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Utility of Splenic Macrophage Aggregates as an Indicator of Fish Exposure to Degraded Environments

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Abstract.—The utility of splenic macrophage aggregates (MAs) as an indicator of fish exposure to degraded environments was evaluated in several species of estuarine fishes as part of the Environmental Protection Agency's Environmental Monitoring and Assessment Program–Estuaries (EMAP-E). Using image analysis, we measured the number and mean size of MAs per square millimeter on tissue sections of spleen from 983 fishes representing seven species from 266 stations scattered across coastal estuaries of the Gulf of Mexico. At 16 stations, at least one fish exhibited a high density of MAs (>40 MAs/mm^2). Densities of MAs that exceeded 40/mm^2 correlated with exposure to either hypoxic conditions or sediment contamination. Fisher's exact test showed that the observed frequencies of joint occurrence between high numbers of MAs and both high sediment contaminants and low dissolved oxygen were significantly greater than the expected background frequencies. For all 16 sites where MAs were greater than 40/mm^2, sediments displayed at least one contaminant at a concentration in the highest 5% of those observed for all Gulf of Mexico stations. Additionally, comparison of subjective visual analyses with the image analysis measurements showed a strong correlation, indicating that similar analyses can be performed without computer image analysis. This study demonstrates that splenic MAs are effective biotic indicators for discriminating between fish exposed to degraded and nondegraded environments.

Macrophage aggregates (MAs) are focal accumulations of macrophages found in the spleen, head kidney, and sometimes liver of teleost fishes. These structures are easily visualized in histologic sections through the presence of three pigments: hemosiderin, melanin, and ceroid/lipofuscin. Many factors are known to affect the accumulation and/or proliferation of these structures, including age (Brown and George 1985; Blazer et al. 1987), nutritional status (Agius and Roberts 1981), and infectious diseases (Agius 1979; Vogelbein et al. 1987). Changes in various MA parameters (e.g., number, size, percent area occupied) in relation to environmental contamination have been reported by several investigators (Wolke et al. 1985; Spazier et al. 1992; Wolke 1992; Blazer et al. 1994; Couillard and Hodson 1996; Meinelt et al. 1997; Facey et al. 1999). Because MAs are known to change in number, size, and pigment content in relation to fish health and environmental degradation, they qualify as anatomical and cytological biomarkers (Wolke 1992). The value of using MAs as histological biomarkers lies in their ubiquity, availability, ease of measurement, and association with degraded environmental conditions.

Although evaluation of MA density may be a sensitive histological indicator of fish health (Wolke 1992), research thus far generally has focused on laboratory exposures or field collections from a specific location. No regional baseline information exists to establish a ''normal'' density of MAs, so comparisons cannot be made with MA densities observed in experiments or those found in fish exposed to specific stressors. Thus, it is important, particularly for indicators of individual health, to characterize the statistical distribution of these indicators within broad regional populations. Only against such baselines can the magnitude of an observed ''increase in density'' be interpreted.

This study was conducted as part of the U.S. Environmental Protection Agency's Environmental Monitoring and Assessment Program-Estuaries (EMAP-E), which was designed to provide a re-

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gional-scale perspective of the ecological condition of the estuarine resources of the continental United States. The strategy of EMAP for the development of biotic indicators requires that an indicator can be used to make spatial assessments of the ecological condition of systems across large geographical areas. In this paper we report on one of the biotic indicators (MAs) utilized by this program. To further substantiate the efficacy of variations in splenic MA number (density) as an indicator of exposure to environmental degradation, we will demonstrate associations between high numbers of splenic MAs in fish and the occurrence of high concentrations of sediment contaminants as well as low proportions of dissolved oxygen, total organic carbon (TOC), or both. More specifically, we will provide a critical value for the number of MAs per square millimeter for certain groups of estuarine fishes that can discriminate between exposure to degraded environments and exposure to nondegraded environments.

Methods

Fish collection.—Fish were collected over a 4-year period from the Louisianian Province, extending along the Gulf of Mexico coast from Anclote Key, Florida, to the Mexican border. In all, 644 stations were sampled between July 1 and August 30, 1991–1994. Fish were collected with a 5-m otter trawl with 2.5-cm-mesh wings and a 1.2-cm-mesh cod end. The net was towed over the bottom for 10 min against the tide at a speed of 1.0 m/s. Duplicate trawls were taken at all stations. All fish caught were identified as to species, counted, and inspected for gross pathological abnormalities (Fournie et al. 1996). All fish with external abnormalities, as well as reference (nonpathology) fish, were preserved in Dietrich's solution (Yevich and Yevich 1994) for laboratory examination and verification. Liver samples and spleens were removed, dehydrated in an ethanol gradient, cleared in xylene or a xylene substitute, and embedded in paraffin. Sections were cut 6 μm thick with a rotary microtome, stained with Harris' hematoxylin and eosin (H&E) or Perl's method for iron (Luna 1968), and examined microscopically.

Sediment analysis.— Before trawling, we collected a composite sediment sample for chemical contaminant analysis at the approximate midpoint of the trawl path at each site, using a 440-cm^2 Young-modified van Veen grab (Holme and McIntyre 1971). A Teflon-coated spoon was used to remove the top 2 cm of sediment from 5 to 10 grabs. These sediments were homogenized, and 100-mL samples were placed in clean glass jars with Teflon-coated lids and stored frozen. Samples were analyzed for the 73 chemicals listed in Macauley et al. (1999) according to standard methodologies (see USEPA 1995; Fournie et al. 1996). Total organic carbon was determined by drying at least 5 g (wet weight) of sediment for 48 h. Weighed subsamples were ground to fine consistency and acidified to remove sources of inorganic carbon (e.g., shell fragments). The acidified samples were ignited at 950°C and the carbon dioxide evolved was measured with an infrared gas analyzer.

TABLE 1.—List of scientific and common names of fishes used in this paper (see Robins et al. 1991).

Common name	Scientific name
Bullhead catfishes	Ictaluridae
Brown bullhead	*Ameiurus nebulosus*
Blue catfish	*Ictalurus furcatus*
Sea catfishes	Ariidae
Hardhead catfish	*Arius felis*
Gafftopsail catfish	*Bagre marinus*
Porgies	Sparidae
Pinfish	*Lagodon rhomboides*
Drums	Sciaenidae
Spot	*Leiostomus xanthurus*
Atlantic croaker	*Micropogonias undulatus*

Measurement of dissolved oxygen.—The concentration of dissolved oxygen (DO) in the water column was determined at each site. Instantaneous vertical profiles from surface to bottom were measured, generally between 0900 and 1600 hours, by using a Hydrolab Surveyor 2 equipped with a DO electrode. Continuous measurements of DO were taken every 15 min for 24 h with a DataSonde 3 electronic monitor deployed 0.5 m off the bottom. Both instruments were calibrated daily by using known solutions, shipboard intermachine comparisons pre- and postdeployment, and weekly air-saturated water tests.

Macrophage aggregate analysis.—Quantitative splenic MA analysis was subsequently performed on tissue sections of spleen from 983 fishes representing seven species. These samples represent a subsample of the total number of stations (266 of 644) sampled by EMAP in the Louisianian Province, based on the occurrence of the target species (see Macauley et al. 1999). Common and scientific names of these target fishes (Table 1) follow Robins et al. (1991). Macrophage aggregate parameters were measured by using a true color (HSI Imaging) particle analysis package (MicroComp Integrated Image Analysis System

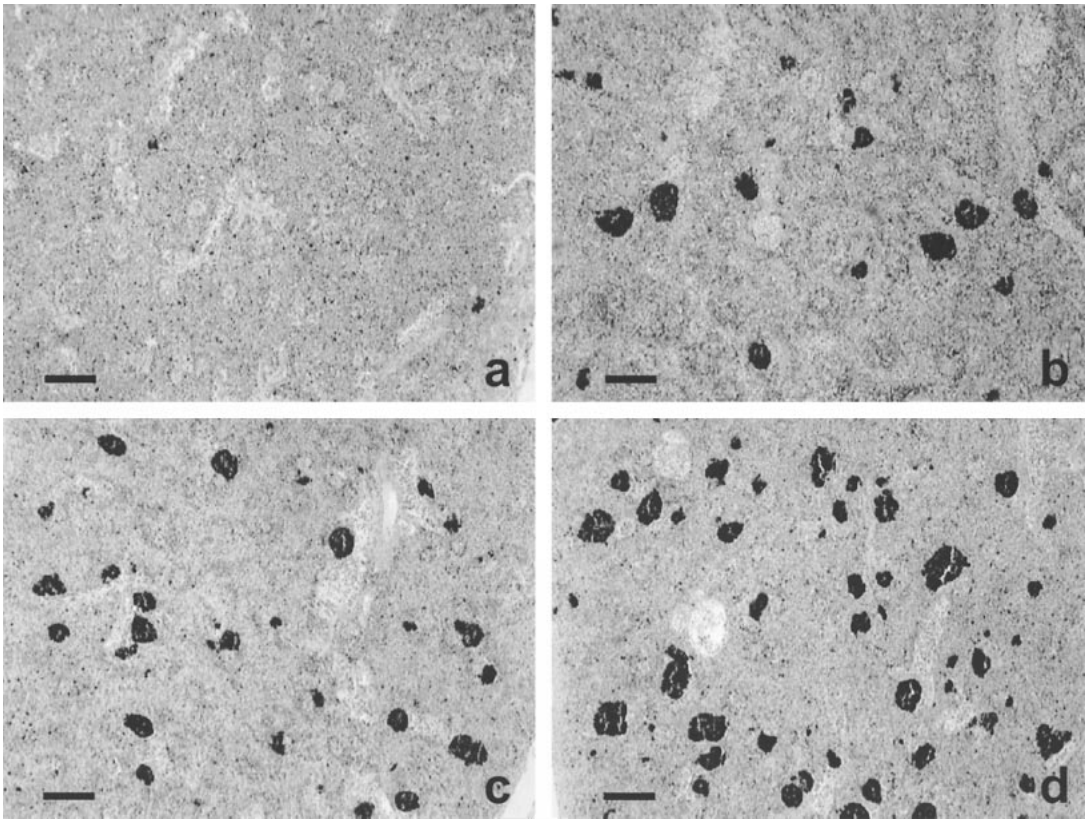

FIGURE 1.—Histologic sections of spleen from Atlantic croaker showing representative examples of the four categories of macrophage aggregate (MA) density: (**A**) very few MAs, rating = 1; (**B**) moderate number of MAs, rating = 2; (**C**) high number of MAs, rating = 3; and (**D**) massive number of MAs, rating = 4. Hematoxylin and eosin stain. Bar = 100 μm.

with Sony 3 charge-coupled device color video camera input). The system was calibrated and measurements taken at 10× magnification. Three fields of view (~0.82 mm^2/screen) were arbitrarily selected for analysis from each spleen. After a screen was selected, minor adjustments of the microscope stage position were made so that no MA was lying on the edge of the counting field. The full-color image was then captured as a digital image. Images generated by computer-produced masks of the MAs in each screen enabled recording of the number of MAs per screen and the area (μm^2) of each MA. A size discriminator was used to eliminate objects smaller than 50 μm^2 (the approximate size of three aggregated macrophages). Total screen area was also determined for calculating MAs per square millimeter.

While screening slides from the EMAP-E collections for the presence of pathological abnormalities, we also performed a subjective evaluation, assessing the number of MAs per spleen. This process had been part of our evaluation protocol from the beginning of the EMAP-E program, before collection of the computer image analysis data. We therefore continued to collect these ''subjective'' data after the computerized image analyses had been implemented so that we could compare the outcomes of the subjective and quantitative methods. Sections of entire spleens were evaluated according to the following subjective scale: 0 = no MAs, 1 = <5 MAs/mm^2, 2 = 5−20 MAs/mm^2, 3 = 20−35 MAs/mm^2, and 4 = >35 MAs/mm^2. (Figure 1 shows examples of splenic tissue from Atlantic croaker that correspond to subjective evaluation rankings of 1 to 4.) Each spleen examined was then assigned an appropriate ranking score derived from the subjective evaluations, and the scores were later compared with the quantitative values derived from the computer image analysis.

TABLE 2.—Ninetieth-percentile and maximum concentrations of sediment contaminants from the Louisianian Province of EPA's Environmental Monitoring and Assessment Program–Estuaries using only probability-based stations sampled from 1991 to 1994.

Major class	Analyte	Concentration	
		90th percentile	Maximum
Metals (μg/g)	Silver	0.19	0.9
	Arsenic	12.50	37.4
	Cadmium	0.41	1.5
	Chromium	74.00	149
	Copper	21.60	104
	Mercury	0.09	0.4
	Manganese	833.20	2,130
	Nickel	30.60	51.2
	Lead	25.60	610
	Antimony	1.06	3.8
	Selenium	0.59	1.8
	Tin	2.78	13.5
	Zinc	122.00	625
Polycyclic aromatic hydrocarbons (ng/g)	Acenaphthene	3.40	147
	Acenaphthylene	3.10	62
	Anthracene	8.00	287
	Benzo(a)anthracene	25.80	279
	Benzo(a)pyrene	24.72	372
	Benzo(e)pyrene	23.10	356
	Benzo(b)fluoranthene	35.17	319
	Benzo(k)fluoranthene	21.78	271
	Benzo(g,h,i)perylene	21.59	260
	Biphenyl	9.9	110
	Chrysene	32.80	434
	Dibenzothiophene	5.51	114
	Dibenzo(a,h)anthracene	8.15	123
	2,6-dimethylnaphthalene	25.30	274
	Fluoranthene	50.19	653
	Fluorene	12.03	126
	(i)1,2,3,-c,d-pyrene	21.56	244
	1-methylnaphthalene	12.38	173
	2-methylnaphthalene	19.88	199
	1-methylphenanthrene	19.60	168
	Naphthalene	13.21	219
	Perylene	91.71	817
	Phenanthrene	48.00	416
	Pyrene	56.20	545
	2,3,5-trimethylnaphthalene	36.80	410
Polychlorinated biphenyls (ng/g)	PCB 101	0.57	35
	PCB 105	0.24	23
	PCB 118	0.41	35
	PCB 126	0.13	1
	PCB 128	0.17	5
	PCB 138	0.80	18
	PCB 153	0.56	14
	PCB 18	0.14	11
	PCB 180	0.41	9
	PCB 187	0.16	7
	PCB 195	0.06	1
	PCB 206	0.08	3
	PCB 209	0.15	6
	PCB 28	0.41	14
	PCB 44	0.25	1
	PCB 66	0.30	32
	PCB 8	0.24	1
Pesticides (ng/g)	Aldrin	0.03	1
	alpha-Chlordane	0.18	3
	cis-Nonachlor	0.10	3
	Endrin	1.05	7
	gamma-Chlordane	0.27	5
	Heptachlor	0.05	1

TABLE 2.—Continued.

Major class	Analyte	Concentration	
		90th percentile	Maximum
	Heptachlor epoxide	0.02	12
	Hexachlorobenzene	0.31	23
	Lindane	0.18	3
	Mirex	0.01	3
	o,p′-DDD	0.15	7
	o,p′-DDE	0.18	8
	o,p′-DDT	0.03	2
	Oxychlordane	0.01	3
	p,p′-DDD	0.98	17
	p,p′-DDE	1.08	32
	p,p′-DDT	0.24	18
	trans-Nonachlor	0.18	3
TBT (ng/g)	Dibutyltin	2.60	12
	Monobutyltin	5.00	18
	Tributyltin	7.90	53

Site characteristics and statistical analyses.—The cumulative distribution function (CDF) of MA densities for each target species was calculated as the weighted distribution of the observed MA densities at all sites at which a target species had been collected. The weighting factor was the product of the inverse of the inclusion probability of the site (area associated with sample site divided by total area associated with all sites where the target species was collected) and the average catch of the species per trawl at each site (number of individuals of a target species collected per trawl divided by total number of individuals of a target species collected from all sites). This weighting factor adjusts the distribution for differences in abundance of target species among sites and differences in the area represented by each sample site. By determining this factor, all collected individuals of each target species can be used in the analysis.

When we noted an individual target fish in the field as having a gross pathology (e.g., skin lesions or ocular, skeletal, or branchial abnormalities; see Fournie et al. 1996), that fish was examined for MA densities. If no target fish at a site was determined to have a gross pathology, a random fish was selected for MA density evaluation. This seemingly biased selection of some fish for having gross pathologies was adjusted in the calculation of the MA density CDF by including in the weighting factor an additional term representing the probability of selecting a target fish with a gross pathology (number of individuals of a target species with gross pathologies divided by total number of individuals of a target species collected).

The formula used for base-level selected target fish:

$$\hat{p} = \left\{(N_{gk}/D_k)\left[\sum_n (P_{si}/F_i)\right]\left[\sum_n (P_{spi}/P_{si})\right]\right\}$$

where:

$\hat{p}$ = the estimated proportion of fish with condition g (macrophage density) in year k

$N_{gk} = \sum z_{gki}x_{ki}$ $\quad D_k = \sum x_{ki}$

n = the total number of hexagons composing the grid

z_{gki} = the response from hexagon i in year k (1 if the condition g is met, 0 otherwise)

x_{ki} = 1 if the hexagon i is sampled in year k, and 0 otherwise

$\sum (P_{si}/F_i)$ = sum over i hexagons of the proportion of the number of fish of species s in hexagon i divided by the total number of fish in hexagon i

$\sum (P_{spi}/P_{si})$ = sum over i hexagons of the proportion of fish of species s in hexagon i with pathology p divided by the number of sish of species s in hexagon i

The cooccurrence of high numbers of MAs with environmental stressors was examined statistically. Contingency tables were used to test the null hypothesis (H_0) that no association exists between MAs and environmental stressors versus the al-

ternative hypothesis (H_A) that a positive association exists between MAs and environmental stressors. Analyses were performed by using the MA density (number/mm^2) and a suite of selected stressors including DO, TOC, and 73 sediment contaminants.

Dissolved oxygen was considered to be stressful if the minimum measured in a 24-h period was less than 2 mg/L; DO greater than 5 mg/L for the entire 24-h period was indicative of nonstressful oxygen conditions (Summers and Engle 1993). More than 2% TOC in sediments was indicative of organic pollution, whereas less than 1% TOC indicated normal sediment conditions (Summers et al. 1993). Of the more than 200 analytes measured in sediments by EMAP over 4 years, 73 were chosen to represent sediment contaminants having the potential to induce stress in fish. This subgroup excluded contaminants for which no guidelines were available (Long et al. 1995), for which quality control measures were not met consistently, or which represented summed concentrations (e.g., total DDTs). The selected analytes were grouped as metals (13), polycyclic aromatic hydrocarbons (PAHs; 25), polychlorinated biphenyls (PCBs; 17), and pesticides (18). To determine whether or not concentrations of any given analyte were ''high,'' they were compared with the highest 10% (the 90th percentile) of concentrations for that analyte found in the Louisianian Province. Only the 395 probability-based sites in the Louisianian Province from 1991 to 1994 were used to compute an unbiased estimate of the 90th percentile concentration for each analyte (Table 2). Ten percent of the probability-based stations had concentrations of a given analyte that exceeded the 90th percentile value. If at least 10% of the analytes in at least one major group had concentrations greater than the 90th percentile value, a station was classified as having high sediment contaminants. On the other hand, a station was classified as having low sediment contaminants if zero analytes in all groups had concentrations exceeding the 90th percentile value.

In a pilot study completed in 1991 and independent from the EMAP probability survey, MA densities were examined as a potential indicator of contamination (Summers et al. 1993). In that study, based on small sample sizes from nonprobabilistic sites, significant differences in MA densities were observed in fish collected from heavily contaminated sites and reference sites ($P < 0.001$). Macrophage densities greater than 40 MA/mm^2 were observed only at heavily contaminated sites; however, densities at reference sites never exceeded 25 MA/mm^2 (Summers et al. 1993). Thus, the appropriate indicator value for MA density appeared to be between 25 and 40 MA/mm^2. We used this pilot study result as the initial criterion for discriminating between stressed and unstressed fish in the present study. On the basis of the pilot study findings, we considered more than 40 MAs/mm^2 as indicative of high density MAs (or stressed fish) and less than 15 MAs/mm^2 as indicative of low density MAs (or less stressed fish). A station at which at least one fish had more than 40 MAs/mm^2 was considered to have high MAs.

By classifying both MAs and stressor variables as high or low, we constructed 2×2 contingency tables with high–low MAs as the row levels and high–low stressor variables as the column levels. When sample size requirements for chi-square measures of association were not met, we used Fisher's exact test to test H_0 (Stokes et al. 1995). Fisher's exact test for a 2×2 contingency table tests the probability of obtaining a higher proportion in the first cell, given the conditional probabilities in the margins. In other words, if the first cell in the 2×2 contingency table represents the probability of occurrence of both high MAs and high sediment contaminants, a significant association is indicated if the observed frequency is significantly higher than the expected frequency, given that the number of stations with high MAs is fixed and the number of stations with high sediment contaminants is fixed. The expected frequency for the first cell ($\mathrm{Exp}[n_{11}] = n_{1+}\, n_{+1}/n$) is calculated as the product of the total number of stations in row 1 (n_{1+}) and the total number of stations in column 1 (n_{+1}) divided by the grand total number of stations (n). In addition, the odds ratio indicates the strength of association, a positive odds ratio indicating that high stressor values are more likely to cooccur with high density MAs. To reject the H_0 of no association in favor of the H_A of a positive association, the probability of obtaining a greater than observed frequency in the first cell should be low (<0.05 is ideal; <0.10 indicates significance at the 90% level).

Results

In all, 29,964 fish were collected throughout the estuaries of the Gulf of Mexico. All were examined for gross pathological disorders, and 213 individuals of target species had at least one pathological abnormality (Fournie et al. 1996). These 213 pathology fish and 770 randomly selected target fish from all sites that had no observed pathologies

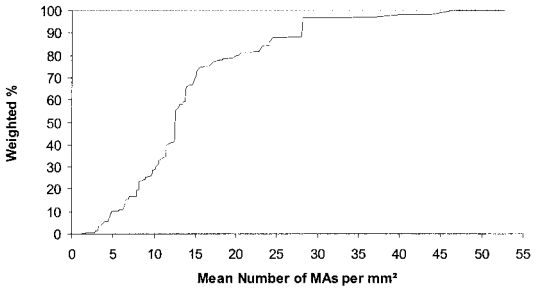

FIGURE 2.—Trawl and station weighted cumulative distribution functions (CDFs) for all species during the period 1992–1994 for mean number of aggregates per square millimeter.

were combined to create the 983 target fish we examined to determine the abundance of MAs in the spleen of each fish. As described earlier, the 213 pathology fish were combined with the randomly selected fish by adjusting the likelihood of selection of the 213 pathology fish by the probability of a target species having a pathology. The trawl- and station-weighted (and pathology-weighted when appropriate) cumulative distribution function (Figure 2) shows that 1.9% of target fish in Gulf of Mexico estuaries had splenic MA densities greater than 40 MAs/mm^2.

The 2 × 2 contingency table and expected frequencies used in Fisher's exact test are presented in Table 3. The observed and expected frequencies in the first shaded area are of interest. They indicate a positive association between high MA densities and high concentrations of sediment contaminants, for which the observed frequency of joint occurrence (11) is greater than the expected frequency (8.46). This positive association is significant ($P = 0.078$), and the odds of exposure to high sediment contaminants are fivefold higher when high densities of MAs are observed. The association between high densities of MAs and low DO (second shaded area) is also significant ($P = 0.0003$); the observed frequency in the first cell is much greater than the expected frequency. The odds ratio (15.257) indicates that a fish with high MAs is 15 times more likely to have been exposed to low DO than a fish without high MAs. The association between high densities of MAs and high concentrations of TOC was not significant.

To confirm that 40 MAs/mm^2 was indeed the most appropriate critical value, we performed the statistical analyses with contingency tables and Fisher's exact test and using 35 and 30 MAs/mm^2 as critical values. When the critical value was less than 40, the strength of association with stressors diminished. Using less than 35 MAs /mm^2 as the critical value gave no significant association between MAs and sediment contaminants or TOC, and the strength of association with DO, although significant ($P < 0.05$), was reduced to an odds ratio of 8.09 (from 15.257 with MAs of 40/mm^2). When MAs exceeding 30/mm^2 was used to represent high numbers of MAs, the association with DO was decreased even further. This confirms our use of 40 MAs/mm^2 as the critical value for MAs.

At 16 of the 266 stations where fish were analyzed for MAs, at least one fish exhibited high densities of MAs (>40 MAs/mm^2) (Figure 3A). Of these 16 stations, 2 stations contained sediments with at least one contaminant at a concentration greater than 99% of that found at all Gulf of Mexico stations; 14 stations had at least one contaminant at a concentration greater than that seen at 95% of all sites; and all 16 sites showed contaminant concentrations exceeding those found at 90% of all sites (Figure 4). Of the 16 stations exhibiting increased numbers of MAs, 11 were associated with one or more stressors, according to the indicator definitions detailed in the Fisher's exact test (Figure 3B). All of the 5 remaining sta-

TABLE 3.—The 2 × 2 contingency tables for Fisher's exact test for association between high-density macrophage aggregates (MAs) and high sediment contaminants ($P = 0.078$), low dissolved oxygen ($P = 0.003$), and high total organic carbon (not significant). Shading indicates the comparisons tested; numbers in parentheses are expected frequencies. High MAs are defined as being greater than 40/mm^2, low MAs as being less than 15/mm^2.

Variables and expected frequency	High MAs	Low MAs	Total
Sediment contamination			
High	11 (8.46)	63 (65.54)	74
Low	1 (3.54)	30 (27.46)	31
Total	12	93	105
Dissolved oxygen			
Low (<2 mg/L)	6 (1.34)	7 (11.67)	13
High (>5 mg/L)	5 (9.67)	89 (84.34)	94
Total	11	96	107
Total organic carbon			
High (>2%)	2 (1.50)	11 (11.50)	13
Low (<1%)	15 (15.50)	119 (118.5)	134
Total	17	96	147

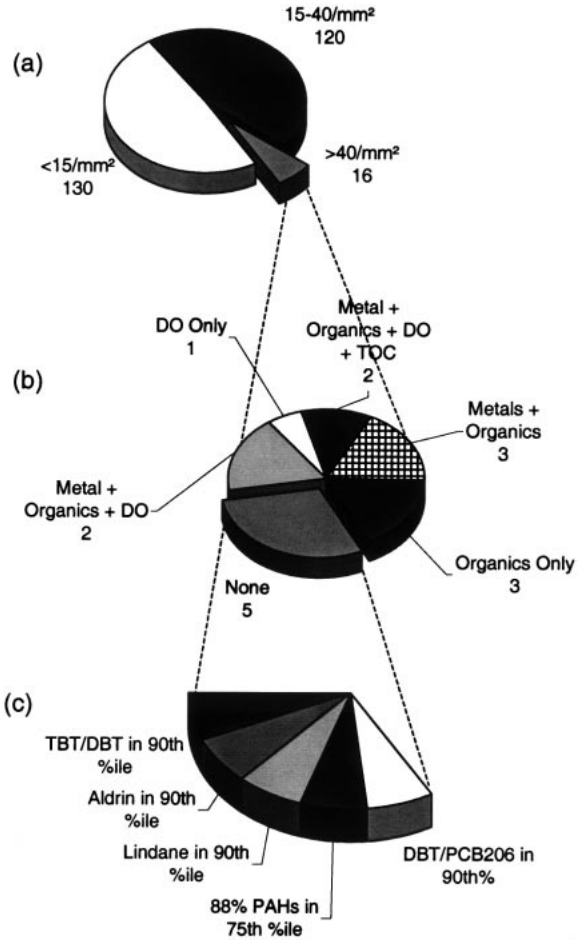

FIGURE 3.—Exploding pie depiction of (**A**) the number of stations having fish with high (>40/mm²), moderate (15–40/mm²), and low (<15/mm²) densities of splenic macrophage aggregates (MAs); (**B**) the number of sites with high macrophage densities with high values (highest 10% observed at all sites evaluated) for various types of environmental stressors; and (**C**) sediment contaminant values observed at those sites with high densities of MAs but with concentrations less than the highest 10% observed at all sites.

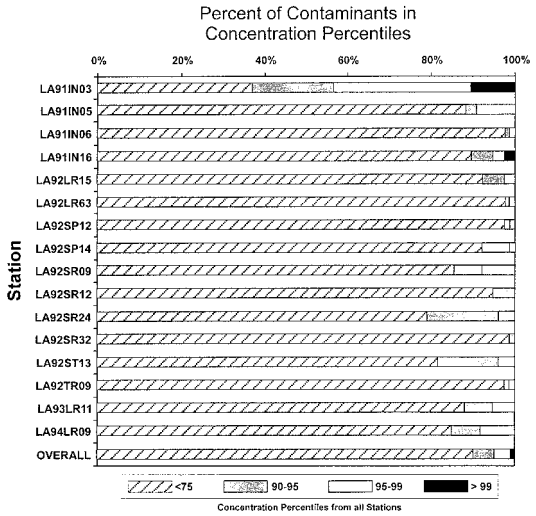

FIGURE 4.—For the 16 stations with > 40 macrophage aggregates/mm², the percentage of contaminants that were <90%, 90–95%, 95–99%, and > 99% of all sediment contaminant concentrations observed in Gulf of Mexico estuaries during the EMAP surveys.

tions indicated by the pie slice labeled ''none'' (Figure 3C) had at least one sediment contaminant concentration in the 90th percentile of all contaminants found at all stations. One of the five had one PAH in the 90th percentile and 88% of all measured PAHs in the 75th percentile. Two stations had one pesticide (lindane or aldrin) in the 90th percentile. The remaining two stations had butyltins in the 75th and 90th percentiles, respectively. Twelve of the 16 sites (75%) with high MAs had at least one contaminant concentration in the 95th percentile. All stations where fish with more than 40 MAs/mm² were observed had one or more environmental stressors at concentrations considered ''high'' (Table 4). Eleven of the 16 sites displayed at least 12% of all contaminants in one of the contaminant groups at greater than the 90th percentile. In fact, half of these sites displayed at least 23% of all contaminants in one of the groupings at the greater than 90th percentile.

The subjective evaluation ratings (i.e., 0, 1, 2, 3, and 4) of splenic MAs was entered into the statistical analysis package with the image analysis data. Comparing the subjective evaluations of MAs with the results of image analysis data for the same fishes by using a chi-squared frequency table revealed a strongly significant relationship between expected and observed results ($P < 0.001$). The subjective rankings versus the mean number of MAs per square millimeter (Figure 5) show a strong relationship between these parameters, the subjective values corresponding to the following MA densities: $1 = 3.8 \pm 0.8$, $2 = 15.1 \pm 0.8$, $3 = 32.7 \pm 3.4$, and $4 = 44.1 \pm 6.8$.

Discussion

This study supports the hypothesis that variations in the number of splenic MAs may be used to monitor fish health as characterized by exposure to environmental degradation. This hypothesis is based on two assumptions regarding the biology

TABLE 4.—Associations between high (>40/mm^2) macrophage aggregates (MAs) and sediment contaminants, dissolved oxygen (DO), and total organic carbon (TOC). The table lists all stations where the maximum MA is above 40/mm^2 for any fish species along with the stressor(s) that were considered to be important (indicated by bold italics). The abbreviation PAH stands for polycyclic aromatic hydrocarbon, PCB for polychlorinated biphenyl, DBT for dibutyltin and TBT for tributyltin.

		Percent of contaminants in 90th percentile						
Estuary	Fish species	Metals	PAHs	PCBs	Pesti-cides	DO	TOC	Other stressors
Watson's Bayou, Florida	Pinfish	***38***	***72***	***82***	***50***	***0.0***	***7.6***	
Choctawatchee River, Florida	Spot	***46***	0	6	***11***	***0.5***	***7.5***	
Wolf Bay, Alabama	Pinfish	8	0	0	6	***1.5***	1.5	
Brazos River, Texas	Spot	0	***16***	6	***17***	3.1	1.3	
Mississippi Sound, Mississippi	Hardhead catfish	***23***	0	***12***	6	***1.6***	1.4	
Corpus Christi Bay, Texas	Pinfish	0	0	6	0	5.9	0.1	DBT or PCB206 in 90th percentile
Dauphin Bay, Alabama	Pinfish	0	4	0	6	4.5	0.5	88% PAHs in 75th percentile
Heron Bay, Mississippi	Hardhead catfish	0	0	0	6	5.4	0.6	Lindane in 90th percentile
Bayou St. John, Florida	Hardhead catfish	0	***36***	***12***	0	4.2	0.7	
Dauphin Bay, Alabama	Pinfish	0	8	***12***	0	4.1	0.2	
Bayou Teche, Louisiana	Blue catfish	***54***	***16***	***12***	***17***	5.4	1.4	
Redfish Bay, Texas	Pinfish	0	0	0	6	5.6	0.4	Aldrin in 90th percentile
Mississippi Sound, Mississippi	Hardhead catfish	***23***	***24***	***12***	***17***	4.4	1.3	
Chandeleur Sound, Louisiana	Pinfish	0	0	0	0	6.5	0.0	TBT or DBT in 90th percentile
Mobile Bay, Alabama	Hardhead catfish, Atlantic croaker	***38***	0	0	***17***	***0.8***	1.0	
Mobile Bay, Alabama	Atlantic croaker	***62***	4	0	***17***	4.1	1.7	

of MAs (Wolke et al. 1985). First, MAs will localize the products of pathological tissue destruction and will display easily observable pigments—such as the lipoproteins, lipofuscin and ceroid, which increase during tissue necrosis and starvation, and hemosiderin, which is a product of hemolysis. The third pigment that accumulates in MAs is melanin, which presumably aids in the removal of substances that have undergone phagocytosis by macrophages. Secondly, variations in the number of MAs indicate stress on the physiological homeostatic mechanisms of the fish and, therefore, an alteration in the health status of the fish. Wolke et al. (1985) suggested that if health is defined as a physiological balance with the environment, then pathological conditions that result in excessive tissue destruction or hemolysis would indicate an upset in this balance and signify a deterioration of health, a situation not conducive to growth, reproduction, or survival of affected fish.

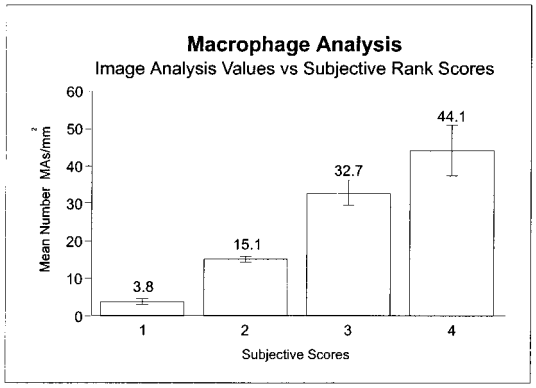

FIGURE 5.—Comparison of macrophage aggregate (MA) densities as determined by computer image analysis (MAs/mm^2) and the rank scores of 0–4 based on the subjective microscopic evaluation of splenic tissues. Note the strong relationship shown between these sets of values.

Although MAs occur in the spleen, head kidney, and liver of most teleosts, it is splenic MAs that best serve as a reliable histopathological bioindicator of fish health and environmental degradation. Studies have demonstrated that, for certain species, both hepatic and splenic MAs can be used to monitor fish health. Blazer et al. (1987) showed that thermally impacted largemouth bass from a cooling reservoir had significantly greater MA densities (MAs/mm^2) in both liver and spleen than did nonstressed fish. Couillard and Hodson (1996)

found greater densities of MAs relative to age in liver, spleen, and kidney of white suckers sampled downstream from a bleached-kraft pulp mill than in fish sampled upstream of the mill. They concluded the density of MAs appeared to be a useful indicator of bleached-kraft mill effluent (BKME) toxicity. However, other studies concerning the use of MAs as bioindicators have utilized only splenic MAs (e.g., Barker et al. 1994; Blazer et al. 1994; Khan et al. 1994). From a physiological standpoint, this is supported by the experimental studies of Ziegenfuss and Wolke (1991) concerning the kinetics of MA formation. Using microspheres in goldfish, they demonstrated that MAs form in greater numbers and more rapidly in the spleen and kidney than in liver. Excessive tissue destruction or hemolysis that could result from exposure to contaminants would therefore lead to an increase in MA formation in the spleen. The present study indicated that, for the marine and estuarine fish species we examined, correlations between the number of MAs per square millimeter and stressors were also observed in spleen.

Although the exact mechanisms involved in MA proliferation are not known, sufficient laboratory and field studies exist to support the hypothesis that increased density of MAs is linked to contaminant exposure. We have suggested that excessive hemolysis could result from exposure to contaminants and would lead to an increase in MA formation in the spleen. Couillard and Hodson (1996) also indicated that hemolytic compounds contained in BKME may accelerate the rate of destruction of red blood cells, leading to an increased density of MAs and splenic hemosiderosis. An increase in splenic MA density and the deposition of hemosiderin in MAs have been induced experimentally by exposure to dioxin (Van der Weiden et al. 1994) and observed in fishes collected in the vicinity of pulp mills (Barker et al. 1994; Khan et al. 1994). A mechanistic model was also proposed by Couillard and Hodson (1996), linking induction of hepatic cytochrome P450 1A (CYP1A) enzymes to MA proliferation. One of the most consistent responses of fish to BKME is increased activity of CYP1A. Couillard and Hodson (1996) suggested that CYP1A induction might cause oxidant damage of cellular membranes, which could lead to an increased rate of cell death, an accumulation of ceroid/lipofuscin, and a subsequent proliferation of MAs. Another possible mechanism could involve impaired macrophage function. Voccia et al. (1994) demonstrated that BKME impairs macrophage phagocytic function in vitro, but the link between impaired macrophage function and MA density has not been investigated. Regardless of the mechanism, the formation of MAs and the accumulation of pigments increase in the spleen and liver of fish from contaminated environments and therefore serve as reliable histopathological bioindicators.

We have established that more than 40 MAs/mm^2 in the spleen of certain fishes occurs only in fish captured from degraded environments. Our preliminary study, designed to evaluate MA densities as a potential indicator of contamination, showed that densities greater than 40 MAs/mm^2 were observed only at heavily contaminated sites. In the present study, spleens from a total of 983 fishes from 266 stations scattered across the Gulf of Mexico were examined and, at 16 stations, at least one fish exhibited MA densities exceeding 40/mm^2. In all cases, these densities correlated with exposure to either hypoxic conditions or sediment contamination. As already stated, we confirmed that MA density exceeding 40 MAs/mm^2 was the most appropriate critical value indicating contamination by performing the same statistical analyses with 35 and 30 MAs/mm^2 as the cutoff points. From our studies, we conclude that finding such densities ($>$40/mm^2) of MAs in certain estuarine fishes indicates the fishes may have been stressed as a result of exposure to either hypoxic conditions or sediment contamination.

Comparison of the subjective and quantitative methods showed that the subjective values corresponded well with the measured values. Overall, the subjective rank score of 4 corresponded to 44.1 $\pm$ 6.8 MAs/mm^2. This rating, therefore, generally indicated that a fish had been exposed to either contaminants or hypoxia, in accordance with our critical value of 40 MAs/mm^2. Similarly, the subjective rank score of 2 generally corresponded to a MA value of 15 MAs/mm^2 or less and therefore identified fish from lightly impacted environments. If a somewhat more objective method is desired, actual counts of MAs within a defined area (e.g., calibrated ocular grid) may be made of several fields per spleen. MA density (numbers of MAs/mm^2) can then be calculated that provide a more quantitative value. This procedure would require considerably more investment in time for evaluation but would presumably reduce the ''observer bias'' in cases where several observers would be required to collect the data.

The value of using MAs (particularly splenic MAs) as histopathological biomarkers lies in their ubiquity, ease of measurement, and association

with sediment contaminants as demonstrated in this survey. In addition, however, several practical considerations make spleen the tissue of choice for evaluating variations in MA parameters. One can easily dissect the intact spleen from most fishes in the field or laboratory and adequately fix the tissue in a small vial; even if the splenic tissue is poorly fixed or badly autolyzed, however, MAs remain intact and may be used for future analysis. Whole sections of spleen can easily be mounted on glass slides for microscopic examination and MAs are readily discernible in H&E-stained sections. Furthermore, relation between the subjective MA evaluations and the quantitative measurements of MA number/mm^2 is demonstrably strong (Figure 5). Therefore, although the use of an image analysis system may generate stronger data that can provide for more varied statistical evaluation, researchers who do not have computer image analysis capabilities may still use MAs subjectively to identify heavily compromised environments.

The utility of MAs as a histopathological bioindicator or biomarker has been criticized by some researchers as being too nonspecific; others consider that too many variables are involved in alterations of MA parameters to be of value. Even though several studies have indicated a limited usefulness of MAs as indicators of pollution (e.g., Haaparanta et al. 1996), more than 50 literature citations have noted increases in various MA parameters in fish collected at contaminated versus those collected at reference sites or in laboratory exposures to individual contaminants. However, statistical demonstration that a clear relationship exists between increased MA density and high amounts of environmental stress (sediment contamination, hypoxia, enrichment) remains difficult because of the rare occurrence of truly high amounts of these stressors (only 16 of 266 sites and $<$2% of fish). Nevertheless, the likelihood of the cooccurrence of these events being due to chance is very small. The probability of intersection of 2% of fish with MA densities greater than 40/mm^2 at 16 of 266 sites, where all 16 sites have sediment concentrations in the highest 10% of contaminated sites, is less than 1 in 1,000,000. Clearly, the increased density of MAs to values more than 40 MAs/mm^2 has a relation, albeit complicated, to environmental stressors.

Additionally, we believe that the nonspecificity of MAs is a positive attribute of this bioindicator. A bioindicator such as MAs, which respond relatively rapidly to several environmental stressors, should be of great value as a first-line indicator or early warning system of environmental problems. Such a bioindicator would serve to notify or warn regulatory agencies of a problem much less expensively than chemical analysis of the sediments. This indicator also indicates the potential, at least, for some change in the physiology of individual fish rather than simply an increased environmental stressor. Then, more specific measurements could be targeted to certain locations to specifically examine population- or community-level fish effects.

Several investigators have tried to develop indices of marine or estuarine fish condition (Deegan et al. 1997). Most of these approaches have been semiquantitative and have lacked the statistical rigor demonstrated in the present analyses. Broad-scale indices of fish condition that can be used over wide geographic expanses provide a tool for environmental decision makers that can be used to gauge the biological condition of estuarine ecosystems rather than their simple chemical or physical state. Combined with indices of the condition of other biota such as benthos (Engle and Summers 1999) and plankton, determinations of ecological condition can be related to effects rather than simple exposure. Once the dosimetry between condition and exposure is well understood, the ability to foresee ecological problems will be greater and our ability to protect the environment will be significantly improved.

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