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SOME EFFECTS OF PETROLEUM ON NEARSHORE MARINE ORGANISMS

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ABSTRACT

The overall objective of this project was to better understand the effects of chronic, low-level oil pollution on nearshore Alaskan marine organisms.

The bivalve mollusc *Macoma balthica* accumulated hydrocarbons during 180 days of continuous exposure to Prudhoe Bay crude oil in flowing seawater dispersions with nominal concentrations of 0.03 mg/l, 0.3 mg/l and 3.0 mg/l. The animal's ability to concentrate oil from seawater increased with decreasing oil in water concentration. Decreases in *M. balthica*'s oil burden began after 30 to 120 days (depending on the oil concentration) and continued for at least 60 days after oiling ceased. Aliphatic and aromatic hydrocarbons were fractionated in markedly different ways by the animal. Branched and cyclic aliphatics in the range dodecane through hexadecane were preferentially retained over their higher homologs; whereas larger and more substituted aromatics were selectively concentrated.

Macoma balthica showed a number of physical, behavioral, physiological and biochemical changes during oil exposure. An oil in seawater concentration of 3.0 mg/l caused severe dysfunction in the clams including a decreased burial rate, increased respiration rate, and inhibition of growth leading to very high mortalities. The lowest concentration of oil tested, 0.03 mg/l, inhibited growth and caused abnormalities in gonad morphology. One group of adverse oil effects which was related to sluggishness and disorientation of the animals appeared after seven days' oiling; another group related to a negative energy balance was not observed until 60 days. We conclude that chronic exposure of *M. balthica* to oil-in-seawater concentrations as low as 0.03 mg/l will in time lead to population decreases.

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CHAPTER 1
INTRODUCTION

This report describes a research project the goal of which was to better understand biological changes at the organism and community levels that result from a defined chronic input of petroleum to an intertidal environment. This project was undertaken in Alaska in 1975 because at that time several development activities were in progress or being contemplated which had the potential to cause chronic oil pollution of the marine environment through low level permitted discharges during normal operations. These activities included oil terminal operations in Port Valdez and outer continental shelf oil exploration in several regions of the state.

The project focused on the effects of chronic low level inputs since these, unlike catastrophic spills resulting from shipwrecks or other accidents, are manageable through the permitting process. Proper setting of permitted discharge levels is a very important matter since levels set too high will not provide society with the degree of environmental quality which it desires but levels set too low will lead to cleanup expenses which produce little benefit. Thus to err in either direction in the setting of discharge levels is likely to lead to wasting of resources. Clearly no single research project can supply sufficient scientific information for the evaluation of the effects of various discharge levels. Yet we believe that the work reported here is an important step in that direction.

The initial approach adopted to reach this project's overall goal of better understanding the biological effects of chronic oil pollution was to examine the effects of natural petroleum seeps on Alaskan intertidal environments. We hoped that by comparing organisms and communities existing under the influence of such seeps to carefully selected controls it would be possible to correlate differences with long term oiling. However, preliminary work at three oil seep areas in South Central Alaska indicated that the number of

uncontrollable and unquantifiable environmental variables was sufficient to make the success of this approach doubtful at best.

Consequently, the approach of this project was modified to focus on laboratory experimentation where closer control of variables was possible. This laboratory approach has two distinct disadvantages in that the practical exposure period is greatly reduced and that there is systematic uncertainty of extrapolating from the simplified model "environment" of the laboratory back to the natural environment. However, it was (and is) our opinion that these disadvantages are out-weighed by the ability to control and manipulate variables which the laboratory provides. The purpose of this experiment was to examine the largest possible number and variety of physical, physiological, chemical and biochemical parameters in a single chronic oiling experiment. The focus of the experiment was the biological <u>effect</u> of oil pollution. Hence the parameters examined concentrated on ones potentially related to oil's mode of toxicity such as membrane function and respiration and excluded ones related to oil's fate such as degradative metabolic pathways.

CHAPTER 2

ACCUMULATION, FRACTIONATION AND RELEASE OF OIL BY THE INTERTIDAL CLAM *MACOMA BALTHICA*

INTRODUCTION

The rate of hydrocarbon accumulation in marine organisms upon exposure to sub-lethal concentrations of oil in seawater and the subsequent loss of those hydrocarbons after termination of oil exposure have been reviewed by Varanasi and Malins (1977). Some of this information has come from *in situ* observations after an oil spill or in a chronically polluted area, and other information has come from laboratory studies with static or flow-through systems. While much work remains to be done, one result of the oil exposure studies is clear: marine organisms, and in particular bivalves, have the ability to concentrate petroleum hydrocarbons from seawater in their tissues.

We have conducted a controlled, laboratory experiment in which the bivalve mollusc, *Macoma balthica*, was exposed to seawater dispersed crude oil for 180 days. In this chapter we describe the apparatus in which this experiment was conducted and report the accumulation, fractionation and release of oil by the test animals.

MATERIALS AND METHODS

In order to provide a realistic simulation of chronic exposure of marine animals to oil-in-seawater dispersions in the laboratory, we constructed a continuous-flow system capable of simultaneously delivering dispersions with nominal oil in water concentrations of 0.03 mg/l, 0.3 mg/l and 3.0 mg/l. Our system is based primarily on the design of Hyland *et al.* (1977). Because we wished to disperse a moderately viscous crude oil, we also incorporated some of the features of Roubal *et al.* (1977).

The components of the system, as shown in Figure 1, are a constant head pressure tank, a mixing and separation tank, manifold to deliver sea water and the oil/seawater dispersion to each of eight exposure tanks, and filter drums to remove the oil. Seawater was maintained at a constant level in the head tank (A) and flowed into the mixing chamber (B) at 10 ℓ/min. A peristaltic pump (D) metered Prudhoe Bay crude oil (C) into the mixing chamber at 0.3 ml/min. Mixing energy was applied by a stainless steel blade rotated at high speed by a stirring motor (F). The oil/seawater dispersion separated during its passage through a 340 ℓ tank (G) and was removed from below the resulting oil slick at 6.66 ℓ/min as it entered the dosing manifold (I). The tanks used were constructed of marine plywood covered with fiberglass resin. All plumbing was done with rigid PVC pipe and fittings. The mixing chamber was constructed of acrylic plastic. The entire system was leached with running seawater for two weeks prior to use. Evidence of contamination from plastics was not detected in either water or animals. Overflow from the separation tank passed through a plastic fibre floss filter (K), two 120 ℓ separation drums and one of the two 80 ℓ polyethylene drum filters (L) containing hydrophobic plastic strips, plastic floss and activated charcoal.

Different exposure concentrations were achieved by dilution of the oiled seawater with non-contaminated seawater (H) from the head tank to give a total flow of 3 ℓ/min through each exposure tank. Seawater was supplemented with diatom enriched seawater (≃ 1:1 mixture) from "upwelling ponds" on the grounds of the Seward marine facility (Nevé *et al.*, 1976) for a period of 4-6 hours per day beginning 9 July and ending 24 November 1977. The average exposure temperature was 8.0° (Range = 7.1-9.0°).

Specimens of *Macoma balthica* obtained from Resurrection Bay near Seward, Alaska (149°27'W; 60°06'N) were maintained in sand filled petri dish bottoms immersed in flowing seawater. Only clams which buried themselves were used for experimentation. On 14 June 1977, after two weeks acclimatation, the 180-day oil exposure experiment was begun. Water samples were collected from all exposure tanks and analyzed for hydrocarbon concentrations at 30-day intervals beginning on day 0 and ending on day 150. Replicate samples of approximately 50 specimens of *Macoma balthica* were obtained from each treatment periodically throughout the 180-day exposure period and one set of replicate

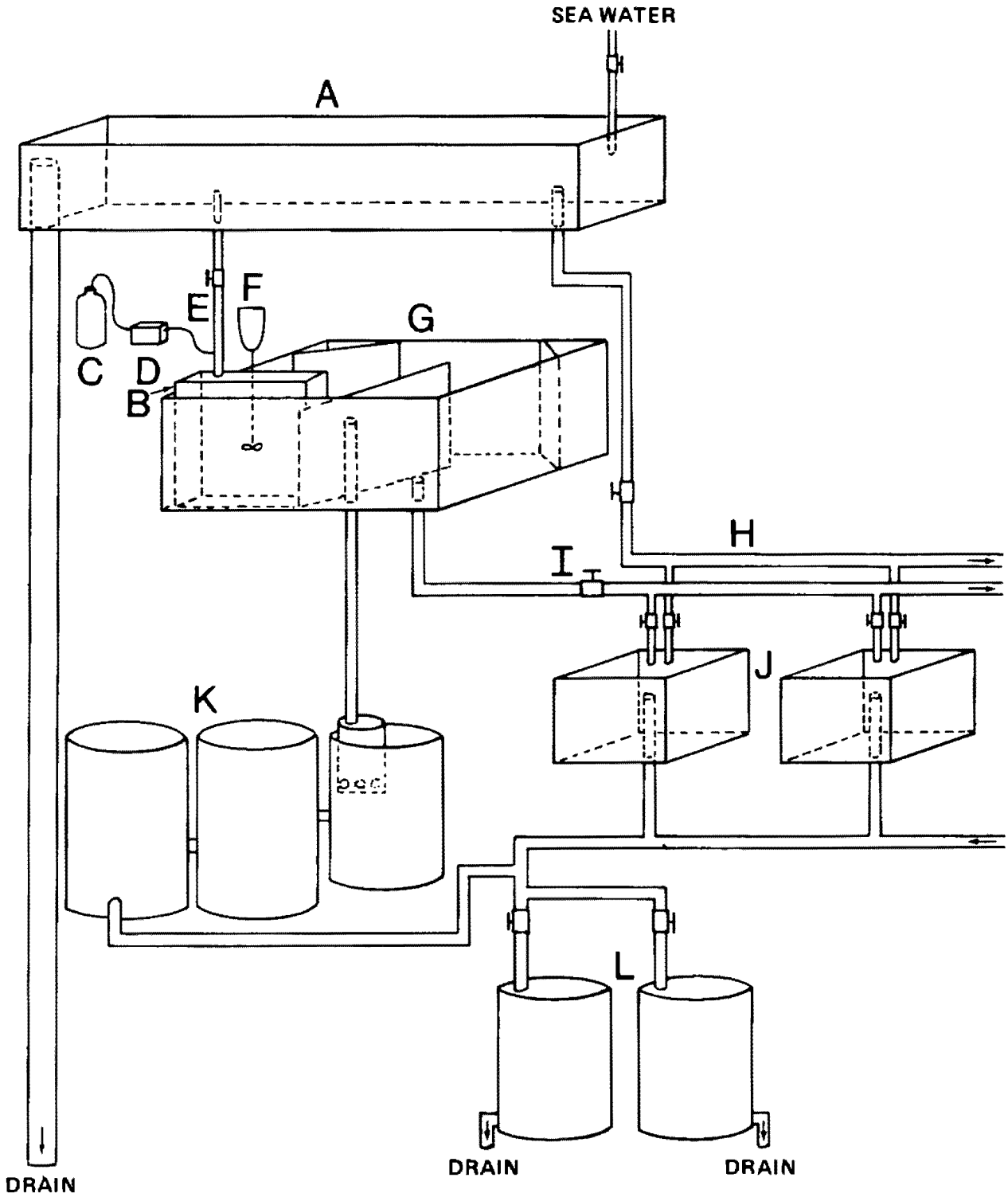

Figure 1. Schematic representation of continuous oil exposure system: A, head tank; B, mixing chamber; C, oil supply; D, oil pump; E, seawater supply; F, stirring motor; G, separation tank; H, non-contaminated seawater manifold; I, oil/seawater dispersion manifold; J, exposure tanks; K, separation tanks; L, final filter.

samples from the control, low oil, and medium oil treatments after 60 days recovery in clean seawater. The clams were sieved from the silica sand, placed in fiberglass mesh buckets and returned to their exposure tanks for 24 h. There was no food available at this time, allowing the clams to clear their intestinal tract. After 24 h they were removed from the tanks, rinsed with clean seawater and immediately frozen.

Water samples were liquid-liquid extracted with three 5% (vol.) hexane washes in a separatory funnel. The hexane extracts were combined, dried over Na_2SO_4, and reduced to 2 ml under vacuum in a rotary evaporator. The evaporation parameters were optimized for the recovery of napthalene. Clam tissue samples were analyzed for hydrocarbons by modification of the method proposed by Warner (1976). Tissue was removed from the shell while still frozen and placed in a tared 50 ml centrifuge tube with teflon-lined screw cap. Approximately 10 g wet weight of tissue was obtained from 50 *Macoma balthica*. Samples were digested with 10 N NaOH at 90° for 3 h, and allowed to cool to room temperature. Hexane was then added, the tube resealed and shaken vigorously for one minute. The sample was then centrifuged at 2400 rpm for 10 min. The organic phase was subsequently removed with a 20 ml syringe and the procedure repeated twice. The hexane extract was dried over Na_2SO_4 and concentrated to between 1 and 2 ml on a rotary evaporator, and further concentrated to 0.5 ml under nitrogen before fractionation on a column of 5 g of 5% deactivated silica gel. A saturate hydrocarbon fraction was eluted with hexane and an unsaturated and aromatic hydrocarbon fraction with 40% benzene/hexane or 20% methylene chloride/hexane.

Quantification and identification of individual compounds was either by gas chromatography (GC) or by gas chromatography coupled mass spectrometry (GC-MS). Samples were concentrated to approximately 0.2 ml and an aliquot injected onto a 30 m x 0.75 mm glass SCOT column coated with OV-101 in either an Hewlett-Packard (HP) 5710 GC with flame ionization detector, or an HP 5930/5933 GC-MS.

Compounds were identified and quantified on the GC-MS by single ion monitoring and calibration with an external standard. Aromatic compounds were quantified by reference to fully deuterated naphthalene and anthracene which were included in the sample as an internal standard. Alkylated aromatics were

assumed to have the same response as the parent compound which results in an increasing but unknown underestimation with increasing alkyl substitution. The percentage recovery after the sample handling process was calculated from the recovery of an alkane, aromatic standard added to one of the two control samples from each sampling date before digestion. All hydrocarbon values are corrected for this handling loss, which averaged 25%.

RESULTS

Total hydrocarbon concentration (mg oil/l seawater) for each exposure level measured at 30 day intervals is presented in Figure 2. The greatest relative variability is encountered at the control and low oil level. The overall pattern is that of high exposure level or undiluted mixing tank output. The increased variability of the lower oil levels is due to the widely varying control levels. Over 150 days, when the water hydrocarbon concentrations were measured (Fig. 2), the levels were relatively constant and an order of magnitude apart at nominal values of 3.0 mg/l, 0.3 mg/l, and 0.03 mg/l. Related work (Shaw and Reidy, 1979) has shown that 86% of the dispersed oil produced in this experiment was contained in particles larger than 5 μm.

The hydrocarbon concentrations in clam tissue over the 180-day period of exposure to oil and the 60-day depuration period are presented in Figure 3. ±These results are expressed on a wet weight basis. Those who prefer dry weight basis will find the information necessary for conversion tabulated in Chapter 9 of this report1. As one of the two control clam samples for each date had a hydrocarbon standard added to determine analytical recovery, only the unaltered control values are presented.

Clams exposed to the highest concentration of oil in water (3.0 mg/l) rapidly accumulated hydrocarbons to a maximum of nearly 1500 μg/g wet weight after 30 days. Their hydrocarbon content then gradually decreased throughout the rest of the exposure period. Initial uptake decreased with decreasing oil exposure levels; however, both at medium and low oil concentrations, clams reached their maximum hydrocarbon content after 120 days of exposure. Hydrocarbon levels in clams exposed to medium concentrations then began to decline at a fairly constant rate throughout the rest of the experiment, including the

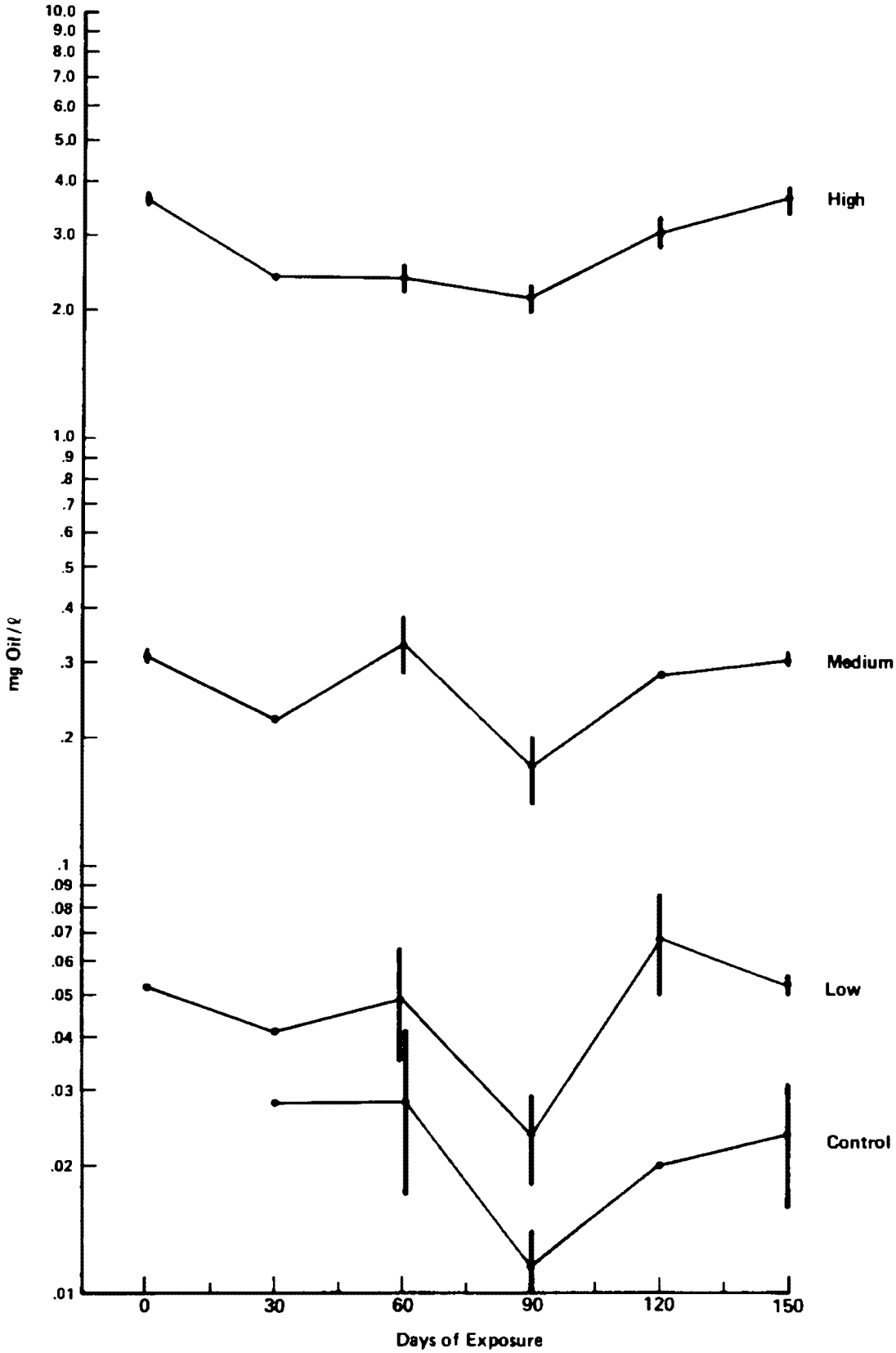

Figure 2. Concentration of total oil in seawater in test and control tanks during the course of the experiment. Bars show the range of duplicate determinations; dots are single determinations.

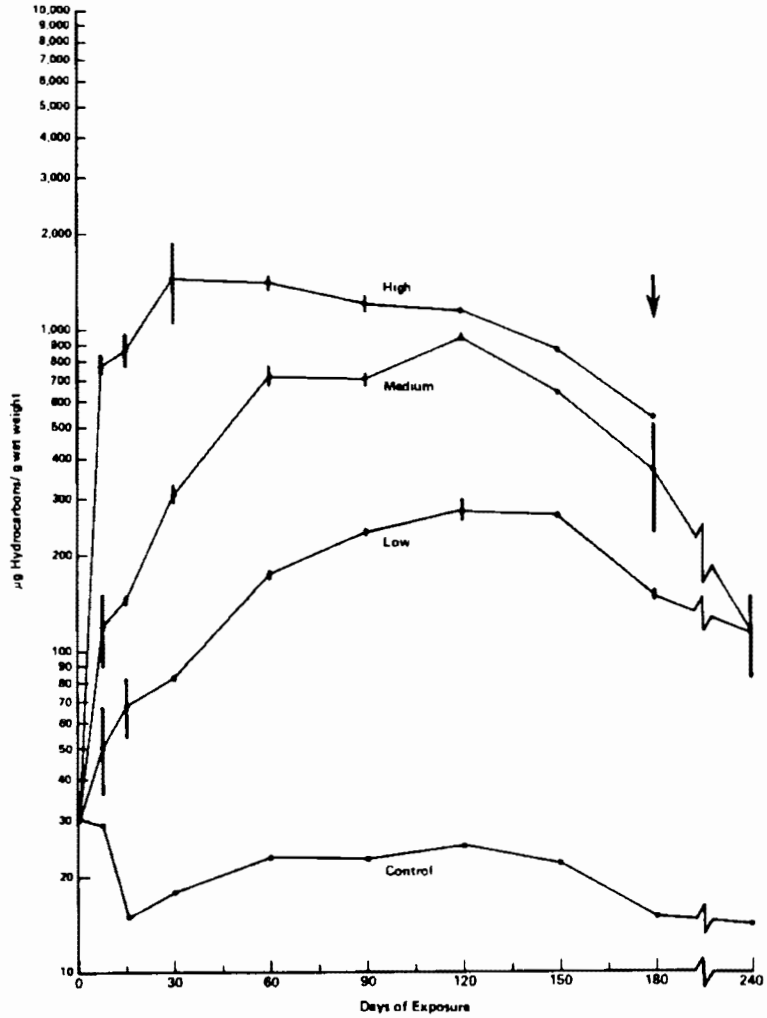

Figure 3. *Macoma balthica* tissue concentrations of total hydrocarbons during 180 days of exposure to oil in seawater dispersions and 60 days of recovery. "High", "medium", "low", and "control" correspond to oil in seawater concentrations shown in Figure 2. Bars show the range of duplicate determinations; dots are single determinations.

60-day depuration period. At the low oil level, clams showed a decline in hydrocarbon content during the last 30 days of exposure and a slower rate of decline after exposure to oil ceased. At the low and medium oil levels, clams approached the same hydrocarbon level during the 60-day depuration period. While there was a 100-fold difference in the amount of oil to which the clams were exposed, after day 60, there was less than a 10-fold difference in hydrocarbon content among the clams exposed to oil.

The concentration of hydrocarbons found in each column chromatographic fraction is summarized in Table 1. Fraction 1 contained saturated hydrocarbons and fraction 2 aromatic hydrocarbons and biogenic olefins. The control clams contained more fraction 2 material than fraction 1 material as did the clams in the low concentration of oil after day 60. At the higher concentrations of oil, clams had more fraction 1 material than fraction 2 material. Chromatograms of a typical oil/water dispersion (Fig. 4 A, B), *Macoma balthica* tissue after 120 days of exposure to oil (Fig. 4 C, D), 180 days of exposure to oil (Fig. 4 E, F), and 60 days of depuration (Fig. 4 G, H) demonstrate that a considerable change occurred in the composition of the oil accumulated by the clams. For the aliphatics, the relative amount of lighter compounds, those in the region bounded by dodecane and hexadecane on the chromatogram, increased from 25% at 30 days of exposure to 56% after 180 days of exposure (Table 2).

The fractionation of aliphatics within the dodecane to hexadecane range was studied further. Normal alkanes, branched alkanes, monocyclic alkanes and bicyclic alkanes were assayed by GC-MS to determine the relative intensity of parent peaks of each class with 12 to 16 carbon atoms. This was done for extracts of the oil in water and oil exposed clams collected after 90, 180 and 240 days. For each sample, the ion intensity data of the five carbon numbers investigated was normalized within each compound class (Table 3). Then the data were regrouped by carbon number and each compound class was renormalized over the sampling sequence (Table 4). The double normalization eliminated the effect of variations in relative abundance of the parent ions in the individual mass spectra of compounds in the four classes; this procedure demonstrated the enhancement or diminution for each carbon number of each compound class relative to the total abundance in that compound class at any given time. These results for the branched, monocyclic and bicyclic alkanes are shown in Figure 5.

TABLE 1. *MACOMA BALTHICA*. HYDROCARBON CONCENTRATIONS (µg/g wet weight basis) IN TISSUES DURING OILING EXPERIMENT FOR EACH COLUMN CHROMATOGRAPHIC FRACTION

	Treatment							
	Control		0.03 mg/l		0.3 mg/l		3.0 mg/l	
Day	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
0	7.9	22						
7	7.7	13	30	22	60	63	480	310
15	8.9	6.4	37	32	80	63	570	310
30	8.2	9.4	48	33	190	120	1020	430
60	9.7	14	91	84	390	340	780	600
90	9.5	13	108	130	350	300	630	570
120	8.8	16	130	140	540	400	630	510
150	9.5	13	150	110	390	250	500	360
180	7.2	8.4	68	81	240	130	160	350
240	6.9	7.4	64	51	61	52		

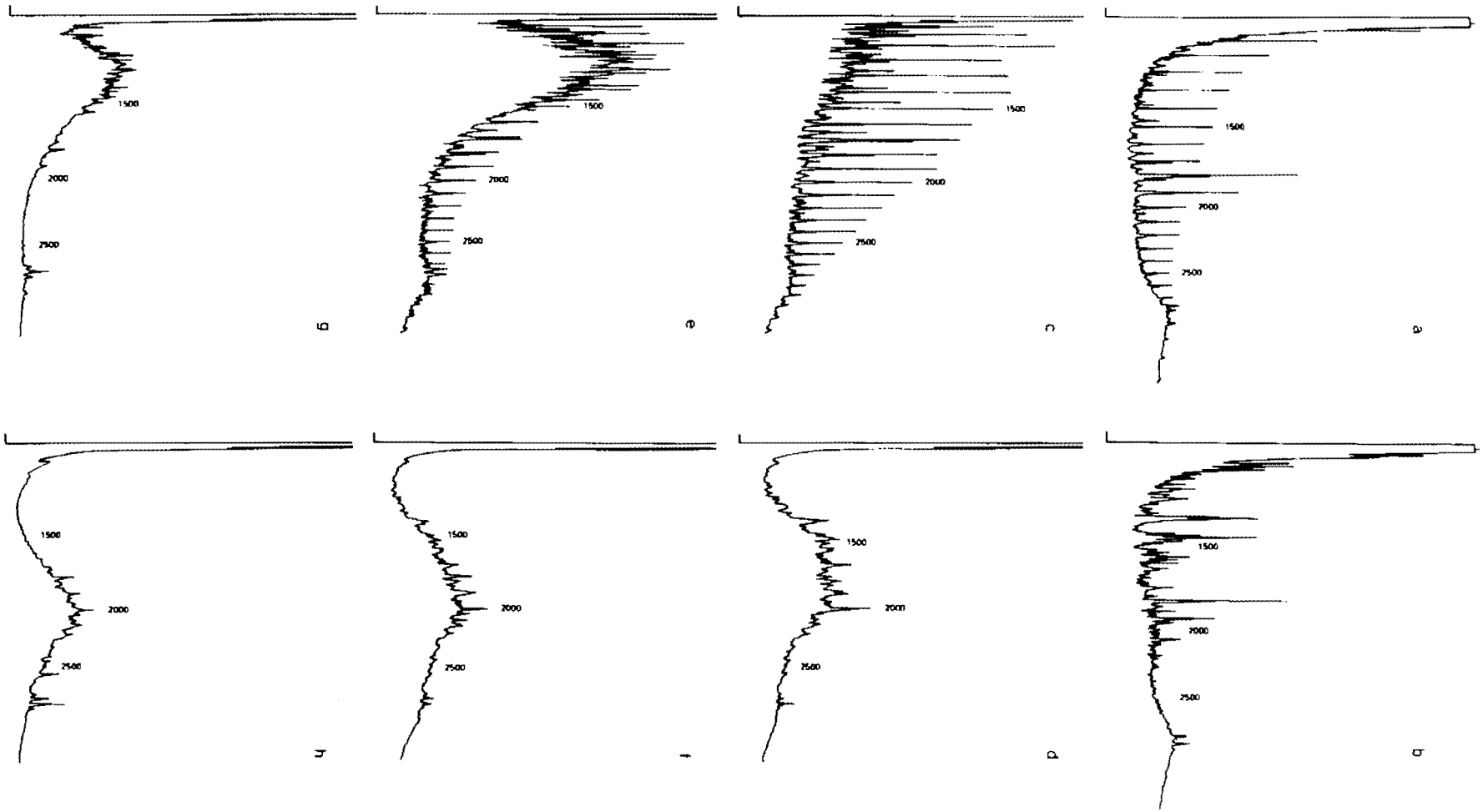

Figure 4. *Macoma balthica* gas chromatograms of oil in water dispersion and tissue accumulations of hydrocarbons. A, saturate fraction of oil in water dispersion; B, unsaturate fraction of oil in water dispersion; C, saturate fraction of tissue after 120 days; D, unsaturate fraction of tissue after 120 days; E, saturate fraction of tissue after 180 days; F, unsaturate fraction of tissue after 180 days; G, saturate fraction of tissue after 240 days; H, unsaturate fraction of tissue after 240 days. Retention indices are shown for each chromatogram.

TABLE 2. *MACOMA BALTHICA*. PERCENTAGE OF TOTAL SATURATED ALIPHATIC HYDROCARBONS ELUTING BETWEEN DODECANE AND HEXADECANE DURING GAS CHROMATOGRAPHY

	Treatment			
Day	Control	0.03 mg/l	0.3 mg/l	3.0 mg/l
0	10			
30	3	25	30	28
60	5	34	33	32
90	2	49	39	36
120	3	43	32	34
150	1	40	33	30
180	4	57	52	44
240	0.5	53	55	

TABLE 3. AREA AND PERCENT OF TOTAL AREA FOR EACH OF FOUR CLASSES OF ALKANES WITH 12 TO 16 CARBON ATOMS IN GAS CHROMATOGRAMS FROM SAMPLES OF THE OIL/WATER DISPERSION AND *MACOMA BALTHICA* TISSUE TAKEN DURING CHRONIC EXPOSURE TO THE DISPERSION

Carbon Atoms	Normal Alkanes		Branched Alkanes		Monocyclic Alkanes		Bicyclic Alkanes	
	area	%	area	%	area	%	area	%
			Oil/seawater Dispersion (o/w)					
12	435	18	394	25	892	37	3216	36
13	587	24	400	25	539	22	2939	27
14	321	13	296	19	306	13	1470	17
15	569	24	255	16	316	13	1063	12
16	492	20	238	15	344	14	724	8
			Macoma balthica 90 day exposure (90)					
12	1381	19	2562	28	8697	38	30748	32
13	1675	24	2218	24	6465	28	28179	30
14	1392	20	1775	20	3766	16	19803	21
15	1295	18	1426	16	2318	10	11185	12
16	1354	19	1098	12	1638	7	5247	6
			Macoma balthica 180 day exposure (180)					
12	1775	19	5342	29	19059	34	32858	25
13	1843	19	4703	25	16349	29	38562	29
14	1809	19	3504	19	10377	18	31585	24
15	2052	21	2807	15	6658	12	20757	15
16	2069	22	2377	13	4282	8	10236	8
			Macoma balthica 180 day exposure, 60 day depuration (240)					
12	0	0	5318	37	21145	33	38305	20
13	0	0	3981	28	19372	31	55203	29
14	0	0	2554	18	12165	19	49800	26
15	0	0	1447	10	6701	11	33119	17
16	0	0	1045	7	3754	6	15221	8

TABLE 4. PERCENT OF TOTAL AREA FOR EACH OF FOUR CLASSES OF ALKANES IN GAS CHROMATOGRAMS FROM SAMPLES OF THE OIL/WATER DISPERSION AND *MACOMA BALTHICA* TISSUE TAKEN DURING CHRONIC EXPOSURE TO THE DISPERSION, REGROUPED BY CARBON NUMBER AND NORMALIZED WITHIN EACH CLASS

Carbon Atoms	Normal Alkanes		Branched Alkanes		Monocyclic Alkanes		Bicyclic Alkanes		Sample
	%	Norm	%	Norm	%	Norm	%	Norm	
12	18	.94	25	.68	37	.97	36	1.0	o/w
12	19	1.0	28	.76	38	1.0	32	.89	90
12	19	1.0	29	.78	34	.89	25	.69	180
12	0	0	37	1.0	33	.86	20	.56	240
13	24	1.0	25	.89	22	.71	27	.90	o/w
13	24	1.0	24	.86	28	.90	30	1.0	90
13	19	.79	25	.89	29	.94	29	.96	180
13	0	0	28	1.0	31	1.0	29	.96	240
14	13	.65	19	.95	13	.68	17	.65	o/w
14	20	1.0	20	1.0	16	.84	21	.81	90
14	19	.95	19	.95	18	.95	24	.92	180
14	0	0	18	.90	19	1.0	26	1.0	240
15	24	1.0	16	1.0	13	1.0	12	.71	o/w
15	18	.75	16	1.0	10	.76	12	.71	90
15	21	.88	15	.94	12	.92	15	.88	180
15	0	0	10	.63	11	.85	17	1.0	240
16	20	.91	15	1.0	14	1.0	8	1.0	o/w
16	19	.86	12	.80	7	.50	6	.75	90
16	22	1.0	13	.87	8	.57	8	1.0	180
16	0	0	7	.47	6	.43	8	1.0	240

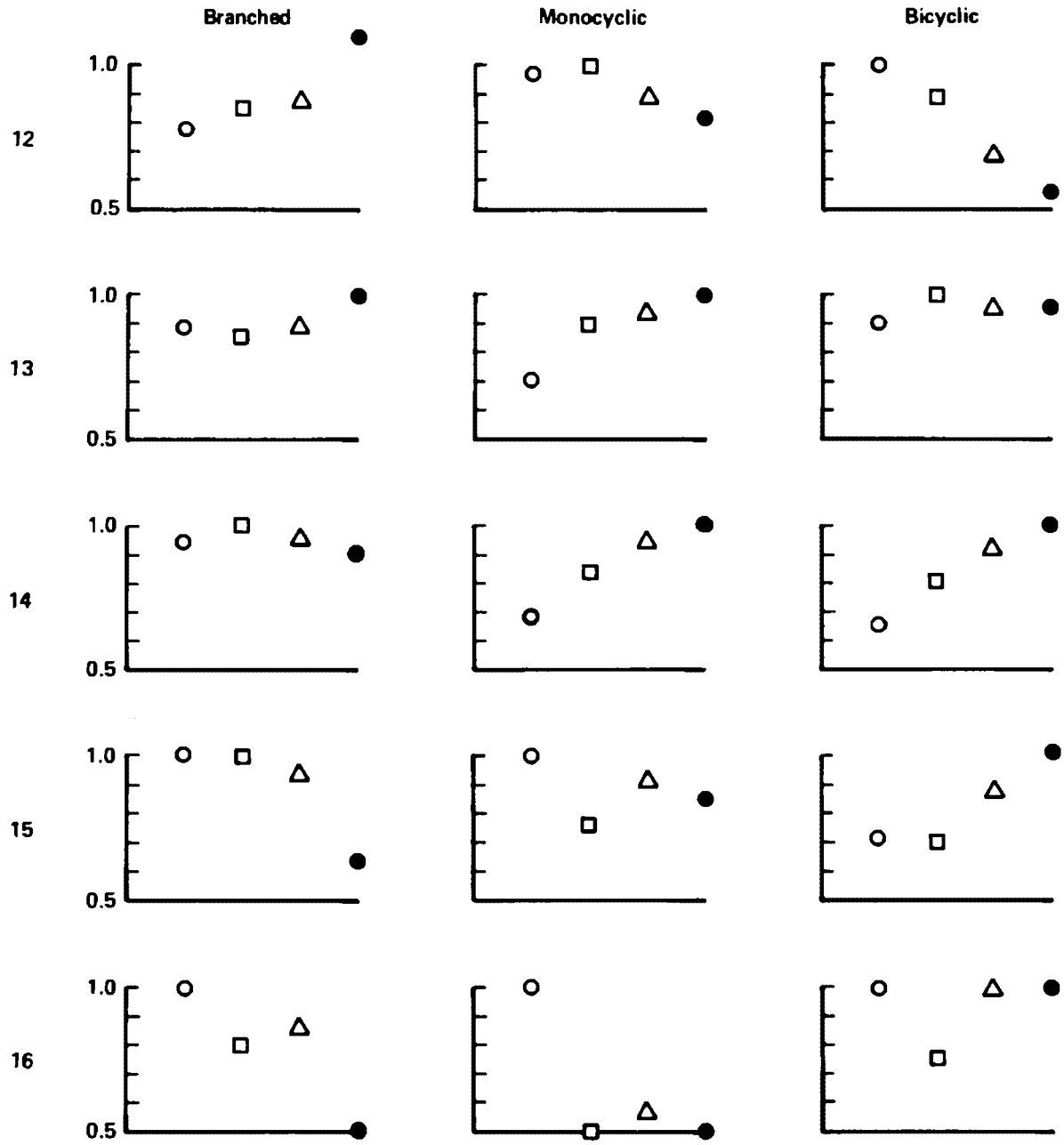

Figure 5. *Macoma balthica* branched, monocyclic, and bicyclic saturated hydrocarbons with 12 to 16 carbon atoms. Each graph in the matrix shows the amounts of the compounds with the indicated structure and carbon number in oiled seawater (○), and in tissue at day 90 (□), 180 (△), and 240 (●) relative to the total abundance of the structural class at the time. The normalization procedure used to derive these results is described in the text.

The results for normal alkanes are not shown since this compound class showed diminution through time for all chain lengths. The branched alkanes showed relative enhancement of 12 and 13 carbon atom compounds through time and relative diminution of larger compounds. In the monocyclic alkanes relative enhancement was observed for compounds with 13 and 14 carbon atoms. While the bicyclics showed relative enhancement only for compounds of 14, 15 and 16 carbon atoms.

Aromatic compounds identified and quantified by single ion MS, and their mean alkyl substitution level are presented in Tables 5 and 6 respectively, for low oil level clams and the oil/seawater dispersion. Relatively high levels of these compounds were found in clams before the start of the experiment, suggesting that *Macoma balthica* in Resurrection Bay are exposed to low levels of aromatic hydrocarbons. The control clams, after 180 days in clean seawater had lost most of the aromatic compounds, but still retained significant amounts of the anthracenes, fluoranthenes, and dibenzothiophenes. (Here and throughout this paper we use the name of one aromatic ring system to indicate that system and its isomers: thus "anthracene" means "anthracene and phenanthrene"; and by "anthracenes" we mean "anthracene, phenanthrene and their alkyl homologs".) The mean substitution level for most of the parent compounds generally increased with continued oil exposure and even upon depuration. There was a considerable difference between the mean substitution level of the oil-exposed clams after 60 days depuration and that of the oil/seawater dispersion for all the aromatic systems except the fluoranthenes. In general, there was selective retention of more substituted compounds (Table 7), except for the chrysenes for which the mean substitution level was lower after depuration than in the oil/seawater dispersion.

The concentration of aromatic compounds determined by GC-MS and the total concentration of hydrocarbons in the second fraction of column chromatography determined by GC were not the same and the concentration found by GC-MS as a percentage of the total varied from 25% in clams before oil exposure to 6% after depuration. The concentration of hydrocarbons determined by GC-MS is an underestimate, as only the parent compounds were calibrated against standards and increased alkyl substitution causes decreasing response in single ion monitoring. The change in the percentage of the total accounted for by MS may

TABLE 5. *MACOMA BALTHICA*. CONCENTRATION OF AROMATIC HYDROCARBONS PRESENT IN ANIMALS AND EXPOSURE WATER

	Macoma balthica (ng hydrocarbons/g wet weight)						Oil/water
Treatment Day	Control 0	0.03 mg/kg 60	0.03 mg/kg 120	0.03 mg/kg 180	0.03 mg/kg 240	Control 180	0.03 mg/l
Compounds							
Naphthalenes	120	1600	2400	980	340	21	350
Biphenyls	160	930	1500	780	330	31	86
Tetralins	5.5	640	1000	440	130	0	43
Fluorenes	470	1400	2100	960	260	93	110
Anthracenes	2200	3000	3200	1900	740	380	200
Fluoranthenes	220	920	960	540	200	130	30
Chrysenes	73	310	510	240	68	26	15
Benzothiophenes	0	140	280	120	44	0	14
Dibenzothiophenes	1800	2300	3000	1300	570	130	140
Benzonaphthenothiophenes	150	570	780	470	210	0	24

TABLE 6. *MACOMA BALTHICA*. MEAN SUBSTITUTION LEVEL OF AROMATIC HYDROCARBONS PRESENT IN ANIMALS EXPOSURE WATER

	Macoma balthica						Oil/water
Treatment Day	Control 0	0.03 mg/kg 60	0.03 mg/kg 120	0.03 mg/kg 180	0.03 mg/kg 240	Control 180	0.03 mg/kg
Compounds							
Naphthalenes	5.0	5.2	5.3	6.3	7.2	4.3	3.6
Biphenyls	4.5	5.0	5.0	5.4	5.6	5.6	3.9
Tetralins	6.1	4.8	4.9	5.4	5.7	0	3.4
Fluorenes	3.3	4.7	4.5	4.8	5.6	2.7	3.8
Anthracenes	2.6	3.3	3.3	3.5	3.8	3.5	3.1
Fluoranthenes	1.7	2.4	2.5	2.5	2.4	2.2	2.4
Chrysenes	0.5	1.1	1.2	1.1	0.9	0.7	1.1
Benzothiophenes	0	6.1	5.6	6.1	7.0	0	4.6
Dibenzothiophenes	2.7	3.2	3.2	3.2	3.3	6.0	3.0
Benzonaphthenothiophenes	1.4	1.9	2.0	2.4	2.5	0	1.9

TABLE 7. *MACOMA BALTHICA*. CONCENTRATION FACTOR[a] OF AROMATIC COMPOUNDS IN ANIMALS EXPOSED TO 0.03 mg/kg OIL IN WATER AFTER 60 DAYS DEPURATION. (MOLECULAR WEIGHT OF COMPOUND)

Alkyl Substitution (No. of carbon atoms)	Naphthalenes	Dibenzothiophenes	Anthracenes	Chrysenes
0	0.05(128)	0 (184)	0.26(178)	4.44(228)
1	0 (142)	0.89(198)	0.55(192)	4.93(242)
2	0.01(156)	2.98(212)	2.82(206)	5.61(256)
3	0 (170)	7.12(226)	5.91(220)	
4	0.63(184)	7.96(240)	5.84(234)	
5	3.01(198)	8.73(254)	10.56(248)	
6	5.40(212)	8.77(268)	10.54(262)	
7	9.30(226)			

[a]Concentration factor = $\frac{\mu g \text{ compound/g wet weight of } \textit{M. balthica}}{\mu g \text{ compound/g seawater}} \times 10^{-3}$

be the result of the different levels of substitution encountered, the presence of hydrocarbon or hetero-aromatic systems not investigated by GC-MS or possibly by the formation of oxygenated metabolites whose column chromatographic behavior is similar to the aromatics.

DISCUSSION

The continuous flow system produced reasonably uniform petroleum exposures at the three test concentrations (Fig. 2). The fluctuations of the measured oil concentrations in the high, medium and low exposures tended to occur together (*e.g.*, all were lowest on day 90). This is to be expected since the two lower concentration solutions were produced by dilution of the high concentration.

Previous work has indicated that non-polar lipids partition between animal tissues and the aquatic environment in an equilibrium fashion (Hamelink *et al.*, 1971) and that the initial uptake rate of hydrocarbons is directly proportional to the aqueous hydrocarbon concentration (Stegeman and Teal, 1973). Expressed symbolically these relationships are:

$$K = [O_A]/[O_W] \qquad (1)$$

$$\text{and} \quad d[O_A]/dt = k_i[O_W] \qquad (2)$$

where K is the equilibrium constant for the partition of oil between animals and water, $[O_W]$ is the concentration of oil in water, $[O_A]$ is the concentration of oil in the animal and k_i is the rate constant for the initial uptake of oil by the animal. Using our data from Figure 3, we have evaluated (1) at 120 days and (2) at seven days for the three values of $[O_W]$. K varied from 9.2×10^3 at 0.03 mg/l down to 3.8×10^2 as the oil concentration rose to 3.0 mg/l and k_i ranged from 95/day at the high oil concentration to 34/day at the low concentration of oil. Both "constants" had intermediate values at 0.3 mg/l oil. Both k_i and K increased as $[O_W]$ decreased; that is *M. balthica* is more efficient at extracting and retaining oil from water at low oil-in water concentrations.

One explanation for this observation is that the concentration of hydrocarbons in the clams is related not only to the hydrocarbon concentration in

the water, but also to the amount and efficiency of water filtration accomplished by the clams at the various oil exposure levels. Stainken (1975) has observed the mode of accumulation of an oil/water dispersion in a filter-feeding bivalve (*Mya arenaria*). Oil droplets are processed as food and are accumulated in the gut and digestive diverticula and from there are transferred to the rest of the body, probably as individual molecules. No food was administered to the *Macoma balthica* in the present study for 24 h prior to sampling for hydrocarbon analysis, however the animals were left in seawater containing oil droplets. No attempt was made to segregate the gut from the other tissues, thus the hydrocarbon data are for entire clams, including gut contents without food. At higher oil concentrations in this experiment, the rate of uptake declined probably as a result of the greatly reduced siphon activity of the animals exposed to the high oil concentration (Chapter 3). The uptake rate response of *M. balthica* over the large range of concentration of this experiment demonstrated that other behavioral patterns in addition to shell closure at very high exposure levels may influence the rate at which hydrocarbons are taken up by organisms.

Bivalves exposed to oil usually fail to return rapidly to pre-exposure hydrocarbon levels during depuration (Lee *et al.*, 1972; Stegeman and Teal, 1973; Clark and Finley, 1975; Fossato, 1975; Boehm and Quinn, 1977). Stegeman and Teal (1973) hypothesized a stable compartment in an organism, which after saturation with hydrocarbons, released those hydrocarbons very slowly. In our experiment, the low and medium exposure clams returned to approximately the same hydrocarbon level after 60 days of depuration. It may be that this final oil concentration 110 mg/g (wet weight) represents the size of this "stable compartment" for *Macoma balthica*. However, in the absence of information from even longer depuration periods, this conclusion is quite tentative.

As illustrated in Figure 4, our experimental work shows that fractionation of Prudhoe Bay crude oil occurred during both accumulation and depuration by *Macoma balthica*. Because of the extreme molecular complexity of the crude oil, we have not examined the fractionation process for individual compounds but have investigated the behavior of several classes of compounds present. Table 1, which records the concentrations of aliphatic and aromatic hydrocarbons in *M. balthica* shows that aromatics were enhanced in the animals' tissues throughout

the experiment. The ratio of aliphatics to aromatics (f_1/f_2) in the oil in water dispersion was 3.7. However this ratio in the tissues was generally less than 1.5 and always less than 2.5. Clearly *M. balthica* selected in favor of aromatics both in initial uptake rate and in equilibrium partition.

Figure 5 indicates that within the aliphatic fraction cyclic compounds were generally retained to a greater extent than branched chains which in turn were preferentially held over linear alkanes. Similar fractionation has been observed by other workers (*e.g.* Blumer *et al.*, 1970; Stegeman and Teal, 1973). Figure 5 and Table 2 also show that molecular size (carbon number) influenced retention. Although these results are too complex for detailed interpretation, it seems reasonable that selective metabolism, tissue transport, membrane transport or final deposition site may be related to the size, solubility or configuration of the compound (Tanford, 1978). The apparent preference for accumulation and retention of lighter aliphatic compounds (Table 2), was unexpected in view of decreasing aqueous solubility with increasing molecular weight. This may indicate that uptake of aliphatics occurs *via* the dissolved phase with attendant fractionation, but that release involves some other process.

While the relative percentage of the aromatic ring systems remained fairly constant during the course of our experiment, the mean alkyl substitution level increased, most noticeably in the lighter aromatic compounds. Given the inherent change in sensitivity of MS with increased levels of alkyl substitution, it is difficult to demonstrate a change in relative amount of the sum of each parent compound and its alkyl substituants. Changes in the relative amount of alkyl substituted aromatic compounds have been observed in a benthic anemone following an oil spill (Grahl-Nielsen *et al.*, 1978). Only aromatic compounds with up to 3 carbon alkyl substitution were examined, but there appeared to be a pattern favoring higher alkyl substitution with time. A field study of *Modiolus demissus* chronically exposed to oil (Lake and Hershner, 1977) demonstrated a loss of lighter aromatic compounds upon depuration and an apparent relative increase in 2,3,6-trimethylnaphthalene, fluoranthene, and pyrene. These authors concluded that most diaromatics are lost upon depuration and tri- and tetra-cyclic aromatics are retained. When a larger range of alkyl substituted compounds is available, as in the present study, a pattern of increasing

retention with increasing molecular weight emerges, both among unsubstituted aromatic compounds and among various alkyl substitutions of any given aromatic ring system. However, for compounds of a given molecular weight, the more substituted one has a higher concentration factor (Table 7). Thus, in contrast to the behavior of aliphatic hydrocarbons, retention of aromatic compounds appears to decrease with increasing aqueous solubility, and the long period of slow depuration observed in many bivalves may result from relatively high concentrations of less water soluble aromatic compounds.

In studies of aromatic sulfur compounds in organisms upon recovery from oil exposure, Grahl-Nielsen *et al.* (1978) found an increase in the relative amount of dibenzothiophene and its alkyl derivatives, whereas Lake and Hershner (1977) found a decrease in aromatic sulfur compounds that paralleled the decrease in aromatic compounds as a whole. In the present study there was little or no enhancement of the aromatic sulfur compounds relative to other aromatic compounds. The concentration factor for C_6 substituted dibenzothiophenes (Table 7) fell between C_6 naphthalenes and C_6 anthracenes, about where one might expect it on the basis of molecular weight.

It thus appears from our laboratory data, that when *Macoma balthica* is exposed to chronic low levels of an oil in water dispersion, the characteristics of the oil are extensively modified within the clam. The aliphatic compounds are handled quite differently than the aromatics. We have suggested selective metabolism as one of the possible causes of fractionation of petroleum by *M. balthica*. Although much work under short-term acute conditions (Varanasi and Malins, 1977) indicates that molluscs have little or no ability to metabolize hydrocarbons, we believe that the presently available information does not exclude the possibility of selective metabolism during longer accumulation and release periods.

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CHAPTER 3

BIOCHEMICAL AND PHYSIOLOGICAL EFFECTS OF OIL ON *MACOMA BALTHICA*

One of the more noticeable events that follows severe oil pollution in a marine system is mass mortality in number of species of plants and animals (Burk, 1977; Krebs and Burns, 1977; American Petroleum Institute, 1977). However, mortality, although easy to measure, is the grossest of all effects of oil pollution. Numerous workers have investigated a variety of more subtle sub-lethal effects of oil pollution including respiration (Eldridge *et al.*, 1977; Hargreave and Newcombe, 1973), cellular morphology (Gardner *et al.*, 1975), reproductive development (Berdugo *et al.*, 1977; Byrne and Calder, 1977), feeding (Atema and Stein, 1974; Morton and Wu, 1977) growth and maturation (Griffin and Calder, 1977; Keck *et al.*, 1978), behavior (Donahue *et al.*, 1977; Hargreave and Newcombe, 1973), and even some biochemical effects (Davavin *et al.*, 1975; Heitz *et al.*, 1974; Manwell and Baker, 1967). To date only a few parameters have been measured for any single species. It has been difficult to extrapolate what effects will show up in one particular organism exposed to a particular pollutant.

Many oil pollution studies performed in recent years have been concerned primarily with either uptake and retention of hydrocarbons (Boehm and Quinn, 1977; Clark and Finley, 1975; Corner, 1975; Fossato and Canozonier, 1976; Harris *et al.*, 1977) and with short term, effects of crude or refined oils on various parameters of marine organisms (e.g., Atema and Stein, 1974; Anderson *et al.*, 1974; Gilfillan, 1975; Griffin and Calder, 1977; Hargreave and Newcombe, 1973; Lee *et al.*, 1978; Lindén, 1978; Malins, 1977). The major rationale for these studies is to attempt to understand what effects an acute oil spill will have on various marine ecosystems.

There is, however, need for studies which involve the effects of low levels of oil pollution over an extended period of time. Such a

chronic pollution situation is now in existence in Port Valdez, Alaska, the terminus of the Trans Alaskan pipeline. The ballast water treatment plant at Valdez is currently adding a low level of oil into that harbor at a more or less continuous rate. The effect of this kind of oil input on the biological populations in the harbor is largely unknown. Since large populations of the intertidal clam *Macoma balthica* reside in the harbor this organism was chosen as an experimental animal in these studies.

Previous experiments have shown that *M. balthica* is sensitive to oil treatment. Shaw *et al.*, (1976) have shown that this species will burrow to the surface of the sediment after the mud flats have been subjected to an oiling regime. High concentrations of oil ($5\mu l/cm^2$) caused significant mortalities. Further studies conducted in the laboratory have confirmed these results (Shaw *et al.*, 1977). Taylor and Karinen (1977) have exposed *M. balthica* to oiled sea water by three different methods. They reported that oiling caused increased mortality, inhibited the burrowing response of the clams, and also caused the clams to burrow to the surface of the mud. *Macoma balthica* is therefore a good candidate for studying the effects of oil on intertidal, benthic marine organisms. Further the clam is circumpolar in distribution (Gilbert, 1973), and its biology has been studied and reported by several researchers (Brafield and Newell, 1961; Bubnova, 1972; Chambers and Milne, 1975; Gilbert, 1973, 1977, 1978).

It was our hope that by measuring several different parameters for an organism we would be able to arrive at a better understanding of how oil pollution can affect that organism and possibly its entire population. We subjected *Macoma balthica* to continuous levels of Prudhoe Bay crude oil suspended in sea water from June to December 1977. Various behavioral, physiological, and biochemical parameters were assayed in an attempt to define the potential effects of long-term oil pollution on this marine organism.

METHODS

Experimental Set-up

Approximately 12,000 *Macoma balthica* were obtained from the mud flats of Resurrection Bay near Seward, Alaska at low tides during May 3-5, 1977.

The clams were placed in fresh running sea water for about two weeks at 6°C. Approximately two weeks before the commencement of the experiment the clams were screened through hardware cloth into various size classes. Clams 11-13 mm in length were selected for feeding rate studies. Clams 6-11 mm long were selected for physiological, behavioral and biochemical studies. The clams were placed in plastic petri dish bottoms (90 x 25 mm) filled with 170 grams of pre-washed silica sand (0.1 to 0.7 mm). Fifteen clams of 6-11 mm in length were placed in each dish. Only ten clams of the 11-13 mm size were used per dish.

The clams were placed in the exposure tanks which measured 110 cm by 50 cm by 36.5 cm deep (about 200 l total volume of water). Two tanks were used for each exposure level. Fresh sea water flowed into and through the tanks at approximately 3.0 l/min. After one week clams which remained on the surface were replaced until all individuals had buried themselves.

On 14 June 1977 oil was introduced at various rates by a continuous-dosing apparatus as described in Chapter 2. Three different oiling levels plus a control were used in the experiment. The average levels of hydrocarbons in each treatment were approximately control, 0.0 mg/l; low oil, 0.03 mg/l; medium oil, 0.3 mg/l; high oil 3 mg/l. Water temperatures, pH, salinity and dissolved oxygen were essentially the same for all treatments (Table 1). Tanks were cleaned periodically and dead animals were counted and removed. The experimental clams were fed an average of 4-6 hours a day by pumping diatom-rich water into the tanks from a nearby "upwelling pond" (Nevé *et al.*, 1976). Feeding began on 9 July and was stopped on 21 November 1977.

Biochemical Assays

Clams were sampled for various biochemical assays by removing randomly selected petri dishes and washing the clams onto a screen. The clams were then placed back into the tanks from which they had been removed to depurate for 24 hours (except clams which were to be assayed for enzymes which were not allowed to depurate but were homogenized at once). After 24 hours depuration the clams were shucked, and weighed. They were then lyophilized for 12-24 hours and weighed again to obtain dry weights. The dried clams were stored in dessicators at -20°C until assayed.

TABLE 1. AVERAGE MONTHLY SALINITIES, TEMPERATURE, pH, DISSOLVED OXYGEN AND PHYTOPLANKTON SPECIES DENSITIES OF WATER FLOWING INTO EXPOSURE TANKS

Day	Salinity °/₀₀	Temp. °C	pH	dO_2 ppm	Phytoplankton Densities cells/m[a]	Dominant Species %
0	32.6	7.0	-	-	20,500	Sc[a] 76 UPD 22
30	31.6	7.7	-	-	7,900	Sc 84 UPD 10
60	34.0	7.7	-	-	1,300	Sc 43 UPD 49
90	33.7	7.4	-	-	900	Ls 57 Cc 26 Sc 13
120	33.0	9.0	-	-	3,400	Bf 71 Rs 18
150	33.0	9.1	8.1	8.8	9,700	Bf 91 UPD 6
180	-	8.0	-	-	-	- -

[a]phytoplankton abbreviations: Sc = *Skeletonema costatum*; UPD = Unidentified Pennate Diatoms; Ls = *Leptocylindricus* spp.; Cc = *Cylintrotheca closterium*; Bf = *Bacteriosira fragilis*; Rs = *Rhizosolenia* sp.

[b]phytoplankton densities of upwelling (feeding) water.

Protein, Glycogen, and Total Carbohydrates

All assays were performed separately on individual clams. The lyophilized clams from each treatment were each soaked in 1.0 ml of cold distilled water for 15 to 30 minutes and then homogenized at 0° in a 5.0 ml glass-Teflon tissue homogenizer at 2250 rpm for 25-30 seconds. The homogenate was made up to 4.0 ml with distilled water and stored frozen until assayed.

Protein was assayed by the method of Lowry *et al.* (1951) directly on the crude homogenate. Bovine serum albumin (BSA, Sigma Chemical Co.) was used as the standard.

Glycogen was measured on the same crude homogenate by a modification of the method of Seifter *et al.* (1950) using 40% KOH digestion for three hours at 90-100°. The glycogen was precipitated with KCl-saturated 95% ethanol according to the procedure recommended by Giese (1967). The resuspended glycogen was assayed by the anthrone procedure (Spiro, 1966) and expressed as milligrams of glucose equivalents. Total carbohydrate in the crude homogenate was assayed by the anthrone procedure using glucose as a standard (Spiro, 1966).

Total Lipid

Total lipid was also determined on individual clams. To the lyophilized clam was added 100 µl of distilled water on ice. After 15-20 minutes 2.0 ml of benzene:menthanol (2:1 v/v) were added and the clam was homogenized in a 5.0 ml glass-Teflon tissue homogenizer at 2000 rpm for 30 seconds at 3-4°. The homogenizer was rinsed with an additional 1.0 ml of benzene:menthanol. The extract was washed with 1.0 ml of 0.9% NaCl in water and centrifuged to separate the layers. The upper lipid layer was transferred, and the water phase was re-extracted with 1.0 ml of benzene. The benzene fraction was added to the lipid fraction. The lipid extract was washed once more with 1.0 ml 0.9% NaCl and the water phase was again back-extracted with 1.0 ml of benzene. All lipid phases were combined in a tared vial. The vials were reweighed to obtain the total lipid weight.

RNA and DNA

RNA was separated from DNA by a modified Schmidt-Thannhauser procedure suggested by Munro and Fleck (1966). RNA was determined by its absorbance at 260 nm using calf liver RNA (Sigma Chemical Co.) as a standard. DNA was extracted in hot (70°) 1.2 N $HClO_4$ and estimated using diphenylamine according to the procedure of Giles and Meyers (1965). It was found unnecessary to correct for turbidity by measuring the absorbance at 700nm. Calf thymus DNA (Sigma Chemical Co.) served as a standard.

Preparation of the Crude Homogenate for Enzyme Assays

At each time point ten clams from each treatment were screened from the sand and placed in cold (2-4°), fresh sea water. The clams were shucked and placed into a 15 ml conical centrifuge tube. Three milliliters of cold "homogenization buffer" (0.25 M sucrose, 1mM EDTA, pH 7.0) were added and the contents mixed and centrifuged. The supernatant was decanted which removed most of the unabsorbed oil droplets from the clams. The clams from such treatment were then homogenized in 10.0 ml of the homogenization buffer on an ice bath at top speed for two, ten second bursts in a Virtis '45' homogenizer. The homogenate was passed through nylon gauze into a 15.0 ml glass-Teflon tissue homogenizer. The solution was further homogenized at 1000 rpm for three strokes. This homogenate was stored frozen at -20° in 2.0 ml aliquots for enzyme assays.

Na^+-K^+-ATPase and Mg^{++}-ATPase (EL No. 3.6.1.4)

For total ATPase the assay mixture contained in a final volume of 1.0 ml: 50 mM imidazole·HCl (pH 7.5), 4 mM $MgCl_2$, 0.5 mM Na_2EDTA, 20 mM NaCl, 20 mM KCl, 3 mM ATP, and 0.100 ml of the crude homogenate. To measure the Mg^{++} dependent ATPase, KCl and NaCl were omitted and ouabain was added at a final concentration of 2 mM. Na^+-K^+-ATPase activity was taken as the difference between the total ATPase and the Mg^{++}-ATPase activities. Controls included a blank without crude homogenate, a phosphate standard, and a blank with crude homogenate added after the reactions were stopped with trichloroacetic acid (TCA). Incubations were carried out at 20° for 10 minutes and stopped

with 2.0 ml of cold ascorbate:TCA (2.5%: 12.5% w/v). The precipitated protein was pelleted by centrifugation. Inorganic phosphate was measured in the supernatant by the method of Baginski *et al.* (1974). Results were expressed as nanomoles Pi released/minute/mg protein.

5'-Nucleotidase (EL No. 3.1.3.5)

This assay was run essentially as described by Aronson and Touster (1974) with the following modifications. A volume of 0.100 ml of crude homogenate was assayed in 0.500 ml total. The reaction was run at 30° for 15 minutes and stopped with 1.0 ml of ascorbate:TCA (2.5%:12.5% w/v). Inorganic phosphate was measured by the method of Baginski *et al.* (1974). Results were expressed as nanomoles Pi released/minute/mg protein.

Phosphodiesterase (EL No. 3.1.4.1)

The substrate used in this assay was Thymidine 5' monophospho-p-nitrophenol ester (Sigma Chemical Co.). The enzyme was assayed according to the method of Aronson and Touster (1974). A total volume of 0.5 ml was used, including 50 µl of the crude homogenate. Assays were run at 30° for 15 minutes. Results were expressed as changes in absorbance at 400nm/minute/mg protein (ΔA_{400}/min/mg protein). Protein concentrations in the crude homogenates were assayed by the method of Lowry *et al.* (1951). The assays of the enzymes were linear with respect to protein concentration under the conditions employed.

Physical, Behavioral, and Physiological Assays

Wet and Dry Weights

Wet and dry weights were determined on approximately 30 *Macoma balthica* from each treatment. Wet weights were obtained from depurated and shucked clams. Dry tissue weights were measured after lyophilization for 12-24 hours.

Growth and Condition Index

One hundred clams from each treatment (10 clams per petri dish) were selected to monitor growth and condition. These clams (10.5 mm long, SD = 0.15 mm) were left undisturbed from 14 June to 12 October 1977. The measurements

taken at the beginning and the end of the experiment were shell length and dry tissue weight. Dry weight was obtained by drying the tissue in an oven at 80°C until a constant weight was reached. A condition index relating dry weight to shell length was calculated by:

$$\text{Condition Index} = \frac{\text{Dry Weight (g)}}{\text{Shell Length (mm)}} \times 100$$

Burying Experiment

One hundred clams (6-11 mm in length) from each treatment were used in experiments to determine the relative burrowing rates. The clams were screened from the sand prior to each experiment and replaced on top of the sand in a pyrex baking dish (25.3 cm x 15.2 cm x 4.4 cm deep) set 4 cm below the surface of the water. At timed intervals over the next 24 hours the numbers of unburied clams were recorded. The same clams were utilized for each burying experiment. Results were expressed as the percent of the clams which remained unburied after one hour.

Feeding Rates

For feeding experiments one hundred clams for each treatment ranging in size from 11-13 mm were divided into groups of ten and put into 9 cm diameter plastic petri dishes containing 170 grams of silica sand and placed into the treatment tanks. Each month the clams were starved for 24 hours after which three dishes were selected from each treatment and placed on racks in 2.5 ℓ plastic cylinders. The cylinders were filled with 1400 ml of water from the treatment tanks originally containing the clams. One hundred milliliters of fresh sea water containing phytoplankton densities of 1.4×10^5 to 7.5×10^6 cells were then added. The species compositions of the phytoplankton were similar to those listed in Table 1. Water temperature was maintained at 8-9°C. During the first two monthly observations the water in the cylinders was mixed by aeration for five minutes per hour. At the end of six hours a phytoplankton sample was withdrawn for counting. During the last three monthly observations phytoplankton collection occurred after one hour. Phytoplankton were counted and identified at 600x utilizing the Utermohl technique (Lund, *et al.*, 1958).

Respiration

Oxygen uptake by *Macoma balthica* was measured in a smaller version of the modified Scholander respirometer described by Steen and Iverson (1965). The air volume was 15 ml and the water volume 100 ml which allowed changes in air volume of 1 µl to be detected. The respirometer readings were stable to ± 2 µl O_2/hr after equilibration.

Two or three *Macoma balthica* were used for each respiration rate measurement. Clams were removed from the exposure tanks before feeding, screened from the sand, returned to the exposure tanks for two hours, and then placed in the respirometer chambers. Respirometers were allowed to equilibrate for one hour and then oxygen uptake was measured at half-hourly intervals for three or four hours. Clam respiration was measured at 10°, which was close to the average daily exposure temperature. After the final measurement, clams were removed from their shells, weighed, lyophilized, and reweighed. Respiration rate is expressed as µl O_2 consumed (STP)/mg dry weight-hour.

Reproduction

Macoma balthica at day 120 of the experiment were depurated and then placed in formalin. Ten clams were used from each treatment. The gonads were cut out and dehydrated in ethanol (Davenport, 1960). The tissues were cleared in xylene and embedded in paraffin. Sections of 20µm thickness were cut on a microtome, stained with Ehrlich's acid alum hematoxylin, and examined under a microscope for any anomalies in the gonad tissue.

Electron Microscopy of Gill Tissue

Gill tissue was removed from two *Macoma balthica* from each treatment after 120 days of oil exposure. Tissues were fixed in 5% gluteraldehyde buffered in 0.1 M cacodylate, pH 7.3, rinsed in buffer and sent to the Electron Microscope Laboratory at the University of Alaska, Fairbanks, for further processing. Post-fixation was in 1% osmium tetroxide in cacodylate. All fixitive and buffer solutions were adjusted to 980 milliosmols with sucrose to approximate as closely as possible the osmoality of the tissues *in vivo*. Tissues were dehydrated in alcohol and acetone, embedded in Epon-Araldite, and sectioned

with glass or diamond knives. These sections were post-stained with uranyl acetate and lead citrate and viewed in a JEM 6AS electron microscope.

RESULTS

General

When oiling first began, all of the clams were buried in the petri dishes. During the first week of exposure, clams in both the high (3.0 mg/l) and the medium (0.3 mg/l) oiled treatments burrowed to the surface. Approximately 10% surfaced in each treatment. By day 16 almost all of the clams in the medium exposure had reburied themselves. The clams in the high oiled treatment, however, remained at the surface of the sand with more clams surfacing throughout the experiment. The numbers of unburied clams were monitored for each treatment at monthly intervals. The results are shown in Figure 1. It can be seen that 3.0 mg/l oil causes over 90% of the surviving clams to burrow to the surface after six months (180 days) of exposure. It was not until day 90 that the 0.3 mg/l oiled clams began to burrow to the surface in significant numbers ($p < 0.01$).

We also noted that clams which were unburied at 3.0 mg/l oil seemed unable to orient themselves with respect to the surface of the sand. Clams would extrude their feet which would probe in various directions, but were unable to penetrate the sand. Thus the clams were unable to rebury themselves once they had come to the surface.

During feeding periods the clams in the control and the low oiled (0.03 mg/l) tanks would extend their siphons and begin very active feeding behavior, both suspension-type and deposit feeding (Brafield and Newell, 1961). The clams in the medium oil treatment displayed less feeding activity, and those in the high oil treatment were very sluggish in any type of feeding behavior. It was rare that any extended siphons were seen. In general, it appeared that oiling caused a degree of narcosis and/or disorientation in the clams at 3.0 mg/l. These clams remained in this condition throughout the six months of oil exposure.

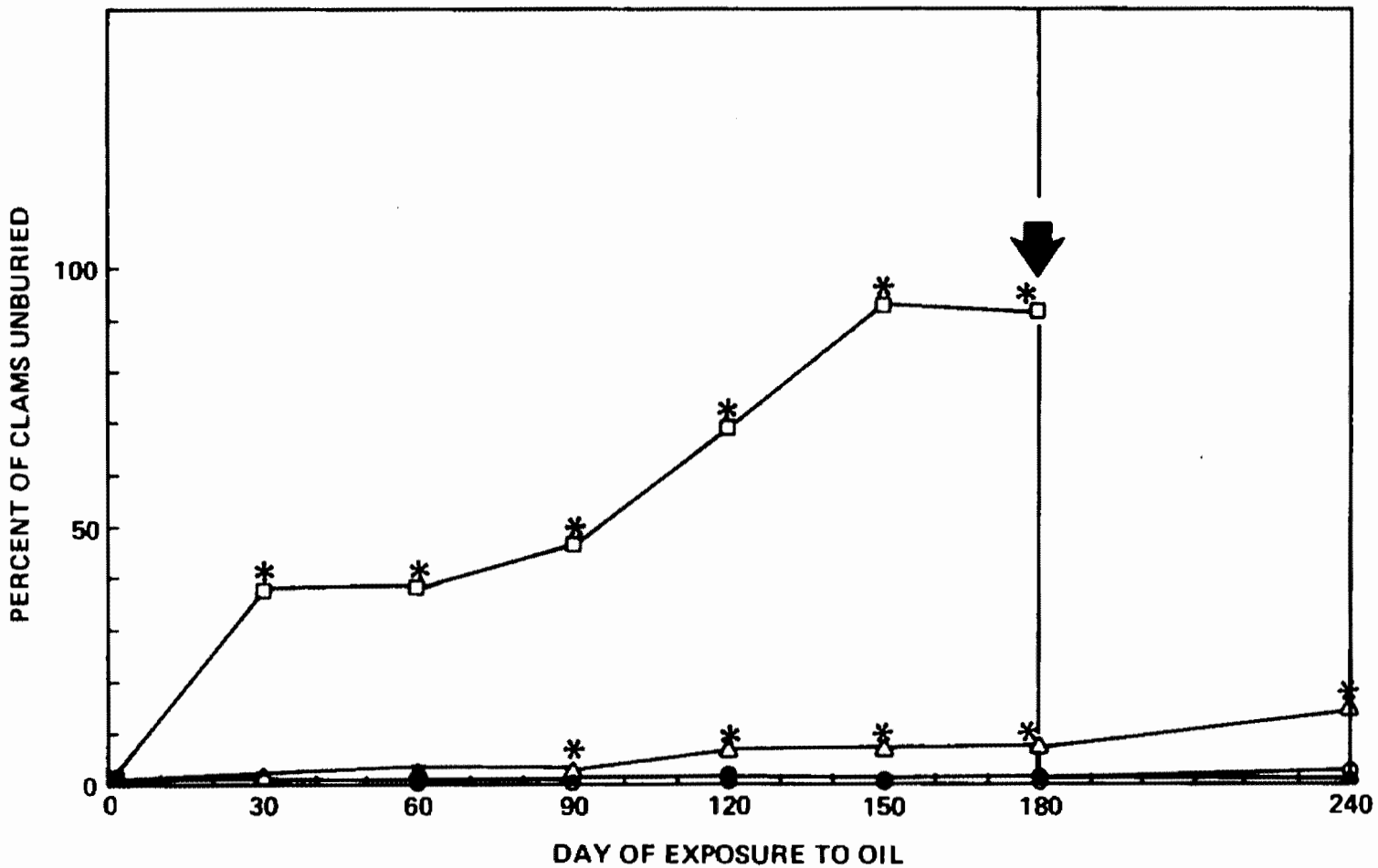

Figure 1. Percentage of unburied clams in oil-exposed seawater. Points marked with an asterisk (*) are significantly different from the control values at $p < 0.01$ (arcsin t-test). (●) control; (○) 0.03 mg/l; (△) 0.3 mg/l; (□) 3.0 mg/l; (↓) end of oiling regime.

Feeding

Experiments performed to determine relative feeding rates of the clams verified the apparent inactivity of the oiled clams. The results of the feeding experiments are shown in Table 2. The values for days 30 and 60 were taken after six hours of feeding. Obviously, this time period was too long since the clams cleared almost all the algal cells in each treatment. Therefore, we used one hour feeding periods subsequent to day 60. There is a general trend of lowered feeding activity with increasing oil dosage. It is likely that even the one hour time period is too long for an accurate estimate of the rate of feeding. The differences between treatments are, therefore, probably underestimates of the actual situation.

Rates of Burrowing

When the clams were measured for their ability to rebury themselves, it was found that oil exposure has almost immediate effects on this behavior pattern. Figure 2 shows the results of a typical experiment at day 90. We picked a time period of one hour to measure the percentage left unburied. This was based on experiments such as the one shown in Figure 2. Figure 3 summarizes the results for all the burying experiments throughout the exposure period. At day 1, the first day of oil exposure the high oiled clams showed a significant decrease in their burying rate with respect to the control ($p < 0.02$, arcsin t-test). This decrease became even greater as the experiment progressed ($p < 0.001$ at days 5-180). Clams at 0.3 mg/l also began to show significant effects by day 5 of the experiment ($p < 0.001$), and they also remained consistently slower than the controls except for the point at day 60. (The point at day 60 was high for the controls due to a power outage and subsequent cessation of the water flow for 12 hours. Many clams in the control tanks burrowed to the surface at this time, probably due to decreased dissolved oxygen levels.) Clams at 0.03 mg/l oil were also slightly affected with respect to the controls, showing significant difference in burrowing rates at days 5, 30 and 90 ($p < 0.05$) (Figure 3).

TABLE 2. A COMPARISON OF THE PERCENTAGE OF ALGAL CELLS FILTERED FROM THE WATER BY *MACOMA BALTHICA* SUBJECTED TO CHRONIC OIL TREATMENT

Days of Exposure to Oil	Initial Cell Count (in 500 ml)	% of Cells Filtered by 30 *Macoma*			
		Control	Low (0.03 mg/1)	Med. (0.3 mg/1)	High (3.0 mg/1)
30[a]	5.2 x 10	98	79	81	67
60[a]	7.3 x 10	99	99	99	99
90[b]	0.14 x 10	99	50	14	0
114[b]	1.6 x 10	86	86	69	69
150[b]	1.0 x 10	36	44	6.6	2.7

See Table 1 for algal compositions: experiment details are given in Methods.

[a]six hour feeding study

[b]one hour feeding study

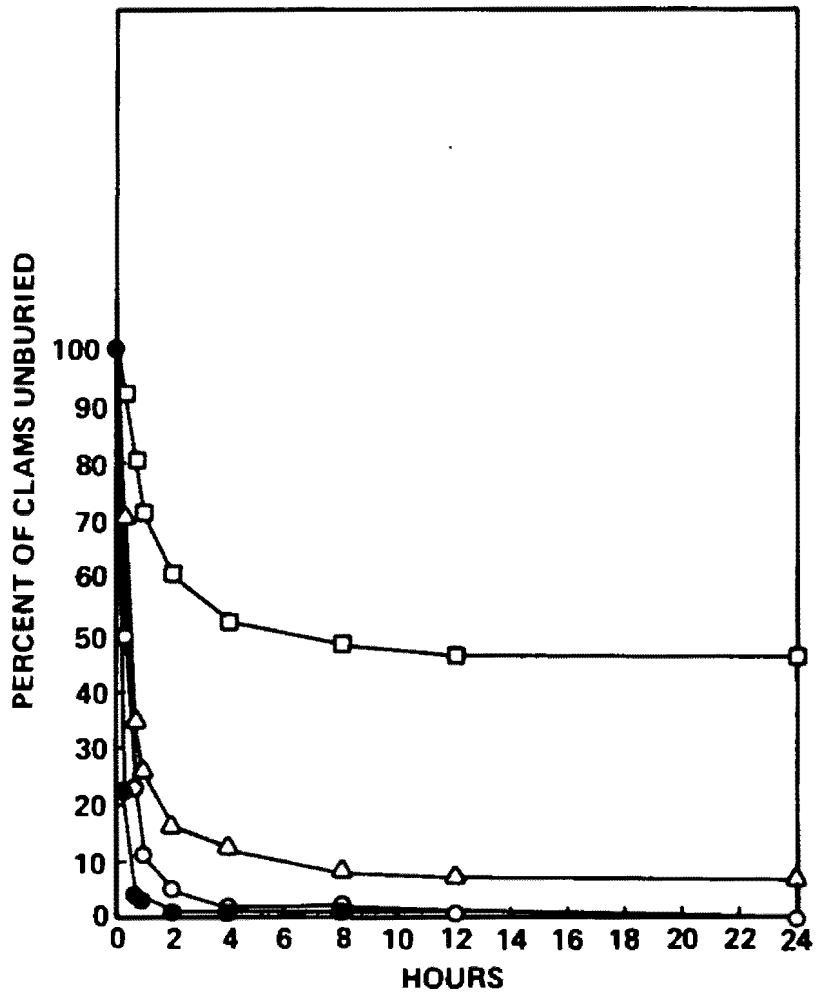

Figure 2. Burying behavior of *Macoma* in different concentrations of oiled seawater for day 90. The number of clams for each treatment were: control (●) 98; 0.03 mg/l (○) 98; 0.30 mg/l (△) 99; 3.0 mg/l (□) 65. Time at 0 hours denotes the beginning of the experiment with all the clams lying flat on the surface of the sand.

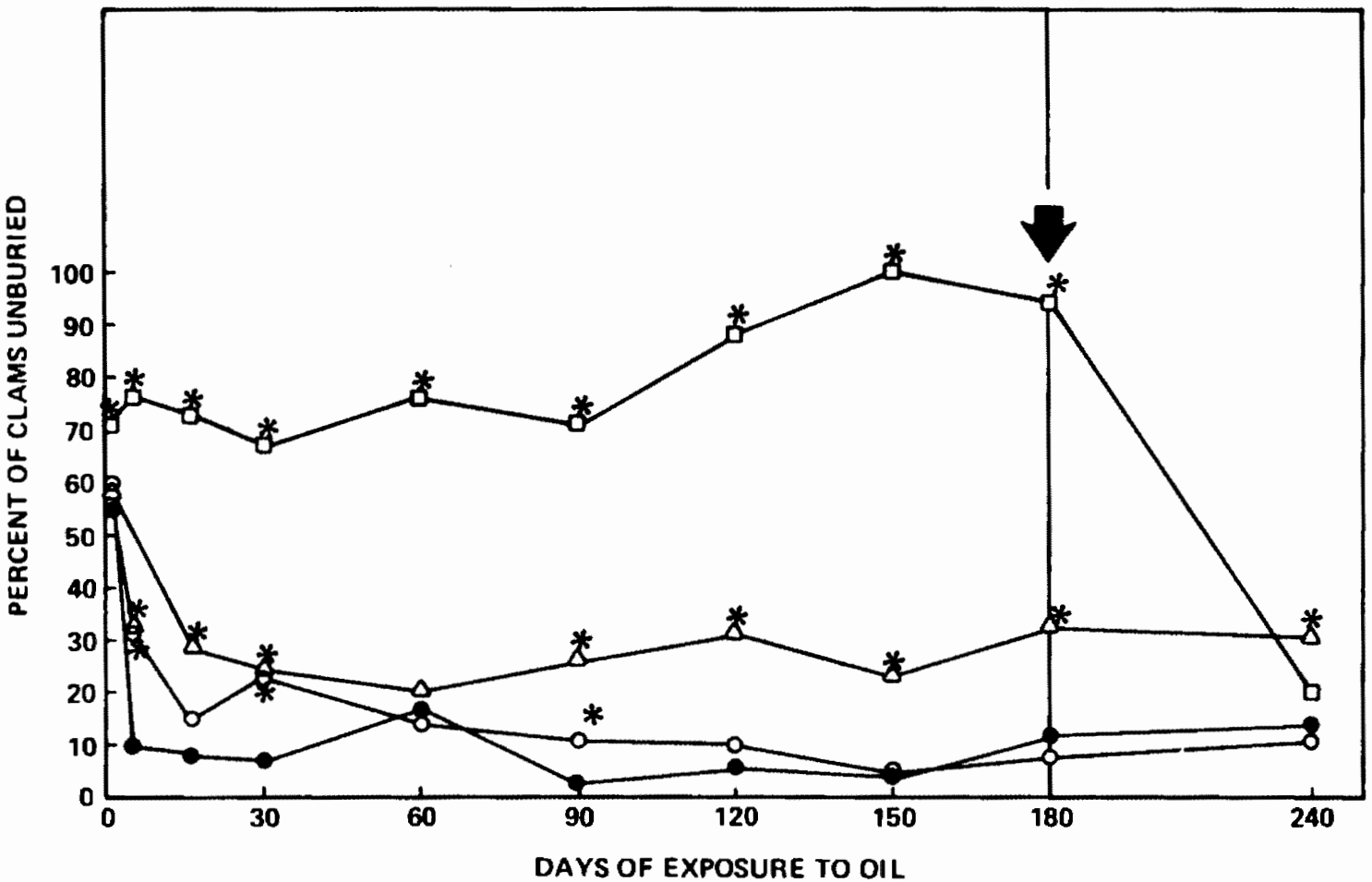

Figure 3. Percentage of *Macoma* which remain unburied after one hour of being placed on the surface of the sand. Asterisk (*) denotes values which are significantly different from the control values at $p < 0.01$ (except for 3.0 mg/l at day 0, $p < 0.02$ and 0.05 mg/l for day 90, $p < 0.05$). Arcsin t-test was used to evaluate the data. Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

Mortalities

Although the major concerns in this experiment were with sublethal effects, there were significant mortalities in the oiled treatments with respect to the controls. The total cumulative mortality in the control tanks after six months was about 3.1% (Figure 4). The low oiled (0.03 mg/l) clams had a similar mortality of 3.2% after the six months of treatment. In the 0.3 mg/l treatment mortalities reached 8.0% by day 180 and were significantly different from the controls by day 120 ($p < 0.01$, arcsin t-test). The 3.0 mg/l exposed clams began to die by day 30 (2.9%, $p < 0.001$). By day 180 the mortality had reached 81%. In fact there were not enough clams left after day 180 in the 3.0 mg/l treatment to take a recovery point two months later. The trend in mortality is approximately in linear proportion to dosage. That is, ten times the oil concentration gave about a ten-fold mortality rate. (EC_{50} for 180 days = 1.8 mg/l).

Wet and Dry Weights

The initial wet tissue weight for the clams was about 153 mg per clam (Table 3). Over the course of the next six months this value decreased for all four treatments. The control values dropped to 118 mg/clam by day 180. The clams at 0.03 mg/l showed little change in wet weight until day 150 when a large decrease took place. The clams at 0.3 mg/l oil had values similar to the controls up to day 120 after which the treated clams showed a greater loss in wet weight. The most marked decrease was in the 3.0 mg/l treatment. The wet weight began to decrease immediately. By day 180 the clams averaged only 99 mg wet weight each.

When the dry weights for the lyophilized tissues are compared, again decreases are shown over the six month period (Table 3). Here again the 3.0 mg/l and the 0.3 mg/l treated clams show a greater decrease in weight compared to the controls and the 0.03 mg/l treated clams. When the dry weights are divided by the wet weights to correct for size differences, a clear pattern emerges. The controls and 0.03 mg/l clams maintain their dry/wet ratio at around 17-18% throughout the exposure period. The clams at 0.3 mg/l initially have higher values, but by day 120 these values are significantly lower than

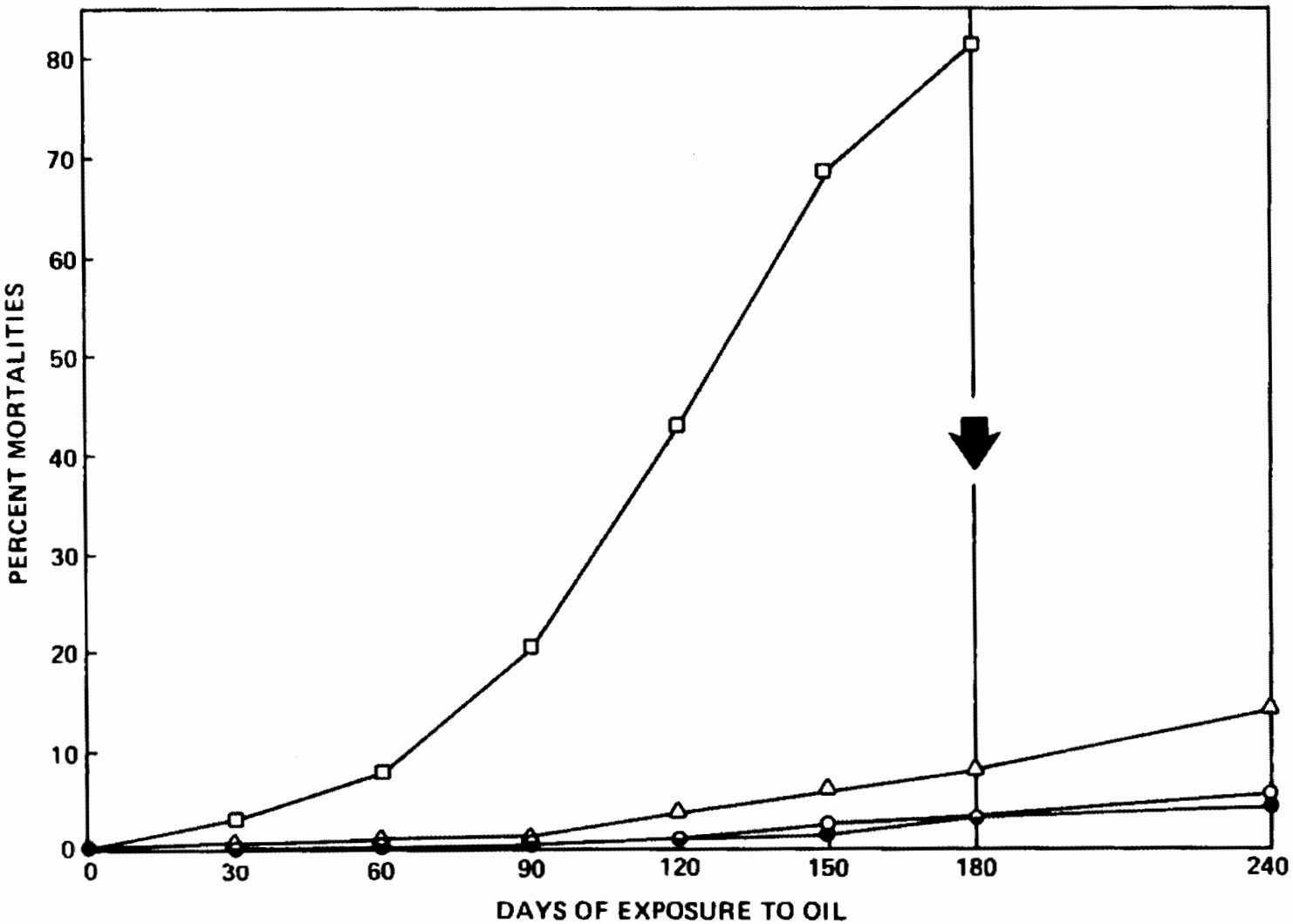

Figure 4. Cumulative mortalities of *Macoma* for the various oil treatments during six months of exposure. Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

TABLE 3. AVERAGE VALUES (± S.D.) FOR WET AND DRY TISSUE WEIGHTS OF CLAMS EXPOSED TO CONTINUOUS LOW-LEVEL OIL

Treatment		Day of Exposure to Oil									
		0	5	16	30	60	90	120	150	180	240 (recovery)
0.0 ppm	wet	153±32	153±46	151±41	151±40	148±39	131±36	145±39	129±34	118±25	120±37
	dry	27.1±6.1	28.8±9.5	28.1±8.0	26.9±7.3	26.2±7.4	22.5±6.5	25.9±7.3	22.1±4.7	20.0±5.7	20.9±6.1
	dry (%)[a]	17.7±1.1	18.6±1.2	18.2±1.0	17.8±1.0	17.7±1.1	17.2±1.3	17.9±1.6	17.4±1.9	16.9±1.9	17.6±1.6
	N	30	30	19	30	30	30	30	30	30	30
0.03 ppm	wet	-	148±36	147±38	147±37	146±36	145±48	150±40	145±33	128±42	116±35
	dry	-	27.2±7.0	27.6±7.0	27.0±6.9[b]	25.9±6.5	24.9±7.9	25.7±7.3	24.9±6.2	21.0±6.6	20.3±5.8
	dry (%)	-	18.4±1.2	18.9±1.2	18.3±1.1[b]	17.8±1.1	17.3±1.2	17.1±1.7	17.1±1.9	16.4±1.6	17.6±1.7
	N	-	30	30	30	30	30	30	30	30	30
0.30 ppm	wet	-	153±40	155±44	147±47	149±40	137±42	136±51	112±34	105±28	96±30[c]
	dry	-	28.8±8.0	30.2±8.2	27.4±8.6[b]	26.0±6.9	23.1±7.0	23.2±8.8[b]	18.0±4.4[c]	16.7±4.0	16.7±4.4[c]
	dry (%)	-	18.7±1.1	19.1±1.1[c]	18.4±1.3[b]	17.4±1.1	16.9±1.3	16.9±1.7[b]	16.2±1.9[b]	16.0±1.5	17.7±1.9
	N	-	30	30	30	30	30	30	29	28	30
3.0 ppm	wet	-	150±29	140±38	139±32	112±36[c]	108±29[b]	112±40[c]	97±38[c]	99±45	-
	dry	-	27.9±6.9	26.3±7.3	24.3±5.8	18.0±6.0[c]	17.5±5.6[c]	15.4±5.8[c]	13.6±6.3[c]	13.4±6.7[c]	-
	dry (%)	-	18.6±1.5	18.9±1.3	17.5±1.0	16.1±1.4[c]	16.1±1.7[b]	14.0±2.8[c]	14.2±3.6[c]	13.3±1.5[c]	-
	N	-	30	30	30	27	18	22	15	12	-

[a] day 0 values were assayed before the oil was added to the tanks. The values for dry (%) are expressed as percent of the wet weight.

[b] significant difference with respect to the control at $p < 0.05$.

[c] significant difference with respect to the control at $p < 0.01$.

in the control (Table 3). The clams in the 3.0 mg/l treatment have significantly lower values for dry to wet weight ratios than the controls for most of the points measured. These trends can be visualized by referring to Figure 5.

Growth

Lengths of *Macoma balthica* shells were measured at the beginning of the experiment and after exposure to four months of oiling (Table 4). Although the experiment took place during a time of probable slow growth (Beukema and deBruin, 1977; Gilbert, 1973), the control group of clams showed an average increase of 0.50 mm in shell length during this period. Clams from the oiled treatments showed significantly less growth with increases of 0.10 mm, 0.00 mm and 0.01 mm from the 0.03 mg/l, 0.30 mg/l, and 3.0 mg/l treatments respectively. Initially all of the clams for this experiment measured 9.8-11.2 mm in length.

Condition Index

The condition index was calculated for the clams after four months of exposure to oil. This time period was chosen rather than after six months due to the decrease in algal numbers in the upwelling pond used for feeding the clams (Table 1). The values in Table 4 show that the condition index for the control clams was lower than that of the clams exposed to 0.03 mg/l, but was significantly higher than those for the 0.3 mg/l and the 3.0 mg/l clams.

Respiration

Respiration rates of *Macoma balthica* after 120 and 150 days of oil exposure are summarized in Table 5. Significant increases in respiration rate were observed in the clams at the 0.3 mg/l and 3 mg/l exposure level. The slight increases in rate observed in clams at the lower exposure level, although not statistically significant may in fact be real as there were too few determinations to detect small changes in respiration data.

Gill Sections

Examination of *Macoma balthica* gill tissue electron micrographs did not reveal any gross morphological differences between oil treatments and controls.

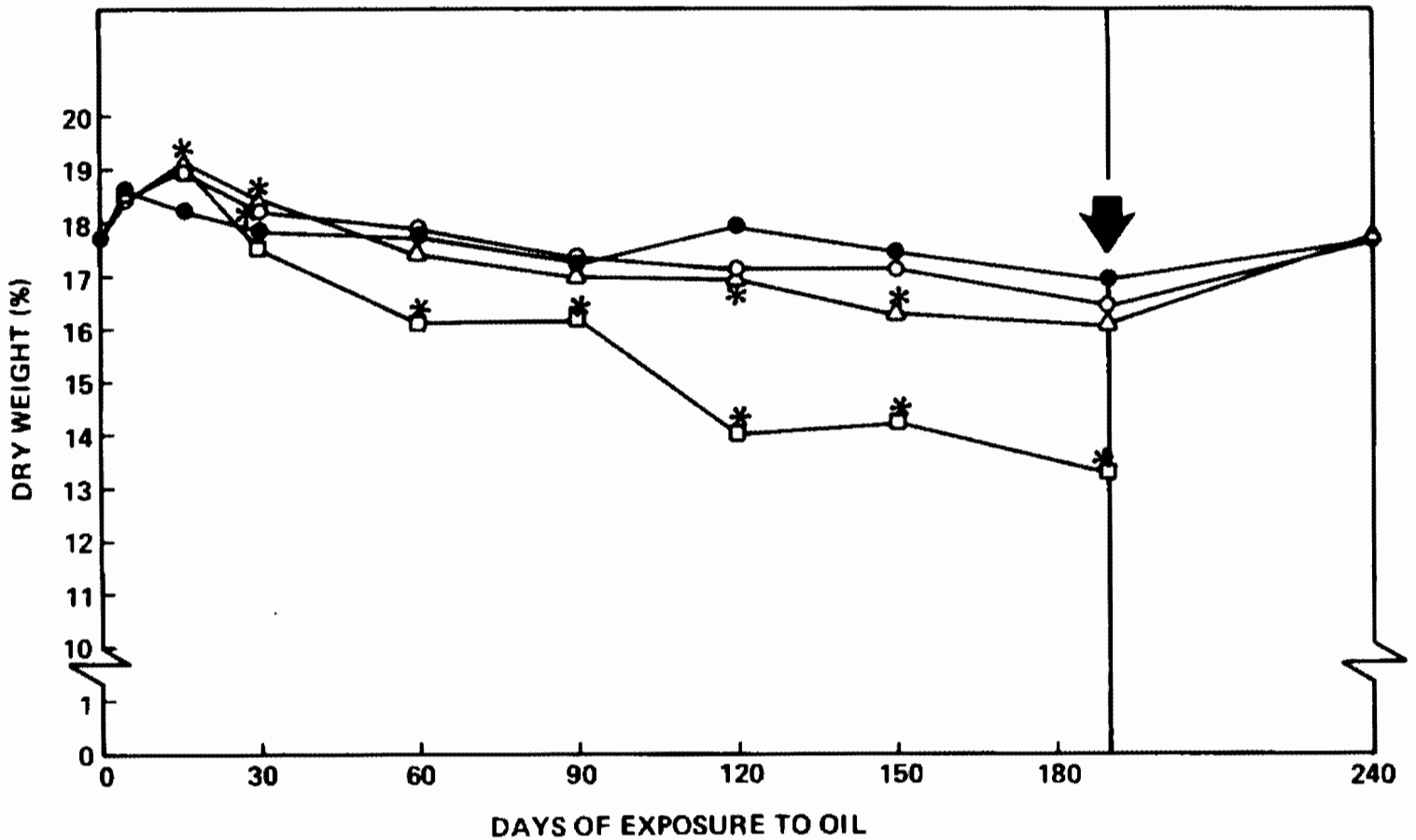

Figure 5. Percent dry weight for *Macoma* for points throughout the six month oiling experiment. Asterisks (*) indicate values significantly different from the control (see Table 5). Controls (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

TABLE 4. GROWTH AND CONDITION INDEX FOR *MACOMA BALTHICA* AFTER EXPOSURE TO OILED SEA WATER

Treatment	Day	Mean Length of Shell (mm ± SE)	N	Condition Index[a] (± SE)	N
Control	0	10.50 ± 0.015	100	0.165 ± 0.0007	100
Control	120	11.00 ± 0.028	99	0.142 ± 0.0012	99
0.03 mg/l	120	10.60 ± 0.017[b]	95	0.149 ± 0.0007[b]	90
0.30 mg/l	120	10.46 ± 0.094[b]	98	0.138 ± 0.0008[b]	80
3.0 mg/l	120	10.51 ± 0.054[b]	47	0.109 ± 0.0010[b]	47

[a] condition index = $\frac{\text{dry weight (g)}}{\text{shell length (mm)}} \times 100$

[b] values significantly different from controls (day 120) at $p < 0.01$ (student t-test)

TABLE 5. OXYGEN CONSUMPTION RATES OF *MACOMA BALTHICA* AFTER EXPOSURE TO OILED SEA WATER[a]

	Oxygen Consumption $\mu l O_2$/mg dry wt. hr ± SE	Mean Dry Weight/clam mg ± SE	N
Control	0.406 ± 0.027	30.7 ± 2.4	6
0.03 mg/l	0.477 ± 0.036	30.1 ± 2.8	5
0.3 mg/l	0.486 ± 0.034[b]	24.2 ± 1.6	5
3.0 mg/l	0.578 ± 0.039[c]	17.1 ± 3.9[c]	6

[a] 2 determinations of control and 3.0 mg/l clams after 120 days oil exposure
4 determinations of control and 3.0 mg/l clams after 150 days oil exposure
5 determinations of 0.03 mg/l and 0.3 mg/l clams after 150 days oil exposure

[b] values significantly different from controls $p < .05$

[c] values significantly different from controls $p < .01$ (student t-test)

Sample size was too small to assess subtle changes that may have taken place. There may have been some destruction or disorganization of the latero-frontal cilia cell cytoplasm in animals from the highest exposure level, but it was not possible to delineate the range of variation in control clams. *Macoma balthica*'s method of feeding (Gilbert, 1977) may have removed most of the oil from the water before exposure of the gill. If this did indeed occur, then the palps, not the gills might have been adversely affected. Unfortunately the palps were not microscopically examined.

Reproduction

Three different stages in the female reproductive cycle of *Macoma balthica* could be recognized from histological examination of gonads removed from individuals after 120 days of oiling. Females were categorized as mature if the follicles were completely full of mature ova, spawning or spent if the follicles were only partially full or completely empty, and resorbing gametes if the ova were being digested by amoebocytes. The number of females in each category and the sex ratio are summarized for each treatment in Table 6. Although this was a small sample, there appears to be some resorption of gametes in all of the oil treatments, but none in the controls. There were no obvious differences, between treated males and controls and the sex ratio was close to unity for all groups.

Protein Content

There was no definite trend for values of protein/dry weight during the exposure period. Control values ranged from 44.4% to 51.3% of the dry weight (Figure 6a). At day 30 (and day 60 for the 0.3 mg/ml treatment) all the oil-treated clams had significantly lower values for percent protein compared to the control, but the clams appeared to recover this deficiency during the remainder of the experiment. The absolute values of the protein for all the treatments, in mg protein per clam, did decrease in the same fashion as the corresponding dry weight (Table 7).

TABLE 6. SEX RATIO AND FEMALE REPRODUCTIVE CONDITION OF *MACOMA BALTHICA* AFTER 120 DAYS OF EXPOSURE TO OILED SEA WATER

Treatment	Sex Ratio ♂♂/♀♀	Female Reproductive Condition (# of ♀♀)			Total ♂♂ + ♀♀
		Mature	Spawning	Egg Resorption	
Control	.86	6	1	0	13
0.03 mg/l	.86	1	4	2	13
0.3 mg/l	1.17	2	2	2	13
3.0 mg/l	.75	3		1	7

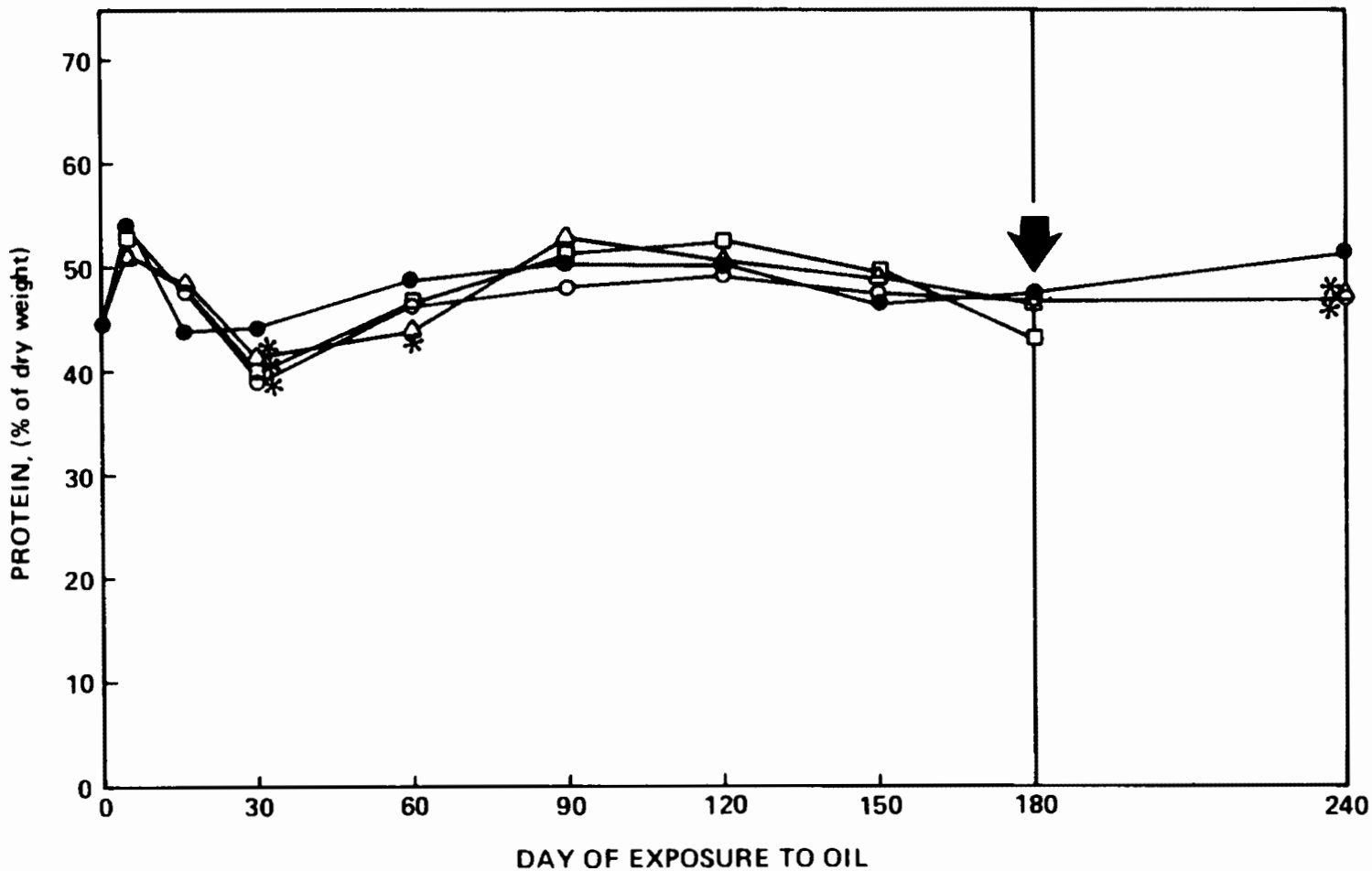

Figure 6a. Protein (percent of dry weight) for *Macoma* exposed to oiled seawater for six months. Asterisks (*) indicate values significantly different from the control (see Tables 7, 9 and 10). Control (•); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

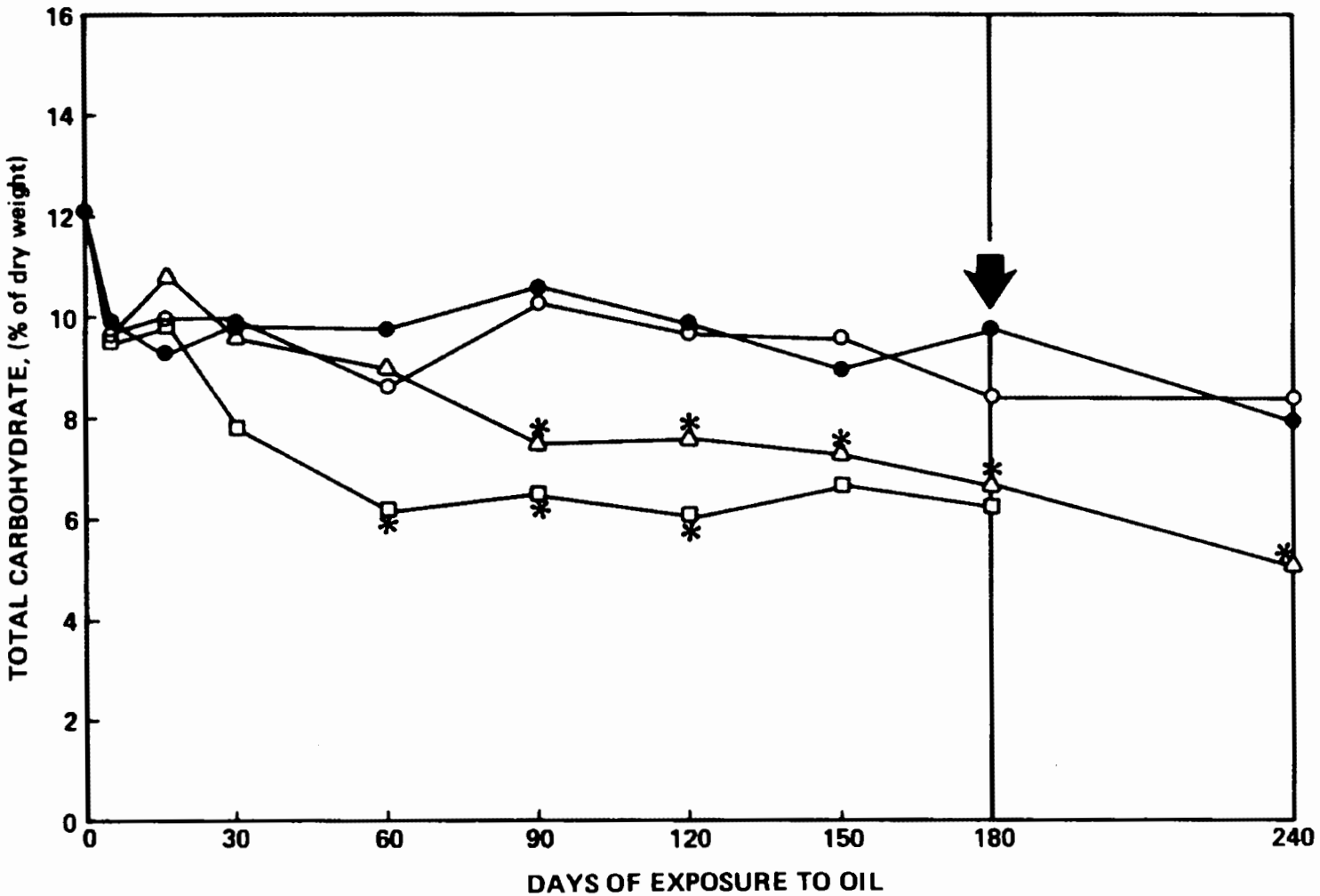

Figure 6b. Total carbohydrate (percent of dry weight) for *Macoma* exposed to oiled seawater for six months. Asterisks (*) indicate values significantly different from the control (see Tables 7, 9 and 10). Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

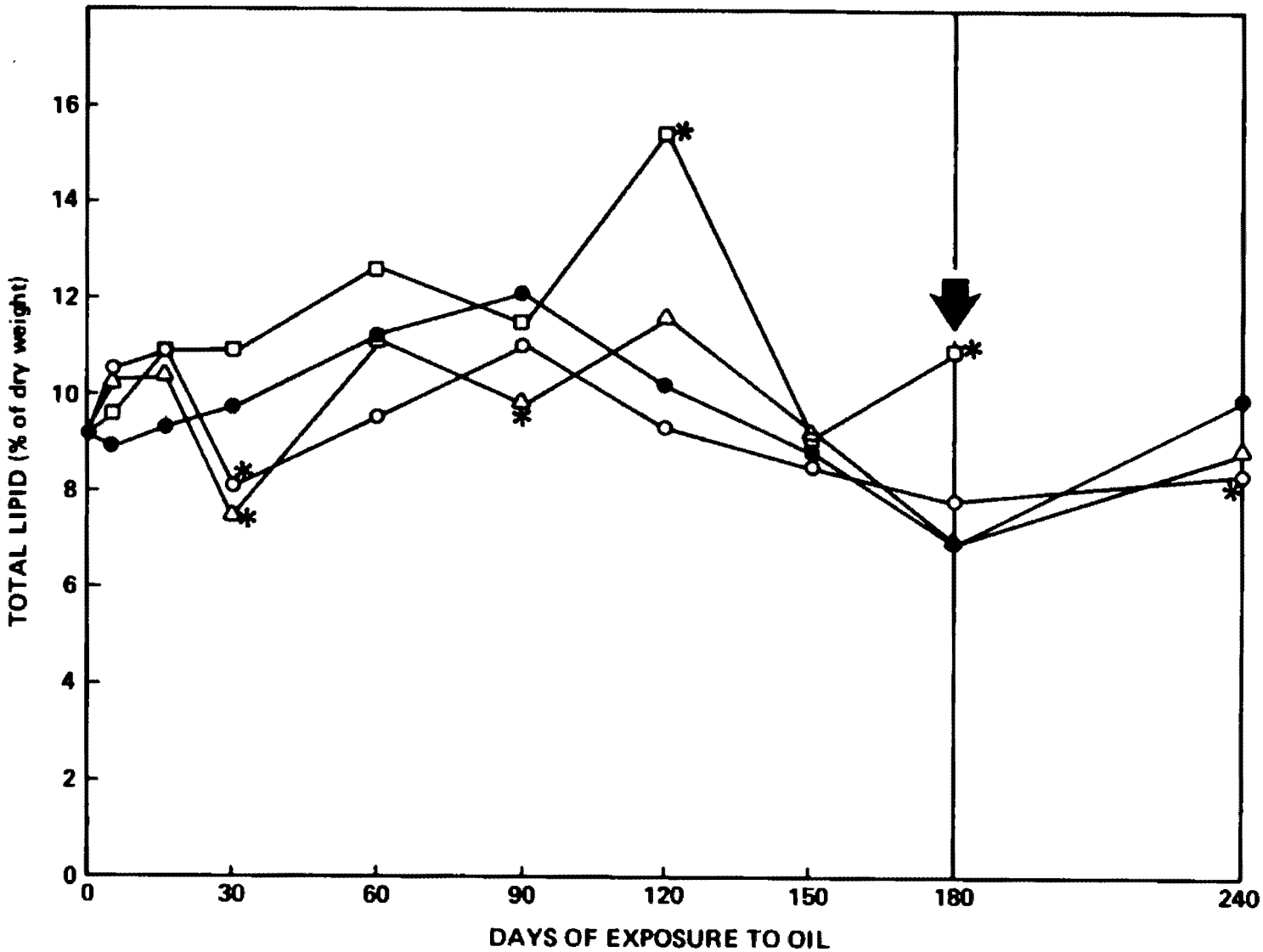

Figure 6c. Total lipid (percent of dry weight) for *Macoma* exposed to oiled seawater for six months. Asterisks (*) indicate values significantly different from the control (see Tables 7, 9 and 10). Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

TABLE 7. PROTEIN VALUES (mg/clam and % of dry weight, d.w.) FOR CLAMS EXPOSED TO OILED SEAWATER FOR SIX MONTHS

Treatment	Day of exposure to oil									
	0	5	16	30	60	90	120	150	180	240 (recovery)
Control										
mg/clam	11.0±1.8	14.1±3.8	11.9±3.6	12.7±3.1	11.0±3.2	12.1±3.0	14.1±4.1	11.6±1.7	10.6±2.9	11.2±3.5
% of d.w.	44.4±7.4	54.0±5.7	43.7±6.9	43.9±2.9	48.7±2.8	50.2±4.7	50.2±3.0	46.5±3.5	47.5±3.9	51.3±3.7
N	10	10	7	10	10	10	10	10	10	10
0.03 mg/l										
mg/clam	-	13.4±3.6	12.4±3.4	11.2±2.3	11.8±2.8	13.4±4.4	12.2±2.7	13.3±2.5	11.7±2.8	10.4±2.1
% of d.w.	-	51.4±5.1	47.7±3.4	39.0±6.3[a]	46.1±4.4	48.1±3.3	49.4±1.5	47.1±2.8	47.2±2.5	47.0±4.2[a]
N	-	10	10	10	10	10	10	10	10	10
0.30 mg/l										
mg/clam	-	13.3±4.2	15.5±3.5	11.2±3.5	12.3±4.1	11.9±3.2	13.2±2.9	9.6±2.6	7.7±2.1	8.5±2.1
% of d.w.	-	51.1±6.2	48.6±3.1	41.4±1.3[a]	43.8±4.6[a]	52.8±2.9	50.3±4.0	48.6±5.3	46.6±2.9	47.2±4.4[a]
N	-	10	10	10	10	10	10	9	10	10
3.0 mg/l										
mg/clam	-	15.4±2.8	11.0±2.0	10.1±3.2	8.8±2.0	11.0±3.6	9.5±3.6	7.9±5.5	6.7±2.0	-
% of d.w.	-	52.4±8.2	47.9±5.3	39.8±3.1[a]	46.7±3.9	51.2±4.5	52.4±4.1	49.5±4.2	43.1±1.5	-
N		10	10	10	9	6	6	4	3	-

All values are means ± S.D.

[a]significantly different from the control at $p < 0.05$ (student t-test)

Glycogen and Total Carbohydrate

The values obtained for glycogen showed much scatter. There was no discernible pattern in the data, and the values appeared atypically low compared to those reported in the literature (Beukema and deBruin, 1977). It was determined that the initial procedure used to extract the glycogen did not sufficiently inactivate breakdown of glycogen to glucose. The homogenate lost 90% of the reactive glycogen when it was stored at -20° for one week. These values are reported in Table 8. We relied on the values obtained for total carbohydrate as estimates of energy reserves.

The values for total carbohydrate per milligram of dry weight in Figure 6b indicate that the oil-stressed clams were depleting their carbohydrate reserves more rapidly than either the protein or lipid (Figure 6a,b,c). By day 60 the clams exposed to 3.0 mg/l had lost half of their carbohydrates as indicated in Table 9. The clams exposed to 0.3 mg/l also showed a slower, but still significant, decrease in total carbohydrate content. Again the low-oiled clams (0.03 mg/l) showed essentially an identical pattern to the control group.

Total Lipid

The third major biochemical constituent of *Macoma balthica* the lipid content, showed considerable scatter (Table 10). The lipid content of the control clams started out at about 9.2% of the dry weight, rose to a peak at day 90 (12.0%), then fell to 7.0% by day 180 (Figure 6c). The clams from 0.03 mg/l and 0.3 mg/l showed essentially the same patterns with significant differences from the controls at day 30 for both, and at day 90 for the 0.3 mg/l treatment ($p < 0.05$). The lipid pattern for the highest oiled treatment (3.0 mg/l) generally showed an increased lipid content with respect to the control. There were highly significant differences between the 3.0 mg/l oiled clams and the controls at day 120 ($p < 0.005$, 15 d.f.) and day 180 ($p < 0.01$, 12 d.f., student t-test).

RNA and DNA

RNA values were measured periodically as an estimate of relative ongoing protein synthesis in the clams. Figure 7 and Table 11 show that the RNA values

TABLE 8. GLYCOGEN VALUES FOR *MACOMA BALTHICA* EXPOSED TO OILED SEA WATER FOR SIX MONTHS

Treatment	Day of exposure to oil									
	0	5	16	30	60	90	120	150	180	240 (recovery)
Control										
mg/clam	0.31±0.27	0.35±0.20	0.36±0.20	0.53±0.37	0.35±0.22	0.17±0.09	0.17±0.10	0.16±0.09	0.21±0.09	0.33±0.29
% of d.w.	1.2±1.2	1.4±0.9	1.4±0.7	1.9±1.4	1.6±0.9	0.67±0.26	0.66±0.43	0.66±0.38	1.1±0.5	1.4±1.1
N	10	10	7	10	10	10	10	10	9	10
0.03 mg/l										
mg/clam	-	0.39±0.20	0.44±0.50	0.52±0.15	0.24±0.14	0.18±0.09	0.17±0.08	0.24±0.21	0.15±0.11	0.36±0.29
% of d.w.	-	1.5±0.7	1.5±1.1	1.9±0.7	0.94±0.46	0.71±0.50	0.71±0.38	0.80±0.59	0.56±0.32[a]	1.6±1.2
N	-	10	10	10	10	10	10	10	10	10
0.3 mg/l										
mg/clam	-	0.34±0.34	0.72±0.48	0.44±0.27	0.33±0.13	0.10±0.02	0.08±0.05	0.13±0.07	0.17±0.09	0.19±0.14
% of d.w.	-	1.3±1.4	2.2±1.5	1.6±1.0	1.3±0.6	0.44±0.07[a]	0.33±0.21[a]	0.70±0.42	1.0±0.4	1.0±0.7
N	-	10	9	10	10	10	10	9	10	10
3.0 mg/l										
mg/clam	-	0.39±0.25	0.42±0.26	0.32±0.27	0.14±0.09	0.09±0.03	0.03±0.02	0.14±0.15	0.11±0.08	-
% of d.w.	-	1.3±0.8	1.8±1.0	1.2±0.9	0.75±0.40[a]	0.43±0.08	0.19±0.09[a]	1.0±1.0	0.76±0.52	-
N	-	10	10	10	9	6	7	5	3	-

All values are means ± S.D.

[a]values significantly different from control at $p < 0.05$ (student t-test).

TABLE 9. TOTAL CARBOHYDRATE IN *MACOMA BALTHICA* DURING SIX MONTHS OF OILED SEA WATER

Treatment	Day of exposure to oil									
	0	5	16	30	60	90	120	150	180	240 (recovery)
Control										
mg/clam	3.07±0.99	2.54±0.62	2.56±0.93	2.70±0.84	2.27±0.62	2.60±1.61	2.81±0.74	2.22±0.35	2.19±0.78	1.84±1.05
% of d.w.	12.1±3.2	9.93±1.90	9.29±1.44	9.81±3.36	9.75±1.74	10.6±2.23	9.88±2.01	8.94±1.36	9.74±2.62	7.92±3.03
N	10	10	7	10	9	10	9	10	10	10
0.03 mg/l										
mg/clam	-	2.53±0.87	2.71±1.45	2.80±0.35	2.21±0.71	2.77±0.81	2.36±0.61	2.71±0.67	2.18±1.12	1.89±0.64
% of d.w.	-	9.66±2.07	10.0±2.31	9.86±1.73	8.58±1.53	10.3±3.25	9.69±1.95	9.64±1.55	8.44±2.78	8.44±2.39
N	-	10	10	10	10	10	10	10	10	10
0.3 mg/l										
mg/clam	-	2.54±0.78	3.58±0.75	2.68±1.25	2.47±0.80	1.68±0.56	2.01±0.59	1.45±0.40	1.15±0.62	1.13±0.50
% of d.w.	-	9.78±2.26	10.8±2.15	9.60±2.83	8.98±2.76	7.49±1.83[a]	7.60±1.14[a]	7.33±1.26[a]	6.67±2.21[a]	5.13±2.30[a]
N	-	10	10	10	10	10	10	9	10	10
3.0 mg/l										
mg/clam	-	2.86±0.89	2.33±0.95	2.10±1.32	1.16±0.40	1.39±0.52	1.04±0.52	0.85±0.57	1.01±0.45	-
% of d.w.	-	9.56±2.35	9.83±2.83	7.79±2.65	6.16±1.62[a]	6.49±1.59[a]	6.07±1.97[a]	6.64+5.25	6.23±1.63	-
N	-	10	10	10	9	6	7	5	3	-

All values are means ± S.D.

[a]significantly different from the control at $p < 0.05$ (student t-test)

TABLE 10. TOTAL LIPID IN *MACOMA BALTHICA* DURING SIX MONTHS EXPOSURE TO OILED SEA WATER

Treatment	Day of exposure to oil									
	0	5	16	30	60	90	120	150	180	240 (recovery)
Control										
mg/clam	2.61±0.65	2.51±1.27	2.41±0.83	2.52±0.85	2.91±0.71	2.42±0.74	2.44±0.37	1.78±0.63	1.34±0.46	2.04±0.74
% of d.w.	9.19±1.28	8.89±2.06	9.31±1.50	9.65±2.04	11.2±2.30	12.1±2.31	10.2±1.82	8.81±1.34	6.92±1.75	9.87±1.45
N	10	10	6	10	10	10	10	9	10	10
0.03 mg/l										
mg/clam	-	2.65±0.86	3.03±0.71	2.10±0.56	2.37±0.81	2.72±1.20	2.74±1.00	2.08±0.71	1.71±0.85	1.61±0.49
% of d.w.	-	10.5±1.17	10.9±1.55	8.13±0.93[a]	9.50±1.25	11.0±1.76	9.34±1.66	8.53±1.81	7.75±1.76	8.35±1.21[a]
N	-	9	10	10	10	10	10	10	10	10
0.30 mg/l										
mg/clam	-	3.08±0.97	2.74±0.93	2.31±1.41	2.87±0.97	2.52±1.47	2.76±0.97	1.87±0.68	1.16±0.19	1.42±0.26
% of d.w.	-	10.2±1.38	10.4±1.61	7.49±1.74[a]	11.1±1.77	9.79±2.15[a]	11.6±2.14	9.23±1.96	6.98±1.35	8.90±1.73
N	-	10	10	10	10	10	10	10	9	10
3.0 mg/l										
mg/clam	-	2.76±0.42	2.59±0.83	2.75±0.62	2.70±0.72	1.91±0.60	1.96±0.63	1.13±0.47	1.85±0.96	-
% of d.w.	-	9.62±1.33	10.9±2.03	10.9±1.62	12.6±2.56	11.5±1.56	15.4±2.77[a]	9.14±1.44	10.9±2.11[a]	-
N	-	10	9	10	9	6	7	5	4	-

All values are expressed as means ± S.D.

[a] values significantly different from control at $p < 0.05$ (student t-test)

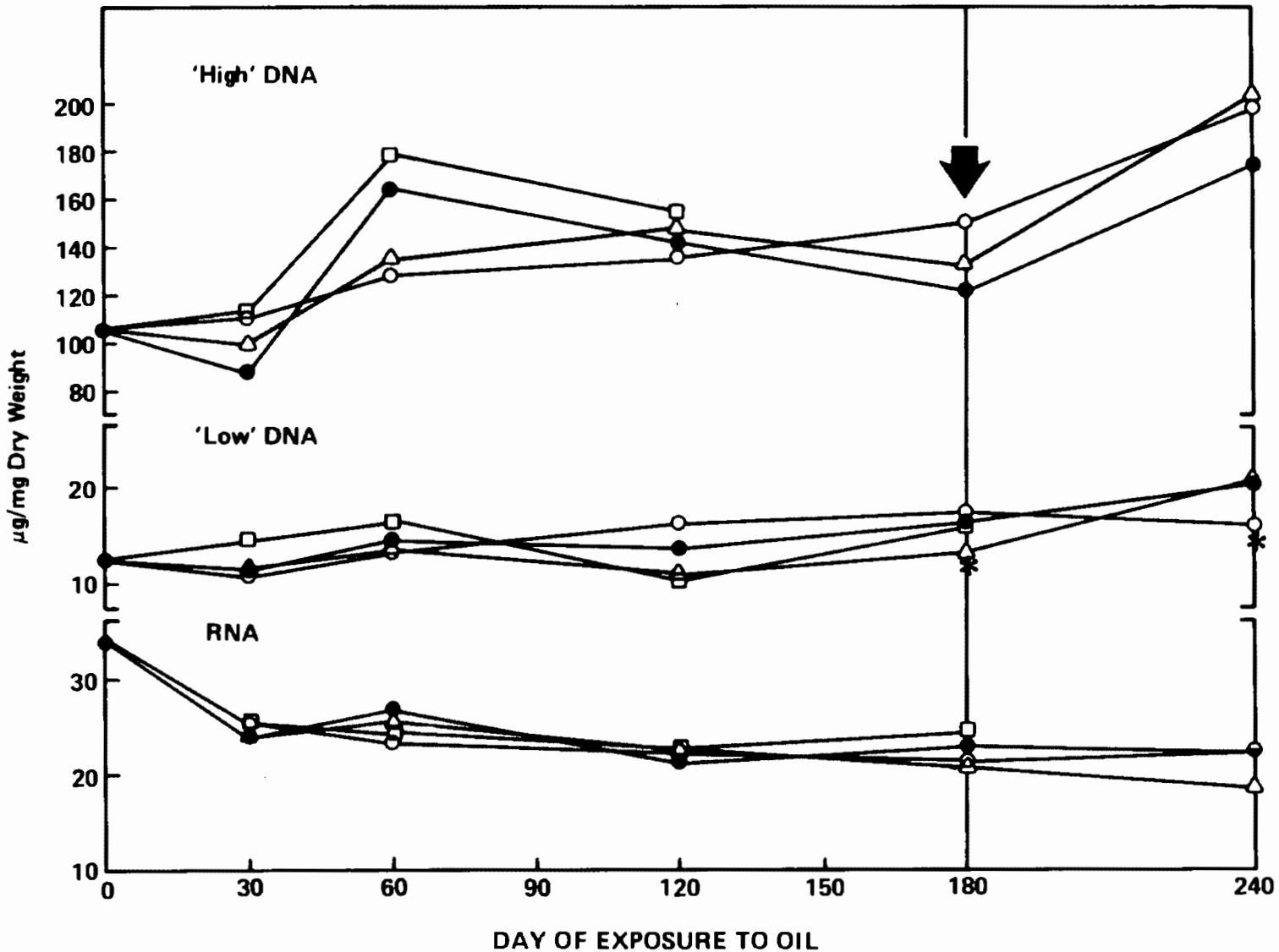

Figure 7. RNA and DNA values for *Macoma* during exposure to six months of oiling. Asterisks (*) indicate values which are significantly different from the controls at $p < 0.05$ (see Tables 11-13). Note change in scale on y-axis (µg/mg). Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

TABLE 11. RNA VALUES FOR *MACOMA BALTHICA* DURING EXPOSURE TO SIX MONTHS OF OILED SEA WATER

Treatment	Day of exposure to oil					
	0	30	60	120	180	240 (recovery)
Control						
mg RNA/clam	0.96±0.37	0.62±0.29	0.76±0.22	0.52±0.18	0.40±0.10	0.44±0.09
µg/mg d.w.	33.9±8.0	23.8±5.0	26.7±7.4	21.2±3.6	23.0±7.2	22.4±5.2
N	10	10	10	10	10	10
0.03 mg/l						
mg RNA/clam	-	0.67±0.31	0.61±0.23	0.52±0.25	0.35±0.65	0.44±0.20
µg/mg d.w.	-	25.6±6.2	23.5±7.0	22.0±6.6	21.4±3.7	22.5±4.3
N	-	10	10	10	10	10
0.3 mg/l						
mg RNA/clam	-	0.61±0.25	0.66±0.36	0.39±0.12	0.34±0.11	0.30±0.12
µg/mg d.w.	-	23.7±7.7	25.3±7.6	22.2±5.2	20.8±4.2	18.7±4.0
N	-	10	10	10	9	10
3.0 mg/l						
mg RNA/clam	-	0.57±0.27	0.33±0.09	0.34±0.07	0.18±0.03	-
µg/mg d.w.	-	25.3±7.7	24.8±5.4	22.7±6.7	24.6±3.6	-
N	-	10	9	7	4	-

All values are means ± S.D.; d.w. = dry tissue weight of the clams

TABLE 12. VALUES FOR "LOW" DNA IN *MACOMA BALTHICA* DURING EXPOSURE TO PRUDHOE BAY CRUDE OIL IN SEA WATER

Treatment	Day of exposure to oil					
	0	30	60	120	180	240 (recovery)
Control						
µg/clam	381±120	289±80	397±107	293±163	264±33	380±92
µg/mg d.w.	12.4±2.2	11.4±2.1	14.6±3.5	13.6±2.8	16.5±2.0	20.2±3.1
N	6	3	8	3	6	8
0.05 mg/l						
µg/clam	-	277±91	344±35	371±101	271±53	271±122
µg/mg d.w.	-	11.0±1.6	13.1±3.0	16.3±1.7	17.3±4.9	16.1±2.9[a]
N	-	8	3	4	5	6
0.30 mg/l						
µg/clam	-	297±100	371±42	184±116	234±54	249±75
µg/mg d.w.	-	11.5±1.8	13.7±3.2	10.8±2.2	13.1±1.1[a]	20.4±2.4
N	-	4	4	4	5	2
3.0 mg/l						
µg/clam	-	311±76	225±43	164±74	120±4	-
µg/mg d.w.	-	14.5±3.6	16.4±2.0	10.4±2.2	16.2±2.4	-
N	-	6	5	5	4	-

All values are means ± S.D.; d.w. = dry weight of clam

[a]significantly different from the control at $p < 0.01$ (student t-test)

TABLE 13. VALUES FOR "HIGH" DNA IN *MACOMA BALTHICA* EXPOSED TO SIX MONTHS OF OILED SEA WATER

Treatment	Day of exposure to oil					
	0	30	60	120	180	240 (recovery)
Control						
µg/clam	2340±440	2130±650	6570±4500	3570±840	2570±920	4520±140
µg/mg d.w.	105±34	88±19	164±49	142±34	123±40	174±17
N	4	6	2	6	4	2
0.03 mg/l						
µg/clam	-	3060±680	2920±710	3290±1400	2600±680	4500±1100
µg/mg d.w.	-	110±5	128±33	136±27	151±32	197±9
N	-	2	6	6	5	4
0.3 mg/l						
µg/clam	-	2640±1200	3020±660	2870±220	2330±1000	3430±1600
µg/mg d.w.	-	100±32	136±30	147±18	133±56	204±76
N	-	6	6	5	3	8
3.0 mg/l						
µg/clam	-	2490±600	2170±260	2730±2100	-[a]	-
µg/mg d.w.	-	111±14	177±17	153±68	-	-
N	-	4	4	2	-	-

All values are means ± S.D.; d.w. = dry tissue weight of clams

[a]there were no 'higher' out clams in the treatment at day 180.

for all the treatments are virtually the same throughout the entire experiment. There was an initial drop of RNA from 34 µg RNA per mg dry weight to about 24 µg/mg by day 30. After this point the values remained essentially constant.

The relative amount of DNA per mg of dry tissue weight can give an estimate of the relative size and numbers of cells in the clams. If the dry weight is decreasing, but the DNA content remains the same or increases, the ratio of DNA to dry weight will increase, indicating that the average cell size is shrinking. If the total DNA content drops as well as the dry weight, then the clams are probably reducing the numbers of their cells. The results we obtained were complicated by a bimodal distribution of DNA in the clams (Tables 12 & 13). One group of values ('low' DNA) fell within 10-30 µg DNA/mg and the other group ('high' DNA) had much higher values ranging from 90 to 120 µg DNA/mg. Very few values fell in between these two groups. Based on histological studies made on the clams at day 120, we concluded that the low value DNA clams represent females and the high values represent males, since we found no evidence of clams with immature gonads.

Figure 7 shows the plot of 'low' DNA values for each of the three treatments and the control. Overall there were no differences between the control values and the values for any of the oiled treatments, indicating relatively little effect of oil on DNA content in the clams. However, there appears to be a general trend in all of the treatments to higher values of DNA from day 0 to day 180. Since all the clams had decreased dry weights by day 180, this trend indicates that the average cell size of the animals was getting smaller.

Figure 7 also shows the plot for the 'high' values of DNA. The pattern is similar to that of the 'low' DNA clams with no significant differences between the controls and any of the oiled treatments.

Enzyme Activities

It has become evident in recent years that many membrane-bound enzymes require an intact membrane in order to function normally (White *et al.*, 1973). There is evidence also that hydrophobic molecules which are soluble in the membrane matrix may disturb the lipid structure (Roubal, 1974). Such disturbances could have profound effects on the associated membrane-bound enzymes.

We picked four enzymes to study which are known to be associated with the plasma membrane. If oil did in fact penetrate the plasma membrane of the clams, we expected to see alterations in the specific activities of some of these enzymes. The four enzymes we assayed were the Na^{+}-K^{+}-ATPase, Mg^{++}-ATPase, 5'- nucleotidase, and phosphodiesterase.

For the Na^{+}-K^{+}-ATPase we saw no effect of oil treatment on the specific activities (Figure 8a). Two enzymes were noticeably affected by at least one of the oil treatments. The Mg^{++}-ATPase in the control clams (Figure 8a) showed a gradual increase in activity throughout the duration of the experiment. This increase was inhibited by the oil treatment. Linear regressions were fitted to the data points and an analysis of covariance was calculated for the treated clams with respect to the controls. The 0.03 mg/l treatment showed no significant effect of oil treatment, but the 0.3 mg/l and the 3.0 mg/l treatments had slopes significantly different from the control with $p < 0.05$ and $p < 0.01$ respectively. This corresponds to an inhibition by day 180 of about 24% at 0.3 mg/l and 41% at 3.0 mg/l oil.

The level of activity of phosphodiesterase in the control clams remained more or less constant during the experiment (Figure 8b). Oiling the clams caused an increase in specific activity that was significantly different from the control for the 3.0 mg/l treatment ($p < 0.01$). The activity of the phosphodiesterase was increased about 55% with exposure to 3.0 mg/l oil.

Because the specific activities of the 5'nucleotidase showed no correlation with respect to day of exposure (Figure 8c), the values for each treatment were pooled, averaged, and the means tested for significance against the controls using a two-tailed t-test. For days 5 through 90 the 3.0 mg/l treatment showed significantly higher values at $p < 0.02$ (d.f. = 8). For days 5-180 the 0.03 and 0.3 mg/l treatments had significantly higher values than from the controls at $p < 0.05$ (d.f. = 15). By day 180 all of the values from all of the treatments were identical.

Recovery Period

After 180 days of oiling, the clams were allowed to remain in the tanks with fresh flowing sea water. The same parameters were measured after two months of

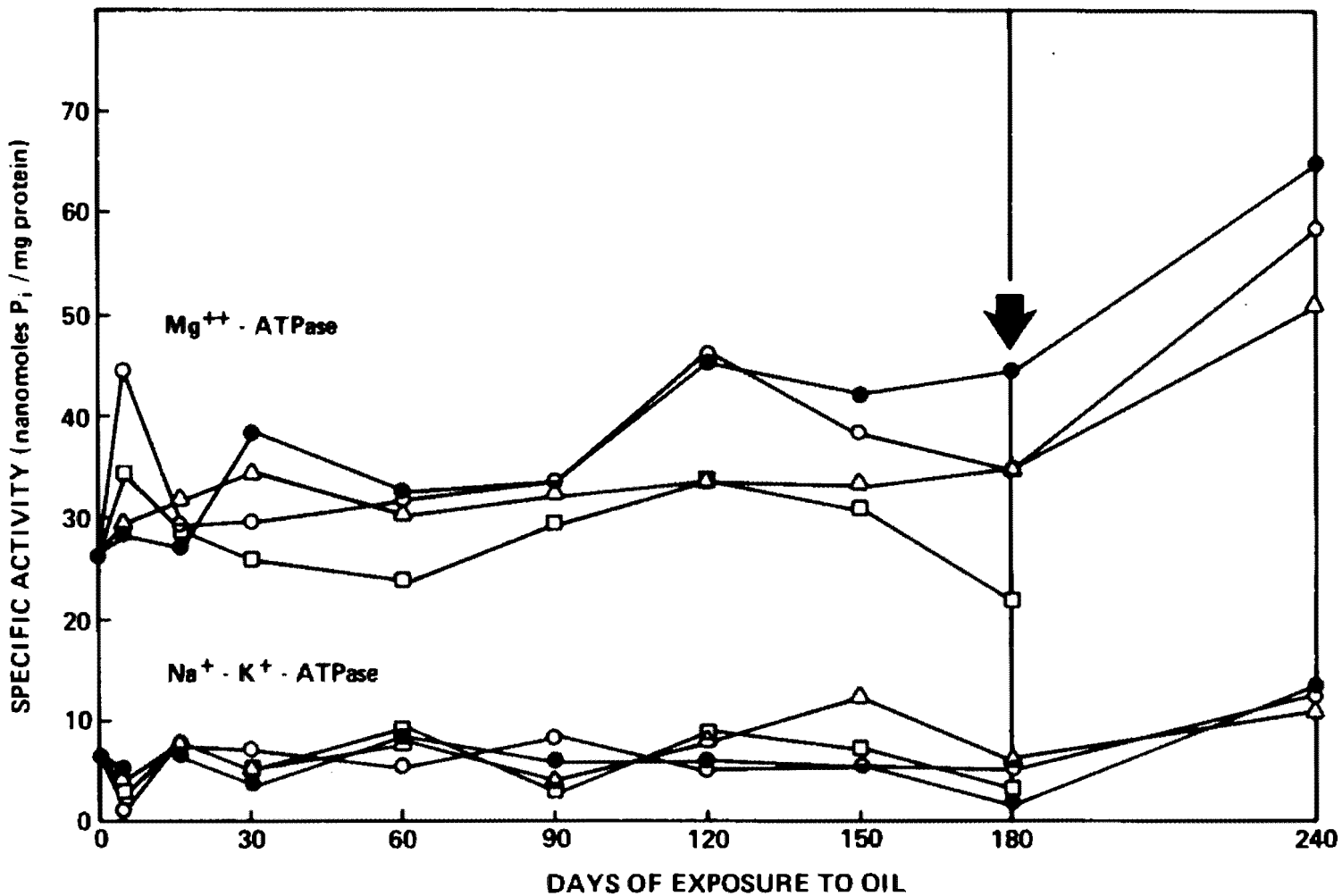

Figure 8a. Enzyme (ATPases) activities in *Macoma* as a function of six months of oiling. Assays were performed on pooled samples of ten clams each. For experimental details see Methods section. Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

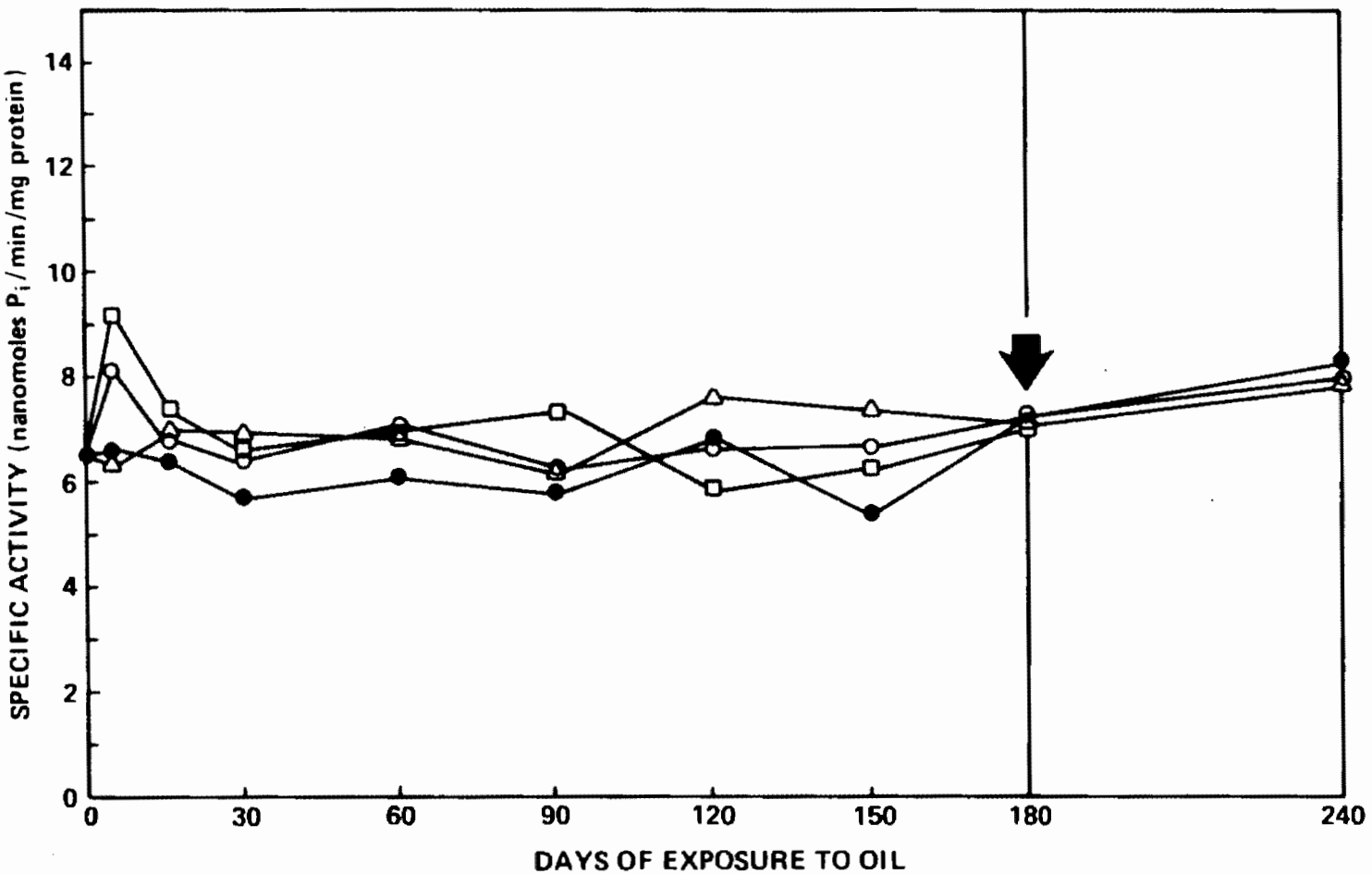

Figure 8b. Enzyme (5'-Nucleotidase) activities in *Macoma* as a function of six months of oiling. Assays were performed on pooled samples of ten clams each. For experimental details see Methods section. Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

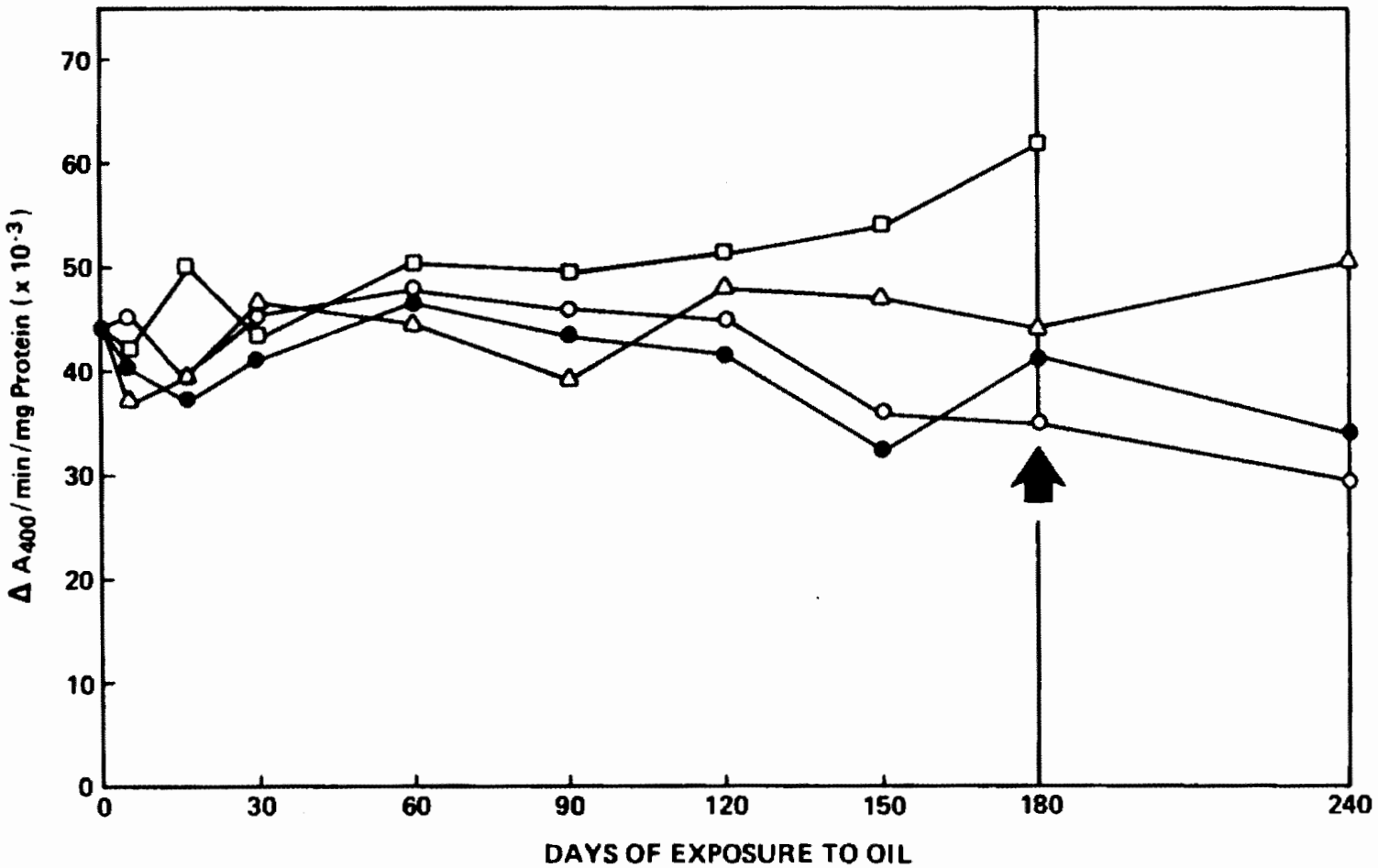

Figure 8c. Enzyme (Phosphodiesterase) activities in *Macoma* as a function of six months of oiling. Assays were performed on pooled samples of ten clams each. For experimental details see Methods section. Control (●); 0.03 mg/l (○); 0.3 mg/l (△); 3.0 mg/l (□); end of oiling regime (↓).

recovery. This is noted as day 240 in the figures and tables. There were not enough clams remaining in the 3.0 mg/l treatment after day 180 to assay, and so the recovery point was taken only for the control and the two lower oil concentrations with the exception of the burying experiment.

In general, the clams showed only partial recovery after two months in fresh sea water. The rate of burrowing of the 3.0 mg/l treated clams increased significantly, but the clams at 0.3 mg/l remained at the day 180 level (Figure 3). Mortalities also continued to increase in the 0.3 mg/l treatment (Figure 4). Wet weights continued to decline for the oil-treated clams (Table 3). The dry weights, however, leveled off which caused the dry to wet weight ratios to regain the same value as for the controls (Table 3, Figure 5). The number of clams unburied, lying on top of the sand also continued to increase for the 0.3 mg/l treatment (Figure 1).

Protein values dropped significantly from the controls at day 240 for the oil-treated clams (Figure 6a). Total carbohydrate also continued to decline in the 0.3 mg/l oiled clams, remaining significantly lower than the values for the controls (Figure 6b). Lipid levels showed a significant difference between the 0.03 mg/l and the control clams ($p < 0.05$), but no differences between the 0.3 mg/l and the controls (Figure 6c). RNA and DNA values were not significantly different (Figure 7), except for the 0.03 mg/l treatment where there is a significant decrease in the relative amount of 'low' DNA ($p < 0.05$).

The phosophodiesterase activity of the 0.3 mg/l oiled clams appeared to remain higher than the control value (Figure 8b). Likewise the Mg^{++}-ATPase activities, although increased, were still below the value for the controls (Figure 8a).

DISCUSSION

The results of exposing *Macoma balthica* to six months of Prudhoe Bay crude oil in seawater are summarized in Table 14. Before we can compare the effects of the oiling treatment on the clams, it is important to establish that the clams in the control treatment were not in turn subjected to an unusual stress in the laboratory. If one uses mortalities as an index of clam stress, it would seem that with only 3% mortalities over six months, the clams were not

TABLE 14. SUMMARY OF THE EFFECTS OF SIX MONTHS OF OILED SEAWATER ON VARIOUS PARAMETERS OF *MACOMA BALTHICA*

Parameters measured	Level of oil in exposure tanks 0.03 mg/l	0.30 mg/l	3.0 mg/l
1. Behavior			
general activity	normal[a]	decreased	decreased
burying rate	normal	decreased	decreased
% unburied	normal	increased	increased
feeding rate	normal	decreased	decreased
2. Mortalities	normal	increased	increased
3. Respiration rate	normal	normal?	increased
4. Physical			
wet weight	normal	normal	decreased
dry weight	normal	decreased	decreased
dry/wet ratio	normal	decreased	decreased
growth	decreased	decreased	decreased
condition index	increased	decreased	decreased
5. Morphology			
gonads[b]	reabsorbed	abnormal	abnormal
gills[b]			
6. Biochemical			
protein	normal	normal	normal
carbohydrate	normal	decreased	decreased
lipid	normal	normal	increased[c]?
DNA	normal	normal	normal
RNA	normal	normal	normal
Mg^{++}-ATPase	normal	normal	decreased
Na^{+}-K^{+}-ATPase	normal	normal	normal
5'-nucleotidase[b]	increased?	increased?	increased?
phosphodiesterase	normal	normal	increased

[a] normal, decreased, increased - are levels relative to those in the controls

[b] see Results section for discussion of the effects on these parameters

[c] see Table 10 and Figure 6C

highly stressed. However, changes in wet and dry weights, condition factor, and carbohydrate content (in mg/clam) indicate a lack of sufficient energy input for the control clams requiring a reliance on energy reserves.

There are two factors that might have made significant differences between the controls in the laboratory and normal clams in the field. One is the absence of tides in our experimental system and this is related to the second factor: the type of sediment in which the clams were buried. In natural conditions around the coast of Alaska, *Macoma balthica* is found primarily in intertidal mud flats. The species also seems to be adapted to deposit feeding as opposed to suspension feeding (Gilbert, 1977). The movement of the tides brings fresh sediment to the *M. balthica* in the mud flats on a periodic basis. In our experiment the clams were placed in sand, which for the most part, had a larger grain size than the sediment from the mud flats from which the animals were taken. Most of the grains in the experiment were larger than those *M. balthica* is reported to ingest (Gilbert, 1977). We, therefore, were forcing the experimental animals to suspension feed on the incoming algae from the upwelling pond. If *M. balthica* is truly adapted primarily for deposit feeding, and if it therefore takes more energy to feed on suspended material, it is to be expected that the control clams were under more stress than they would have been under natural conditions.

The histological sections done on control *M. balthica* at day 120 revealed full guts in most of the animals. Thus they were apparently eating well even while their dry weights were declining. Beukema and deBruin (1977) described seasonal variations in physical and chemical composition of *Macoma balthica* in the Dutch Wadden Sea. They discovered that growth takes place mainly during April through June. For the rest of the year the rate of growth is nearly zero and the ash-free dry weight falls to 55% of the June value by the end of December. The condition index, therefore, was also found to drop a similar amount during the same period. The experiment presented here was performed from the middle of June to the middle of December in 1977. This coincides with the period of low growth and decreased weight found in normal clams in the Dutch Wadden Sea. Resurrection Bay at 60°N latitude is obviously a different habitat than Dutch Wadden Sea at 53°N, but it seems not unlikely to us that the control clams in our experiment were acting in an essentially normal seasonal pattern.

It is obvious from Table 14 that higher oil concentrations cause many more disturbances than the lower oil concentrations. The clams exposed to low oil (0.03 mg/l) showed significant differences from the controls in growth and condition index. They also showed greater anomalies in the histology of the gonads. But, otherwise, the low oiled clams appeared to be little different from the controls during the six months of oiling.

The condition index at day 120 for the low oiled clams was significantly greater than that for the controls, but the condition indices for medium (0.3 mg/l) and high (3.0 mg/l) oiled clams were lower than controls (Table 4). The increased condition index at the low oil concentration was due to a decrease in the rate of shell growth during oil exposure and the same weight as controls at the end of the exposure period. Shell deposition in *M. balthica* begins with the formation of the periostracum composed of tanned protein (Bubel, 1973). The quinone tanning process hypothesized for periostracum formation is similar to the process of byssal thread formation in mussels (Hillman, 1961) which has been shown to be adversely affected by oil exposure (Swedmark *et al.*, 1973; Dunning and Major, 1974; and Eisler, 1975). This process appears to be extremely sensitive to petroleum exposure, as out of the many parameters examined in *M. balthica*, it was one of only two which showed a significant difference from controls at the lowest level of oil exposure.

Clams exposed to the higher oiling levels (0.3 mg/l and 3.0 mg/l) showed many signs of stress resulting from a negative energy balance (Table 14). Losses in dry weight, decreases in carbohydrate content, resorption of gametes, and decreases in shell growth are all indicative of a state of energy utilization rather than storage. This condition arose primarily from a decreased food intake (Table 2), but was compounded by the increased oxygen demand of a higher metabolic rate (Table 5).

The amount and efficiency of water filtration need to be examined when there is a decrease in food intake. A noted reduction in apparent feeding behavior of *Macoma balthica* upon oil exposure suggests that the volume of water processed for food was probably reduced. A reduction in filtering efficiency cannot be ruled out however, as petroleum products have been observed to affect cilia behavior (Johnson, 1977).

Increased oxygen consumption has been observed in many organisms due to petroleum exposure, but decreases in oxygen consumption have also been observed (Johnson, 1977; Stainken, 1978). It is well known that total oxygen consumption is the sum of a basal metabolic rate and a rate due to activity such as feeding or locomotion. Each of these processes may be affected differently by oil exposure. Percy (1977) has hypothesized that decreases in oxygen consumption are the result of activity reduction which may mask a general increase in basal metabolic rate. To support this hypothesis, he has shown that whereas intact oiled amphipods showed considerable variation in magnitude and direction of respiration rate change from controls, cell-free homogenates of oil exposed individuals all had a higher metabolic rate than the corresponding control cell-free homogenates. The *Macoma balthica* from the present set of experiments were not being fed at the time of oxygen consumption measurements, thus the increase in metabolism after oil exposure probably represents a change in the basal metabolic rate.

Increased oxygen demand could result from enzyme degradation of petroleum constituents, however Lee *et al.* (1972) could detect no metabolic products of any of a number of hydrocarbons accumulated by *Mytilus edulis* over short periods of time and, in general, bivalves seem to be lacking in aryl hydrocarbon hydroxylase (Varanasi and Malins, 1977). As Stainken (1978) has suggested, increased metabolic rate may be the result of oxidative phosphorylation uncoupling. In such a situation, metabolic rate is dependent only on the amount of substrate available for oxidation and not on the concentration of ATP, as no ATP is produced. Thus the animals receive little benefit from energy utilization. If some constituent or group of constituents of the ingested or absorbed petroleum were acting as an uncoupler of oxidative phosphorylation, this would be an extremely deleterious effect of oil exposure and certainly worthy of further investigation.

All of the *Macoma balthica* experienced a decline in biomass, those exposed to 0.3 mg oil/l showing a greater decline than controls on day 150 and those exposed to 3 mg oil/l on day 60 (Table 3). The average feeding rate over 120 days of oiling was depressed only slightly at 0.03 mg oil/l, but 51% at 0.3 mg oil/l and 61% at 3 mg oil/l (Table 2). This was coupled with a 20% increase in metabolic rate at 0.3 mg oil/l and a 42% increase at 3 mg oil/l (Table 5).

With little energy available for growth or reproduction and a greater expenditure than energy intake, resorption of gametes as an energy reserve can occur, which appears to have happened in *Macoma balthica* from all oil treatments by day 120 (Table 6). The exhaustion of energy reserves was the probable cause of the increased mortality of *M. balthica* from the 0.3 mg oil/l and 3 mg oil/l treatments.

Starvation cannot explain all of the results in the experiment. That explanation will not clarify why the oiled clams had reduced burying rates within 24 hours after exposure to the oiled seawater. Nor does it explain why the clams came to the surface of the sand in the presence of oiled seawater.

The slow burying rate appears to be the result of either a general narcotization as described by others (Morton and Wu, 1977; Linden, 1976; Percy and Mullin, 1977), or a disorientation mechanism. The clams in the high oiled (3.0 mg/l) treatment would make efforts to bury themselves by protruding their feet, but they seemed to have an impaired sense of orientation. It is possible that along with being narcotisized, causing sluggish movements of the foot, the statocysts in the clams were affected by the oiling, causing them to be disoriented with respect to the gravitational pull of the earth, thus retarding the burying rate of the clams. The ability to burrow is of critical importance to the clams. *Macoma balthica* have been reported to surface periodically and move about over the mud and then rebury themselves. This may be a behavior pattern performed to find a better site in the mud for feeding (Brafield and Newell, 1961) or as a result of infection by trematodes (Swennen, 1969; Hulscher, 1973). In the course of our studies, we observed trematode infection in some clams. However, we did not investigate the frequency of this infection or its relation to other parameters. Whatever the reason, if the clams are inhibited in their reburial rate, they expose themselves longer to the dangers of predation plus the chance of being transferred to an area of unsuitable substrate by the action of the tides.

The same hazards caused by being slow to rebury are also presented to clams which spend more of their time on the surface. Feder *et al.* (1976) reported that oiling *M. balthica* caused them to come to the surface of the sediment. Shaw *et al.* (1977) found that their tendency to come to the surface after three

days of exposure to an oil slick seemed to be a function of sediment depth. Animals in 3.5 cm of sediment did not surface after the oiling treatment, but those clams in only 1.0 cm thick sediment did come to the surface in significant numbers. The sediment in the petri dishes in the present experiment was 2.5 cm deep and the clams came to the surface again in significant numbers as shown in Figure 1. However, in order to verify the results of Shaw *et al.* (1977), we placed groups of ten clams in large beakers filled with sand to a depth of about 9 cm. These beakers were placed in the tanks along with the petri dishes. Although the clams were slower to surface in the beakers, after 150 days of oiling eight of the ten clams in the beaker in the high oiled treatment had surfaced. This indicates that under conditions of chronic oil pollution the *Macoma balthica* will eventually burrow up and out of the sediment. Further, they will also be slower to rebury themselves again. This behavior pattern will certainly cause a serious decrease in the population in an oil polluted area.

It was part of our hypothesis that oiling marine organisms could have serious consequences on membrane structures due to the fact that both membranes and oil are hydrophobic. It was our belief that if the oil was able to dissolve into the membranes of the clam, then enzyme functions which depended on an intact membrane would be impaired. Enzymes such as the Na^+-K^+-ATPase (sodium pump) are well known to require associated lipid for proper function (White *et al.*, 1973). It has also been shown that compounds found in crude oil are able to enter the membranes of fish (Roubal, 1974).

The work done to date on enzyme systems with respect to oil pollution has been concentrated in two areas. One area is concerned with aryl hydrocarbon hydroxylase (AHH) activity in animals after exposure of various marine organisms to aromatics found in crude oil (e.g. Gruger *et al.*, 1977; Kurelec *et al.*, 1977). The emphasis in these experiments is on the exposed organism's ability to degrade aromatic hydrocarbons by mixed function oxidases. To date there have been no reports for such AHH activity in bivalve molluscs. The other area of interest has been in screening a large number of enzyme activities from a number of organisms as a function of either short term exposure to oil (Heitz *et al.*, 1974) or *in vitro* exposure of tissue samples (Manwell and Baker, 1967).

In this experiment, we have specifically looked at enzyme activities that are associated with the plasma membranes. Our results, however, do not support a model of general membrane disruption caused by oiling. Only the Mg^{++}-ATPase activity was significantly reduced by exposure to both 3.0 mg/l and 0.3 mg/l oil in seawater. The Na^{+}-K^{+}-ATPase appeared to be unaffected by oiling, and the 5'-nucleotidase and phosphodiesterase appeared to be activated slightly by the oiling regime. It is likely that these enzymes occupy different sorts of environments in the membranes which are differentially affected by oiling (Roubal, 1974).

A decrease in the activity of an enzyme caused by oiling could be a result of direct action on the enzyme or a result of action at another level such as on synthesis or degradation of the enzyme or even effects on the relative levels of modifiers of the enzyme. We performed a series of experiments *in vitro* on crude homogenates from *Macoma balthica* which showed that the Mg^{++}-ATPase activity is decreased after crude oil and other hydrocarbons are mixed with the homogenates (Table 15). This lends support to the idea that the oil can directly interfere with membrane-bound enzyme activity. More work needs to be done in this area in order to understand the effect of oiling on membranes in living systems.

It is unclear to us exactly what the Mg^{++}-ATPase activity represents. Most of the activity (90%) was found to be associated with the membrane fraction as determined by differential centrifugation. About 50% of this membrane activity was associated with the mitochondrial pellet (15,000 xg pellet of 1,600 xg supernatant). It is likely that half of the ATPase activity is from mitochondrial electron transport, but the rest is associated with the plasma membrane fraction. Since we did not differentiate between membrane fractions in this experiment, it is unknown whether the decrease in Mg^{++}-ATPase activity is due to mitochondrial or plasma membrane interference by the oil or both. If the ATPase in the mitochondria were affected, this might be a result of the effect of oil on membrane permeability causing dysfunction in coupling ionic and pH membrane potentials to ATP synthesis (Hinkle and McCarty, 1978). It is possible that such interference would cause uncoupling of oxidative phosphorylation leading in turn to an increase in the respiration rate. In fact, the

TABLE 15. *IN VITRO* EFFECTS OF CRUDE OIL AND COMPONENTS OF CRUDE OIL ON Mg^{++}-ATPase FROM CRUDE HOMOGENATES OF *MACOMA BALTHICA*

Hydrocarbon treatment	Amount added per ml of crude homogenate (in µl/ml)	% inhibition with respect to the control
H_2O	100	(0)
Prudhoe Bay Crude oil	10	20
	20	22
Benzene	1	0
	10	31
	100	50
Toluene	1	0
	10	23
	100	60
Tetradecane	1	13
	10	16
	100	38

Crude homogenates from *Macoma* were prepared and assayed according to the procedure in Methods. Test hydrocarbons were added after preparation of the crude homogenate in the amounts specified in the table. Water was added to obtain a final volume of 1.1 ml. These solutions were then vortexed to mix the hydrocarbons with the crude homogenate. From this 100 µl were assayed for Mg^{++}-ATPase activity. Controls contained 100 µl of water per ml of crude homogenate.

oiled clams in this experiment did have an increased rate of oxygen consumption (Table 5). On the other hand, the Mg^{++}-ATPase in the plasma membrane may be instrumental in supplying the energy needed to generate the ciliary movement necessary for pumping water through the clam over the labial palps and the gills. If this ATPase activity were decreased by the oiling, we would expect a decrease in the filtering rate of the clams. The observed decrease in the feeding rate of the clams may reflect such a decrease in the pumping rate, although it should be pointed out that there could be a difference between the rate that water is pumped through the clams and the rate at which particles are cleared from the incoming water.

Another question raised by this experiment is why there was a decrease in dry weight relative to wet tissue weight in the oiled clams. It is reasonable to conclude that as the clams use up their energy reserves, their dry weight will decrease. The controls displayed such a decrease in dry weight. Yet their dry to wet weight ratio remained at around 17.5% (Figure 5). The high oiled (3.0 mg/l) clams, however, lost dry weight faster than wet weight, showing a ratio of under 14% by day 180. It seems possible that a relative increase in wet weight to dry weight would indicate that the clams are becoming osmotically unbalanced. That is, they are becoming hypotonic with respect to the seawater and by day 180 are acting as though they are in lower salinity than they in fact are. Thus they have the problem of regaining ionic balance with the surrounding high salinity seawater. Such response to altered salinities is usually accomplished in bivalves by changing the levels of free amino acids in their tissues (Schoffeniels, 1976; Gainey, 1978). Jeffries (1972) has reported that the hard clam, *Mercenaria mercenaria* shows significant changes in free amino acid composition in clams that have come from a chronic hydrocarbon polluted area. Clams from the polluted area had less free amino acids (on a µmole/dry weight basis) and different ratios of taurine to glycine than the unpolluted controls. We did not measure free amino acids in this experiment, but would like to suggest this as another area for future investigation.

The results reported here have shown the potential deleterious effects of crude oil on *Macoma balthica* which had remained in a fairly constant, uniform environment. The clams were not subjected to any of the sorts of stresses that normally occur in their natural environment in the mud flats, such as changes

in salinity, temperature, oxygen availability, and wave action. If oiling the clams under constant conditions causes a general slow-down of activity and weakening of the clams, then they may not be able to respond to a change which normally occurs on the mud flats. For example, when the tides recede from the mud flats in Resurrection Bay, the salinity of the water remaining on the flats is lowered due to the freshwater run-off. As mentioned above, to compensate for a change in salinity, bivalves alter the composition and amounts of their free amino acids in their tissues. If the clams are already in a weakened state induced by oiling, such natural changes in their environment would probably lead to a much higher mortality rate than one would have projected based on the laboratory data reported in this paper. Experiments of this nature, an artificial stress imposed with natural stresses on biological systems, are just beginning.

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