SUBLETHAL EFFECTS OF BIOLOGICALLY TREATED

PETROLEUM REFINERY WASTEWATERS ON

AGONISTIC BEHAVIOR OF MALE

ORANGESPOTTED SUNFISH,

LEPOMIS HUMILIS

(GIRARD)

By

JAMES CARROLL PETERSEN

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University of South Dakota

Vermillion, South Dakota

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Thesis Approved:

S. L. Burks
Thesis Adviser

Rudolph J. Miller

P. Larry Claypool

Norman N. Durham
Dean of the Graduate College

1043032

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CHAPTER I

INTRODUCTION

Objectives of Research

In recent years the petroleum refining industry has improved the

quality of its wastewaters by installing activated sludge systems,

biological waste stabilization lagoon systems, or combinations of both

in order to meet 1977 effluent criteria set as a result of the Federal

Water Pollution Control Act of 1972 (PL 92-500). This has resulted in

the reduction of acute lethal effects upon organisms in receiving waters.

In the past the majority of bioassays of toxic substances, includ-

ing petroleum refinery wastewaters, have investigated the short term

lethal effects of these substances on organisms. Improvement in the

quality of refinery wastewaters has stimulated the need for investiga-

tions of various chronic and sublethal physiological and behavioral

responses in order to more adequately assess the total effect of these

wastewaters. The primary objective of this research was to investigate

sublethal effects of biologically treated petroleum refinery wastewaters

through observations of the agonistic behavioral responses of fish.

The second major objective of this study was to develop a relatively

fast method for detection of sublethal deleterious effects without the

use of time consuming chronic or life-cycle bioassays. Dicks (1976)

recognized the significance of using behavioral responses to bridge the

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gap between acute lethal bioassays and more time consuming chronic bio-

assays and field investigations.

Significance of Behavioral Responses

Several authorities have stressed the necessity for research on the

effects of sublethal levels of pollutants on avoidance reactions,

reproduction, and other normal behavior patterns in order to more accu-

rately assess effects of contaminants for establishment of ecologically

sound water quality criteria (Warner et al., 1966; Stickel, 1969;

Sprague, 1971; Baker, 1976; Sprague, 1976).

For several reasons, it is important that consideration be given to

behavioral changes in response to pollutants. First, it is likely that

evolutionarily stable behavior has distinct survival value to organisms

in their natural habitat and that any changes in behavior are likely to

be deleterious (Warner et al., 1966). Behavioral changes also appear to

be very sensitive indicators of pollution (Sprague, 1971). Weir and

Hine (1970) found that a very small fraction of the 48 h LC50 for various

metals was sufficient to cause significant impairment of the ability of

goldfish (Carassius auratus) to respond to a previously conditioned

response. Concentrations of less than 1/1570 (0.066%) of the 48 h

LC50 for lead were sufficient to cause impairment of the ability of

goldfish to respond to a flashing light and avoid a mild electric shock.

Warner (1967, p. 191) called behavioral changes ". . . the most sensitive

indicator yet developed of toxicant-induced change in living systems."

Warner et al. (1966) and Scherer (1977) further support the use of

behavioral bioassays by stating that behavioral changes are more com-

prehensive than physiological or biochemical changes.

Previous Studies of Sublethal

Behavioral Changes

In recent years there has been an increase in the emphasis placed upon the study of sublethal behavioral changes resulting from exposure to pollutants. Many of these studies involved changes in behavior as a result of pesticide exposure. These studies include investigations of the effects of fenitrothion on locomotion, feeding and social behavior of coho salmon (Bull and McInerney, 1975), fenitrothion on the ability of juvenile Atlantic salmon to hold territories (Symons, 1973), DDT on exploratory behavior on goldfish (Davy et al., 1973), DDT on light discrimination and learning by rainbow trout (McNicholl and MacKay, 1975a and 1975b), sevin on schooling behavior of Menidia (Weis and Weis, 1975), and parathion on susceptibility of shrimp to predation (Farr, 1977).

Although the majority of research dealing with sublethal behavioral changes appears to be associated with pesticide exposures, there have been several studies investigating sublethal behavioral effects of exposure to various metals. Effects include disruption of Atlantic salmon migration by copper and zinc (Sprague et al., 1965), reduced settlement of oyster spat as a result of zinc exposure (Boyden et al., 1975), extinction of a previously conditioned response in goldfish caused by several heavy metals (Weir and Hine, 1970), and increased susceptibility of Gambusia to predation as a result of sublethal exposure to mercury (Kania and O'Hara, 1974).

Studies of behavioral changes of aquatic organisms following exposure to various petrochemicals have been concerned primarily with

the effects of oil spills. Krebs and Burns (1977) investigated the
effects of a fuel oil spill on locomotor and burrowing behavior, molt-
ing coloration, and molting of the crab, Uca pugnax. Limpets exposed
to a simulated crude oil spill detach from the substrate at higher than
normal rates (Dicks, 1973). A lengthy review of the behavioral effects
of various petroleum components has been compiled by Clark and Brown
(1977). These include narcosis caused by volatile normal paraffins,
interference with nutrition and chemoreception after exposure to non-
volatile paraffins and numerous chronic effects of aromatic hydro-
carbons. Johnson (1977) presents an extensive review of various
changes in behavior of bacteria, algae, and invertebrates. These
include inhibition of chemosensory attraction of bacteria to prey and
color changes, disruption of locomotor behavior, changes in respiratory
movement rates, narcosis and elimination of reproductive behavior in
invertebrates. Pattern (1977) reviews the sublethal effects of petroleum
hydrocarbons on fish behavior. These effects include changes in avoid-
ance reactions, cough responses, increases and decreases in swimming
activity, disruption of schooling behavior, and narcosis.

Fewer studies have investigated the sublethal behavioral effects of
oil refinery wastewaters. Dicks (1976) reported a lowered settlement
density of the cyprid stage of the barnacle, Balanus balanoides, near a
refinery outfall. Laboratory studies of the effects of this wastewater
on the earlier nauplii stage of B. balanoides indicated that there was
a reduction of swimming activity. However, the lower salinity of the
wastewater (9 p.p.t. vs. 34 p.p.t. for seawater) was indicated to be at
least as important as other characteristics of the wastewater in reduc-
ing swimming activity. Also in laboratory tests, Parsons (1972; cited

by Dicks, 1976) found that refinery wastewaters reduced the locomotor
activity of the grooved periwinkle, _Littorina saxatilis_. Dicks (1976)
reports large numbers of the oligochaete _Nereis diversicolor_ leaving
their burrows in response to an accidental discharge of refinery waste-
water. The _Nereis_ were then extensively fed upon by sea birds. This
sublethal behavioral response resulted in an observable ecological
effect and was found to be due to factors other than salinity change
(Baker, 1976).

Three petroleum refinery wastewater treatment methods were evaluated
using bioassays by Burks and Wilhm (1978) and Kleinholz (1978). Fathead
minnows (_Pimephales promelas_) and assemblages of benthic macroinver-
tebrates were exposed to the wastewaters during 32-day static and con-
tinuous flow bioassays. The treatment methods evaluated were: (1)
activated sludge treatment, (2) activated sludge treatment followed by
dual media (sand and anthracite coal) filtration, and (3) activated
sludge treatment and dual media filtration followed by adsorption on
activated carbon. Male fathead minnows displayed spawning behavior
consisting of establishment, defense, and cleaning of spawning sites
during one of the bioassays. Vertical color bars and rostral tubercles,
which are secondary sexual characteristics, were seen in these males.
These spawning behaviors were observed only in minnows exposed to con-
trol water or to wastewater which had been treated by the activated
sludge-dual media-activated carbon method. Other sublethal behavioral
effects of treated petroleum refinery wastewaters on fathead minnows
have been reported by Graham (1963) and Graham and Dorris (1968). Graham
observed loss of schooling behavior, loss of appetite, sluggishness, slow

or absent response to a disturbance, and lowered index of condition (even for fish which seemed to be feeding normally).

Sprague et al. (1978) investigated sublethal behavioral effects of treated petroleum refinery wastewaters in a laboratory study dealing with rainbow trout (Salmo gairdneri), flagfish (Jordanella floridae), and the invertebrate Daphnia pulex. The wastewater used was generally in compliance with Canadian regulations governing physicochemical parameters and non-lethality to rainbow trout. Sublethal effects examined included effects on growth, avoidance reactions, locomotor reactions and cough responses of rainbow trout; effects on growth and reproduction of flag-fish; and effects of reproduction of Daphnia. The threshold for most of the effects just mentioned was generally calculated to be at about 10% effluent/90% dilution water. Dilutions of as low as 0.52% effluent were calculated to be the threshold for 5% inhibition of Daphnia pulex reproduction.

Significance of Agonistic Behavior
in Bioassays

Agonistic behavior, which involves fighting and competitive behavior, attacks and escapes has recognizable benefits for individuals and populations (Johnsgard, 1967; Johnson, 1972). It may be used for establishment of territories for breeding, feeding and shelter. Crook (1970) notes that territory operates as a "social mortality factor," allowing holders of territory to escape predation and increase success of breeding activities. In other situations limited space for ter-ritories can limit overpopulation and favor the survival of healthier individuals (Johnson, 1972). The common repertoire of agonistic

behaviors of various centrarchids has already been studied extensively both in the laboratory (Miller, 1963; Hadley, 1969; Dennis, 1970; Powell, 1972) and in the field (Barney and Anson, 1923; Breder, 1936; Witt and Marzolf, 1954; Hunter, 1963; Miller, 1963; Boyer, 1969). Ecological significance and existing knowledge of centrarchid agonistic behavior, coupled with the previously discussed sensitivity and comprehensiveness of behavioral bioassays, make observation of agonistic behavior changes an appropriate and attractive sublethal bioassay method.

<div align="center">Selection of the Orangespotted

Sunfish for Bioassay</div>

The orangespotted sunfish, Lepomis humilis (Girard), was selected for use in this behavioral bioassay for several reasons. First, it is widely distributed, from North Dakota to Ohio southward to Alabama and Louisiana and throughout the Great Plains from Texas to the Dakotas (Miller and Robison, 1973). In addition, Gould (1962) found L. humilis to be a good fish for oil refinery effluent bioassays. Gould reported that L. humilis was not statistically different from the fathead minnow (Pimephales promelas) in resistance to oil refinery effluents. The fathead minnow is a widely used bioassay organism. Irwin (1965) also ranked the resistance of the orangespotted sunfish and the fathead minnow to oil refinery effluents. In terms of 96 h TL50, Irwin found the orangespotted sunfish to be slightly more resistant than the fathead minnow. On a scale of 1 to 100, with 100 representing the most resistant fish tested (the common guppy, Lebistes reticulatus), L. humilis ranked at 61.80 while P. promelas ranked at 49.19.

L. humilis are sexually dichromatic. The males have orange to orange-brown spots on their sides; females have brownish spots (Miller, 1963). This allows males and females to be distinguished so that variations in behavior due to sex can be eliminated (Greenberg, 1947; Allee et al., 1948; Erickson, 1967; Hadley, 1969).

Finally, agonistic behavior of the orangespotted sunfish has been studied in the laboratory previously (Miller, 1963; Dennis, 1970; Powell, 1972). This eliminated the need for preliminary investigations of agonistic behavior patterns before they could be used as a measure of sublethal stress from contaminants in petroleum refinery wastewaters.

Description of the Refinery

The refinery chosen for this study was a class B refinery which processed about 50,000 barrels of crude oil per day. The wastewater treatment system consisted of an API gravity oil separator, activated sludge, sludge clarifier, and a sequence of three polishing lagoons (Burks and Wilhm, 1978). The wastewater was collected near the outlet leaving the final lagoon.

Wastewater from the refinery was selected because it consistently caused low or no acute mortality to fathead minnows in 96 h static bioassays conducted by the Reservoir Research Center, Oklahoma State University (Burks, S. L., Reservoir Research Center, Oklahoma State University, Personal communication, September 1976).

CHAPTER II

MATERIALS AND METHODS

Collection and Handling of Fish

Fish to be used in the bioassays were captured with a throw net
from Theta Pond on the campus of Oklahoma State University, Stillwater,
Oklahoma. To minimize variation in agonistic behavior due to size
(Greenberg, 1947; Erickson, 1967; Hadley, 1969; Dennis, 1970) and sex
(Greenberg, 1947; Allee et al., 1948; Erickson, 1967; Hadley, 1969),
only fish which were between 4.0 and 6.5 cm in standard length and
which were considered to be males were kept to be used for bioassay.
Males were selected on the basis of coloration. To assess the accuracy
of selection 20 fish thought to be males were collected from Theta Pond
on 30 April 1979. Squash mounts of gonadal tissue were made for micro-
scopic examination. Nineteen (95%) were males and one fish (5%)
appeared to contain some ova.

After capture, a sample of fish to be used in bioassays were
examined for parasites as recommended by APHA (1976). Only about half
of the fish examined were infected with monogenetic trematodes (1-2
per fish) and no internal parasites were found. Fish were allowed to
acclimate to laboratory conditions in a 830 liter fiberglassed holding
tank for a period of at least one month before being used in a bioassay.
This tank was supplied with dechlorinated water which had been filtered

through activated carbon (Table I). During this time and during the

bioassays fish were fed at least twice per day with a dried flake food.

TABLE I

CHEMICAL ANALYSES OF CONTROL WATER

Alkalinity (total, mg/l $CaCO_3$)*	148
Conductivity (μmhos/cm)*	513
Hardness (mg/l $CaCO_3$)*	198
pH*	8.25
Cr (total, mg/l)	0.08
Cu (total, mg/l)	0.14
Pb (total, mg/l)	0.04
Zn (total, mg/l)	0.01

*Values are means of measurements obtained from water in control
aquaria on Day 11 (the first day of second half of bioassay) of each
of Bioassays 1 through 8. Metal analyses were of dechlorinated,
activated carbon filtered tap water obtained in late 1976.

Description of Aquaria

The six glass aquaria used for the bioassays were 51 cm long by

26.5 cm wide by 31 cm high and could contain a volume of approximately

42 liters. In order to separate individual fish before behavioral

observation, each aquarium was divided into four cubicles by three

partitions of stainless steel sheeting. The sheeting was held in place

by guides constructed of sections of microscope slides attached to the aquaria walls with silicone sealant. These partitions allowed water exchange among the cubicles although visual contact between the fish was nearly eliminated. Similar aquaria with only one partition were used for behavioral observation aquaria (Figure 1).

Bioassay Methods

The six bioassay aquaria were placed in two rows of three aquaria-- one row above the other. Cardboard dividers were placed between the aquaria and at the ends of each row in order to prevent visual contact between fish in adjacent aquaria and also to attempt to keep the amount of light entering each aquarium equal. A 16 h photoperiod (0700 to 2300 h) was maintained in the room where the bioassays and behavioral observations were conducted. To minimize any influence of sunlight, windows in the room were covered with sheets of black plastic.

Prior to each bioassay 24 fish were removed from the holding tank and their standard lengths measured. Pairings were determined from these measurements--the shortest fish paired with the second shortest fish, the third shortest paired with the fourth shortest, etc. Six of the pairs were then randomly designated control pairs and the remaining six pairs were designated treatment pairs. At this time, for the purpose of distinguishing one individual from the other within a pair, a portion of either the upper or lower lobe of the caudal fin was removed from each fish. Since caudal fin lobes were removed from control and treatment fish, changes in observed agonistic behavior would not be attributed to fin clipping.
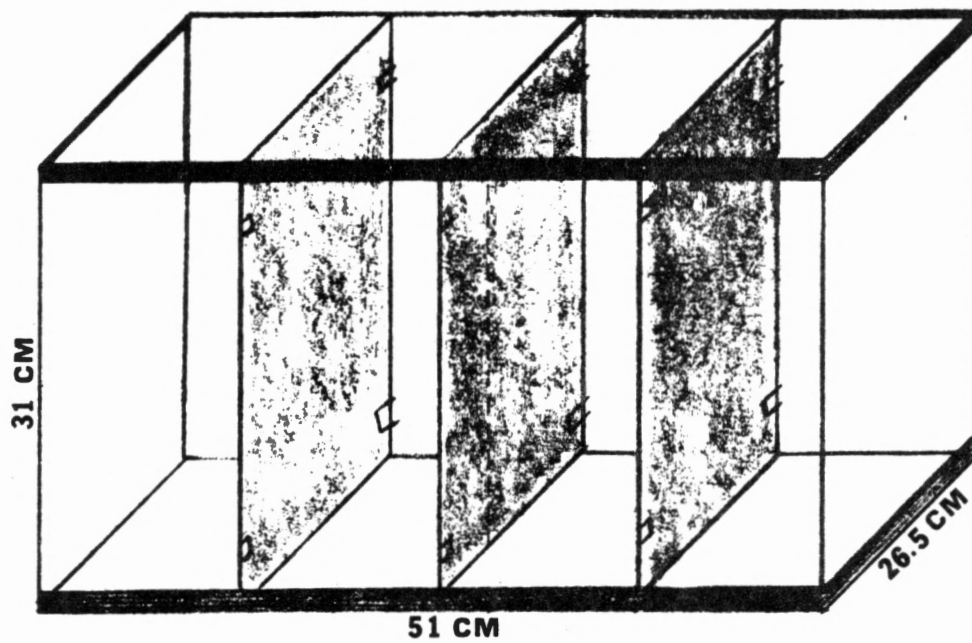
TREATMENT/CONTROL AQUARIUM
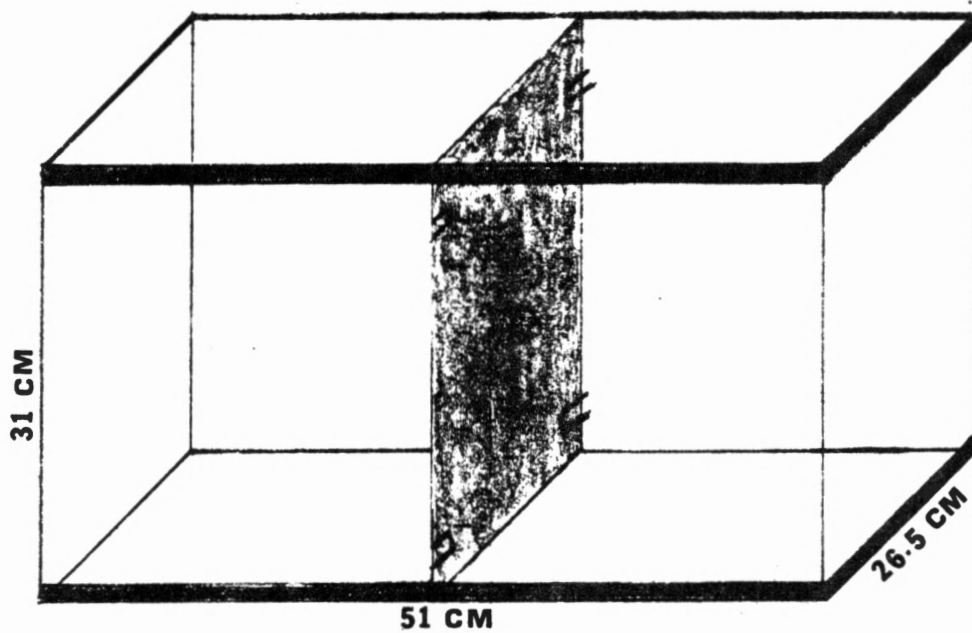
OBSERVATION AQUARIUM

Figure 1.   Control, Treatment, and Observation Aquaria
with Removable Partitions

Since the agonistic behavior frequencies of pairs were so variable, it was necessary to establish a "baseline" level of behaviors for each pair of fish. The first 10 days of the bioassay were used for this purpose. All 24 fish were placed in aquaria containing 15 liters of control water which consisted of dechlorinated tap water filtered through activated carbon. The second 10 days of the bioassay were used to expose the fish pairs to either the treatment or control condition.

The selection of 10 day periods was somewhat arbitrary, although it was influenced by Graham and Dorris (1968) who reported that fathead minnows exposed to low toxicity refinery wastewaters exhibited a sharp increase in stress behaviors and deaths after 7 to 10 days. From the standpoint of an agonistic behavioral study, this 10 day period also corresponds favorably with pre-observation isolation periods of three days used by Dennis (1970) and Powell (1972) and 14 days used by Hadley (1969).

On the same day (Day 0) that the fish were measured, they were placed in the control or treatment aquaria—one fish per cubicle. Aquaria were randomly designated as control or treatment aquaria. To minimize the possibility of intrapair auditory or olfactory communication, individuals of any one pair were placed in separate aquaria. Fish within a single aquarium were either all control or all treatment fish.

It was impractical to transport quantities of water adequate for recommended flow rates (APHA, 1975) for continuous-flow bioassays. However, Clemens and Summers (1952) have reported that toxicity of oil refinery wastewaters to red shiners (Notropis lutrensis) did not change for 20 days when the wastewater was stored at 6°C in a capped glass container. It was decided that a static bioassay with periodic

replenishment with stored wastewater would be used. Biologically treated wastewater was collected from the last of a series of three polishing lagoons. Wastewater was then transported to the laboratory in air-tight 20 liter glass jugs and stored at approximately 6°C. Fresh wastewater was collected for each bioassay.

On the first day of the "baseline" establishment period (Day 0) 15 liters of control water was placed in each aquaria. Then, at two day intervals, an additional two liters of control water was added to each of the aquaria. The water was introduced into the aquaria through four rubber tubes attached to a plastic dispenser. Each tube emptied into a separate cubicle to keep conditions in all cubicles as uniform as possible.

On Day 10, four control pairs and five treatment pairs were randomly chosen from the 12 treatment and control pairs. At approximately one hour intervals these nine pairs were placed in observation aquaria and observed as described in the following section. The fifth treatment pair served as an alternate in the event of mortality among the first four treatment pairs.

On Day 11, the fish were again placed in the same cubicle occupied during the first 10 day period. However, the treatment fish were placed in aquaria containing 15 liters of biologically treated petroleum refinery wastewater, while the control fish were again placed in 15 liters of control water. At two day intervals an additional two liters of control water or wastewater were added to the appropriate aquaria.

On Day 21, after a 10 day exposure, the agonistic behavior of eight previously observed pairs of fish was again observed. If no mortalities occurred among the originally observed treatment pairs then the first

four treatment pairs observed on Day 10 were again observed. If there was a mortality among the first four treatment pairs, the fifth pair was used as an alternate and observed again. If there were mortalities among more than one pair of the previously observed treatment pairs, the bioassay was not considered in any statistical comparison of agonistic behavior changes.

Behavioral Observations

In pairing the fish for behavioral bouts, it was decided to use pairs of fish in which both individuals were exposed to the wastewater or to the control water in order to eliminate the possibility that a non-affected control fish would stimulate or inhibit the behavior of a treatment fish. This decision was prompted by the investigations of Hale (1956) on the effect of forebrain lesions on the behavior of green sunfish. Hale found that lesioned fish were much less aggressive, but if groups of these fish were placed with some normal fish their aggressive behavior rates were much closer to the rates of normal fish. It is also likely that most of the fish present in a receiving stream would have been exposed to the pollutant.

On Day 10, four pairs of control fish and five pairs of treatment fish were randomly chosen to be observed for one hour periods. The order of observation was also random. Each pair in turn was placed in an observation aquarium (one fish on each side of the partition) and allowed to acclimate for at least one hour but no more than two hours. At the end of the acclimation period the partition was carefully lifted and the fish were observed for one hour.

Seven behavior patterns were chosen for observation. These behaviors, and definitions (modified from Dennis, 1970, and Powell, 1972) used as criteria for recording each, are listed below:

1. Approach (AP): An approach was recorded whenever one fish swam into the vicinity of the other and either displayed or elicited a display from the second fish.

2. Fin erection (FE): A fin erection was recorded whenever the medial fins were erected.

3. Bite (BT): A bite was recorded whenever mouth contact was made with an opponent. (Occasionally mutual mouthlocks occurred and a bite was recorded for both fish in these cases.)

4. Chase (CH): A chase was recorded whenever one fish was in direct pursuit of the other.

5. Opercle spread (OP): An opercle spread was recorded whenever the opercle covers were spread or flared away from the head.

6. Tail beat (TB): A tail beat session was recorded whenever the caudal peduncle of a fish was swung from side to side pushing water against the body of the other. As long as the individual beats occurred in succession without pause, only one tail beat was recorded. When the beats were separated by a pause of approximately one second or longer, this pause marked the end of a tail beat session.

7. Avoid (AV): An avoid was recorded whenever a fish, after being approached by the other fish, moved slowly away from the second fish and was not pursued.

8. Total (TOT): A sum of the first six behaviors listed above was computed. Avoids (AV) were not included because they were considered to be generally more submissive than the other six behaviors.

All observations were recorded on an Esterline-Angus event recorder wired to a keyboard (Frey and Miller, 1972). This allowed a record of frequency, duration and temporal spacing of each behavior for each fish. The appropriate key on the keyboard was pressed for the duration of each behavior observed. Data were then transcribed from the chart paper to a notebook. Cumulative totals at five minute intervals were recorded for each behavior type for individual fish. This allowed behavioral frequencies to be compared at several temporal levels between 5 and 60 minutes.

On Day 21, after exposure to either a second 10 days in control water or to the 10 days in the wastewater, the pairs of fish were again observed. To avoid an effect due to time of day, each pair of fish was observed at approximately the same time on Days 10 and 21.

The frequency of each behavior for each pair of fish per hour following the first 10 day exposure was designated as AP_{10}, FE_{10}, etc. This frequency served as a "baseline" to compare with the frequencies observed following the second 10 day exposure (AP_{20}, FE_{20}, etc.).

For each pair of fish the change in frequency of each behavior was calculated:

$$AP_{20} - AP_{10} = \Delta AP$$
$$FE_{20} - FE_{10} = \Delta FE, \text{ etc.}$$

The "Δ" values for the control pairs and treatment pairs were then compared statistically using a SAS analysis of variance computer program (Barr et al., 1976, see Appendix).

Chemical and Physical Observations

On Days 11, 13, 15, 17, and 19, following the introduction of

wastewater or control water, water samples were collected from each aquarium. Samples were analyzed for alkalinity, hardness, and pH. Alkalinity and hardness were determined as recommended by APHA (1975). A Beckman Zeromatic pH meter was used to determine pH. Carbon dioxide concentrations were calculated using the following formula (Lind, 1974):

$$mg\ CO_2/l = 1.589 \times 10^6\ [H^+] \times mg/l\ alkalinity\ as\ HCO_3^-.$$

On Days 11 through 20, dissolved oxygen and temperature were measured in each aquarium with a YSI Model 51B dissolved oxygen meter and a YSI Model 5738 probe. Conductivity was measured with a YSI Model 33 Salinity-Conductivity-Temperature Meter.

On Days 11 and 21, water samples were collected from each treatment aquarium in glass bottles for total organic carbon analysis. On Day 21, samples were collected in polyethylene bottles for analysis of chromium, lead and zinc. Samples for metal and TOC analysis were acidified to pH 2 with concentrated nitric acid immediately after collection. Total organic carbon analyses were performed on a Beckman 915 TOC Analyzer. For total metal (i.e., suspended + dissolved) analyses a volume of 100 ml of sample was reflux digested twice in 3 ml of concentrated nitric acid and dissolved in 3 ml of 50% hydrochloric acid for five minutes. The samples were then diluted to 100 ml with 0.2 N nitric acid (EPA, 1974). Samples were then analyzed with a Varian Techtron Type AA-5 atomic absorption spectrophotometer equipped with a Perkin Elmer HGA-70 heated graphite atomizer accessory.

CHAPTER III

RESULTS

Changes in Agonistic Behavior

The major objective of this investigation was to evaluate the use-
fulness of changes in fish behavior for detecting sublethal effects of
wastewaters. Therefore, exposures of test fish to oil refinery waste-
waters which resulted in acute mortality of more than one fish were not
subjected to statistical analysis of the effects of the wastewater on
agonistic behavior. Eight bioassays were performed. Three tests
(Bioassays 4, 5, and 6) resulted in two or more mortalities and were not
included in the statistical analysis. In four tests, no acute mortality
occurred during the exposure. In a fifth exposure only one mortality
occurred. In these five bioassays (Bioassays 1, 2, 3, 7, and 8) a total
of 80 fish were observed. During 160 hours of observation 10,453
approaches, fin erections, tail beat sessions, chases, bites and avoids
were recorded.

The Δ values (i.e., the per pair frequency on Day 21 minus the per
pair frequency on Day 10) obtained for each pair of fish were compared
statistically. Highly significant ($P < 0.01$) differences were found
between control and treatment Δ values for approaches, bites, and the
sum of all behaviors except avoids (i.e., TOT). A significant ($P < 0.05$)
difference was found between treatment and control Δ values for chases.

and avoids (Table II). For all types of agonistic behavior, except tail beats, the control pairs were generally more active during the second observation period (Day 21) than during the first observation (Day 10). This resulted in positive Δ values. In contrast, among the treatment pairs, the frequencies were generally reduced on Day 21 (following the 10 day wastewater exposure). This resulted in negative Δ values for the treatment pairs (Figure 2). The highest Δ values among the control group were those for fin erections and chases. The most negative Δ values among the treatment group were those for tail beats (as they were for the control group), chases and bites. Chases and bites would have to be considered the most overtly aggressive behavior types of those observed. It may be significant that these behaviors deviated most from a Δ value of zero. The greatest deviations from the control Δ values were again for chases, bites, and also fin erections.

A 60 minute observation period was considered to be more time con-suming than desirable for use in routine bioassays for wastewater monitoring. To investigate the feasibility of using shorter observation periods, the cumulative frequencies obtained at the end of 15 and 30 minutes of the 60 minute observation periods were compared using the same methods utilized for the 60 minute observation period. For the 30 minute observation period, significant (P < 0.05) differences were found between treatment and control Δ values for approaches, bites, and avoids. A highly significant (P < 0.01) difference in Δ values of total (TOT) behaviors was found (Table III). A comparison of Δ values for the various behavior types shows that Δ values are lower in the treatment group (Figure 3). For the 15 minute observation period, significant (P < 0.05) differences were found between the treatment and control Δ

TABLE II

RESULTS OF STATISTICAL ANALYSIS OF TREATMENT,
RUN, AND INTERACTION EFFECTS AFTER
60 MINUTES OF OBSERVATION

Behavior	Mean Square of Sources of Variation			
	MS_{TRT}	MS_{RUN++}	$MS_{RUN \times TRT}$	MS_{ERROR}
Approaches	940.90**	202.60	59.90	109.12
Fin Erections	4040.10$^+$	2718.63	1176.35	1053.98
Tail Beats	62.50	99.16	164.06	176.03
Chases	3385.60*	204.09	541.41	699.18
Bites	3348.90**	445.15	381.28	299.37
Opercle Spreads	1050.63	170.40	786.13	667.28
Avoids	96.10*	37.34*	28.67	13.32
Total	62805.62**	4380.06	6790.19	6555.73

*Significant (P < 0.05) probability of effect.

**Highly significant (P < 0.01) probability of effect.

$^+$Approaches significant (P < 0.05) probability of effect.

$^{++}$Run effect refers to variation between individual bioassays.
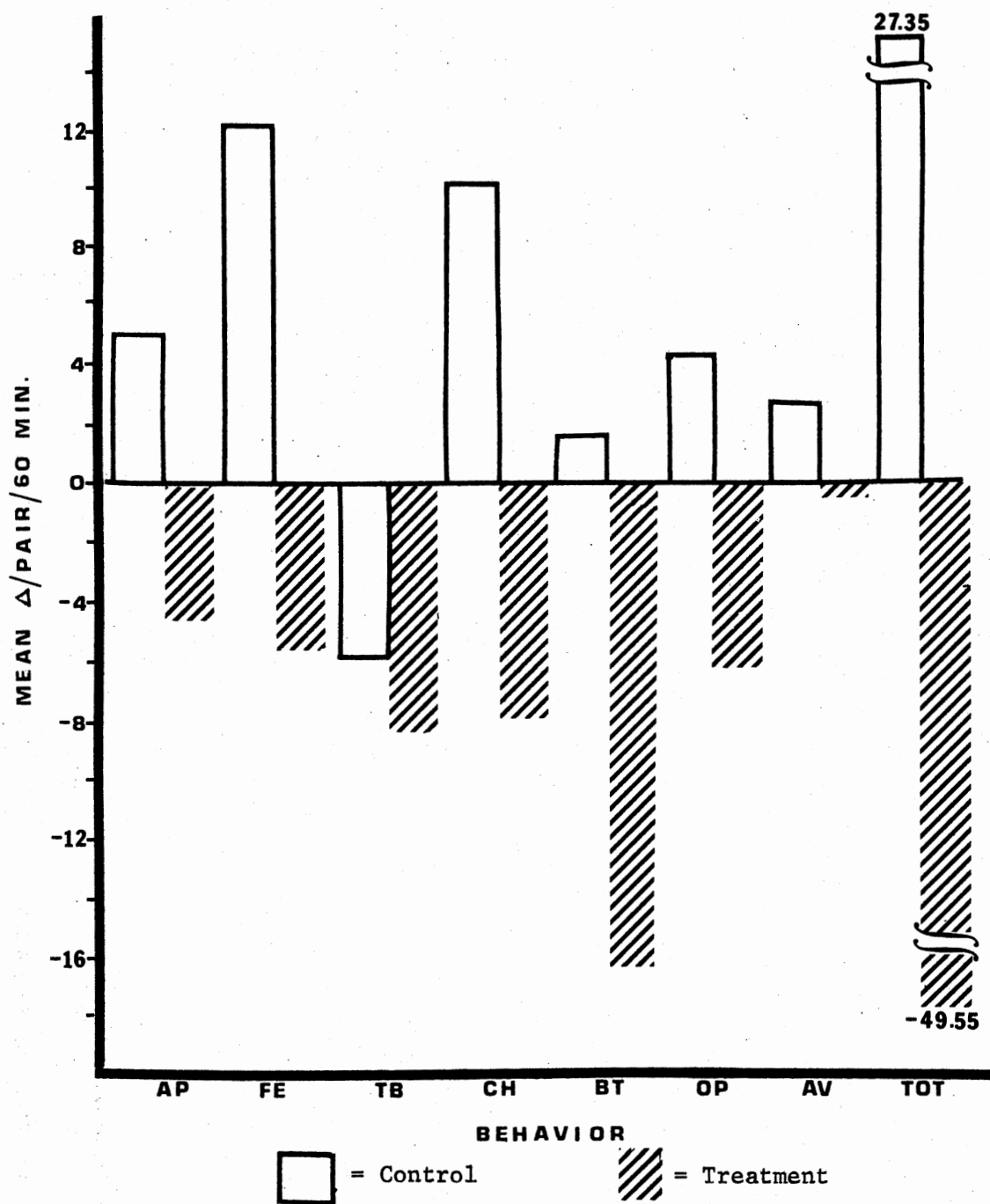
Figure 2.   Mean Treatment and Control Δ Values After
60 Minutes of Observation

TABLE III

RESULTS OF STATISTICAL ANALYSIS OF TREATMENT,
RUN, AND INTERACTION EFFECTS AFTER
30 MINUTES OF OBSERVATION

Behavior	Mean Square of Sources of Variation			
	MS_{TRT}	MS_{RUN}++	$MS_{RUN \times TRT}$	MS_{ERROR}
Approaches	291.60*	82.96	37.04	41.93
Fin Erections	1562.50+	762.10	217.63	392.48
Tail Beats	46.23	210.46+	38.79	81.59
Chases	455.63	147.13	50.13	156.66
Bites	462.40*	110.90	56.90	73.25
Opercle Spreads	40.00	123.28	118.88	105.42
Avoids	48.40*	15.29	12.96	7.17
Total	12673.60**	1324.44	751.29	1637.32

*Significant (P < 0.05) probability of effect.

**Highly significant (P < 0.01) probability of effect.

+Approaches significant (P < 0.05) probability of effect.

++Run effect refers to variation between individual bioassays.
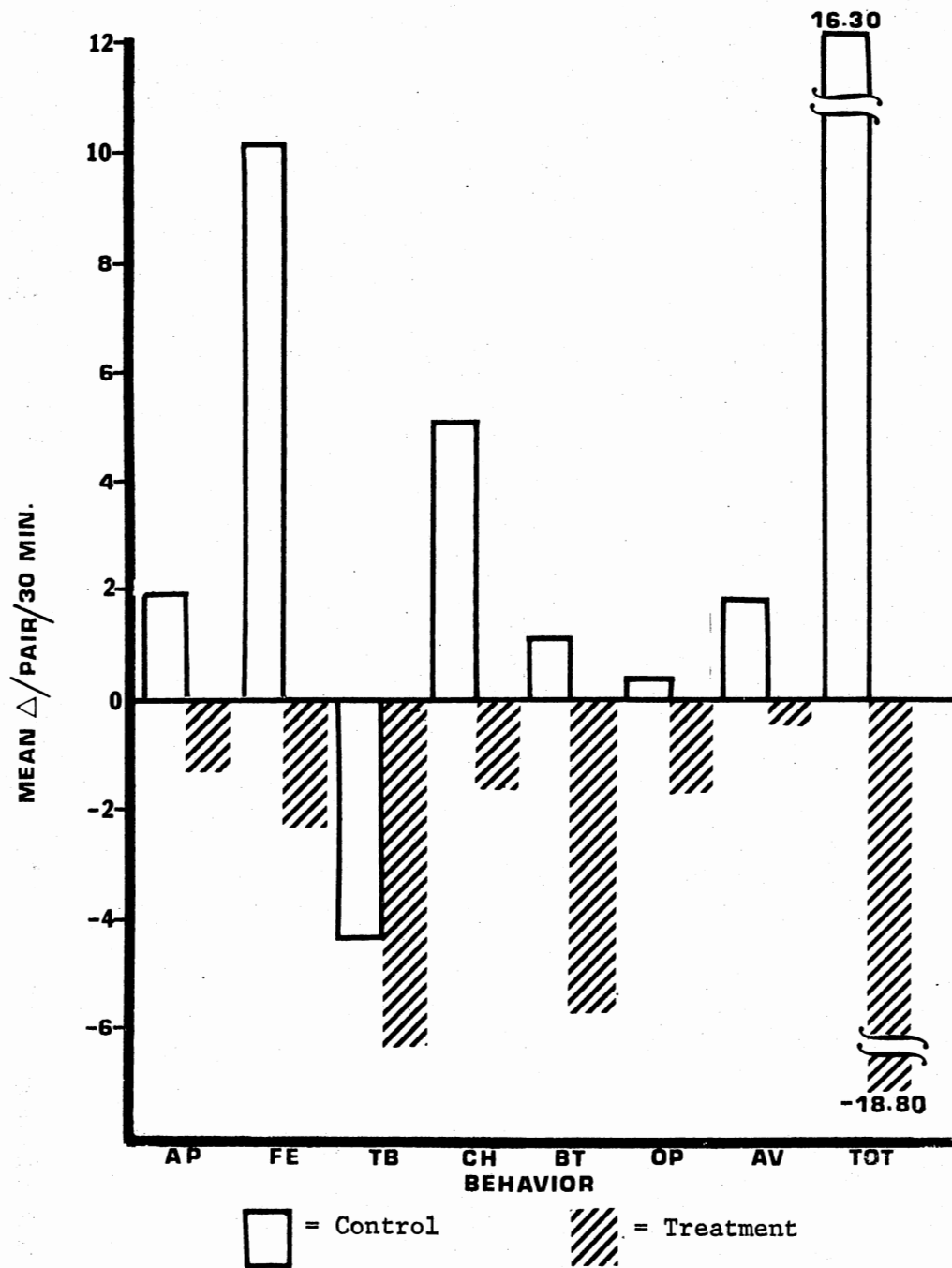
Figure 3.   Mean Treatment and Control Δ Values After
30 Minutes of Observation

values for approaches and avoids. Highly significant (P < 0.01) differences were found for Δ values of fin erections and TOT (Table IV). Treatment Δ values are lower than control Δ values (Figure 4).

Seasonal Variation in Agonistic
Behavior Rates

To test for differences in frequency of agonistic behavior rates due to season, the frequency of all behavior types of all pairs of fish observed on Day 10 of Bioassays 1 through 8 were compared using a SAS computer program to perform Duncan's new multiple range test (Steel and Torrie, 1960; Barr et al., 1976). On Day 10 treatment and control fish had been exposed to control conditions only and so both of these groups were considered together. For two types of behaviors, tail beats and bites, the fish of Bioassay 7 were significantly more active than the fish of other bioassays (Figure 5). The number of chases in Bioassay 7 was significantly greater than the number in any of the other bioassays except Bioassay 8. Bioassays 7 and 8 were both conducted during mid-summer. Bioassay 4, conducted during mid-December, usually had the lowest mean frequency of the eight bioassays.

Mortalities and Non-Quantified Sublethal
Effects in Bioassays 4, 5 and 6

The wastewaters used in Bioassays 4, 5 and 6 were the most toxic of the wastewaters collected. In addition to mortalities, several sublethal and pre-lethal effects were observed. These were changes in eye color, ability to retain equilibrium, feeding habits, and irritability. Except for loss of equilibrium, these changes were observed in non-lethal

TABLE IV

RESULTS OF STATISTICAL ANALYSIS OF TREATMENT,
RUN, AND INTERACTION EFFECTS AFTER
15 MINUTES OF OBSERVATION

Behavior	Mean Square of Sources of Variation			
	MS_{TRT}	$MS_{RUN}+$	$MS_{RUN \times TRT}$	MS_{ERROR}
Approaches	75.63*	27.03	13.88	10.83
Fin Erections	1010.03**	146.94	15.96	105.36
Tail Beats	8.10	127.06	63.29	60.33
Chases	34.23	22.85	5.73	26.08
Bites	48.40	18.67	15.09	28.63
Opercle Spreads	1.60	22.15	8.73	14.27
Avoids	4.90*	0.46	0.84	0.98
Total	3294.23**	217.34	291.42	365.85

*Significant (P < 0.05) probability of effect.

**Highly significant (P < 0.01) probability of effect.

+Run effect refers to variation between individual bioassays.
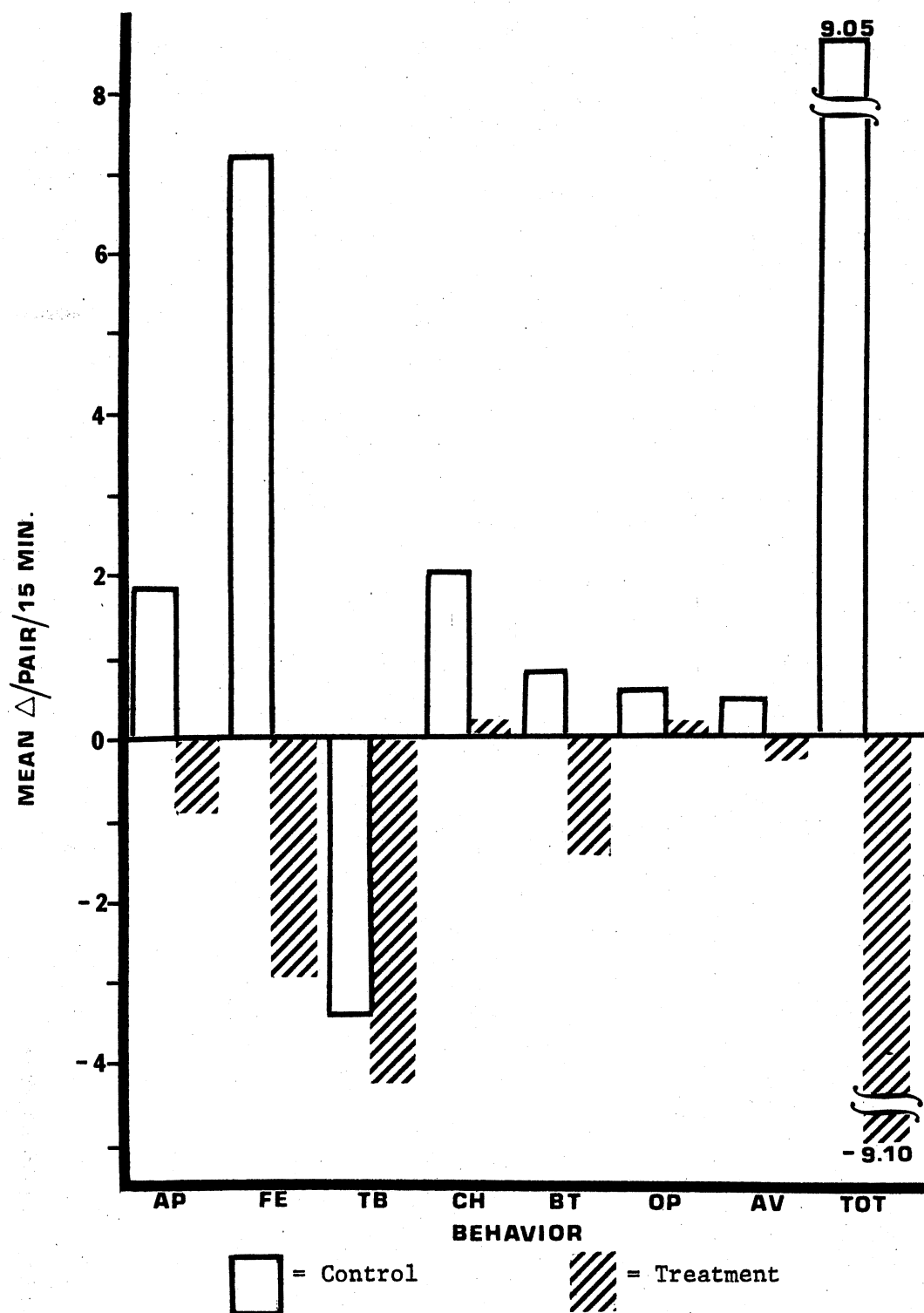
Figure 4. Mean Treatment and Control Δ Values After
15 Minutes of Observation

Bioassay 1—late October   Bioassay 5—early March
Bioassay 2—mid-October   Bioassay 6—mid-May
Bioassay 3—late October   Bioassay 7—early July
Bioassay 4—mid-December   Bioassay 8—early August

Approaches   7   3   6   5   1   8   2   4

Fin erections   3   7   1   2   8   6   5   4

Tail beats   7   8   1   3   5   2   6   4

Chases   7   8   5   1   3   6   2   4

Bites   7   8   1   5   6   3   2   4

Opercle spreads   8   5   7   6   2   3   4   1

Avoids   5   7   6   3   8   4   2   1

Totals   7   3   8   1   5   6   2   4

◄——— Higher Mean Frequency

Lower Mean Frequency ——►

Any two means not underscored by the same line are significantly different ($p < 0.05$).

Figure 5. Seasonal Comparison of Behavioral Frequencies

bioassays also. These observations are mentioned here to illustrate that there are other effects of sublethal petroleum refinery wastewaters on orangespotted sunfish and to provide possible insights for future researchers.

The wastewater used in Bioassay 4 was lethal to five fish. Several stress symtpoms were noticed after fish had been exposed to the wastewater for less than one hour. The wastewater was quickly diluted at that time by adding 6 liters of control water to the 15 liters of wastewater. The most noticeable stress symptoms were loss of equilibrium, exaggerated ventilatory movements, and gulping at the water surface (even though D.O. concentration was 6.4 mg/1 and CO_2 concentration was 3-4 mg/1). They also were extremely sensitive to sounds and movements, darting away very quickly when the sides of the aquaria were tapped with a finger. Occasional erratic swimming and loss of bouyancy were observed. By the end of the second day stress symptoms were reduced and no mortalities occurred after that time.

After a wastewater exposure of only six days, two pairs of fish were observed for a one hour period. These fish had not been previously paired with each other so there was no "baseline" with which to compare this observation. Agonistic behavior of these fish was limited primarily to approaches, and to fin erections which were generally not very pronounced. Also, the usual orange to red iris coloration was totally lacking in all four fish. Aside from lack of iris coloration and low agonistic behavior frequency, none of the four fish appeared to be stressed by the wastewater.

During Bioassay 5 five deaths occurred between the third day and tenth day of exposure to the wastewater. No evident stress behavior,

other than lethargy, was observed before the deaths.

During Bioassay 6, two deaths occurred. Both fish died during the tenth day of exposure and were in the same aquarium. As in Bioassay 4, CO_2 and D.O. concentrations should not have been lethal. None of the other fish of the bioassay appeared to be unusually stressed.

During the wastewater exposure periods of most of the bioassays, treatment fish appeared to be less willing to accept food. This was especially true during the first few days of an exposure.

<center>Physicochemical Parameters</center>

Calculation of Daily Means

Various parameters were measured daily in each of the three control and three treatment aquaria. Other parameters were measured in the six aquaria on alternate days, or only in treatment aquaria at the beginning and end of the wastewater exposure period. For each day that a parameter was measured, a daily mean for control aquaria and a daily mean for treatment aquaria were calculated by summing the three measurements and dividing by three.

Temperature

Water temperature was measured daily in all three control aquaria and in all three treatment aquaria (Table V). During the eight bioassays the temperatures ranged from 16.1 to 27.5°C in the control aquaria and from 16.0 to 27.8°C in the treatment aquaria. The difference between the means of the control aquaria and the treatment aquaria on any one day was never more than 1.4°C.

TABLE V

MEAN DAILY TEMPERATURE IN BIOASSAY AQUARIA

Bio-assay Number	Control or Treatment	Day									
		11	12	13	14	15	16	17	18	19	20
		°C ($\pm$ Std. Dev.)									
1	(C)	–	21.0+.1	20.8+.6	19.3+.1	–	21.1+.2	22.0+.0	21.0+.0	21.0+.5	21.3+.2
	(T)	–	20.9+.1	20.2+.1	19.0+.0	–	20.8+.0	22.0+.0	21.0+.0	21.0+.2	21.0+.0
2	(C)	–	–	–	–	20.8+.8	–	22.0+.1	22.7+.3	–	21.3+.2
	(T)	–	–	–	–	20.5+.4	–	21.5+.5	22.0+.7	–	20.9+.2
3	(C)	22.9+.2	–	23.4+.4	21.5+.0	19.0+.1	–	–	–	–	19.9+.0
	(T)	22.2+.2	–	23.0+.1	21.0+.0	18.9+.2	–	–	–	–	19.8+.2
4	(C)	18.9+.0	–	–	–	–	–	–	–	–	–
	(T)+	19.3+.2	19.8+.4	–	–	19.4+.5	–	–	–	–	–
5	(C)	–	–	–	–	–	17.7+1.0	–	–	16.6+.5	17.4+.5*
	(T)	–	–	–	–	–	16.3+.4	–	–	17.8+.3	17.0+.5*
6	(C)	20.4+.4	–	21.0+.1	21.4+.3	22.2+.3	22.1+.1	22.0+.1	22.3+.4	22.1+.3	22.2+.1
	(T)	20.6+.1	–	21.0+.1	21.1+.1	22.0+.0	21.8+.2	21.9+.1	22.0+.1	22.0+.1	21.4+.6
7	(C)	23.5+.0	23.9+.1	23.0+.1	25.0+.1	27.3+.4	25.6+.4	22.5+.4	22.9+.1	23.9+1.0	22.4+.1
	(T)	23.4+.1	23.5+.3	23.4+.0	25.0+.0	27.5+.5	26.2+.1	23.0+.1	22.7+.2	23.0+.0	22.5+.1
8	(C)	21.3+.3	21.7+.2	21.2+.2	21.0+.0	–	21.6+.2	21.7+.0	22.7+1.0	23.1+.2	21.1+.1
	(T)	21.3+.1	21.4+.1	21.2+.0	21.3+.1	–	21.8+.2	20.9+.4	21.8+.2	23.7+.1	21.6+.1

+Treatment temperatures measured following dilution of wastewater.

*Temperatures measured on Day 21.

Dissolved Oxygen

Dissolved oxygen concentrations ranged from 4.0 to 8.2 mg/1 in the control aquaria and from 1.8 to 8.6 mg/1 in the treatment aquaria during the eight bioassays (Table VI). In most cases the D.O. of the waste-water was less than that of the control. The maximum difference between control and treatment aquaria means on a single day was 3.1 mg/1. Generally there was a decrease in D.O. in both control and treatment aquaria during Days 11 through 21. The water replenishments made on alternate days usually temporarily reversed the decrease in D.O. The decrease in D.O. was more pronounced in the treatment aquaria. Aeration was used only on one occasion and then only for approximately five minutes.

The D.O. concentrations in the treatment aquaria of the most lethal bioassays (Bioassays 4, 5 and 6) were not considered to be directly responsible for mortality since they compared favorably with concentrations measured in non-lethal bioassays. However, low D.O. concentrations have been reported to increase toxicity of various pollutants such as lead, copper, zinc, phenols, and ammonia (Lloyd, 1961; Pickering, 1968).

Conductivity

Conductivity ranged from 480 to 620 μmhos/cm in the control aquaria and from 1990 to 3003 μmhos/cm in the treatment aquaria during the eight bioassays (Table VII). The conductivity generally increased during the 10 days that it was monitored. Since conductivity is related to ionic concentration this would be expected to occur as a result of evaporation.

TABLE VI

MEAN DAILY DISSOLVED OXYGEN CONCENTRATION IN BIOASSAY AQUARIA

Bio-assay Number	Control or Treatment	Day									
		11	12	13	14	15	16	17	18	19	20
						mg/1 (± Std. Dev.)					
1	(C)	–	6.4+.1	7.0+.1	6.9+.1	–	6.6+.2	6.1+.2	6.1+.2	5.9+.1	5.6+.4
	(T)	–	6.1+.3	5.5+.1	5.7+.1	–	5.7+.1	4.7+.1	4.7+.1	4.9+.2	4.7+.2
2	(C)	–	–	–	–	6.3+.1	–	6.4+.2	7.0+.1	–	6.7+.1
	(T)	–	–	–	–	6.0+.0	–	6.2+.0	6.4+.2	–	5.6+.7
3	(C)	–	–	6.5+.1	6.4+.0	6.8+.2	–	–	–	–	6.8+.1
	(T)	–	–	4.9+.5	4.2+.2	5.0+.5	–	–	–	–	4.3+.7
4	(C)	8.2+.0	–	–	–	–	–	–	–	–	–
	(T)	6.7+.1	6.7+.1	–	3.9+.9	5.8+.4	–	–	–	–	–
5	(C)	–	–	–	–	–	7.5+.4	–	–	7.9+.4	7.2+.4*
	(T)	–	–	–	–	–	7.3+.4	–	–	6.5+.2	5.9+1.0*
6	(C)	7.6+.2	–	6.9+.1	6.8+.2	6.4+.1	5.4+.3	5.8+.3	5.6+.1	5.9+.1	5.6+.1
	(T)	8.5+.1	–	6.4+.4	5.8+.5	5.3+.3	3.6+.1	4.1+.1	3.6+.2	4.4+.2	4.1+.3
7	(C)	6.9+.2	6.0+.4	6.2+.3	5.6+.1	4.3+.2	4.1+.1	5.0+.2	5.2+.1	5.4+.2	5.5+.2**
	(T)	5.3+.3	5.1+.3	5.1+.2	4.4+.6	2.9+1.0	2.3+.5	2.4+.1	2.2+.0	2.3+.5	3.4+1.5**
8	(C)	6.6+.1	6.5+.1	6.6+.1	6.3+.1	–	5.6+.1	6.0+.1	5.7+.1	5.6+.1	5.5+.1
	(T)	6.3+.2	6.2+.2	6.1+.4	5.7+.4	–	4.6+.9	4.8+.9	4.3+.6	4.0+.7	3.7+1.0

+6.4°C prior to dilution.

*Measured on Day 21.

**Measured following five minutes of aeration.

TABLE VII

MEAN DAILY CONDUCTIVITY IN BIOASSAY AQUARIA

Bioassay Number	Control or Treatment	Day									
		11	12	13	14	15	16	17	18	19	20
		μmhos/cm ($\pm$ Std. Dev.)									
1	(C)	–	–	482±2	481±2	–	587±25	597±6	560±17	567±12	577±12
	(T)	–	–	2000±0	2007±6	–	2043±6	2110±10	2475±21	2050±0	2110±0
2	(C)	–	–	–	–	557±29	–	590±0	590±0	–	583±12
	(T)	–	–	–	–	2330±30	–	2403±6	2457±21	–	2443±42
3	(C)	533±6	–	567±6	560±0	510±0	–	–	–	–	543±15
	(T)	2303±6	–	2390±10	2390±6	2210±10	–	–	–	–	2260±0
4	(C)	500±0	–	–	–	–	–	–	–	–	–
	(T)	2013±32[+]	–	–	–	2110±10	–	–	–	–	–
5	(C)	–	–	–	–	–	573±31	–	–	590±36	590±26*
	(T)	–	–	–	–	–	2750±87	–	–	2920±147	3003±159*
6	(C)	–	–	490±0	580±0	583±6	587±6	583±6	590±0	513±6	570±0
	(T)	–	–	2400±0	2443±12	2497±6	2507±6	2500±0	2533±21	2107±6	2587±23
7	(C)	510±0	500±0	500±0	510±0	580±20	580±10	507±6	513±6	510±0	520±0
	(T)	2013±12	2020±0	2070±0	2150±0	2267±12	2253±6	2110±10	2107±6	2110±0	2110±10
8	(C)	507±6	510±0	510±0	510±0	–	520±0	533±6	570±0	597±6	583±6
	(T)	2407±12	2427±6	2460±10	2480±0	–	2520±0	2480±17	2587±23	2710±20	2607±12

[+]2700 μmhos/cm prior to dilution.

*Measured on Day 21.

The wastewaters used in Bioassays 4 and 5 were the most toxic, each lethal to five fish, and also were at least 200 to 300 µmhos/cm higher in conductivity than the wastewaters used in the other bioassays. The importance of this is questionable since, depending on the ions involved, a conductivity of 3000 µmhos/cm is not extremely high even for naturally occurring waters.

pH

During the eight bioassays, pH ranged from 7.2 to 8.7 in control aquaria and from 6.2 to 8.7 in the treatment aquaria (Table VIII). The pH in control aquaria was almost always higher than the pH of the waste-water aquaria. Both the control water and the wastewater decreased in pH during the 10 day period.

Alkalinity

In all cases total alkalinity was determined to be due to bicar-bonate ions since no hydroxide or carbonate ions were indicated by phenolpthalein titrations. Total alkalinity ranged from 137 to 160 mg/l in the control aquaria and from 4 to 102 mg/l in treatment aquaria (Table IX). Alkalinity of control water was nearly constant over the 10 day period while the alkalinity of the wastewaters generally de-creased.

Hardness

The water hardness ranged from 176 to 229 mg/l in the control aquaria and from 345 to 470 mg/l in the treatment aquaria (Table X).

TABLE VIII

MEAN DAILY pH IN BIOASSAY AQUARIA

Bio-assay Number	Control or Treatment	Day									
		11	12	13	14	15	16	17	18	19	20
		pH (± Std. Dev.)									
1	(C)	–	–	8.2+.1	8.0+.2	–	8.0+.2	8.0+.2	8.0+.1	8.0+.1	7.9+.1
	(T)	–	–	7.5+.2	7.2+.1	–	7.2+.1	7.1+.2	7.2+.1	7.2+.1	7.0+.2
2	(C)	–	–	–	–	8.0+.1	–	8.1+.0	8.1+.0	8.1+.0	8.0+.1
	(T)	–	–	–	–	7.4+.1	–	7.4+.1	7.3+.1	7.2+.1	7.0+.1
3	(C)	8.1+.2	–	7.8+.2	8.1+.1	8.0+.1	–	–	–	–	8.0+.1
	(T)	7.4+.1	–	7.2+.1	7.0+.2	7.0+.1	–	–	–	–	6.3+.2
4	(C)	–	–	–	–	–	–	–	–	–	–
	(T)	7.5+.0[+]	7.6+.1	–	–	–	–	–	–	–	–
5	(C)	–	–	–	–	–	–	–	–	–	–
	(T)	–	–	–	–	–	–	–	–	–	–
6	(C)	8.4+.1	–	8.0+.2	7.9+.1	7.8+.1	7.8+.1	7.9+.1	7.8+.1	7.8+.1	7.8+.1
	(T)	8.6+.1	–	7.3+.1	6.8+.1	6.7+.1	6.5+.1	6.4+.1	6.4+.1	6.4+.1	6.3+.1
7	(C)	8.5+.0	8.6+.1	8.6+.1	8.5+.1	8.5+.1	8.5+.0	8.4+.0	8.5+.0	8.5+.0	7.8+.0
	(T)	8.2+.1	8.2+.0	8.2+.0	8.1+.1	7.9+.1	7.9+.1	7.7+.2	7.7+.1	7.6+.0	6.9+.0
8	(C)	8.0+.0	7.9+.1	7.9+.1	8.0+.0	–	7.8+.0	7.2+.0	7.8+.0	7.8+.1	7.6+.1
	(T)	7.3+.0	7.3+.1	7.3+.1	7.3+.1	–	7.3+.0	6.8+.3	6.8+.3	6.7+.3	6.7+.5

[+]7.2 prior to dilution.

TABLE IX

MEAN DAILY TOTAL ALKALINITY IN BIOASSAY AQUARIA[+]

Bioassay Number	Control or Treatment	Day				
		11	13	15	17	19
		mg/l as $CaCO_3$ (± Std. Dev.)				
1	(C)	150+2	154+2	149+10*	155+1	156+1
	(T)	21+1	24+1	23+2*	25+1	23+1
2	(C)	–	–	150+0	157+0*	159+1
	(T)	–	–	35+0	34+1*	31+3
3	(C)	145+1	149+1	152+1	–	152+2**
	(T)	35+1	31+2	26+7	–	7+5
4	(C)	–	–	–	–	–
	(T)	65+0[++]	66+2**	–	–	–
5	(C)	–	–	–	–	–
	(T)	–	–	–	–	–
6	(C)	153+1	153+3	158+2	155+0	157+1
	(T)	24+1	19+2	19+5	11+1	11+2
7	(C)	147+4	144+2	146+1	147+1	151+1
	(T)	88+12	82+2	77+11	64+2	57+9
8	(C)	146+1	149+1	–	157+2	159+1
	(T)	52+2	52+2	–	41+13	34+18

[+]No carbonate or hydroxide alkalinity indicated by titration.

[++]32 mg/l prior to dilution.

*Measured one day later than indicated.

**Measured one day earlier than indicated.

TABLE X

MEAN DAILY HARDNESS IN BIOASSAY AQUARIA

Bioassay Number	Control or Treatment	Day				
		11	13	15	17	19
		mg/1 as $CaCO_3$ (+ Std. Dev.)				
1	(C)	223+7	199+5	221+8*	197+5	197+8
	(T)	399+43	356+6	358+14*	356+6	358+5
2	(C)	-	-	188+0	201+5*	212+4
	(T)	-	-	395+9	418+9*	413+8
3	(C)	200+4	197+5	200+4	-	202+4*
	(T)	383+5	388+4	384+4	-	400+4*
4	(C)	-	-	-	-	-
	(T)	349+0+	383+8**	-	-	-
5	(C)	-	-	-	-	-
	(T)	-	-	-	-	-
6	(C)	203+2	200+4	212+12	211+5	214+8
	(T)	402+16	399+12	404+11	403+2	470+0
7	(C)	184+0	188+4	191+2	193+2	203+8
	(T)	368+4	380+4	381+6	391+6	389+2
8	(C)	182+6	188+0	-	201+2	207+2
	(T)	391+2	395+2	-	418+8	413+6

+412 mg/1 prior to dilution.

*Measured one day later than indicated.

**Measured one day earlier than indicated.

The hardness of both control water and wastewater generally increased during the 10 day period.

Carbon Dioxide

During the eight bioassays CO_2 concentration ranged from 0.4 to 15.9 mg/l in control aquaria and from 0.1 to 13.6 mg/l in treatment aquaria (Table XI). Concentrations were generally higher in treatment aquaria and increased with time in both treatment and control aquaria.

Total Organic Carbon

During the eight bioassays total organic carbon concentrations in treatment aquaria on Day 11 ranged from 8.02 to 196.82 mg/l (Table XII). TOC concentration on Day 21 ranged from 8.62 to 77.60 mg/l. Comparison of TOC with number of deaths indicates a possible but inconsistent correlation between TOC and mortality in the bioassays. TOC concentrations were greater than 20 mg/l in three of the four bioassays in which mortality occurred. Concentrations greater than 20 mg/l were measured in all three of the bioassays which, because of excessive mortality, were not considered in statistical comparisons of agonistic behavior changes. The high TOC concentrations measured during Bioassay 6 were partially a result of a large population of phytoplankton. Only one of the four bioassays in which no mortalities occurred had a TOC concentration greater than 20 mg/l. Burks and Wilhm (1978) reported a correlation between TOC and fathead minnow mortality which was better than any correlation that could be shown between mortality and ammonia or metals.

TABLE XI

MEAN DAILY CO_2 CONCENTRATION IN BIOASSAY AQUARIA

Bioassay Number	Control or Treatment	Day				
		11	13	15	17	19
		mg/1 ($\pm$ Std. Dev.)				
1	(C)	–	1.7+.3	2.5+.8*	2.5+.6	2.2+.3
	(T)	–	1.4+.5	2.2+.7*	2.7+.6	2.6+1.0
2	(C)	–	–	2.1+.2	2.0+.0*	2.0+.0
	(T)	–	–	2.4+.4	2.4+.4*	3.1+.4
3	(C)	1.8+.6	3.8+1.9	2.6+.4	–	2.5+.6*
	(T)	2.2+.5	3.4+.6	4.0+1.1	–	5.7+2.9
4	(C)	–	–	–	–	–
	(T)	3.0+.0+	3.0+.5**	–	–	–
5	(C)	–	–	–	–	–
	(T)	–	–	–	–	–
6	(C)	1.0+.2	2.8+.9	3.8+1.1	3.4+.5	4.0+1.0
	(T)	0.1+.0	1.6+.3	6.1+2.4	6.8+1.3	6.7+.5
7	(C)	0.7+.0	0.6+.2	0.8+.1	0.9+.0	0.8+.0
	(T)	1.0+.1	0.8+.0	1.4+.2	1.9+.1	2.2+.4
8	(C)	2.3+.1	3.2+.4	–	15.7+.2	4.3+.6
	(T)	4.2+.1	4.3+.4	–	9.6+3.6	9.6+1.7

+3.6 mg/1 prior to dilution.

*Measured one day later than indicated.

**Measured one day earlier than indicated.

TABLE XII

MEAN TOTAL ORGANIC CARBON AND NUMBER OF
MORTALITIES IN TREATMENT AQUARIA

Bioassay Number	TOC (mg/l)		Number of Mortalities
	Day 11	Day 21	
1	18.57 ± 2.53	17.01 ± 2.30	0
2	–	31.23 ± 16.9	0
3	18.21 ± 1.77	18.55 ± 0.21	0
4	31.93[+], 47.05 ± 1.03	–	5*
5	33.86 ± 21.13	25.16 ± 4.99	5
6	104.16 ± 81.29	46.26 ± 27.35	2
7	13.19 ± 3.28	10.19 ± 1.22	1
8	9.58 ± 2.27	10.36 ± 2.97	0

[+]TOC of diluted wastewater on Day 12. Second number is TOC of undiluted wastewater taken from a collection jar on Day 12.

*Wastewater was diluted after approximately one hour when severe stress was noticed. Mortalities occurred in the diluted wastewater. (15 liters wastewater:6 liters control water).

Heavy Metals

Total chromium concentrations in water sampled from treatment aquaria during the bioassays ranged from <0.02 mg/l to 0.15 mg/l (Table XIII). Highest concentrations were found in the three most lethal bioassays. Pickering and Henderson (1966) obtained static hardwater (360 mg/l) 96 h bioassay LC50 values of approximately 30 mg/l for fathead minnows and 133 mg/l for bluegill sunfish. A proposed EPA criterion for freshwater aquatic life is 0.1 mg/l (EPA, 1976).

Total lead concentrations ranged from <0.01 mg/l to 0.02 mg/l. Concentrations found in water collected from the three most lethal bioassays were as low or lower than concentrations found in non-lethal bioassays. Pickering and Henderson (1966) report a 96 h LC50 of 482 mg/l for fathead minnows in hardwater static bioassays. For rainbow trout (Salmo gairdneri) in hardwater (300 mg/l), the 96 h LC50 was found to be 471 mg/l total lead and 1.38 mg/l dissolved lead (Davies and Everhart, 1973). A proposed EPA criterion for freshwater is 0.01 times the 96 h LC50 of soluble lead using a sensitive resident species (EPA, 1976).

Total zinc concentrations (Day 21) ranged from 0.02 to 0.46 mg/l during the eight bioassays. Again the highest concentrations occurred in the water sampled from the most toxic bioassays. Pickering and Henderson (1966) found the 96 h LC50 of fathead minnows in hardwater to be 33 mg/l. A proposed EPA criterion for freshwater is 0.01 times the 96 h LC50 of a sensitive resident species.

TABLE XIII

MEAN CHROMIUM, LEAD, AND ZINC CONCENTRATIONS AND
NUMBER OF MORTALITIES IN TREATMENT AQUARIA

Bioassay Number	mg/1*			Number of Mortalities
	Cr	Pb	Zn	
1	0.03 ± .02	0.02 ± .01	0.09 ± .05	0
2	0.04 ± .01	0.01 ± .01	0.11 ± .05	0
3	0.04 ± .01	< 0.01	0.08 ± .00	0
4	0.12[+]	< 0.01[+]	0.46[+]	5[++]
	0.14 ± .01	< 0.01	0.11 ± .01	
5	0.04 ± .01	< 0.01	0.20 ± .03	5
6	0.04 ± .01	0.01 ± .01	0.13 ± .02	2
7	0.02 ± .00	0.02 ± .02	0.03 ± .00	1
8	0.03 ± .01	< 0.01	0.03 ± .01	0

*Total metal concentrations in treatment aquaria on Day 21 of Bioassays 1, 2, 3, 5, 6, 7 and 8 and on Day 12 of Bioassay 4.

[+]Concentration in diluted wastewater. Second concentration is of non-diluted wastewater taken from a collection jar on Day 12.

[++]Wastewater was diluted after approximately one hour when severe stress was noticed. Mortalities occurred in the diluted wastewater (15 liters wastewater:6 liters control water).

CHAPTER IV

CONCLUSIONS

The methods used were able to detect decreases in the frequency of several agonistic behaviors as a result of exposure to a sublethal concentration of a biologically treated petroleum refinery wastewater. Statistically significant decreases were found in frequencies of approaches, fin erections, chases, bites and avoids. A parameter composed of all seven behavior types, except avoids, was also significantly affected. For every behavior type and at each of the three temporal levels of observation, the mean Δ value of the wastewater exposed fish was less than the mean Δ value of the control fish, even though these differences were not always statistically significant (Figures 2, 3, and 4).

A 15 minute observation period was sufficient to detect changes in agonistic behavior frequencies. This result was unexpected since it seemed that in many cases during the first five minutes of observation the fish were overcoming the disturbance caused by the lifting of the observation aquarium partition.

There is some indication that fish were more active during the summer months and less active during the winter. For most behavior types, the fish used in Bioassay 7 (early July) were the most active and the fish used in Bioassay 4 (mid-December) were the least active.

Chemical and physical analyses performed during the bioassays revealed that the most lethal bioassays also contained the highest concentrations of chromium, zinc, total organic carbon and also had the highest conductivity readings.

CHAPTER V

DISCUSSION AND RECOMMENDATIONS

Behavioral changes which are potentially deleterious to the organism were detected by the bioassays conducted during this study. Such effects would not have been detected by standard bioassays measuring acute or chronic (up to 10 days) mortality only. While bioassays using acute mortality to measure toxicity of wastewaters serve a useful purpose, it is the responsibility of aquatic biologists to also consider the possibility of sublethal effects of wastewaters. Testing for sublethal effects can be expensive and/or time consuming and therefore is not attractive to industry and biologists involved in monitoring effluents. Changes in growth rates, reproductive rates, mutation rates, etc., may require months to be detected. Equipment needed for conditioned response-learning tests, swimming ability tests, and "cough" response tests probably prohibits more widespread use of these techniques.

The behavioral bioassay technique described in this paper has the advantage of being very inexpensive; it does not require a large amount of expertise, does not require a long exposure period or observation time, and is sensitive. The discovery that two observation periods of only 15 minutes duration were sufficient to detect changes in agonistic behavior improved the attractiveness of this test in terms of sensitivity and time expended. In the simplest case, one observer would be needed

to record on a data sheet the number of fin erections per pair of fish during the 15 minute observation periods. Even though 15-minute observation periods were adequate, an observation period of 30 minutes may be sufficiently more reliable to compensate for the additional observation time required. It may be desirable in some cases to monitor only one type of behavior. It is felt that the best single behavior to observe would be bites. Bites were significantly affected at both 30 and 60 minutes of observation. Also, at 60 minutes, the frequency of bites was statistically the most significantly affected single behavior. Bites are also the most easily recognized behavior and the least subjectively determined. In contrast, fin erections are more difficult to determine since there is a continuum between fully retracted and fully erect fins.

The ecological significance of a change in agonistic behavior rates is unproven at this time. Indeed there is the possibility that the change itself may not be present in a more natural environment and that it is only a laboratory phenomenon. However, if change in frequency of agonistic behavior did occur following exposure to sublethal wastewaters in receiving streams, these changes would potentially be deleterious because of deviation from a pattern which has evolved as a process of natural selection (Warner et al., 1967). Agonistic behavior is known to be a component of centrarchid spawning behavior (Hunter, 1963; Miller, 1963). Male longear sunfish, *Lepomis megalotis megalotis*, have been observed using agonistic behaviors such as chases, fin erections, and opercle spreads to drive other longears, largemouth bass, and other bottom feeding fish away from their nests (Witt and Marzolf, 1954; Keenleyside, 1972). Male orangespotted sunfish have been observed

intruding into the nest of other males to feed on developing embryos (Barney and Anson, 1923).

Conceivably a reduction in frequencies of agonistic behaviors could result in smaller territories--smaller areas defended around the nests of spawning males. If availability of spawning sites is the factor limiting population size, then there would be a potential for an increase in population size. Even if other factors were limiting there would probably be more fry produced. Alternatively, there is the potential loss of more embryos due to intrusions into the nest by other predators if the spawning males were less aggressive. Either of these alternatives, if carried to the extreme, could have a negative effect on the population.

Conditions which may be responsible for alteration of the frequency of agonistic behaviors include subtle changes in color of some body pattern which acts as a "releaser" of aggression by conspecifics (Stacy, 1975), neurophysiological changes, or a decline in general physical condition of the fish.

A comparison of the 60 minute cumulative frequencies of control fish with the cumulative frequencies of the treatment fish indicated that the total number of behaviors exhibited by all treatment fish on Day 10 of the five sublethal bioassays was considerably higher than that exhibited by all control fish. This was surprising since on Day 10 the control and treatment pairs of fish had been treated similarly.

The more frequent activity of the treatment group is not thought to reflect an experimental bias. Fish were paired randomly, designated as treatment or control pairs randomly, and placed in aquaria which had been randomly designated as either treatment or control aquaria.

It is possible that the higher frequencies found for the treatment group are a result of observer bias—especially for some of the more subjectively determined behaviors. However, the possibility of subjectively "inflated" frequencies for treatment pairs on Day 10 was considered during all bioassays and a constant effort was made to avoid this type of bias. Also, of the 35 possible comparisons (7 behavior types x 5 sublethal bioassays), the control pairs actually exhibited a given behavior at least as often as the treatment pairs in 16 cases. In Bioassays 3, 7, and 8 there were a few very active individuals that considerably increased the frequency of agonistic behaviors in the treatment group. These individuals were in part responsible for this unexpected result. The possibility of this type of bias could be eliminated if the observer did not know which pairs were treatment pairs and which were control pairs.

Further investigations of this type could benefit from the use of simultaneous _Daphnia_ bioassays for comparative purposes. The wastewater used for this study was not acutely lethal or chronically lethal over a 10 day period to the sunfish, but a more accurate measure of toxicity using a sensitive organism would be beneficial. Future investigation of the possibility of using shorter exposure periods to reduce the lag time between initial exposure to a pollutant and observation of effect would be helpful. Determination of the effect of observation of wastewater exposed fish in control water should also be made. When investigating effects of pollutants which do not present observation problems because of color exposed fish should be observed in the treatment water to avoid any effect of the transfer from the treatment water to the control water.

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APPENDIXES

APPENDIX A

SAS ANOVA COMPUTER PROGRAM

SAS ANOVA Computer Program

This program is based on SAS ANOVA (Barr et al., 1976).

//EXEC SASBOTH, REGION. GO = 250K

//GO. SYSIN DD*

DATA RAW;

INPUT RUN 1 TRT\$3 PAIR 5 FISH\$7 AP1 9-11 AP2 13-15 FE1 17-19 FE2 21-23
TB1 25-27 TB2 29-31 CH1 33-35 CH2 37-39 BT1 41-43 BT2 45-47 OP1 49-51
OP2 53-55 AV1 57-59 AV2 61-63;

TOT 1 = AP1 + FE1 + TB1 + CH1 + BT1 + OP1;
TOT 2 = AP2 + FE2 + TB2 + CH2 + BT2 + OP2;
AP = AP2 - AP1; FE = FE2 - FE1; TB = TB2 - TB1; CH = CH2 - CH1;
BT = BT2 - BT1; OP = OP2 - OP1; AV = AV2 - AV1; TOT = TOT2 - TOT1;

CARDS;

(Place data cards here)

PROC SORT DATA = RAW; BY RUN TRT PAIR;

PROC MEANS NOPRINT DATA = RAW; BY RUN TRT PAIR;

OUTPUT OUT = TOT SUM = AP FE TB CH BT OP AV TOT;

VARIABLES AP FE TB CH BT OP AV TOT;

PROC SAS72 DATA = TOT; PARMCARDS 4;

PROC ANOVA; CLASSES RUN TRT PAIR; MEANS|TRT;

MODEL AP FE TB CH BT OP AV TOT = RUN TRT

PAIR(RUN TRT);

TEST RUN|TRT BY PAIR(RUN TRT);

;;;;

APPENDIX B

NUMBER OF BEHAVIORS FOR EACH FISH OBSERVED

AFTER 15 MINUTES OF OBSERVATION

FREQUENCY OF BEHAVIORS AFTER
1ST AND 2ND 10-DAY
PERIOD

CONTRUNK #	T	PAIRS #	FISH	AP 1ST	AP 2ND	FE 1ST	FE 2ND	TB 1ST	TB 2ND	CH 1ST	CH 2ND	BT 1ST	BT 2ND	OP 1ST	OP 2ND	AV 1ST	AV 2ND
1	C	1	U	1	0	12	3	0	0	0	0	0	0	0	0	0	0
1	C	1	L	2	4	6	8	0	0	0	0	0	0	0	0	0	0
1	C	2	U	0	0	1	0	0	0	0	0	0	0	0	0	0	0
1	C	2	L	1	1	0	4	1	0	0	0	1	0	0	0	0	0
1	C	4	U	0	0	2	0	0	0	0	0	0	0	0	0	0	0
1	C	4	L	0	5	5	17	0	0	0	2	0	0	0	0	0	0
1	C	6	U	10	4	29	37	12	0	4	0	4	6	0	0	0	0
1	C	6	L	1	0	15	7	10	0	2	0	0	0	0	0	0	2
1	T	2	U	0	0	5	1	0	0	0	0	0	0	0	0	0	0
1	T	2	L	0	0	1	2	0	0	0	0	0	0	0	0	0	0
1	T	3	U	1	1	19	10	0	0	0	0	0	0	0	0	0	0
1	T	3	L	2	1	15	7	1	0	0	0	0	0	0	0	0	0
1	T	4	U	0	0	2	3	0	0	0	0	0	0	0	0	0	0
1	T	4	L	1	5	1	20	0	0	0	0	0	4	0	0	0	1
1	T	5	U	3	1	15	7	2	0	0	0	0	0	0	0	0	0
1	T	5	L	1	0	12	1	0	1	0	0	0	0	1	0	0	0
2	C	1	U	0	2	1	17	0	2	0	7	0	1	0	0	0	0
2	C	1	L	0	0	0	3	0	0	0	0	0	0	0	0	0	0
2	C	2	U	0	2	1	11	0	1	0	0	0	0	0	4	0	0
2	C	2	L	0	2	1	3	0	0	0	0	0	0	0	0	0	0
2	C	4	U	1	0	3	1	0	0	0	0	0	0	0	0	0	0
2	C	4	L	1	0	1	0	12	2	0	0	0	0	0	0	0	0
2	C	6	U	0	2	4	1	0	0	0	0	0	0	0	0	0	0
2	C	6	L	3	1	6	5	0	0	0	0	0	0	0	0	0	0
2	T	1	U	5	2	16	11	8	0	1	0	1	0	3	0	0	0
2	T	1	L	0	0	7	2	1	0	0	0	0	0	0	0	0	0
2	T	2	U	0	0	3	9	0	6	0	0	0	0	0	0	0	0
2	T	2	L	0	1	4	9	0	0	0	0	0	0	0	0	0	0
2	T	4	U	1	0	29	10	0	0	0	0	0	0	0	0	0	0
2	T	4	L	0	3	5	3	0	0	0	0	0	0	0	0	0	0
2	T	5	U	1	0	12	17	0	0	0	0	0	0	0	0	0	0
2	T	5	L	1	0	15	10	0	0	0	0	0	0	0	0	0	0
3	C	2	U	0	0	1	1	0	0	0	0	0	0	0	0	0	0
3	C	2	L	0	1	3	3	0	0	0	0	0	0	0	0	0	0
3	C	5	U	3	1	11	5	3	0	0	0	1	0	1	0	0	0
3	C	5	L	0	1	10	8	10	0	0	0	0	0	0	0	0	0
3	C	6	U	2	3	11	19	0	0	0	0	0	0	0	0	0	0
3	C	6	L	1	0	8	8	0	0	0	0	0	0	0	0	0	0
3	C	4	U	2	0	10	17	0	4	0	5	0	0	0	0	0	0
3	C	4	L	0	4	9	23	0	8	0	0	0	8	0	1	0	0
3	T	1	U	1	0	14	3	2	0	0	0	0	0	0	0	0	0
3	T	1	L	0	0	8	10	4	0	0	0	0	0	0	0	0	0
3	T	2	U	10	0	16	3	4	0	8	0	3	0	0	0	0	0
3	T	2	L	1	0	1	1	3	0	0	0	0	0	0	0	2	0
3	T	4	U	0	3	6	9	0	0	0	0	0	0	0	0	0	0
3	T	4	L	1	0	9	8	0	0	0	0	0	0	0	0	0	0
3	T	5	U	2	2	20	21	3	0	0	0	0	0	0	0	0	0
3	T	5	L	9	1	34	20	1	0	0	0	0	0	0	0	0	0

```
L
U
N P                    FREQUENCY OF BEHAVIORS AFTER
R T A                     1ST AND 2ND 10-DAY
U / I F                        PERIOD
N T R I
    R S    AP      FE      TB      CH      BT      OP      AV
# T # H  1ST 2ND 1ST 2ND 1ST 2ND 1ST 2ND 1ST 2ND 1ST 2ND 1ST 2ND
```

#	T	#	H	AP 1ST	AP 2ND	FE 1ST	FE 2ND	TB 1ST	TB 2ND	CH 1ST	CH 2ND	BT 1ST	BT 2ND	OP 1ST	OP 2ND	AV 1ST	AV 2ND
7	C	2	U	2	6	6	15	10	6	0	0	2	11	2	0	0	0
7	C	2	L	0	0	5	8	10	7	0	0	1	8	0	0	0	0
7	C	4	U	1	0	14	7	15	9	0	0	20	9	0	0	0	0
7	C	4	L	0	13	11	31	14	12	0	18	12	7	0	9	0	3
7	C	5	U	1	0	6	11	6	1	0	0	1	0	0	0	0	0
7	C	5	L	1	6	6	19	5	0	0	0	0	0	0	0	0	0
7	C	6	U	1	1	9	7	0	1	0	0	0	0	0	0	0	0
7	C	6	L	1	5	5	12	1	0	0	0	0	2	0	0	0	0
7	T	1	U	0	0	5	3	4	0	0	0	0	0	0	0	0	0
7	T	1	L	0	1	2	4	2	0	0	0	0	0	0	0	0	0
7	T	3	U	2	3	12	11	13	9	2	0	1	0	0	0	0	0
7	T	3	L	0	0	10	9	1	1	0	0	0	0	0	0	1	0
7	T	6	U	0	2	5	23	26	15	0	16	11	13	1	19	0	0
7	T	6	L	2	1	5	2	38	12	0	0	9	2	0	0	0	0
7	T	2	U	4	0	11	12	7	0	4	0	12	0	0	0	0	0
7	T	2	L	0	1	2	3	7	0	0	0	7	0	0	0	1	0
8	C	2	U	0	0	1	9	0	0	0	0	0	0	0	0	0	0
8	C	2	L	0	1	1	1	0	0	0	0	0	0	0	0	0	0
8	C	4	U	1	1	5	13	2	0	0	0	0	0	0	0	0	0
8	C	4	L	0	1	6	13	0	4	0	0	0	0	0	0	0	0
8	C	5	U	1	7	18	34	3	1	5	19	1	7	10	10	0	0
8	C	5	L	1	0	5	1	10	1	0	0	0	0	0	0	0	4
8	C	6	U	0	1	6	11	0	0	0	0	0	0	0	0	0	0
8	C	6	L	1	1	7	11	1	0	0	0	0	0	1	1	0	0
8	T	1	U	1	1	3	1	0	0	0	0	0	0	0	0	0	0
8	T	1	L	0	0	2	2	0	1	0	0	0	0	0	0	0	0
8	T	2	U	1	0	7	10	0	0	0	0	0	0	0	0	2	0
8	T	2	L	3	5	17	25	9	2	3	4	3	0	19	8	0	0
8	T	3	U	1	1	13	6	0	1	0	1	0	0	0	0	0	0
8	T	3	L	1	1	2	1	1	3	0	0	0	0	0	0	0	0
8	T	6	U	0	0	1	1	0	1	0	0	0	0	0	0	0	0
8	T	6	L	0	1	4	2	0	1	0	0	0	0	0	0	0	0

APPENDIX C

NUMBER OF BEHAVIORS FOR EACH FISH OBSERVED

AFTER 30 MINUTES OF OBSERVATION

				FREQUENCY OF BEHAVIORS AFTER 1ST AND 2ND 10-DAY PERIOD														
				AP		FE		TB		CH		BT		OP		AV	
RUN #	CAT T	PAIR #	FISH	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
1	C	1	J	1	0	22	4	2	0	0	0	0	0	0	0	0	0
1	C	1	L	5	6	15	19	1	0	0	0	1	0	0	0	0	0
1	C	2	J	0	1	12	1	0	0	0	0	0	0	0	0	0	0
1	C	2	L	5	3	5	12	1	0	0	0	1	0	0	0	0	0
1	C	4	J	0	0	10	7	5	0	0	0	0	0	0	0	0	7
1	C	4	L	0	17	10	55	6	0	0	4	1	1	0	1	0	0
1	C	5	J	17	23	44	79	12	3	11	18	6	22	0	3	0	0
1	C	5	L	1	1	16	9	10	0	2	0	0	0	2	0	0	16
1	T	2	J	0	0	6	9	0	3	0	0	0	0	0	0	0	0
1	T	2	L	0	2	1	11	0	0	0	0	0	0	0	0	0	0
1	T	3	J	1	2	31	25	0	0	0	0	0	0	0	0	0	0
1	T	3	L	5	3	20	16	1	0	0	0	0	0	0	0	0	0
1	T	4	J	1	0	13	3	8	0	0	0	5	0	0	0	0	0
1	T	4	L	1	12	8	45	8	0	0	6	11	10	0	0	0	2
1	T	5	J	11	2	45	15	9	1	1	0	1	0	0	0	0	0
1	T	5	L	3	0	17	2	2	1	0	0	0	0	1	0	0	0
2	C	1	J	0	2	10	19	1	3	0	7	0	1	1	0	0	0
2	C	1	L	0	0	3	3	0	0	0	0	0	0	0	0	0	0
2	C	2	J	0	4	11	18	2	3	0	0	0	0	0	4	0	0
2	C	2	L	6	10	29	29	1	4	1	5	2	1	0	0	0	0
2	C	4	J	2	0	7	6	0	0	0	0	0	0	0	0	0	0
2	C	4	L	1	1	19	12	1	0	0	0	0	0	0	0	0	0
2	C	6	J	0	2	9	1	1	0	0	0	1	0	3	0	0	0
2	C	5	L	4	1	12	8	2	0	0	0	0	0	0	0	0	0
2	T	1	J	8	7	45	42	14	0	2	4	8	4	8	0	0	0
2	T	1	L	0	0	15	2	1	0	0	0	0	0	0	0	1	1
2	T	2	J	0	4	7	22	1	6	0	0	0	1	0	1	0	0
2	T	2	L	0	1	10	17	0	1	0	1	0	0	0	4	0	1
2	T	4	J	1	2	42	23	0	0	0	0	0	0	0	0	0	0
2	T	4	L	1	5	7	7	0	0	0	0	0	0	0	0	0	0
2	T	5	J	1	1	21	28	0	0	0	0	0	0	0	0	0	0
2	T	5	L	2	0	22	21	0	0	0	0	0	0	0	0	0	0
3	C	2	J	0	1	5	6	0	1	0	0	0	0	0	0	0	0
3	C	2	L	0	1	8	10	0	1	0	0	0	0	0	0	0	0
3	C	4	J	3	0	15	27	0	5	0	7	0	0	0	0	0	0
3	C	4	L	1	6	12	30	0	9	0	0	0	10	0	4	0	0
3	C	5	J	11	2	27	6	3	0	0	0	7	0	7	0	0	0
3	C	5	L	0	1	15	11	10	0	0	0	0	0	0	0	1	0
3	C	6	J	6	3	22	33	0	0	0	0	0	0	0	0	0	0
3	C	5	L	1	1	14	15	0	0	0	0	0	0	0	0	0	0
3	T	1	J	1	0	24	4	2	0	0	0	0	0	0	0	0	0
3	T	1	L	0	0	13	14	4	0	0	0	0	0	0	0	0	0
3	T	2	J	24	6	59	13	8	0	40	0	23	0	3	0	0	0
3	T	2	L	1	0	3	1	5	0	0	0	1	0	0	0	4	3
3	T	4	J	1	3	15	19	0	0	0	0	0	0	0	0	0	0
3	T	4	L	1	2	12	12	0	2	0	0	0	0	0	0	0	0
3	T	5	J	3	2	34	39	3	0	0	0	0	0	0	0	0	0
3	T	5	L	11	3	60	28	1	0	0	0	0	0	0	0	0	0

FREQUENCY OF BEHAVIORS AFTER
1ST AND 2ND 10-DAY
PERIOD

CONJRTJNR #	CT/TR	PA #	FISH	AP		FC		TB		CH		BT		JP		AV	
#	T	#	H	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
7	C	2	U	4	18	26	47	13	8	0	9	4	12	2	1	0	0
7	C	2	L	1	0	12	9	12	9	0	0	2	8	0	0	0	2
7	C	4	J	4	0	27	8	21	9	0	0	25	9	0	0	0	0
7	C	4	L	2	25	22	62	20	12	0	45	16	11	0	14	0	5
7	C	5	J	1	0	9	15	7	5	0	0	1	1	0	0	1	5
7	C	5	L	4	15	19	55	6	5	1	7	1	6	0	0	0	0
7	C	6	J	2	2	12	9	5	1	0	0	1	0	0	0	0	0
7	C	6	L	10	9	19	20	5	0	6	0	3	2	8	0	0	0
7	T	1	J	0	0	12	7	9	2	0	0	14	0	0	1	0	0
7	T	1	L	0	5	8	25	12	0	0	0	3	0	0	0	0	0
7	T	2	J	12	1	29	27	7	0	14	0	28	0	0	0	0	0
7	T	2	L	0	1	2	23	7	8	0	0	7	2	0	4	4	0
7	T	3	J	8	10	39	35	17	16	10	0	7	0	0	1	0	0
7	T	3	L	0	0	15	19	7	2	0	0	1	0	0	0	1	0
7	T	6	J	0	4	14	61	28	23	0	54	11	39	2	37	2	0
7	T	6	L	9	1	25	3	48	17	20	0	37	2	11	0	0	0
8	C	2	U	1	3	16	10	1	0	0	0	0	0	0	0	0	0
8	C	2	L	0	2	8	1	1	0	0	0	0	0	0	0	0	0
8	C	4	J	4	5	13	27	2	3	0	0	0	1	0	0	0	0
8	C	4	L	1	4	15	26	0	8	0	0	0	0	0	2	0	0
8	C	5	J	3	10	37	63	10	1	11	35	5	13	13	13	0	0
8	C	5	L	1	0	5	1	10	1	0	0	0	0	0	0	2	5
8	C	6	J	0	4	9	18	0	1	0	0	0	0	0	1	0	0
8	C	6	L	1	2	12	18	4	0	0	0	0	0	1	1	0	0
8	T	1	J	1	2	3	2	0	0	0	0	0	0	0	0	0	0
8	T	1	L	0	0	3	6	0	1	0	0	0	0	0	0	0	0
8	T	2	J	1	0	9	14	3	1	0	0	1	0	0	1	3	0
8	T	2	L	8	0	35	51	15	5	27	18	22	6	70	13	0	0
8	T	3	J	3	2	27	23	0	1	0	1	0	1	0	0	0	0
8	T	3	L	1	1	2	1	1	3	0	0	0	0	0	0	0	0
8	T	6	U	0	0	4	1	0	1	0	0	0	0	0	0	0	0
8	T	6	L	0	1	8	4	0	1	0	0	0	0	0	0	0	0

APPENDIX D

NUMBER OF BEHAVIORS FOR EACH FISH OBSERVED

AFTER 60 MINUTES OF OBSERVATION

FREQUENCY OF BEHAVIORS AFTER
1ST AND 2ND 10-DAY
PERIOD

CONTR#	CAT/T	PAIR#	FISH	AP 1ST	AP 2ND	FE 1ST	FE 2ND	TB 1ST	TB 2ND	CH 1ST	CH 2ND	BT 1ST	BT 2ND	OP 1ST	OP 2ND	AV 1ST	AV 2ND
1	C	1	U	1	0	30	9	4	0	0	0	0	0	0	1	0	2
1	C	1	L	20	18	46	55	6	0	0	4	11	1	0	0	0	0
1	C	2	U	1	2	20	1C	0	0	0	0	0	0	0	0	0	1
1	C	2	L	11	15	30	28	1	0	2	9	3	6	0	0	0	3
1	C	4	U	1	0	18	24	6	0	0	0	0	0	1	0	0	9
1	C	4	L	4	28	20	87	11	0	5	5	3	3	2	1	0	0
1	C	6	U	33	47	101	150	12	5	29	63	24	56	0	4	0	0
1	C	6	L	1	1	16	9	10	0	2	0	0	0	2	0	0	22
1	T	2	U	0	1	9	31	1	7	0	0	0	2	0	0	0	0
1	T	2	L	0	4	3	39	1	5	0	0	0	3	0	0	0	0
1	T	3	U	2	3	41	48	0	0	0	0	0	0	0	0	0	0
1	T	3	L	6	5	22	24	1	0	0	0	0	0	0	0	0	0
1	T	4	U	1	0	19	3	11	0	0	0	7	0	0	0	0	0
1	T	4	L	2	24	28	85	16	0	18	29	43	27	0	0	0	2
1	T	5	U	27	10	116	42	9	7	9	2	24	0	0	0	0	0
1	T	5	L	3	0	24	8	3	3	0	0	0	0	1	0	0	0
2	C	1	U	4	10	31	56	12	3	0	8	2	5	2	0	0	0
2	C	1	L	0	0	9	6	12	0	0	0	0	0	0	0	0	1
2	C	2	U	1	4	24	23	2	3	0	0	0	0	0	4	0	0
2	C	2	L	6	10	29	29	1	4	1	5	2	1	0	0	0	0
2	C	4	U	2	0	23	16	0	0	0	0	0	0	0	0	0	0
2	C	4	L	4	1	32	27	2	0	0	0	0	0	0	0	0	0
2	C	6	U	7	5	27	22	11	5	12	1	11	5	22	8	0	0
2	C	6	L	4	3	28	17	8	2	1	0	2	0	0	0	1	3
2	T	1	U	17	13	100	91	14	0	6	10	25	5	8	0	0	0
2	T	1	L	0	0	27	6	1	0	0	0	0	0	0	0	1	1
2	T	2	U	0	5	16	29	0	6	0	1	0	3	0	1	0	0
2	T	2	L	0	1	27	32	0	1	0	1	0	0	0	4	0	1
2	T	4	U	5	2	63	41	0	1	0	0	0	0	0	0	0	0
2	T	4	L	2	5	16	9	0	0	0	0	0	0	0	0	0	0
2	T	5	U	1	1	37	54	0	0	0	0	0	0	0	0	0	0
2	T	5	L	3	1	36	44	1	0	0	0	0	0	1	0	0	0
3	C	2	U	0	1	7	16	0	6	0	0	0	1	0	1	0	2
3	C	2	L	0	5	12	28	0	7	0	1	0	5	0	0	0	0
3	C	4	U	7	1	33	48	0	5	0	7	0	0	0	0	0	5
3	C	4	L	1	24	28	58	0	9	0	12	0	10	0	5	0	0
3	C	5	U	14	6	35	8	5	0	0	0	7	0	7	0	0	0
3	C	5	L	0	1	29	22	14	0	0	0	0	0	6	0	1	0
3	C	6	U	23	5	75	56	0	0	8	0	6	0	0	0	0	0
3	C	6	L	2	2	23	27	0	0	0	0	0	0	0	0	3	0
3	T	1	U	3	0	44	12	15	0	0	0	1	0	2	0	0	0
3	T	1	L	11	2	43	30	19	0	0	0	0	2	0	0	0	0
3	T	2	U	42	11	92	37	8	0	49	1	30	0	3	0	0	0
3	T	2	L	1	0	8	6	5	0	0	0	1	0	0	0	5	5
3	T	4	U	13	7	44	24	1	1	5	0	2	0	0	0	0	0
3	T	4	L	4	2	27	21	0	3	0	0	0	0	0	0	1	0
3	T	5	U	8	4	77	77	3	1	0	0	0	0	0	0	0	0
3	T	5	L	14	9	106	43	2	0	0	0	0	0	0	0	0	0

FREQUENCY OF BEHAVIORS AFTER
1ST AND 2ND 10-DAY
PERIOD

C N T R Y	T R E A T / T	P A I R #	F I S H	AP 1ST	2ND	FE 1ST	2ND	TB 1ST	2ND	CH 1ST	2ND	BT 1ST	2ND	OP 1ST	2ND	AV 1ST	2ND
7	C	2	U	16	30	67	96	17	10	15	33	9	13	2	2	0	0
7	C	2	L	1	0	15	10	13	10	0	0	2	12	0	1	1	3
7	C	4	U	5	0	32	8	23	11	0	0	25	9	0	0	5	0
7	C	4	L	21	39	60	129	23	13	19	118	25	36	1	46	0	6
7	C	5	U	1	1	15	20	10	5	0	0	4	1	0	0	2	10
7	C	5	L	16	27	62	98	10	6	20	26	15	13	0	0	0	0
7	C	6	U	2	3	12	11	5	1	0	0	1	0	0	0	3	0
7	C	6	L	21	13	43	32	5	0	16	3	8	2	8	1	0	0
7	T	1	U	1	0	18	16	9	2	0	0	14	0	0	1	0	0
7	T	1	L	2	7	20	69	12	1	0	0	4	3	0	0	0	0
7	T	2	U	26	3	39	47	7	1	61	0	58	2	0	0	0	0
7	T	2	L	0	1	2	30	7	13	0	0	7	2	0	5	9	0
7	T	3	U	20	18	68	61	26	27	49	0	25	0	18	2	0	0
7	T	3	L	0	1	31	39	13	9	1	0	1	0	0	0	2	0
7	T	6	U	0	12	16	137	28	27	0	127	11	80	2	39	2	0
7	T	6	L	21	1	81	3	49	17	74	0	127	2	21	0	0	0
8	C	2	U	7	9	37	43	6	1	0	1	1	0	0	0	0	0
8	C	2	L	1	3	13	2	6	0	0	0	2	0	1	0	0	0
8	C	4	U	7	13	22	58	2	8	0	25	0	21	0	23	0	0
8	C	4	L	1	6	23	33	0	13	0	0	0	2	0	4	0	0
8	C	5	U	7	19	81	102	10	1	27	41	17	15	13	13	00	0
8	C	5	L	1	0	5	1	10	1	0	0	0	0	0	0	5	6
8	C	6	U	0	10	22	54	0	17	0	4	0	4	0	42	0	0
8	C	6	L	8	3	42	35	8	0	2	0	10	0	8	1	0	1
8	T	1	U	2	2	8	2	11	0	0	0	6	0	0	14	0	0
8	T	1	L	0	0	10	9	8	0	0	0	0	0	0	0	0	0
8	T	2	U	1	12	18	36	3	1	0	6	1	1	0	0	3	0
8	T	2	L	19	9	76	54	27	5	78	18	79	6	140	0	0	5
8	T	3	U	20	10	68	49	1	1	8	2	4	1	1	0	0	0
8	T	3	L	1	1	2	1	1	3	0	0	0	0	0	8	2	2
8	T	6	U	0	0	10	1	0	1	0	0	0	0	0	0	0	0
8	T	6	L	0	0	20	6	0	1	0	0	0	0	0	0	0	0

VITA ^{2}

James Carroll Petersen

Candidate for the Degree of

Master of Science

Thesis: SUBLETHAL EFFECTS OF BIOLOGICALLY TREATED PETROLEUM REFINERY WASTEWATERS ON AGONISTIC BEHAVIOR OF MALE ORANGESPOTTED SUNFISH, LEPOMIS HUMILIS (GIRARD)

Major Field: Zoology

Biographical:

Personal Data: Born in Hot Springs, South Dakota, November 3, 1952, the son of Alfred and Doris Petersen.

Education: Graduated from Hot Springs High School, Hot Springs, South Dakota, in May, 1971; attended University of Northern Colorado, 1971-73; received Bachelor of Science degree in Biology from University of South Dakota in 1975; completed requirements for the Master of Science degree at Oklahoma State University in December, 1979.

Professional Experience: Forestry aide, South Dakota Department of Game, Fish, and Parks, summers of 1974-76; field technician and graduate research assistant, Oklahoma State University, 1976-77; graduate research assistant, Reservoir Research Center, Oklahoma State University, 1978; laboratory technician, Aquatic Life Consultants, Inc., Stillwater, Oklahoma, 1978; graduate teaching assistant, Oklahoma State University, 1978-79; graduate research assistant, Oklahoma Cooperative Fisheries Unit, Oklahoma State University, 1979; hydrologist, U.S. Geological Survey, 1979.

Professional Organizations: North American Benthological Society, American Fisheries Society.