447891

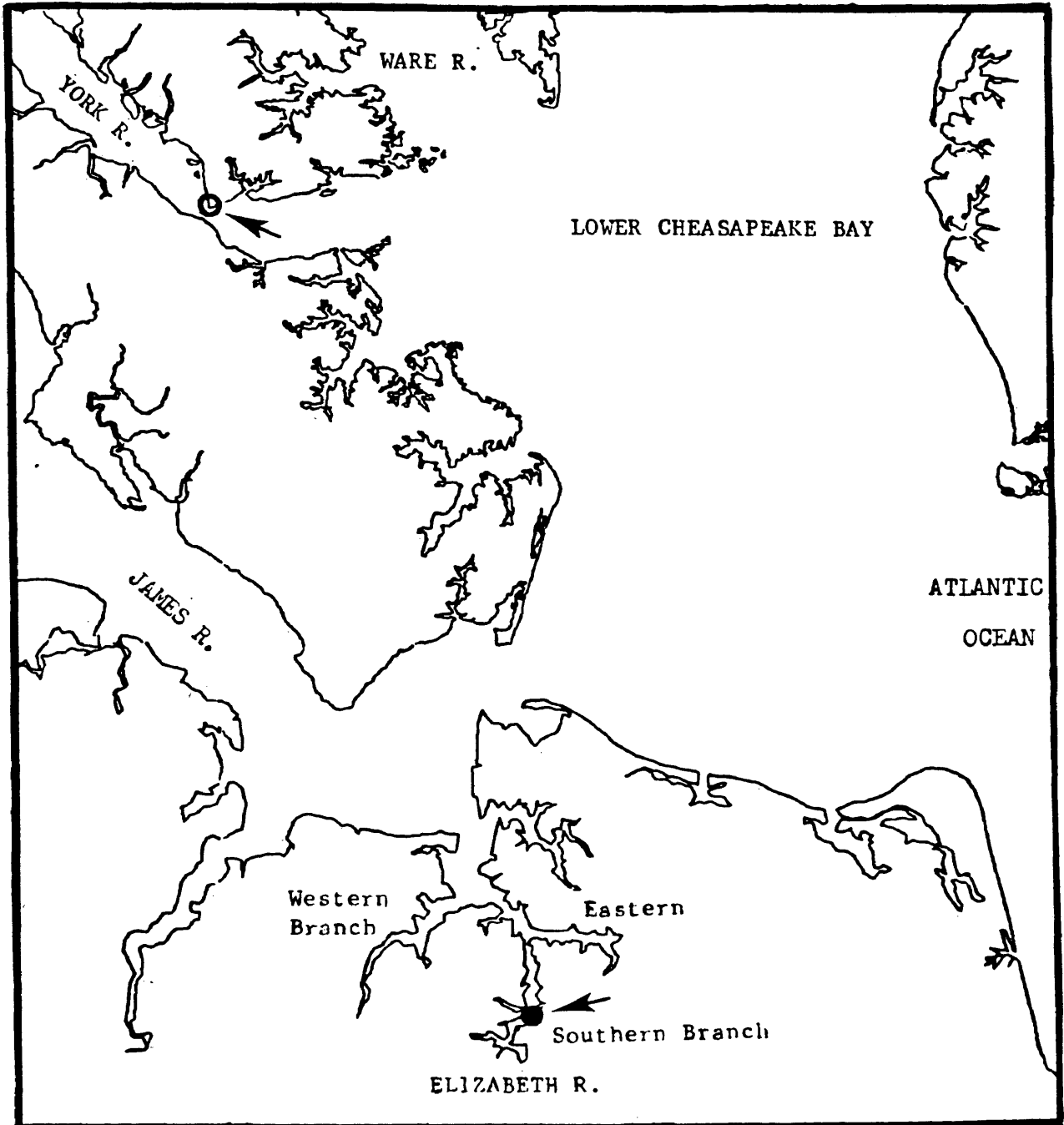
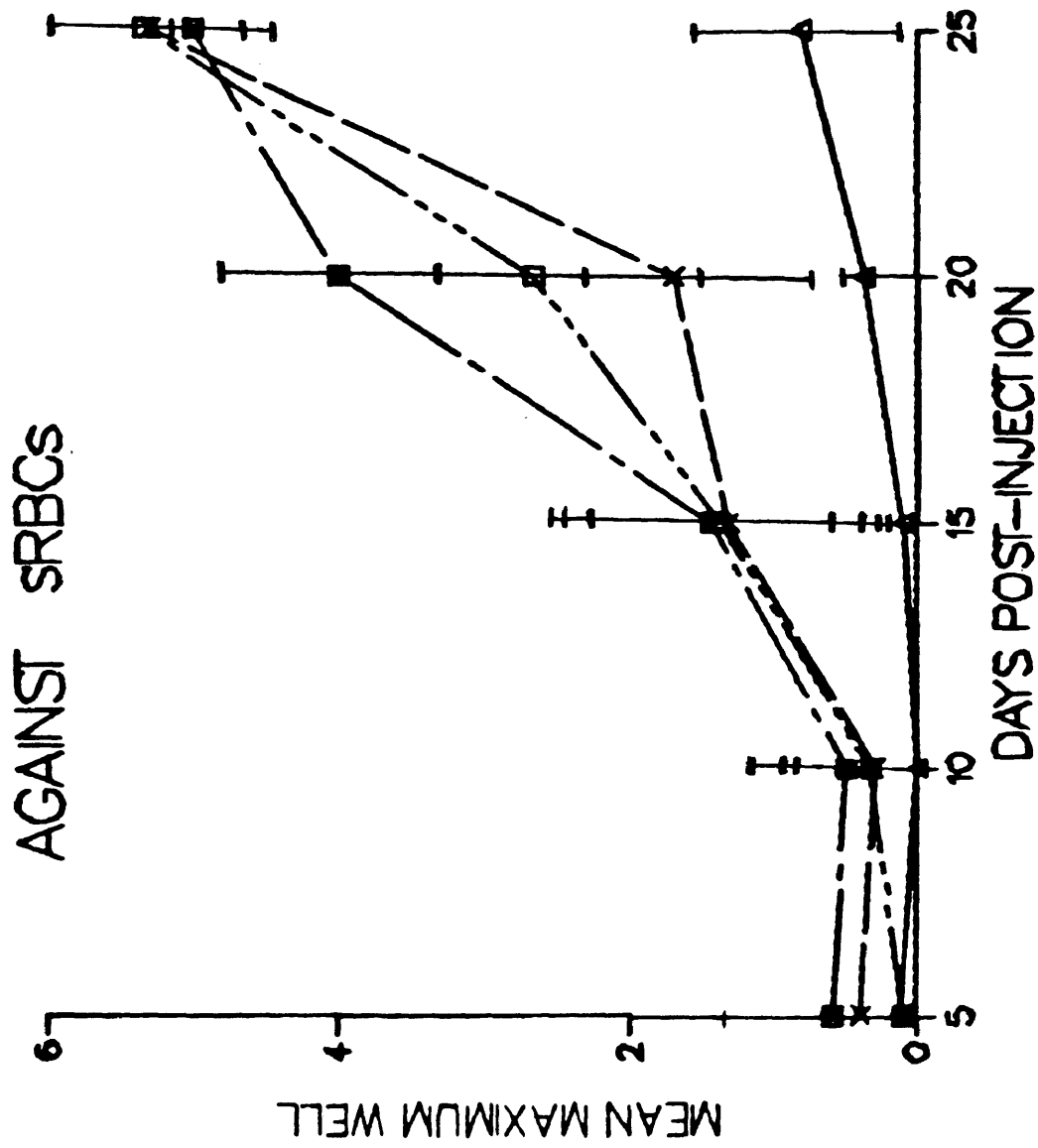


Figure 1: Sampling locations in the Chesapeake Bay. Open circle (○) indicates reference site and closed circle (●) indicates contaminated site.

FIGURE 2. ANTI BODY PRODUCTION OF SPOT



* Temperature increase: 19⁰C to 27⁰C.

FIGURE 3. MEAN DISSOLVED OXYGEN (PHASE 1.)

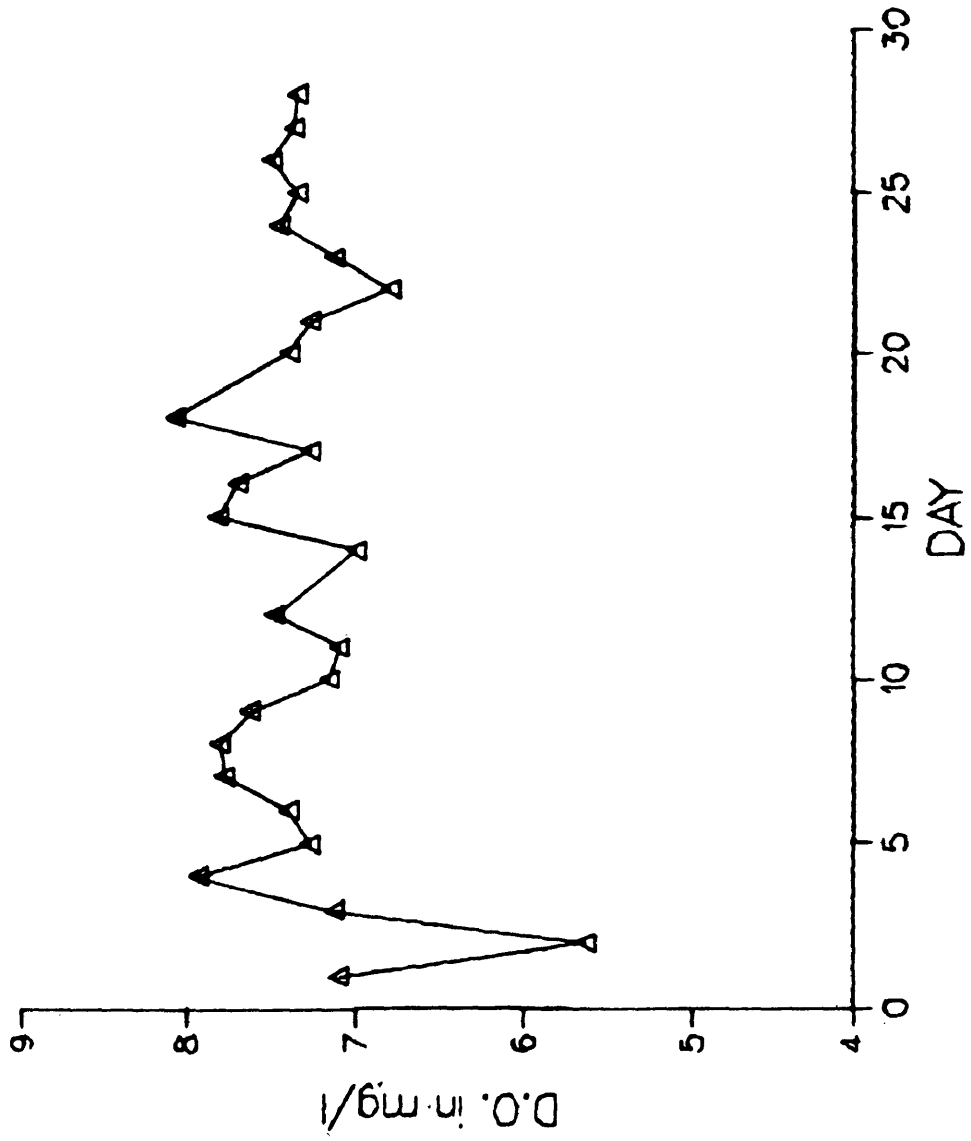


FIGURE 4. MEAN SALINITY (PHASE 1.)

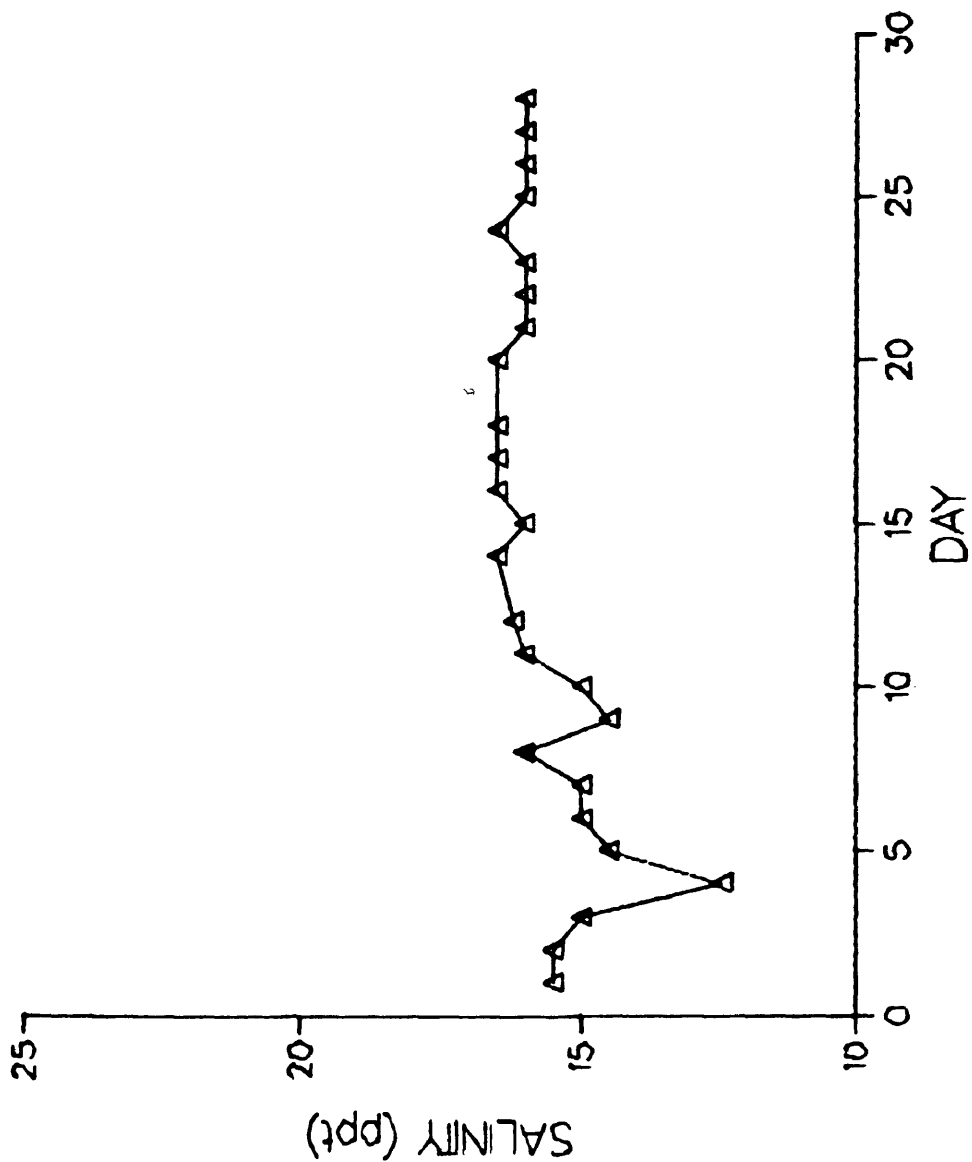


FIGURE 5. MEAN pH (PHASE 1.)

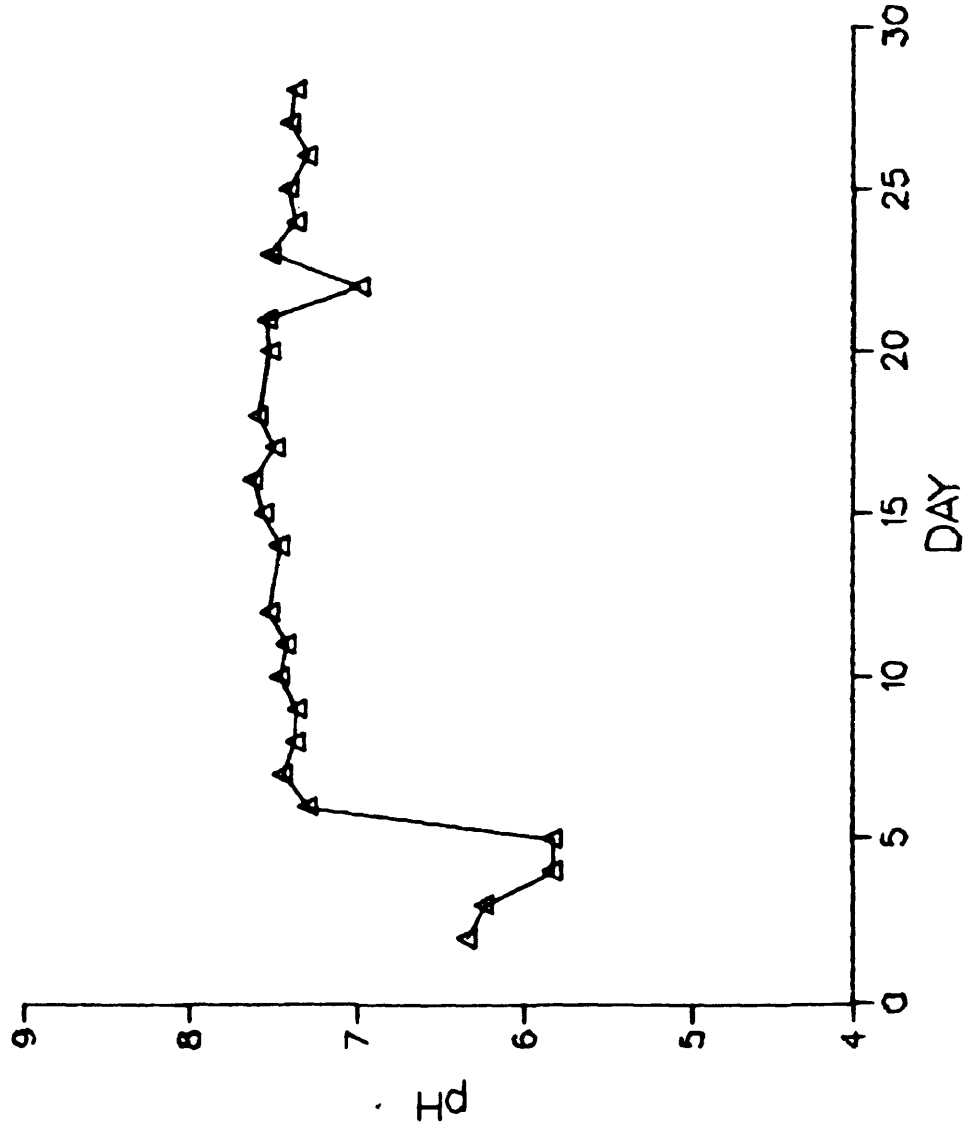


FIGURE 6. MEAN TEMPERATURE (PHASE 1.)

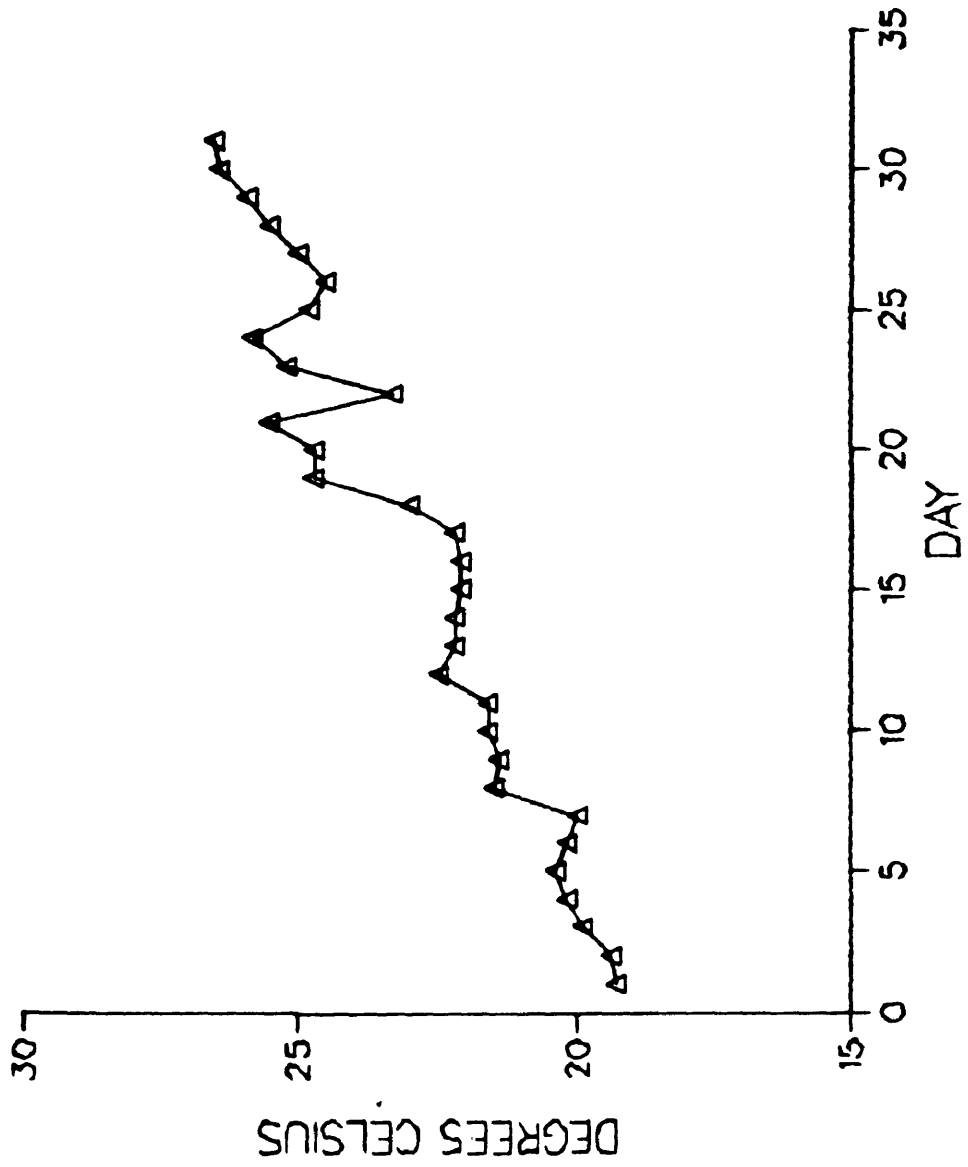
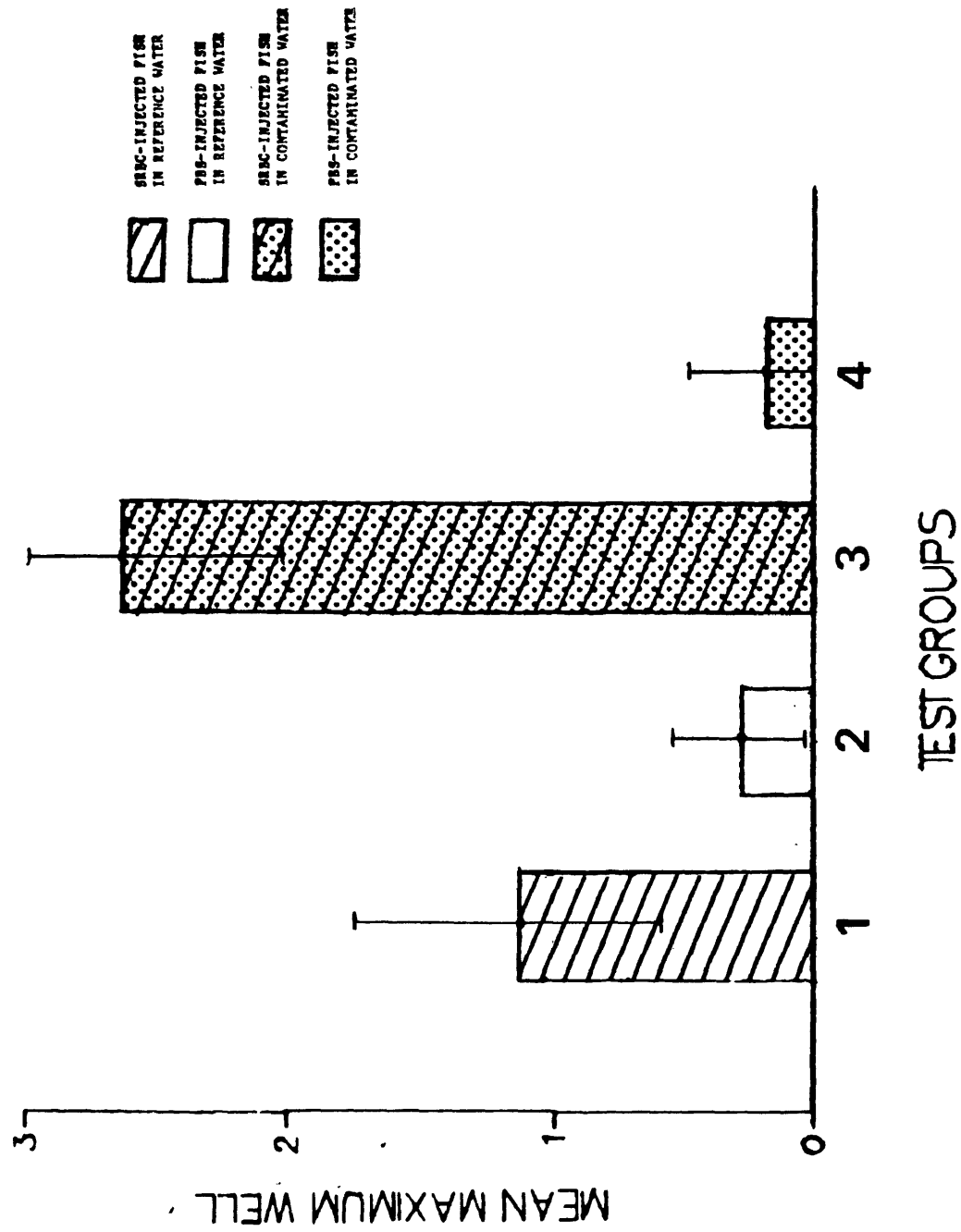


FIGURE 7. ANTI BODY TITERS: PHASE 2



* Time post-injection: 25 days
Temperature range: 25.6 to 28.6°C

FIGURE 8. DISSOLVED OXYGEN (PHASE 2.)

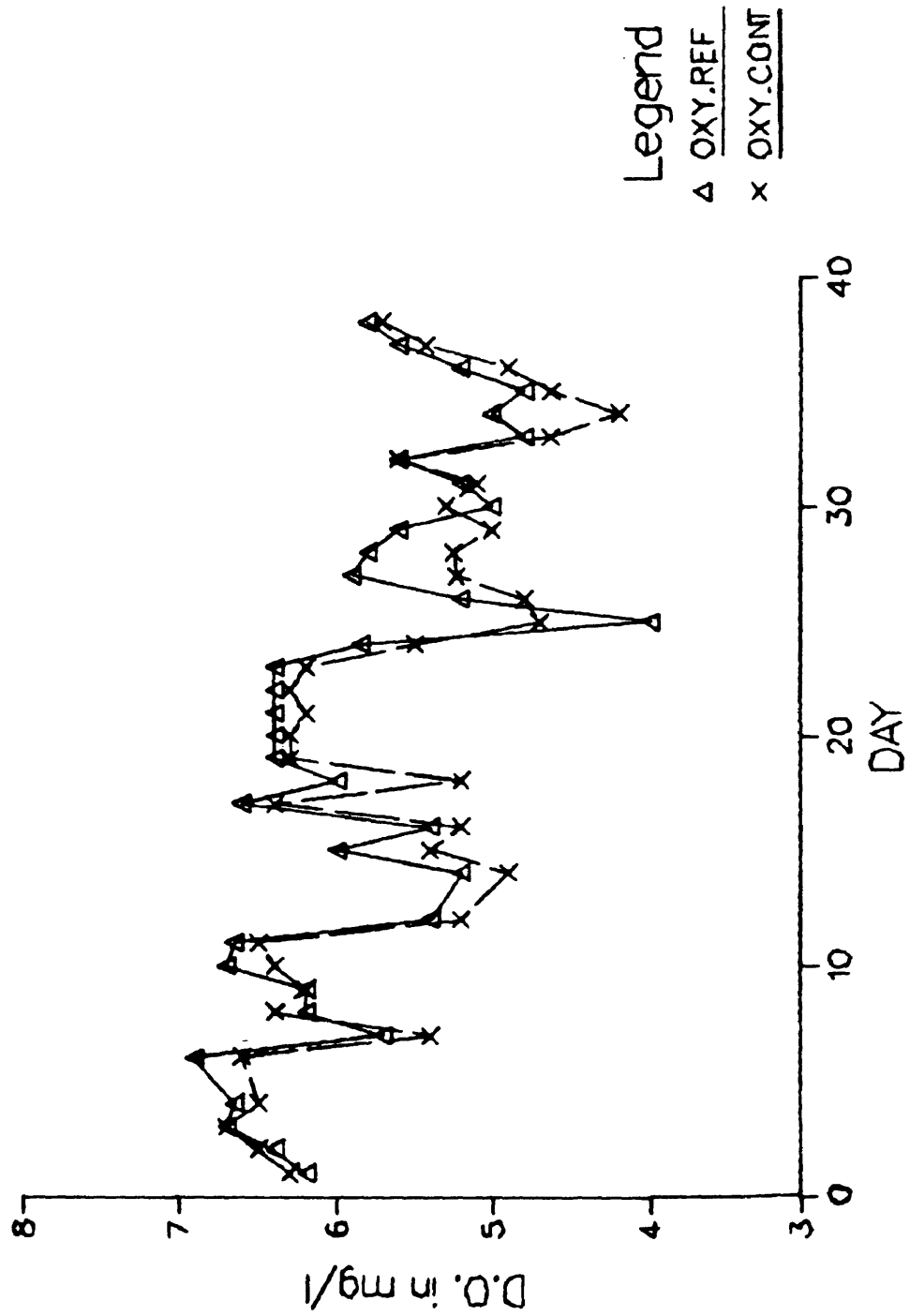


FIGURE 9. MEAN SALINITY (PHASE 2.)

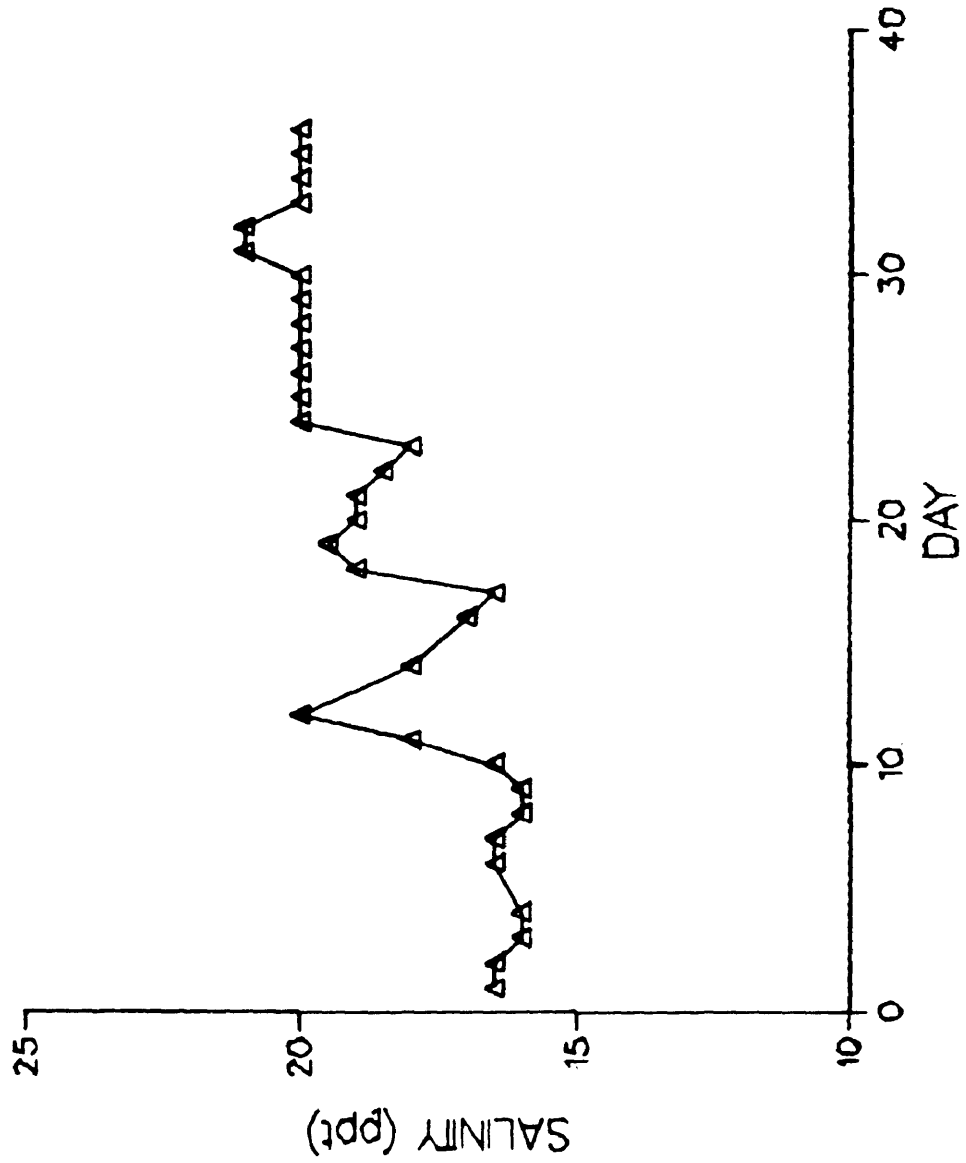


FIGURE 10. MEAN pH (PHASE 2.)

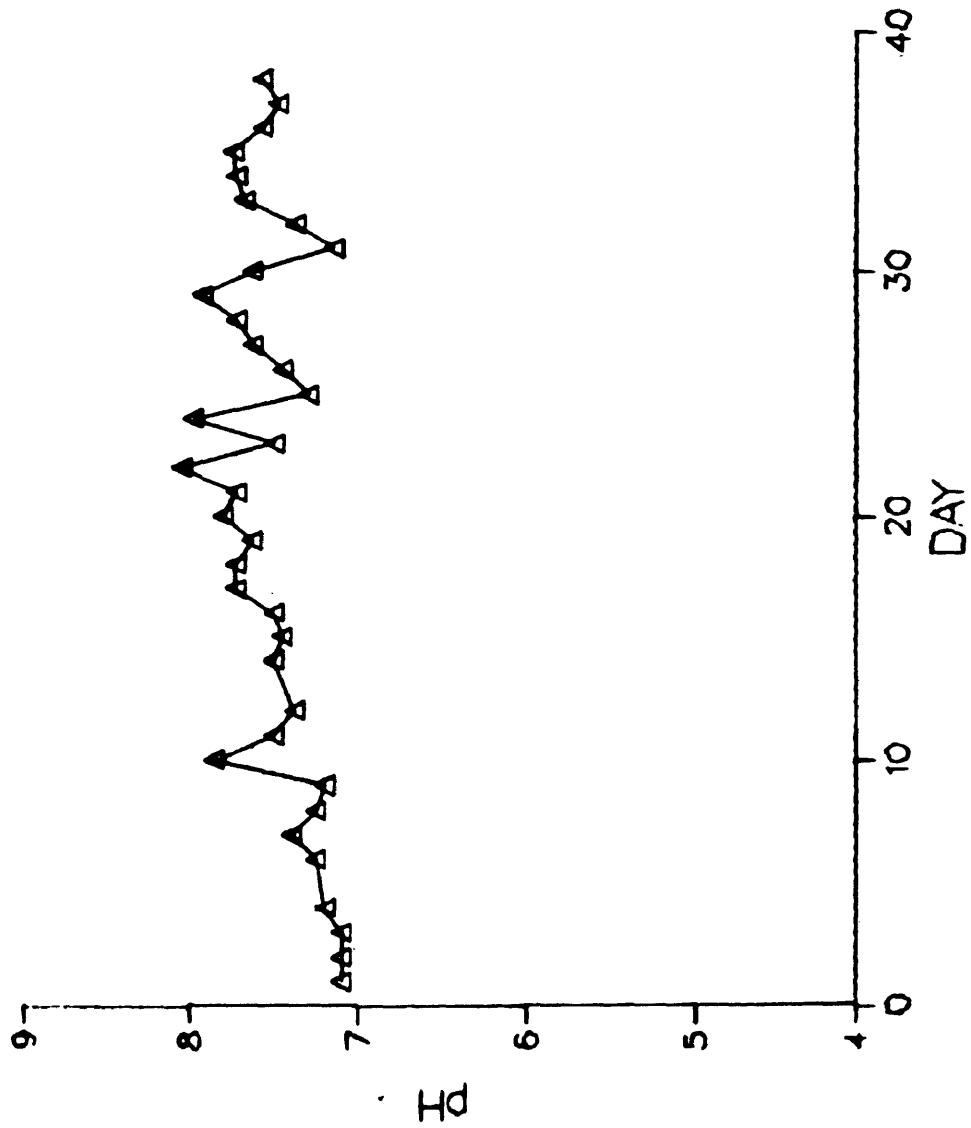
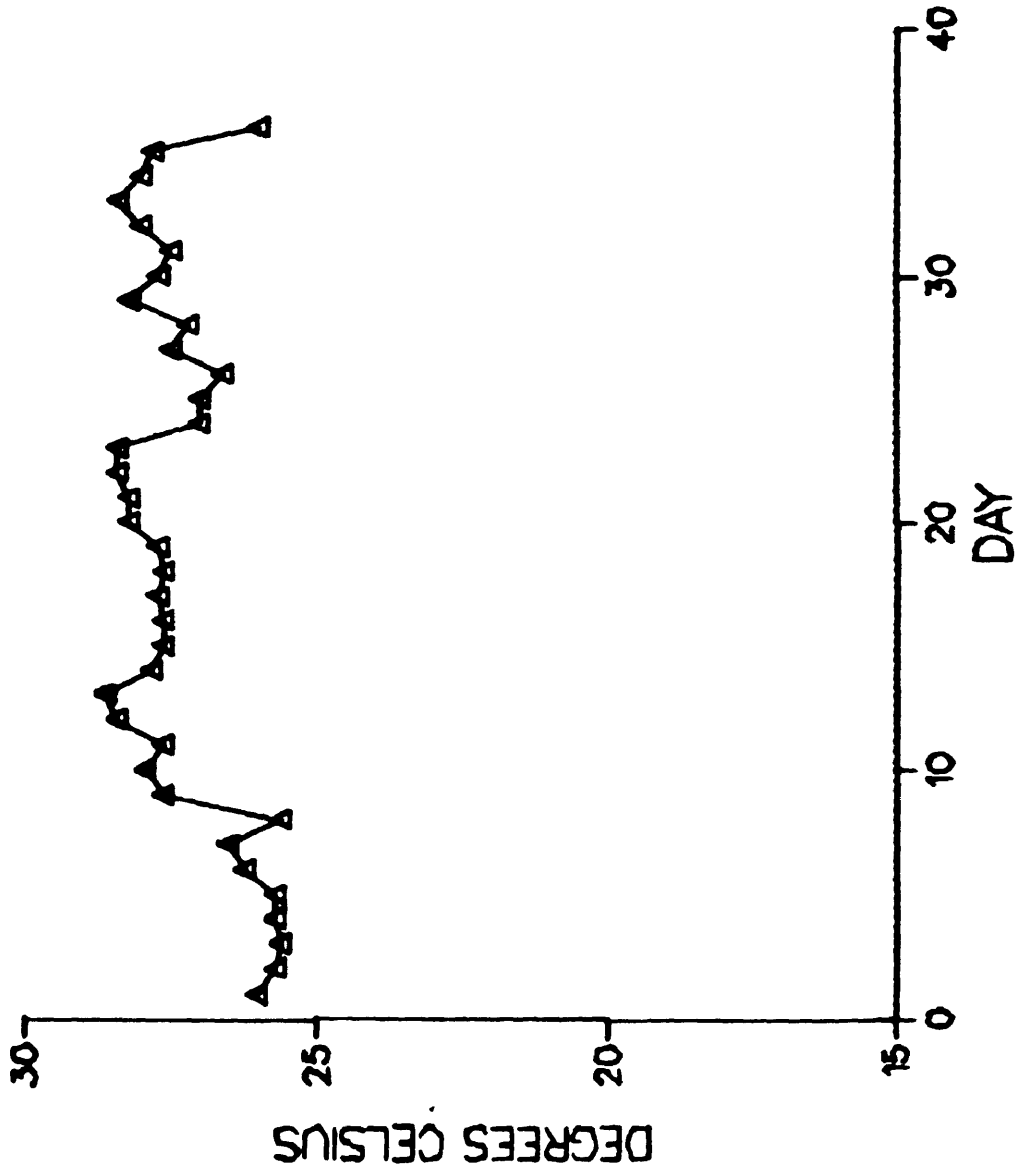


FIGURE 11. MEAN TEMPERATURE (PHASE 2.)



APPENDIX 1.
Antibody levels expressed as
maximum well showing agglutination

Day post- injection	PBS-injected	20%-injected	35%-injected	50%-injected
5	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00
	0.00	2.00	1.00	0.00
	0.00	0.00	0.00	0.00
	1.00	1.00	0.00	0.00
	0.00	1.00	0.00	3.00
	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	1.00
	-	0.00	0.00	2.00
10	0.00	0.00	0.00	0.00
	0.00	0.00	2.00	0.00
	0.00	0.00	0.00	0.00
	0.00	1.00	0.00	0.00
	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	2.00
	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	2.00
	0.00	2.00	1.00	0.00
	0.00	0.00	-	1.00
15	0.00	3.00	2.00	1.00
	0.00	0.00	2.00	2.00
	1.00	0.00	0.00	1.00
	0.00	2.00	0.00	3.00
	0.00	0.00	1.00	2.00
	0.00	4.00	0.00	3.00
	0.00	2.00	3.00	1.00
	0.00	0.00	3.00	0.00
	0.00	1.00	-	-
	20	0.00	3.00	2.00
0.00		1.00	4.00	5.00
0.00		3.00	1.00	4.00
1.00		2.00	3.00	4.00
1.00		0.00	5.00	5.00
0.00		1.00	3.00	2.00
1.00		5.00	0.00	3.00
0.00		2.00	4.00	4.00
-		0.00	2.00	5.00

appendix 1, continued.

Days post- injection	PBS-injected	20%-injected	35%-injected	50%-injected
	-	0.00	-	-
25	1.00	6.00	5.00	4.00
	0.00	4.00	6.00	5.00
	2.00	6.00	5.00	5.00
	1.00	7.00	5.00	5.00
	0.00	5.00	5.00	5.00
	-	4.00	6.00	6.00
	-	5.00	6.00	4.00
	-	-	5.00	6.00
	-	-	5.00	5.00

APPENDIX 2.
Antibody levels expressed as
maximum well showing agglutination

Days post- injection	SRBC-injected Ref. tank	PBS-injected Ref. tank	SRBC-injected Cont. tank	PBS-injected Cont. tank
25	1.00	0.00	3.00	1.00
	1.00	0.00	1.00	0.00
	1.00	1.00	2.00	0.00
	0.00	0.00	3.00	0.00
	1.00	0.00	2.00	0.00
	3.00	0.00	4.00	1.00
	1.00	1.00	3.00	0.00
	0.00	0.00	2.00	0.00
	2.00	0.00	3.00	0.00
	1.00	1.00	4.00	0.00
	2.00	1.00	3.00	0.00
	0.00	0.00	3.00	0.00
	0.00	0.00	3.00	1.00
	2.00	1.00	2.00	0.00
	1.00	1.00	2.00	0.00
	1.00	1.00	4.00	0.00
	1.00	0.00	3.00	0.00
	1.00	0.00	1.00	-
	0.00	0.00	1.00	-
	3.00	0.00	2.00	-
	0.00	0.00	4.00	-
	4.00	0.00	4.00	-
	0.00	0.00	3.00	-
	-	0.00	1.00	-
	-	0.00	-	-

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