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[Article]

Mortality of Centrarchid Fishes in the Potomac Drainage: Survey Results and Overview of Potential Contributing Factors

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Abstract.—Skin lesions and spring mortality events of smallmouth bass *Micropterus dolomieu* and selected other species were first noted in the South Branch of the Potomac River in 2002. Since that year morbidity and mortality have also been observed in the Shenandoah and Monocacy rivers. Despite much research, no single pathogen, parasite, or chemical cause for the lesions and mortality has been identified. Numerous parasites, most commonly trematode metacercariae and myxozoans; the bacterial pathogens *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Flavobacterium columnare*; and largemouth bass virus have all been observed. None have been consistently isolated or observed at all sites, however, nor has any consistent microscopic pathology of the lesions been observed. A variety of histological changes associated with exposure to environmental contaminants or stressors, including intersex (testicular oocytes), high numbers of macrophage aggregates, oxidative damage, gill lesions, and epidermal papillomas, were observed. The findings indicate that selected sensitive species may be stressed by multiple factors and constantly close to the threshold between a sustainable (healthy) and nonsustainable (unhealthy) condition. Fish health is often used as an indicator of aquatic ecosystem health, and these findings raise concerns about environmental degradation within the Potomac River drainage. Unfortunately, while much information has been gained from the studies conducted to date, due to the multiple state jurisdictions involved, competing interests, and other issues, there has been no coordinated approach to identifying and mitigating the stressors. This synthesis emphasizes the need for multiyear, interdisciplinary, integrative research to identify the underlying stressors and possible management actions to enhance ecosystem health.

Fish kills or mortality events of various species have occurred sporadically in the Chesapeake Bay and its tributaries for decades. Often, specific environmental conditions such as low oxygen, blooms of toxic algae, or chemical spills are responsible and many species and age-groups are affected. In other cases, mortality of one or a few species occurs and specific infectious agents

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can be consistently isolated or observed microscopically. Examples of such pathogens include *Aphanomyces invadans*, the oomycete that causes ulcerative mycosis of Atlantic menhaden *Brevoortia tyrannus* (Blazer et al. 2002; Kiryu et al. 2003), and *Mycobacterium* spp. responsible for skin lesions and mortality of striped bass *Morone saxatilis* (Overton et al. 2003; Gauthier et al. 2008). Unfortunately, it is more often the case that no single pathogen, toxicant, or environmental stressor can be definitely identified as "the cause" of skin lesions or mortalities. Numerous opportunistic pathogens, the environmental factors that allow their proliferation, and exposure to complex chemical mixtures combine to cause disease. For this reason, fish diseases in wild populations are integrative, ecologically relevant end points of chronic exposure to environmental stressors, including contaminants. Elevated disease prevalence in general is considered a key indicator of ecosystem dysfunction (Rapport 1999; Vethaak et al. 2009). Skin lesions ranging from ulcers to tumors are used in monitoring programs in marine, estuarine, and freshwater systems (Vethaak and Rheinallt 1992; Fournie et al. 1996; Sanders et al. 1999). An increased prevalence of skin lesions and other gross abnormalities is often associated with poor water quality and/or chemical contaminants and hence considered a good indicator of environmental degradation (Vethaak 1991). In some areas of the North Sea, improvements in water quality have been correlated with a lower prevalence of skin diseases (Vethaak et al. 2009).

The mortalities within the Potomac River drainage of the Chesapeake Bay ecosystem have raised concerns about environmental degradation. Although there are more than 40 fish species in the affected areas of the Potomac River, mortality and skin lesions have only been observed in a few species. These mortalities have involved adult, sexually mature fish, primarily smallmouth bass (SMB) *Micropterus dolomieu*, redbreast sunfish (RBS) *Lepomis auritus*, rock bass *Ambloplites rupestris*, northern hog suckers *Hypentelium nigricans*, and golden redhorses *Moxostoma erythrurum*. To date, no single, specific biological or chemical cause for the skin lesions or mortality has been identified, despite much research by state, federal, and other investigators. Initial histopathological examinations of SMB from the South Branch of the Potomac identified a variety of infectious agents and noninfectious tissue changes, including a high prevalence of testicular oocytes or intersex (Blazer et al. 2007). This raised concerns that contaminants of "emerging concern," specifically the estrogenic or antiandrogenic compounds associated with intersex in fish, may also be associated with immunomodulation in sensitive species. Similarly, initial diagnostic reports of fish from the Shenandoah River kills documented a variety of parasites, bacteria, and fungi, and immunosuppression was suggested (S. Smith, Virginia-Maryland Regional College of Veterinary Medicine, www.deq.state.us/export/sites//default/info/document/vtfishkillreport.pdf).

The mortality events described here occurred between 2002 and 2009, the first being observed in May 2002 when dead and dying fish were reported at several sites on the South Branch of the Potomac in West Virginia. Since that time, skin lesions and mortality of varying severity have occurred in numerous areas throughout the Potomac drainage (Figure 1). While not described in detail in this report, similar mortality events have recently (since 2007) been observed in the James River drainage. Specific observations on the scope of mortality have differed from area to area and there has been no consistent, coordinated response to the various mortality events. As the affected communities became aware of the significance of these events, numerous investigations were launched. A Fish Kill Task Force was formed in Virginia to identify and evaluate potential causes. Members of the task force included state agency representatives, academic experts, professional guides and outfitters, representatives of community groups, and federal researchers. The task force served as a focus for the conduct and reporting of research related to the kills in Virginia (reports are available at www.purewaterforum.org/fishkill/index.php and associated links). The current report provides an overview of the bacteriological, virological, parasitological, and histopathological observations made on centrarchids collected during and prior to the mortality events in selected areas and evaluates the multiple stressors that may contribute to immunosuppression and increased infectious disease susceptibility. It also emphasizes the need for interdisciplinary, integrative research over multiple years, seasons, and sites and that moves beyond the idea of single pathogens and toxicity (acute or chronic) of individual contaminants and toward understanding the complex interactions of multiple stressors in the aquatic ecosystem.

Description of the Morbidity and Mortality Events

The 2002 mortality event in the South Branch impacted a variety of fish species (SMB, RBS, rock bass, golden redhorses, and fallfish *Semotilus corporalis*) inhabiting nearly 113 river kilometers. The mortality of numerous SMB from a world-class fishery quickly attracted the attention of stakeholders. An annual occurrence of chronic, low-level mortality has occurred every spring since 2002. In the spring of 2005, another major fish kill occurred throughout the

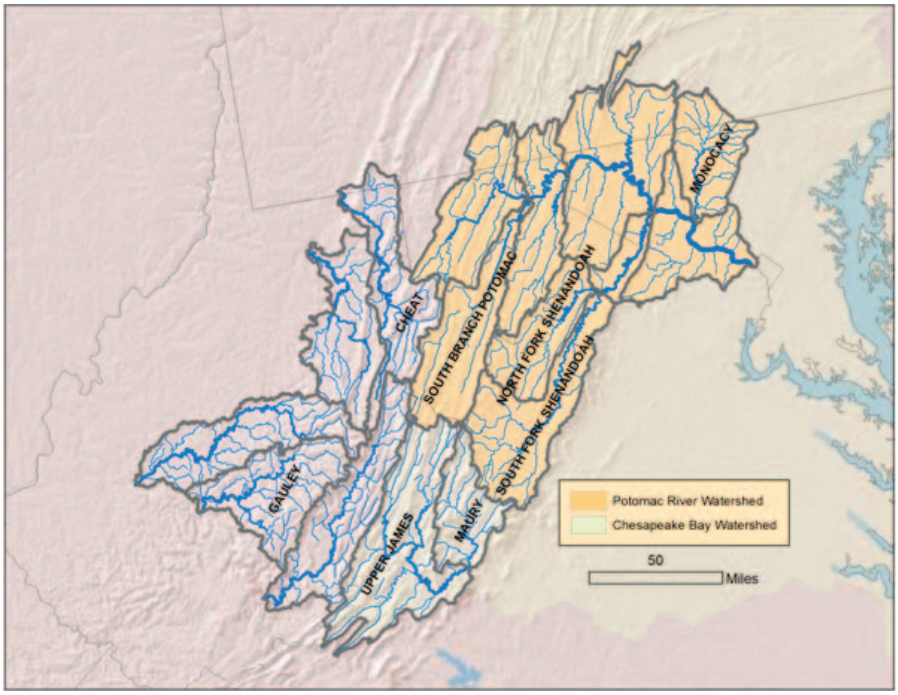

FIGURE 1.—Map of the Potomac River and nearby watersheds.

same stretch of river. This mortality event had its greatest impact on golden redhorses, though other species (including SMB) were affected. During the 2002 and 2005 events, skin lesions were documented on many of the fish involved. The numbers of dead fish and occurrences of skin lesions have been relatively low since 2005. The lesions vary widely in appearance, severity, and body location, with no consistency between species, frequency, or geographical location.

In 2004, the first occurrence in Virginia of similar mortality and morbidity affected nearly the entire length of the North Fork Shenandoah River. In 2005, similar mortality and morbidity occurred along more than 161 km of the South Fork and sporadically on the main-stem Shenandoah River. Anglers and professional fishing guides estimated that 20–90% of mature/catchable SMB were eliminated from various areas of the river during the events in these 2 years. Since those first events, mortality has occurred in hundreds of kilometers of river. The location and severity of the episodes has not been uniform. Counts of dead fish have been as high as 150 fish in 12.9 km, and at times 100% of the SMB observed have had lesions. Fishery biologists observed 20–25% of adult SMB with lesions while conducting a routine electrofishing sample during a suspected mortality period in 2008. Since 2007, the number of dead fish observed at one time has been low; however, mortality may be occurring over a prolonged period. In 2007, mortalities were observed in the nearby upper James River watershed, which includes the Cowpasture River; mortalities continued in 2008 and 2009. The mortality and episodes of morbidity in Virginia's rivers have mainly involved SMB, RBS, and rock bass.

External lesions and deaths of SMB in the Monocacy River were reported by an angler in late May 2009. Observations by state biologists indicated that the mortalities were primarily SMB and golden redhorses. Subsequent electrofishing to assess the prevalence of lesions and obtain fish for necropsy indicated that 29% of the 21 SMB (total length, 92–340 mm) had external lesions. However, all 5 of the quality-size (280 mm and greater) SMB collected had external lesions. Three largemouth bass (LMB) *M. salmoides* were collected, all of which had external lesions. Subsequent population surveys in early fall 2009 indicated a 65% decline in adult SMB compared with population surveys conducted in early spring.

The mortality/morbidity events have all been springtime events that occurred from late March through June. Affected fish were mature adults. It has been suggested that protracted water temperatures of 15°C triggered the mortality events, although a low prevalence of reddened or ulcerative skin lesions has

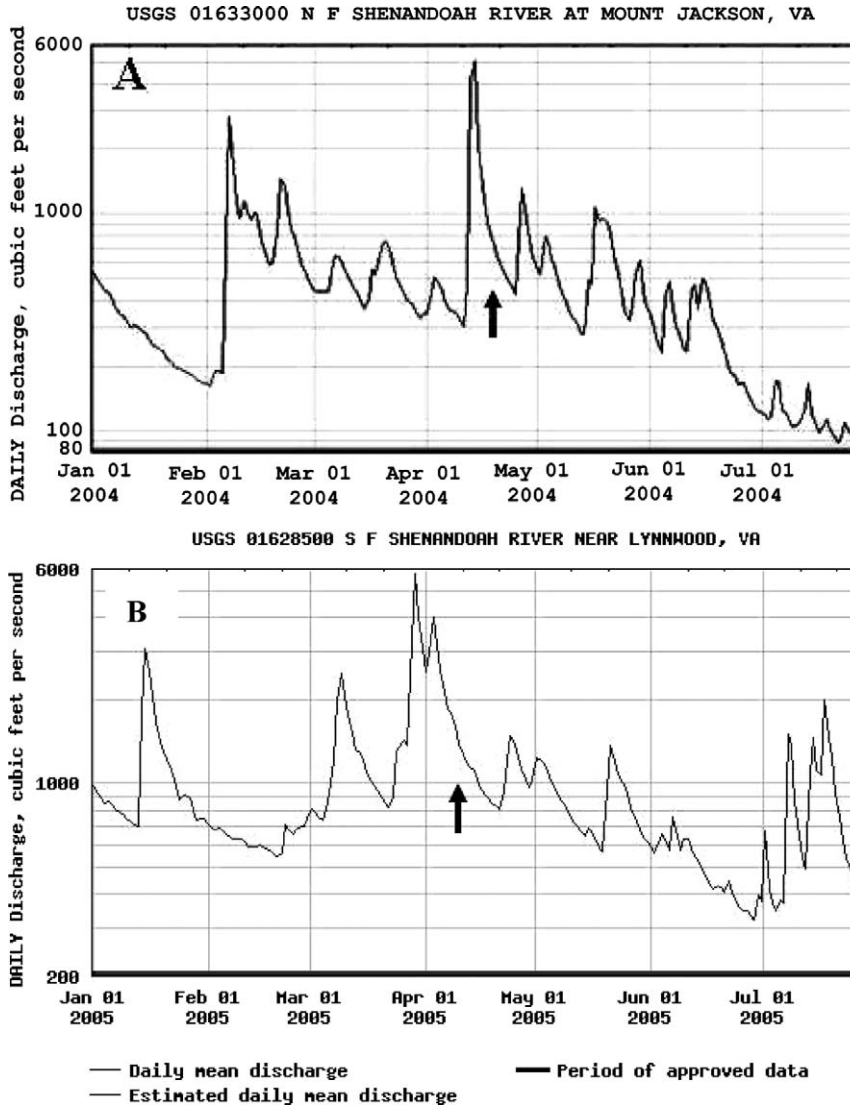

FIGURE 2.—Hydrographs for **(A)** the North Fork Shenandoah River and **(B)** the South Fork Shenandoah River prior to and after the initial observations of fish mortality (arrows) in 2004 and 2005.

been observed earlier in the spring at colder temperatures. There has also been some association between precipitation or runoff and these events. For instance, morbidity and mortality began after high-discharge events in the North Fork in 2004 (Figure 2A) and the South Fork in 2005 (Figure 2B). Similarly, on the Monocacy River, fish appeared to have died during or just after a high-water event. In the early stages of the events fish are sometimes found dead with no external signs of disease. Fish have also been observed acting lethargically, floating with the current at the surface, and exhibiting darkened patches of skin (melanistic spots) or having excess amounts of mucus covering their bodies. By late June the number of fish observed with lesions has decreased. Biologists and anglers have reported capturing adult SMB and RBS later in the summer and early fall that appear to have healed lesions, as evidenced by circular areas on the body surface with regenerating scales. The "initial" mortality event on each of the rivers has been the most severe, with lower numbers of dead fish being observed in subsequent years. However, this may be due to the chronic nature of the mortalities and the lower population density of susceptible individuals in years

TABLE 1.—Watershed size and land use characteristics of fish mortality sites in the Potomac River drainage and a reference site.

River	Watershed size (km^2)	Land cover use (%)			
		Agriculture	Forest	Suburban/urban	Other
Gauley[a]	1,419	1	86	1	12
South Branch Potomac[b]	3,867	24	68	3	5
South Fork Shenandoah[c]	4,331	42	41	13	4
North Fork Shenandoah[c]	2,681	31	62	6	1
Monocacy[d]	1,927	64	25	8	3

[a] From http://www.dep.wv.gov/WWE/watershed/TMDL/grpa/documents/Gauley%202008/Gauley_final_TMLD_report_03_27_08.pdf.
[b] From http://www.dep.wv.gov/WWE/watershed/TMDL/grpa/documents/South%20branch%20of%Potomac/7254_south_branch_potomac-TMLD.pdf.
[c] From ftp-fc.sc.egov.usda.gov/VA/programs/watersheds_info.
[d] From http://dnr.maryland.gov/watershed/surf/proj/wras.html.

following major mortalities, resulting in less accumulation of fish carcasses for the public to notice and report.

Methods

Sites and field collections.—The South Branch of the Potomac River arises in Highland County, Virginia, flows north across the Virginia–West Virginia border into Pendleton County, West Virginia, and continues its flow northward into Grant County, forming a confluence with the North Fork of the South Branch. It flows through Hardy and Hampshire counties, joining the North Branch to form the Potomac. The Shenandoah River is the largest tributary of the Potomac River. It is separated into the North Fork, South Fork, and main-stem Shenandoah. Both the South Fork and North Fork begin in Rockingham County. The South Fork flows through Page and Warren counties, while the North Fork flows through Shenandoah and Warren counties. The Monocacy River, another tributary of the Potomac, lies within Adams County, Pennsylvania, and Frederick, Carroll, and Montgomery counties in Maryland. An out-of-basin reference site was chosen on the Gauley River in the western part of West Virginia near Craigville. The Gauley River arises in Pocahontas County and flows into Webster County. Watershed sizes and land use within each watershed are presented in Table 1.

All fish in this study were collected by boat electroshocking. During the 2002 South Branch mortality event, 2 SMB and 10 golden redhorses were collected by West Virginia Division of Natural Resources personnel and cultured by the U.S. Fish and Wildlife Service's Northeast Fish Health Center (FWS) in Lamar, Pennsylvania, for detection of bacteria and viral pathogens following the procedures used in the National Wild Fish Health Survey (USFWS 2004). In late spring of 2002 (after mortalities had ceased), a variety of skin lesions were excised from the SMB and redhorses and placed in Z-fix (Anatech, Battle Creek, Michigan) for histopathological analyses conducted at the U.S. Geological Survey's National Fish Health Research Laboratory. In June 2005, during a mortality event, seven SMB and eight RBS were collected in the South Fork of the Shenandoah by Virginia Department of Game and Inland Fisheries (VDGIF) personnel and cultured by the FWS. Briefly, kidneys were cultured from freshly euthanized fish through an incision along the ventral–lateral surface. A sample of kidney tissue was inoculated onto individual slants of brain–heart infusion agar (Becton, Dickinson, and Company, Sparks, Maryland) using a sterile 10-μL loop. Inocula of external lesions were plated onto tryptone yeast extract agar prepared without the antimicrobials (Bullock et al. 1986). Small portions of posterior kidney, spleen, and swim bladder were excised and placed in Hanks' balanced saline solution (HBSS; Sigma-Aldrich, St. Louis, Missouri) containing gentamycin (50 μg/mL; Sigma-Aldrich), together with tissues from other specimens to make a five-fish pool. Samples collected in the field were kept on ice and transported back to the FWS for analysis.

In March 2007, prekill samples were collected at sites on the South and North forks of the Shenandoah River as well as the Cowpasture River (Figure 1). The collection time was approximately 1 month prior to the anticipated time of die-offs. The Cowpasture River is a tributary of the James River and was sampled as a possible reference site. However, subsequent to this prekill sampling a high prevalence of intersex (85% or 29/34 males) and parasite-associated external and internal lesions were documented at this site. Twenty SMB were collected by electrofishing at each of the four sites. Fish were euthanized using unbuffered Finquel (Argent, Redmond, Washington), bled from the caudal vessels, weighed, and measured. Any visible

abnormalities, both external and internal, were documented. Pieces of gill, liver, spleen, gonad, anterior and posterior kidney, and any visible abnormalities were preserved in Z-fix for microscopic evaluation. Pieces of liver (two pooled for each sample) were placed in Ziploc bags and stored on dry ice for arsenic analysis. At the North Fork and Cowpasture sites skin and gills were swabbed for bacterial isolations prior to the fish necropsy. Swabs were plated onto tryptic soy agar (TSA; Becton, Dickinson, and Company) and Coomassie Brilliant Blue (CBB) agar plates and incubated at room temperature. The CBB agar is a medium for the detection of the A-layer positive phenotype of *Aeromonas salmonicida* (Cipriano and Bertolini 1988). The periphery of any lesions observed was cultured using sterile loops. Pieces of swim bladder, spleen, and posterior kidney of each fish were placed in a cryovial on dry ice until returned to the laboratory and stored at −80°C.

In April and May of 2007, mortalities occurred in the Shenandoah and James rivers. On April 24, 2007, lesioned and/or moribund SMB ($n = 7$) and RBS ($n = 9$) were collected at sites in the South Fork and North Fork by VDGIF personnel and delivered live to the National Fish Health Research Laboratory. Fish were anesthetized, weighed, measured, and bled. Swabs were taken from the gills and leading edges of skin lesions of six RBS and four SMB and placed on TSA and CBB plates incubated at room temperature. Pieces of gill, liver, spleen, anterior and posterior kidney, gonad, intestine, and skin lesions were placed in fixative for histopathological evaluation. Pieces of spleen, posterior kidney, and swim bladder were placed in HBSS without antibiotics and shipped overnight to the FWS for bacterial and viral isolation. On May 22, 2007, three SMB, one bluegill *Lepomis macrochirus*, and four RBS from the South Fork were necropsied. Pieces of lesions and all of the other tissues listed above were preserved in Z-fix for histopathology.

In May 2007, 17 SMB were collected from the Gauley River for gross and histopathological analyses. This collection was made during the period of the fish mortalities in the Potomac drainage; the resulting data are presented for comparison purposes.

On March 18, 2009, 20 SMB from the South Branch were examined as a prekill sample. Only one fish was observed to have skin lesions, and the leading edge of the lesion and kidney of this fish were cultured using TSA and Cytophaga agar (Anacker and Ordal 1959) and incubated at 16°C until representative bacterial colonies could be distinguished (2–3 d).

In May 2009, SMB mortalities and fish with skin lesions were reported in the Monocacy River. Six SMB and one LMB were collected by Maryland Department of Natural Resources personnel and brought live to the National Fish Health Research Laboratory. Skin lesions and kidneys were cultured as described previously on TSA and Cytophaga agar. Tissues were collected and preserved in Z-fix for microscopic analysis.

Individual tissues (brain, gonad, anterior kidney, spleen, liver, skin, and muscle) from one male and one female SMB collected from the South Fork Shenandoah and one of each sex from the South Branch Potomac in spring 2007 were placed into individual Chem I 2000 vials (Fisher Scientific, Pittsburgh, Pennsylvania) and stored at −80°C until analyzed. Skin and ovary tissues from one female SMB collected in the South Branch Potomac, South Fork Shenandoah, and Gauley (ovary only) rivers in spring 2007 were preserved in the same manner. These tissues were analyzed for a suite of chemicals (Table 2).

A nonprofit group, the Friends of the Shenandoah, obtained funding to have SMB tissues analyzed for arsenic. The 2007 liver samples, 12 individual livers (six pools of two) from each of the South and North Fork Shenandoah sites and eight livers (four pools of two) from the Cowpasture site were analyzed. In 2009, additional samples from the South Fork Shenandoah were analyzed. Samples of skin, liver, testes, ovary, and pooled anterior kidney and spleen weighing 2–3 g were collected during March, April, and May. Sample sizes ranged from one to four; however, in many cases each sample consisted of pooled tissues from two to four fish. Tissues were placed into plastic Ziploc bags and stored at −80°C until shipped on dry ice to the National Water Quality Laboratory, Denver, Colorado, for analysis.

Laboratory analyses.—The tissues preserved in Z-fix were routinely processed for histopathological analyses, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Special stains, including Grocott silver, Gram, Steiner's, Giemsa and periodic acid–Schiff's, were used to visualize infectious agents as indicated by microscopic findings (Luna 1992).

The pure bacterial cultures developed from the primary isolation plates or slants were characterized and identified using standard procedures (Griffin 1992; Koneman et al. 1992; Holt et al. 1994; Janda and Abbott 1998; Murray et al. 1999; MacFaddin 2000; AFS–FHS 2007). Tissues for virological analysis conducted by the FWS were diluted 1:10 (weight : volume) in HBSS and homogenized within 48 h of collection. Homogenates were centrifuged at 2,000 × gravity for 20 min at 4°C. A final dilution of 1:100 (supernatant : Earle's 1000 medium; Fisher Scientific)

TABLE 2.—Analytes of chemical contaminants in individual tissues of smallmouth bass.

Fake head
Polychlorinated biphenyls
PCB 101
PCB 110
PCB 118
PCB 138
PCB 146
PCB 149
PCB 151
PCB 170
PCB 174
PCB 177
PCB 180
PCB 183
PCB 187
PCB 194
PCB 206
Fungicides
Pentachloronitrobenzene
Flame retardants[a]
BDE 47
BDE 66
BDE 71
BDE 85
BDE 99
BDE 100
BDE 138
BDE 153
BCE 154
BDE 183
Pentabromotoluene
Fire master
Dechlorane plus
Dinitroaniline herbicides
Trifluralin
Benfluralin
Organochlorine pesticides
Dieldrin
Endosulfan 1
Tetradifon
Octachlorostyrene
Oxychlordane
trans-chlordane
cis-chlordane
trans-nonachlor
cis-nonachlor
p,p′-DDE
p,p′-DDD
p,p′-DDT
Hexachlorobenzene
Pentachloroanisole
Other pesticides
DCPA (dacthal)
Oxyfluorfen
Personal care products and metabolites
Triclosan
Methoxytriclosan
Pyrethroid insecticides
Cyhalthrin
Cyfluthrin
Tefluthrin
Organophosphate pesticides
Chlorpyrifos
Phenyl pyrazole insecticides
Fipronil
Fipronilsulfide
Desulfnylfipronil

[a] BPE = brominated diphenyl ether.

was inoculated onto replicate wells of 24-well flat-bottom plates (Fisher Scientific) containing preformed monolayers of the following cell lines: Chinook salmon embryo, epithelioma papillosum cyprini, fathead minnow, and bluegill fry. Inoculum was allowed to absorb for 30 min and then overlaid with Earle's 5000 tissue culture medium (Fisher Scientific). Plates were sealed and incubated at 15°C for a minimum of 14 d, with observation for cytopathic effect (CPE) beginning after the third day of incubation. All plates with no signs of CPE were subject to blind pass. Wells showing CPE were analyzed for toxicity. Viruses causing CPE observed following the blind pass and/or toxicity check were identified using serum neutralization and/or polymerase chain reaction.

A subsample (10) of the pooled tissue pieces of swim bladder, spleen, and posterior kidney collected in March 2007 were analyzed for the detection of largemouth bass virus (LMBV) by quantitative polymerase chain reaction analysis as described by Getchell et al. (2007) at the Cornell University Aquatic Pathology Laboratory.

Halogenated compounds (polychlorinated biphenyls [PCBs], brominated flame retardants, and chlorinated and fluorinated pesticides) were extracted, isolated, and analyzed from tissue using a method similar to the procedure outlined by Burkhardt et al. (2005). Briefly, wet tissue was extracted twice, using isopropanol–water mixtures on a commercially available accelerated solvent extraction system (Dionex, Sunnyvale, California). These extracts were diluted with a phosphate buffer (pH 7), and method compounds were extracted from the buffer solution using OASIS-based solid-phase extraction cartridges (SPE; Waters Corp., Milford, Massachusetts). A mixture of dichloromethane and diethyl ether was used to elute the method compounds from the SPE cartridge. These SPE extracts were then reduced in volume and an internal standard was added. The sample extracts were analyzed for method compounds by hydrogen carrier gas–based, capillary column gas chromatography with detection by negative ion (ammonia as the reaction gas) selected ion monitoring mass spectrometry. The presence of 56 individual compounds was determined. Quality assurance was provided by analyzing duplicate samples (environmental samples split at the laboratory), laboratory blanks, and spiked sand samples, and through monitoring recovery of surrogate compounds. Arsenic concentrations were determined using collision–reaction cell inductively coupled plasma mass spectrometry as described by Hoffman (1996) and Garbarino et al. (2006), with quality assurance provided by laboratory blanks and monitoring recovery in known samples.

TABLE 3.—Morphometric results for smallmouth bass collected in spring 2007. Data are presented as means ± SDs.

Site[a]	Sample size	Age (years)	Total length (mm)	Weight (g)	Condition factor[b]	Relative weight[c]
South Fork Shenandoah	20	3.7 ± 1.0	288.4 ± 41.9	316.7 ± 167.1	1.19 ± 0.10	83.8 ± 6.2
North Fork Shenandoah	20	3.1 ± 1.2	273.9 ± 39.8	272.2 ± 141.5	1.21 ± 0.09	83.3 ± 5.4
Cowpasture	20	5.2 ± 1.1	250.2 ± 29.6	180.5 ± 78.1	1.09 ± 0.09	76.9 ± 4.8
Gauley	17	[d]	230.1 ± 33.0	147.2 ± 59.2	1.13 ± 0.06	82.8 ± 5.4

[a] Samples were collected in March (prior to mortality events) in the South Fork Shenandoah, North Fork Shenandoah, and Cowpasture rivers; samples were collected in May (during the period of mortalities in the Potomac drainage) in the Gauley River.
[b] [(total weight − gonad weight)/length^3] × 100.
[c] Weight/($10^{(3.2\times\log \text{ of total length} - 5.329)}$) × 100.
[d] No data.

Results

Morphometric Observations

In March 2007, SMB from the North Fork and South Fork Shenandoah sites had mean ages of 3.1 and 3.7 years, respectively. Their condition factors, calculated as 100 × (total body weight [g] – gonad weight [g])/length [mm]^3 were greater than one at both sites, and their mean relative weights were 83–84%. Smallmouth bass collected from the Cowpasture River were somewhat older (mean age, 5.2 years); however, their mean body weight, condition factor, and relative weight were all less than those observed in fish from the Shenandoah. Smallmouth bass collected at the Gauley River during the same time period as the Shenandoah mortality events were somewhat smaller than those from the Potomac River; however, age data were not available. Condition factors were similar to those observed in fish from the Shenandoah (Table 3).

The SMB sampled during the mortality event in the North Fork Shenandoah in 2007 ranged from 182 to 288 mm in length and from 72 to 322 g in weight. Mean condition factor was 1.21 (range, 1.09–1.35). The RBS ranged from 101 to 176 mm in length and from 110 to 191 g in weight. Mean condition factor was 1.95 (range, 1.60–2.21). Morphometric results indicated that prior to and during the mortality events the condition (condition factor and relative weight) of the fish were normal.

Gross Lesions Observed

Of the SMB collected in 2007 prior to lesion/mortality events, 45% and 35% at the South and North Fork sites, respectively, were normal by gross observation. Observable external abnormalities included leeches, black spot (induced by encysted unidentified trematodes), small erosions, and pale, raised lesions (Table 4). Most of the pale, raised lesions (7/8 at the South Fork, 1/2 at the North Fork) were discrete and mucoid-appearing (Figure 3A). Two fish at the North Fork and three at the South Fork had small reddened areas or erosions, despite the fact that water temperatures had not reached 15°C. Internally, the most common gross observations were white spots within liver, spleen, and kidney tissue (Table 4). White spots in internal organs were also the most common internal observations during the mortality events.

Skin lesions observed during mortality events included a generalized pale discoloration of the body surface, small raised reddened or pale areas (Figure 3B), large reddened areas with the skin intact (Figure 3C), moderate to large ulcerated areas (Figure 3D, E), pale areas with fungal or oomycete growth (Figure 3F),

TABLE 4.—Prevalence (%) of grossly visible lesions on smallmouth bass collected in spring 2007. Sample size was 20 at each site except Gauley (17).

	Gill lesions		External fin and body surface lesions					
Site	Grubs[b]	Eroded	Leech	Black spot[c]	Raised mucoid	Eroded, red, raised	Liver[d]	Spleen[d]
South Fork	25	10	20	5	40	10	95	80
North Fork	45	20	20	25	10	15	100	65
Cowpasture	20	25	15	35	25	10	85	40
Gauley	0	0	24	12	24	0	24	18

[a] Samples were collected in March (prior to mortality events) in the South Fork Shenandoah, North Fork Shenandoah, and Cowpasture rivers; samples were collected in May (during the period of mortalities in the Potomac drainage) in the Gauley River.
[b] Grubs were grossly visible as white cysts and contained trematode metacercariae.
[c] Black spots were slightly raised cysts containing trematode metacercariae.
[d] White spots.

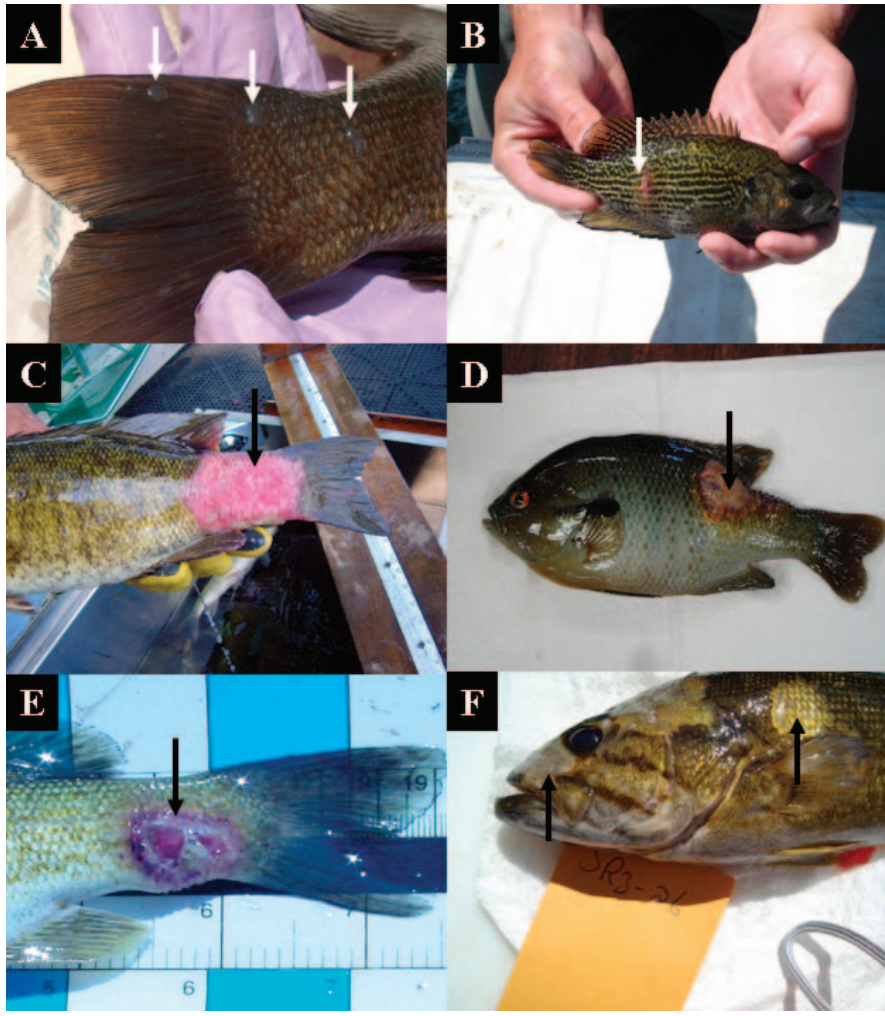

FIGURE 3.—Examples of gross lesions observed during mortalities: **(A)** raised, pale lesions (arrows) on the body surface and fins of a smallmouth bass (SMB), **(B)** small, raised, pale to reddened areas with epidermal erosion (arrow) on the lateral body surface of a redbreast sunfish, **(C)** large, reddened area on the body surface (arrow) of an SMB, **(D)** area of epidermal and dermal ulceration and necrosis with a reddened margin (arrow) on the lateral surface of a bluegill, **(E)** a deep eroded area extending into the underlying muscle (arrow) of an SMB, and **(F)** pale areas with patches of fungal hyphae (arrows) on the skin of an SMB.

and occasionally erosions around the mouth. Some SMB showed moderate to severe erosion of gill tissue, which in severe cases was covered with oomycete fungi. Certain lesions, such as raised, pale, mucoid-like areas (Figure 3A), and the presence of ectoparasites, including metacercariae of trematodes (black spot, white grubs), leeches, and gill myxozoans (grossly observable as small white spots) were observed during mortality events and at other times of the year, while the reddened and ulcerative lesions were primarily observed during lesion/mortality events. None of the SMB collected in May 2007 from the Gauley River had erosions or ulcerative skin or eroded gill lesions. There were skin parasites (leeches and trematodes inducing black spots) as well as raised mucoid lesions. White spots were observed in the liver and spleen but at a lower prevalence than in SMB from the Shenandoah or the Cowpasture River in March (Table 4).

Two of the SMB collected during mortalities on the North Fork Shenandoah in April 2007 had no external lesions, while the other five had skin lesions including raised mucoid-like areas (3), red ulcerated lesions with a pale center (1), and epithelial sloughing (1). Three of the RBS had reddened, raised lesions, two had pale,

raised lesions, one had an ulcerative lesion with fungus; one had areas of excess mucus. All of the RBS examined from the May 2007 mortality event on the South Fork Shenandoah had ulcerative lesions on the body surface with fungal hyphae present. All of the SMB had ulcerative lesions, two with obvious fungal growth.

The SMB collected during the mortality event on the Monocacy River had lesions that included small to large raised, red areas or pale, yellowish areas on the body surface and erosions around the mouth. Most had eroded gills, some with apparent oomycete fungal growth.

Pathogens Observed

During the mortality event in the South Branch in 2002, *Flavobacterium columnare* and *Aeromonas hydrophila* were isolated from skin lesions of SMB and *A. hydrophila* from kidney tissue. In 2005, during mortalities in the Shenandoah, *A. salmonicida* was isolated from RBS kidney and *A. hydrophila*, *A. sobria*, and *Enterobacter* sp. were isolated from both SMB and RBS kidneys.

In spring 2007, prior to the anticipated mortality, neither bacterial nor viral growth was observed from internal organs at any site. However, LMBV was detected by quantitative polymerase chain reaction in 1 of 10 healthy SMB at the North Fork site and in 2 of 10 SMB (both with skin lesions) at the South Fork site. At the North Fork site *A. hydrophila* and other motile aeromonads were cultured from the skin of five healthy-appearing SMB. From the three SMB with red raised or eroded skin lesions *Pseudomonas* spp., *Staphylococcus* sp., and *Moraxella* sp. were cultured. At the Cowpasture River, two SMB had red, raised skin lesions; from one *A. punctata*, *P. fluorescens*, *Plesiomonas shigelloides*, and *Moraxella* sp. were isolated, and from the other *Pseudomonas* spp. and *P. shigelloides* were isolated. From the 18 SMB without skin lesions collected at this site, motile aeromonads were isolated from 17 of the mucus samples and *A. salmonicida* from 1 sample.

During the North Fork Shenandoah mortalities in 2007, *A. salmonicida* was isolated from both gills and lesions of three SMB. In addition, a number of motile aeromonads were present on these fish. The fourth SMB had *A. hydrophila*, *P. shigelloides*, and *Shewanella putrefaciens* on the gills and *P. shigelloides* on the skin. All five RBS cultured had *A. salmonicida* on both gills and lesions. In three of the lesions pure cultures of *A. salmonicida* were obtained. Motile *Aeromonas* spp. and *Pseudomonas* were also cultured from some gills and skin. From the internal organ pools, *A. salmonicida* was isolated from one pool of SMB and one pool of RBS. No viruses were cultured.

From the six SMB cultured and necropsied from the Monocacy River in 2009, motile *Aeromonas* spp. were the predominant bacteria cultured from three of the skin lesions, *A. salmonicida* was recovered in heavy growth-pure culture from two other fish, and *F. columnare* was recovered from the kidney of one of the fish with an external infection of *A. salmonicida*. Except for the one fish from which *F. columnare* was recovered, there was no growth on the primary bacteriological plates from kidney tissues. *Shewanella putrefaciens* was also recovered from lesions of two of the fish from which the motile *Aeromonas* spp. were cultured. Motile *Aeromonas* spp. were cultured from the leading margin of the lesion on the LMB.

Histopathological Observations

The microscopic pathology provided further evidence for the multifactorial etiology of the skin lesions as well as mortality. There were a variety of pathological responses within the epidermis, dermis, and underlying musculature of the fish with external lesions. Most commonly observed were areas of epidermal sloughing with acute inflammation in the dermis and hypodermis (Figure 4A). In some cases, inflammation extended down into the muscle. Often these lesions had both small (consistent with *Aeromonas* sp.) and long, thin (consistent with *Flavobacterium* sp.) rods. Some lesions had a more chronic inflammatory response (Figure 4B) and many of these had long, thin rods present. The pale ulcerative lesions were covered with fungal hyphae which only penetrated into the necrotic epidermis and dermis (Figure 4C). Some of the fish, particularly the RBS, exhibited inflammation and necrosis in the dermis with foci or microcolonies of bacteria typical of *A. salmonicida* infections (Figure 4C). The raised mucoid lesions were areas of epidermal thickening or papillomas (4D). One of the SMB skin lesions had acute inflammation with eosinophilic granular cells within the epidermis, and trematode metacercariae were present in the underlying muscle and small rods, consistent with motile *Aeromonas*. In approximately 60% of the fish collected during mortality events the gills appeared normal (Figure 4E). Two of the RBS and one SMB had eroded gills with severe sloughing of the epithelium; the remaining fish had focal areas of epithelial lifting and lamellar fusion (Figure 4F).

Both RBS and SMB had signs of systemic *A. salmonicida* infections in the liver, kidney, and spleen (Figure 5A) in approximately 20% of the fish collected during mortality events. Three of the RBS had lesions in the gastrointestinal tract associated with the presence

FIGURE 4.—Microscopic changes observed in skin and gill sections of centrarchids collected during mortality events in the Shenandoah and South Branch Potomac rivers. Panel **(A)** shows a raised, pale area on the body surface of a smallmouth bass (SMB) in which the epidermis (a) is still intact but there is underlying inflammation (arrow) within the dermis and hypodermis; scale bar = 150 μm. Panel **(B)** shows a lesion on an SMB in which the epidermis on the periphery is thickened (a) and that closer to the center of the lesion (arrow) is eroded. The underlying tissue (b) is hemorrhagic and inflamed; scale bar = 150 μm. Panel **(C)** shows a chronic response with inflammation, necrosis, and fibrosis throughout the dermis and hypodermis (a) and microcolonies of bacteria (arrows) characteristic of *Aeromonas salmonicida* present in a lesion on a redbreast sunfish. The epidermis is sloughed and fungal hyphae (b) are apparent; scale bar = 100 μm. Panel **(D)** shows the proliferation of epidermal cells leading to papillomatous projections (arrows) in a mucoid lesion on an SMB; scale bar = 150 μm. Panel **(E)** shows a section of a gill of an SMB in which the lamellae (arrows) have normal structure; scale bar = 100 μm. Panel **(F)** shows a gill section in which epithelial lifting (arrows) and focal areas of fusion (a) are evident; scale bar = 150 μm. All sections were stained with hematoxylin and eosin.

of trematodes and acanthocephalans, and two RBS and one SMB had large areas of bile duct proliferation and necrosis associated with myxozoan parasites (Figure 5B, C). Four of the SMB had unidentified parasites in the lumen of the kidney tubules and collecting ducts (Figure 5D). Myxozoan spores were also present in the macrophage aggregates of the anterior kidney and spleen (Figure 5E) of the SMB. All of the SMB had helminth parasites, primarily trematode metacercariae in the liver, spleen (Figure 5F), and kidney, ranging from moderate to heavy loads.

There were also lesions more often associated with contaminant exposure than infectious disease. Two of the three male SMB from the South Fork mortality

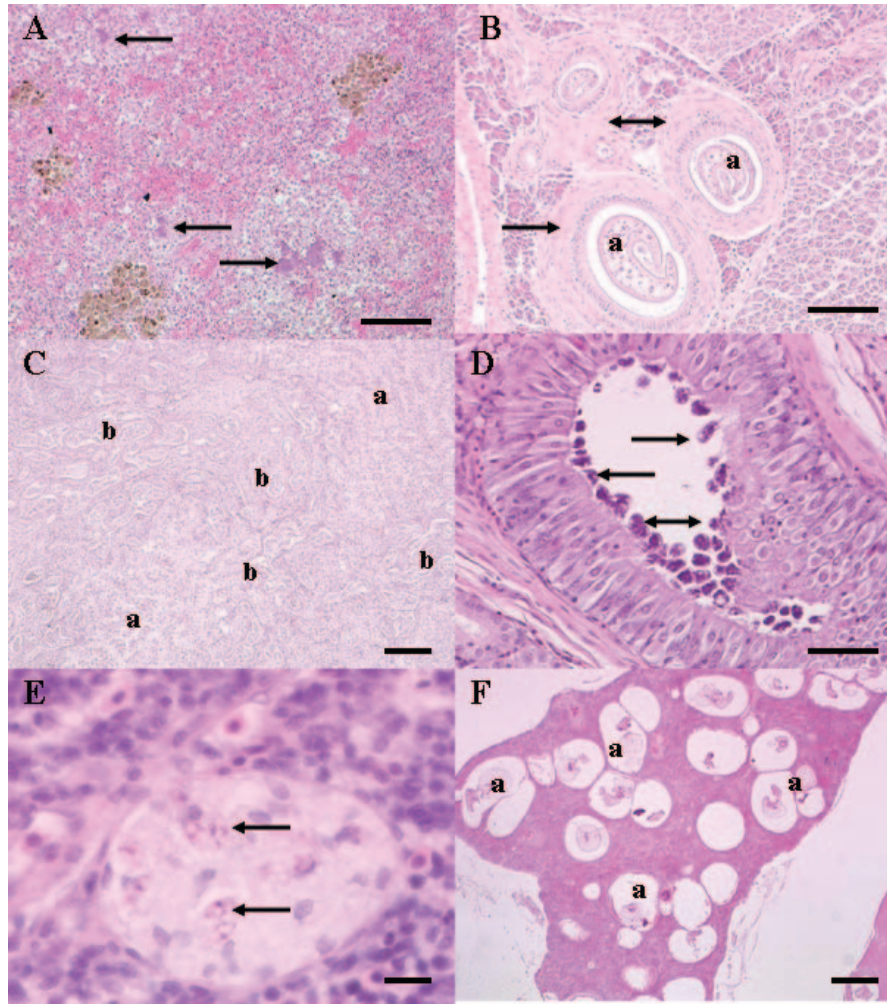

FIGURE 5.—Internal microscopic lesions observed in smallmouth bass (SMB) and redbreast sunfish (RBS) collected during mortality events. Panel **(A)** shows foci of bacterial microcolonies (arrows) typical of *Aeromonas salmonicida* systemic infections in the splenic tissue of a RBS; scale bar = 100 μm. Panel **(B)** shows bile duct proliferation and fibrosis (arrows) in response to myxozoan sporoplasms (a) within the bile ducts of an SMB; scale bar = 100 μm. Panel **(C)** shows foci of normal hepatocytes (a) within a liver with massive bile duct proliferation (b) in response to myxozoan parasites in an RBS; scale bar = 100 μm. Panel **(D)** shows unidentified parasites (arrows) attached to the tubular epithelium of the kidney tubules in an SMB; scale bar = 50 μm. Panel **(E)** shows myxozoan spores (arrows) within macrophage aggregates of the anterior kidney of an SMB; scale bar = 10 μm. Panel **(F)** shows spleen tissue of an SMB with numerous encysted trematode metacercariae (a)' scale bar = 150 μm. All sections were stained with hematoxylin and eosin.

event and all four males from the North Fork mortality event in 2007 had testicular oocytes (Figure 6A). Internally, the SMB had focal areas of liver necrosis, regenerative foci, bile duct proliferation, areas of ceroidlike accumulations and/or large, numerous macrophage aggregates in the liver, kidney, and spleen (Figure 6B). Proliferation of interrenal tissue in the anterior kidney (Figure 6C, D) and gill hyperplasia and hypertrophy leading to the fusion of lamellae (Figure 4F) were also observed.

Tissue Chemical Analyses

Only a very limited data set is available for a suite of halogenated chemicals measured in selected tissues of one male and one female SMB collected in the South Fork Shenandoah River (Table 5) and in ovary and skin samples from a female SMB collected in the South

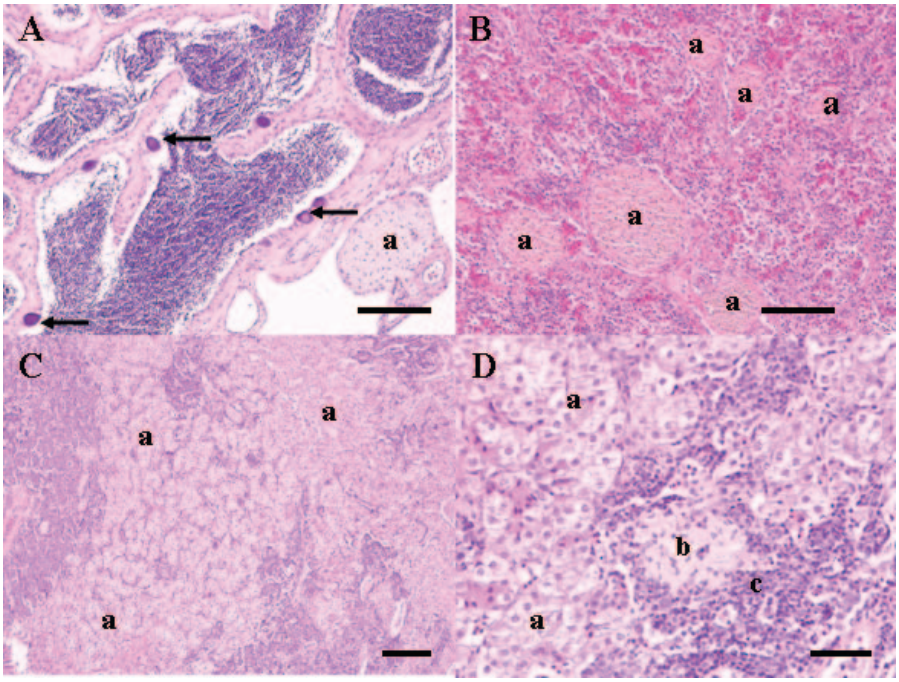

FIGURE 6.—Internal microscopic pathology observed in a smallmouth bass during mortality events. Panel **(A)** shows oocytes (arrows) and macrophage aggregates (a) within the testes; scale bar = 100 μm. Panel **(B)** shows numerous macrophage aggregates (a) in the spleen; scale bar = 100 μm. Panel **(C)** shows proliferation of the interrenal tissue (a) within the anterior kidney; scale bar = 100 μm. Panel **(D)** presents a higher magnification of the tissue shown in (C), illustrating the hypertrophied interrenal cells (a) and macrophage aggregates (b) within the anterior kidney tissue (c); scale bar = 50 μm.

Fork Shenandoah, South Branch Potomac, and Gauley (ovary only) rivers (Table 6). These data are presented to illustrate the differential accumulation of contaminants in the various tissues and potential differences among sites. All of the polychlorinated biphenyl congeners listed in Table 2, the polybrominated diethyl ether (BDE) compounds PBDE-47, −66, −99, −100, −153, and −154, and pentabromotoluene; triclosan and the metabolite methoxytriclosan; the organophosphate pesticide chlorpyrifos; the phenyl pyrazole pesticides fipronil, fipronilsulfide, and desulfnylfipronil; and the organochlorine pesticides hexachlorobenzene, pentachloronitrobenzene, pentachloroanisole, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor, dieldrin, *p,p*'-DDE, *p,p*'-DDD and *p,p*-DDT (DD_x) were measured in at least one tissue. The PCBs, BDE, and organochlorine pesticides were found in all tissues of both sexes. In some tissues (i.e., male gonads), the majority (41.71 versus 47.83) of the total organochlorine pesticide concentration was due to the DD_x compounds. In most tissues these compounds made up a much smaller proportion (Table 5). Fipronil and metabolites were found in the gonads of both sexes and the liver of the male SMB. Triclosan or its metabolite was found in the brain from both sexes and in the gonad, liver, and skin of the female SMB. In general, the highest concentrations of contaminants were measured in the ovary, brain, testes, and male spleen. The lowest concentrations were present in the muscle.

Site differences were observed in ovary concentrations of some chemicals, particularly the sum of organochlorine pesticides and BDE. The highest concentrations were observed in SMB from the Shenandoah River, intermediate concentrations in those from the South Branch Potomac River, and the lowest concentrations in those from the Gauley River. Higher concentrations of organochlorine pesticides, PCBs, and BDEs were observed in the skin samples of SMB from the Shenandoah River than in those from the South Branch Potomac River (Table 6).

Mean arsenic concentrations in the livers of SMB collected in late March and early April 2007 were similar at all three sites (North and South Fork Shenandoah and Cowpasture rivers), ranging from 0.45 to 0.62 mg/kg dry weight. In 2009, tissues were

TABLE 5.—Chemical contaminant concentrations (μg/kg) in selected tissues of smallmouth bass collected in the South Fork Shenandoah River in spring 2007. Abbreviations are as follows: PCBs = polychlorinated biphenyls; BDEs = brominated diethyl ethers; DD_x = DDT, DDE, and DDD; and MDL = minimum detectable level.

Tissue	PCBs	BDEs	Pestidicides	DD_x	Fipronil[a]	Triclosan[b]
Brain						
Male	48.62	216.75	64.85	17.60	<MDL	51.40
Female	18.63	37.50	24.75	4.38	<MDL	28.40
Gonad						
Male	7.74	11.92	47.83	41.71	0.12	<MDL
Female	98.80	134.20	125.28	29.80	0.54	133.10
Anterior kidney						
Male	5.57	75.49	11.69	5.12	<MDL	<MDL
Female	9.29	44.70	7.06	2.16	<MDL	<MDL
Spleen						
Male	82.01	291.55	55.75	14.00	<MDL	<MDL
Female	6.43	26.52	2.06	<MDL	<MDL	<MDL
Liver						
Male	17.12	52.28	7.68	1.05	0.08	<MDL
Female	4.32	0.51	5.44	<MDL	<MDL	24.40
Skin						
Male	26.93	12.06	9.43	2.39	<MDL	<MDL
Female	11.21	48.22	17.26	3.32	<MDL	19.10
Muscle						
Male	2.58	3.41	3.57	<MDL	<MDL	<MDL
Female	2.05	0.39	2.13	<MDL	<MDL	<MDL

[a] Fipronil and the metabolites fipronilsulfide and desulfnylfipronil.
[b] Triclosan and the metabolite mthoxytriclosan.

collected during the 2 months prior to anticipated mortality as well as during the time skin lesions and mortalities were occurring. Liver concentrations were lower in 2009 than in 2007 from the same site on the South Fork Shenandoah. Most interesting was the increase of arsenic concentrations over time, particularly in the skin and hemopoietic (pooled spleen and anterior kidney) tissue (Figure 7).

TABLE 6.—Comparison of biological effects on smallmouth bass and chemical concentrations in water and fish tissue. The abbreviation EEQ stands for estradiol equivalent concentration (ng/polar organic chemical integrative sampler); other abbreviations are given in Table 5.

Variable	North Fork Shenandoah	South Fork Shenandoah	Monocacy below WWTP	South Branch Potomac	Gauley
Fish kills	Severe	Severe	Moderate	Moderate	None
Intersex					
Prevalence (%)	100[a]	80[a]	100[b]	50–100[a]	17[a]
Severity index	1.2 ± 0.3[a]	1.2 ± 0.3[a]	1.9 ± 0.3[b]	0.6–1.4[a]	0.2 ± 0.2[a]
Water quality from passive samplers					
EEQ	4.1 to 79[c]	5.9[c]	14.0[d]	3.6	0[d]
Atrazine (ng/L)	210–650[c]	40[c]; 100[d]	2,100[c]; 690[d]	21d	2[d]
Organochlorine pesticides (pg/L)	1,643[c]	692[c]	639[c]	116[d]	126[d]
PCBs (pg/L)	1,600[c]	560[c]	790[e]	<MQL[d]	<MQL[d]
Tissue contaminants (μg/kg)					
Ovary					
Organochlorine pesticides		125.3		30.5	26.3
DD_x		29.8		13.6	13.5
PCBs		98.8		13.1	58.1
BDEs		134.2		119.6	83.2
Skin					
Organochlorine pesticides		17.3		6.4	
DD_x		3.3		2.6	
PCBs		11.2		6.4	
BDEs		48.2		13.0	

[a] Data from Blazer et al. (2007).
[b] Data from Iwanowicz et al. (2009); collected fall 2005.
[c] Data from Alvarez et al. (2008b, 2008c); collected spring 2007.
[d] Unpublished data, spring 2007.
[e] Data from Alvarez et al. (2009); collected spring 2006.

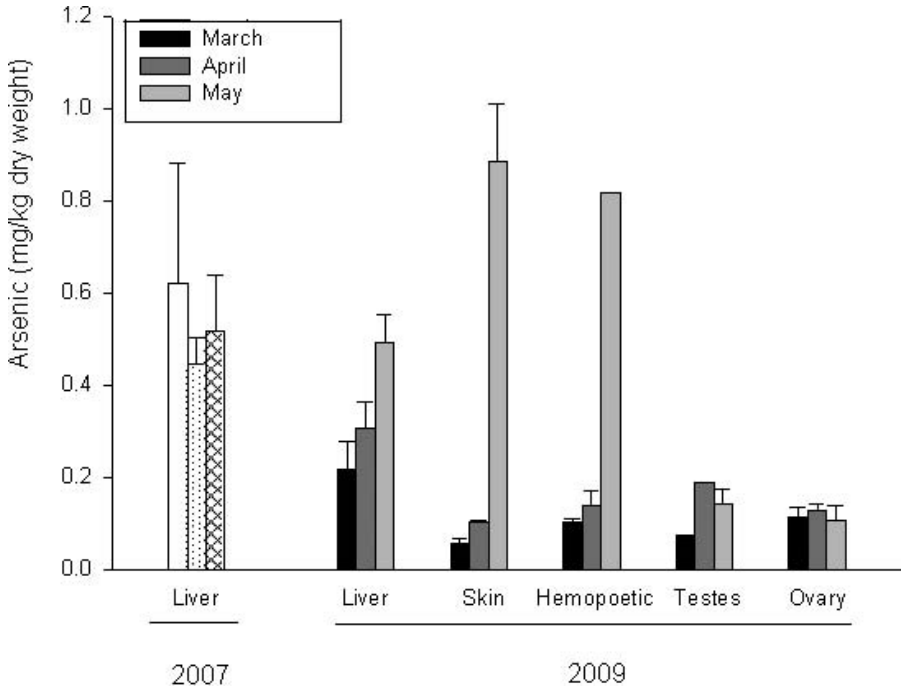

FIGURE 7.—Concentrations of arsenic in tissues of smallmouth bass (SMB) collected in spring 2007 and 2009. In 2007, concentrations were measured in the livers of SMB from the South Fork Shenandoah River (white bar), North Fork Shenandoah River (cross-hatched bar), and Cowpasture River (dotted bar). In 2009, concentrations were measured in multiple tissues of SMB collected from the South Fork Shenandoah River in March (black bars), April (dark gray bars), and May (light gray bars). Data are means + SEs.

Discussion

The data presented here suggest a scenario in which multiple stressors eventually overwhelm the ability of sensitive fish species to maintain homeostasis. Karr (1999) presented the concept of a continuum of human influence on biological condition and river health. As the gradient of human disturbance moves from minimal to severe disturbance the biological condition moves from healthy (sustainable) to unhealthy (not sustainable). The mortality of SMB and other species within the Potomac drainage very likely indicates that, due to numerous stressors, certain fish species are continually close to the threshold between sustainable and non-sustainable physiologic functions. Periodic stressors that alone seem relatively insignificant then tip the balance and lead to mortality. The ulcerative skin lesions and mortality affect adult fish and not the juveniles that might be thought to be more susceptible to bacteria and other pathogens. Hence, increases in parasite loads and tissue contaminant concentrations with age and perhaps spawning behavior, or physiological changes during spawning, may contribute to the cumulative stressors.

A number of microbial pathogens and parasites were observed in the SMB and RBS, both during mortality events and at other times of the year. While it is common for healthy fish to harbor parasites and a normal bacterial flora, the extensive variety of opportunistic and primary pathogens identified in clinically diseased SMB strongly suggests a generalized immunosuppression. No single infectious organism was consistently observed at all sites, and the bacterial and viral pathogens isolated are considered opportunistic and are frequently isolated from healthy-appearing carrier fish. The onset of disease caused by these organisms is most often associated with acute or chronic stress. To date, the motile *Aeromonas* spp. and *Flavobacterium columnare* have been cultured from lesioned SMB in the South Branch of the Potomac, while motile *Aeromonas* spp., *A. salmonicida*, and LMBV have been observed from SMB and RBS in the Shenandoah. In SMB from the Monocacy kill, motile *Aeromonas* spp., *A. salmonicida*, and *F. columnare* were all isolated. Hence it appears that the fish succumbed to whatever microbial pathogens were present. It is important to identify the various pathogens and the environmental factors that lead to

their proliferation, spread, and possibly enhanced virulence. However, perhaps more important is understanding the environmental stressors contributing to increased susceptibility and/or immunosuppression that can be managed for a healthier ecosystem. Hence, we review both the infectious agents observed and the factors known to affect the susceptibility of fish to these pathogens, as well as some of the environmental stressors identified as possible contributing factors to the mortalities of centrarchids within the Potomac drainage, in the hope that further research can be better directed toward management strategies.

Viral Pathogens

Largemouth bass virus is a DNA virus in the family Iridoviridae (genus *Ranavirus*) that is believed to have existed in the United States since 1991 (Grizzle et al. 2002). The first mortalities of largemouth bass associated with this viral agent occurred in Santee–Cooper Reservoir in South Carolina (Plumb et al. 1996). Although the virus has been isolated from other centrarchids (Woodland et al. 2002), to date it has been associated with epidemic mortality only in largemouth bass and Florida largemouth bass *M. s. floridanus* (Goldberg 2002). Naturally occurring outbreaks of LMBV disease primarily affect adults (Plumb et al. 1996; Hanson et al. 2001). It has now been reported throughout the eastern, southern, and midwestern United States (Goldberg 2002; Groocock et al. 2009). Clinical variability among infected populations has been noted; some infected populations experience large-scale fish kills, while others appear to be healthy and experience little to no mortality (Goldberg 2002). Both genetic and phenotypic strain differences, along with environmental and host-related factors, significantly contribute to the clinical manifestation of LMBV infection in the wild (Goldberg et al. 2003). Coinfections with bacterial pathogens are common and may complicate diagnosis (Hanson et al. 2001; Grizzle and Brunner 2003), suggesting that the viral infection could lead to increased bacterial disease susceptibility. In the current study LMBV was only occasionally observed; however, its virulence in SMB and role as a stressor in the mortalities are not known.

Bacterial Pathogens

Bacteria appeared to play a more important role in initiating the reddened or ulcerative skin lesions and in contributing to mortality. Three significant bacterial pathogens of fish were isolated from lesioned or dying fish: *F. columnare*, *A. hydrophila* (and other motile *Aeromonas* spp.), and *A. salmonicida*. *Flavobacterium columnare* is a long, thin, gram-negative, rod-shaped bacterium that causes columnaris disease in freshwater fish; the disease is characterized by erosion and necrosis of the gills and skin, which lead to osmotic and electrolyte imbalances and eventually death (Tripathi et al. 2005). Lesions often start as pale areas on the head, gills, fins, or body surface, usually surrounded by an area with a reddish tinge. Lesions may be covered with a yellowish-white material (Austin and Austin 2007). Although *F. columnare* is considered a ubiquitous bacterium, experimental production of columnaris can be difficult and individual strains differ in their virulence (Decostere et al. 1998). In both salmonids and koi (a form of common carp *Cyprinus carpio*), experimental cutaneous infections were accomplished by removing cutaneous mucus or damaging the epithelium (Tripathi et al. 2005). In cultured fish, the risk factors identified for *F. columnare* infection include water temperatures above 20°C, high rearing density, and skin damage (Suomalainen et al. 2005). *Flavobacterium columnare* has been reported as the cause of disease outbreaks in wild populations of a variety of fish species (Bowser 1973; Wobeser and Atton 1973; Morris et al. 2006). In the hypereutrophic Upper Klamath Lake, high pH ($\geq$10) and elevated ammonia concentrations ($\geq$1 mg NH_3-N/L) occur during blooms of the cyanobacterium *Aphanizomenon flosaquae*. Following these conditions, massive kills of Lost River suckers *Deltistes luxatus* and shortnose suckers *Chasmistes brevirostris* have occurred and *F. columnare* has been isolated from them. In addition, the suckers were frequently parasitized by anchor worms *Lernia* sp. and leeches (Morris et al. 2006). It is interesting to note that high pH and parasitism by leeches and other external parasites are also observed in the areas of the Potomac fish kills.

Although *A. salmonicida* was traditionally considered a salmonid pathogen, causing furunculosis in a variety of trout and salmon species, it infects numerous other freshwater and marine fishes (Austin and Austin 2007). While originally thought to be a primary, obligate pathogen, it has also been described as a free-living, opportunistic pathogen that causes disease in stressed hosts (Pickering 1997). Numerous studies have shown that asymptomatic carrier fish may harbor the pathogen in the intestine, external mucus, gills, and kidney (although it is difficult to isolate in carriers), and clinical disease occurs after stress-induced immunosuppression (Bullock and Stuckey 1975; Hiney et al. 1994; Cipriano et al. 1997). Various stressors or predisposing factors have been shown to increase the susceptibility of a number of fish species to experimental *A. salmonicida* infections. In a study with rainbow trout *Oncorhynchus mykiss*, disease only occurred when the fish integument was damaged or

abraded (Hodgkinson et al. 1987). In experimental bath exposures to 5×10^7 bacteria/mL (levels unlikely to occur in the environment), stressed (experimental handling stress) goldfish *Carassius auratus* experienced 90% mortality, versus 40% for unstressed fish. Early lesions were described as small reddened areas, while later lesions were deep, large cutaneous ulcers penetrating the skin. Interestingly, *A. salmonicida* was only successfully reisolated from early-stage lesions. In late-stage ulcers mainly *A. sobria* and *A. hydrophila* were isolated, suggesting that *A. salmonicida* is essential for the initiation of goldfish ulcer disease but that other aeromonads represent the major factors responsible for skin ulceration (Dror et al. 2006).

Similar stressors and immunosuppression may also account for outbreaks in wild populations. A previous outbreak of *A. salmonicida* in wild SMB was associated with skin damage from commercial nets. The bacteria were most commonly isolated from the skin lesions, but as fish became moribund it was isolated from spleen and kidney in pure cultures (LeTendre et al. 1972). Other reported outbreaks in wild populations have been species specific. In Clear Lake, Iowa, only yellow bass *Morone mississippiensis* experienced extensive losses, despite the presence of other fish species. It was postulated that rising water temperatures, proximity to the spawning season, and possible nutritional deficiency provided the necessary stress to initiate the epizootic (Buckley 1969). Recently, spring skin lesions and kills of common carp in recreational lake fisheries that were caused by *A. salmonicida*, motile *Aeromonas* spp., and *Pseudomonas* spp. have been associated with management practices such as high stocking density, catch-and-release fishing, and suboptimal habitat (Hewlett et al. 2009).

Although many motile *Aeromonas* spp. are considered normal inhabitants of freshwater environments and the external surfaces of healthy fish, when these bacteria are recovered from internal organs in pure culture (heavy growth status) they are considered pathogenic to fish. Motile aeromonads, including *A. hydrophila*, *A. sobria*, and *A. caviae*, are difficult to reliably identify by phenotypic characteristics (Martinez-Murcia et al. 1992; Orozova et al. 2009) and are often considered opportunistic pathogens, causing disease in stressed individuals. Both *A. hydrophila* and *A. sobria* have been associated with disease and skin lesions in a number of fish species, causing heavy mortalities in both farmed and wild fish (Harikrishnan and Balasundaram 2005; Wahli et al. 2005). Strain differences in the pathogenicity of *A. hydrophila* isolates are related to chemotaxis and adhesion to and growth in fish mucus (van der Marel et al. 2008) and the presence of a type III secretion system (Yu et al. 2004; Froquet et al. 2007). Recently, certain bacterial isolates identified by phenotypic methods and sequencing of the 16s rRNA as *A. hydrophila*, *A. hydrophila* subsp. *dhakensis*, and *A. sobria* demonstrated the same virulence factors (siderophore production; production of elastase, hemolysin, lecithinase, and lipase; and the ability to absorb Congo red for detecting extracellular protein A-layer) as *A. salmonicida*. In addition, the minimum lethal dose by injection into rainbow trout for *A. hydrophila* subsp. *dhakensis* (3.3×10^2) and selected *A. sobria* strains (2.5×10^4) was significantly less than that for *A. salmonicida* strains (2.3×10^5) (Orozova et al. 2009). These results indicate that certain strains of motile *Aeromonas* spp. may be significant pathogens.

The epithelium of fish (skin and intestinal) is protected from damage (chemical, enzymatic, and mechanical) by an overlaying mucus layer that also plays an important role in disease resistance (Shepard 1994). Certain pathogenic *A. hydrophila* strains modulate the secretion and glycosylation of intestinal mucus in carp (Schroers et al. 2009). Results from cultures of the skin mucus of apparently healthy SMB prior to the ulcerative lesion and mortality events in the Shenandoah River in 2007 showed the presence of motile *Aeromonas* spp. in skin mucus samples. During the subsequent mortalities that year, motile *Aeromonas* spp. were present in the skin or gill samples of all five fish and *A. salmonicida* was isolated from four of the five fish. In the cultures of RBS from the Cowpasture River with skin lesions (prior to mortality), *A. hydrophila* was the most prevalent bacteria. The only other isolate from lesioned RBS at this time was *P. fluorescens*. Similarly, only *A. hydrophila* and *A. sobria* were cultured from the one SMB with lesions collected premortality in the South Branch Potomac River. These observations suggest that certain environmental stressors are both affecting the disease resistance mechanisms of the skin and disrupting the normal flora and thus allowing potential pathogens to proliferate. A number of genera of bacteria present in normal intestinal flora inhibited the growth of a pathogenic strain of *A. sobria* identified as the cause of ulcerative disease in Eurasian perch *Perca fluviatilis* (Goldschmidt-Clermont et al. 2008). Hence, changes in the normal flora may allow more pathogenic bacteria to proliferate. Motile *Aeromonas* spp. may initiate early skin lesions by destroying the mucus layer, allowing for infection by other pathogens that can then invade underlying tissue. Alternatively, or in conjunction with these changes, environmental factors may influence bacterial communities. Microhabitat changes, particularly resource enrichment and increased nutrient

concentrations, have been documented to turn benign microbe communities (with opportunistic fish pathogens) into virulent ones (Wedekind et al. 2010).

Parasites

A variety of parasites were observed in SMB and RBS. Parasitism is by definition detrimental, as the parasite survives at some cost to the health and/or nutritional status of the host. However, a successful parasite uses the required benefits from the host, completes its life cycle, and is not fatal (McKay 2006). A variety of ectoparasites were noted on the SMB that may enhance infections with secondary or opportunistic bacterial pathogens. These included leeches, trematode metacercariae, myxozoans, and monogenes. Parasites, both protozoan and metazoan, can cause damage to the epithelial layer of skin, gill, or intestine and create a route of entry for bacterial or viral pathogens (Thoney and Hargis 1991; Bandilla et al. 2006; Pylkkö et al. 2006). They may also serve as vectors for viruses and bacteria (Cusack and Cone 1986) and enhance bacterial infections via decreased host immunocompetence (Bowers et al. 2000; Tully and Nolan 2002). The bacteria isolated from SMB in the Potomac River have been shown to be enhanced by parasite infections in other fish species. Increased mortality due to *F. columnare* occurred when fish were infected with the ectoparasite *Argulus corregoni* (Bandilla et al. 2006). Atypical *A. salmonicida* infections in grayling *Thymallus thymallus* were associated with the digenetic parasite *Diplostomum spathaceum* (Pylkkö et al. 2006). *Aeromonas salmonicida* has been isolated from the sea louse *Lepeophtheirius salmonis* (Nese and Enger 1993), *Argulus corregoni* (Shimura et al. 1983), and *Tetrahymena pyriformis* (King and Shotts 1988). *Aeromonas veroni* biovar. *sobria* is commonly isolated from leeches (Phillips 2006). Metacercariae of a trematode, *Posthodiplostomum minimum centrarchi*, a digene that parasitizes centrarchid fish, were observed in liver, spleen, and anterior and posterior kidneys. *Posthodiplostomum minimum centrarchi* has snail *Physa* spp. intermediate hosts, and final hosts include herons and loons (Hoffman 1999). Grizzle and Goldsby (1996) compared the prevalence and density of *P. m. centrarchi* in liver tissue of largemouth bass collected in two Florida lakes. Prevalence (percent of infected fish) was similar between the two lakes, ranging from 83.3% to 100%. However, the amount of liver tissue replaced by parasites was different, 0.83% versus 4.55%. The effect of parasite intensity on fish health (as assessed by hematocrit, relative weight, and growth) was not great at these intensities, although the authors considered the amounts of tissue replaced to be high (Grizzle and Goldsby 1996). In the SMB from the Potomac, the intensity of infection varied greatly; however, as much as 60% of the liver tissue of some fish was replaced by parasites. The high number of metacercarial cysts in tissue, particularly in tissues associated with disease resistance (spleen and anterior kidney) and detoxification (liver), could certainly be detrimental.

Myxozoans (currently unidentified) were observed in both SMB and RBS. The myxozoan spores and/or sporozoites observed within the kidney, spleen, and liver macrophage aggregates were present in SMB examined during moralities as well as at other times of the year. The massive bile duct proliferation and necrosis seen in association with a myxozoan parasite was only observed during mortality events. A number of studies have reported increased myxozoan infections at polluted sites (Overstreet 1988; Khan and Thulin 1991; Bucher et al. 1992). It has also been shown that levels of myxozoan infections (particularly those with benthic oligochaetes as intermediate hosts) will increase under eutrophic or organically enriched conditions caused by municipal effluent or agricultural runoff (Marcogliese and Cone 2001).

Environmental factors can affect parasite populations in numerous ways, and the interactions between contaminants and parasites are often complex. Parasitism can increase if environmental stressors reduce host resistance or increase intermediate or definitive hosts. Alternatively, parasitism may decrease if environmental stressors adversely affect intermediate or final hosts or there are toxic effects on the parasites themselves or infected hosts suffer differentially high mortality (Lafferty 1997; Lafferty and Kuris 1999). Chemicals found at the sites with SMB or RBS mortality may indirectly affect trematode or other parasite infections. Exposure of snails to estrogenic compounds such as bisphenol A and octylphenol induced a syndrome referred to as "superfemales"—a stimulation of oocyte and spawning mass production—but also increased mortality (Oehlmann et al. 2007). Embryo production by the mudsnail *Potamopyrgus antipodarum* was significantly enhanced by exposure to low to moderate dilutions of estrogenic effluent and decreased at higher dilutions (Jobling et al. 2004). Hence, the same chemicals that contribute to the induction of testicular oocytes may act to increase snail populations.

Environmental Stressors

A number of studies conducted by members of the Fish Kill Task Force were directed toward determining if contaminant exposure or other environmental stressors (dissolved oxygen, ammonia) were responsible for the fish kills. Unfortunately, the data were

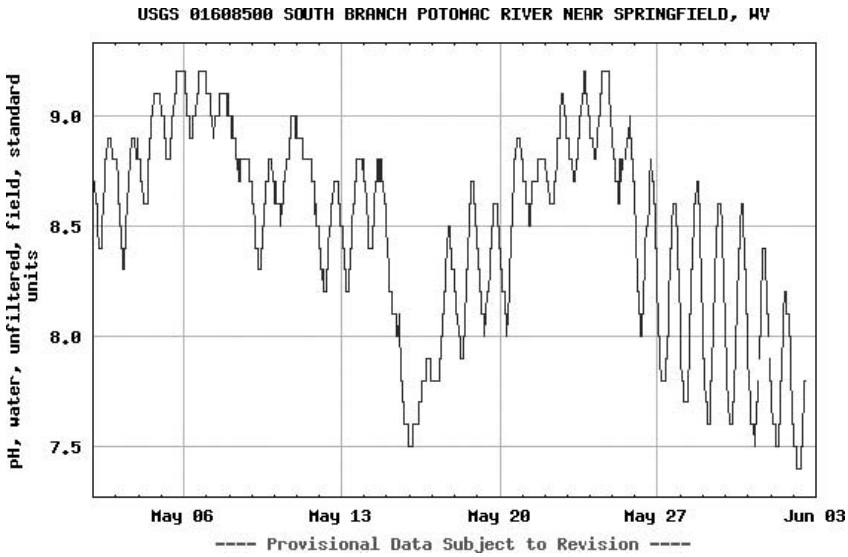

FIGURE 8.—Graph of pH from a continuous monitor placed in the South Branch of the Potomac River in spring 2006 during the time of year when mortality events are observed.

evaluated in terms of assessing individual contaminants or stressors as "the cause" and chemical exposures were evaluated in terms of lethal effect levels or published benchmark screening criteria (Suter 1996; U.S. EPA 2007). Most benchmarks are acute or chronic toxicity or sublethal effects such as a reduction in growth or recruit abundance. This approach does not address the more sensitive responses such as disease resistance and endocrine modulation, the potential additive or synergistic effects of chemicals on these responses, the influence of age, differential organ or tissue accumulation and the effects of specific chemicals, heavy parasite loads, or other physiological conditions present in wild fish that may contribute to a more severe response to various chemicals. For instance, a much overlooked consequence of parasite infections is an increased susceptibility of the host fish to contaminant effects (Khan and Thulin 1991). So, infected animals often suffer mortality at concentrations that normally do not kill uninfected conspecifics (Boyce and Yamada 1977; Pascoe and Cram 1977; Moles 1980). No chemical or environmental "cause" has been identified; however, a number of environmental factors and contaminants were identified as potential contributors to the fish mortalities. These included nutrients (particularly nitrogenous wastes such as ammonia), high pH, suspended sediment, mercury, arsenic, pesticides, and endocrine-modulating substances or contaminants of emerging concern.

Water Quality

It is well documented that both natural (temperature, pH, and dissolved oxygen fluctuations) and anthropogenic environmental stressors affect the physiological and immune functions of fish and may contribute to infectious diseases (Bly et al. 1997; LeMorvan et al. 1998; Snieszko 1974; Sunyer et al. 1995). Dissolved oxygen concentrations have been measured during mortality events in both the South Branch Potomac and Shenandoah rivers and have not been observed to be low. However, significant pH and temperature fluctuations that could contribute to stress for some fish species have been recorded. The pH shows daily swings from close to neutral to highs that often exceed 9.0 (Figure 8). While the pH swings and high pH itself may adversely affect certain fish species, pH also influences the toxicity of many other compounds and may play a role in the release of sediment-associated contaminants. Ammonia is present in the aquatic environment principally as a consequence of agricultural runoff and decomposition of biological waste. While temperature and ionic strength affect the toxicity of ammonia, the main influence is pH. Total ammonia in the environment is the sum of ammonium ion (NH_4^+) and NH_3 (the form that enters organisms through membranes and that is toxic). As the pH increases, the proportion of NH_3 increases. While acute toxicity and derived criteria have been established for many organisms, these are based on exposure of starved, resting, unstressed animals. However, internal

ammonia levels increase following feeding, swimming, and stress (Randall and Tsui 2002). In addition, since ammonia is excreted via the gills, gill hyperplasia and fusion may decrease the ability of the fish to excrete it. The acute toxicity exposure to ammonia of young SMB ranged from 371 mg/L total ammonia at pH 6.5 to 7.4 mg/L at pH 8.7 (Broderius et al. 1985). While we know of no studies on the chronic toxicity of ammonia to SMB, slimy sculpin *Cottus cognatus* exhibited severe gill lamellar fusion and epithelial lifting at levels as low as 0.834 mg/L (Spencer et al. 2008). These lesions are not specific for ammonia toxicity, and although both were noted in some fish collected during mortalities (Figure 6D), they were not consistent findings.

Metals

Two metals identified as potential concerns are mercury and arsenic. Mercury is a widespread problem, not only in the Potomac drainage but in streams across the United States (Scudder et al. 2009). Studies in the South River and South Fork of the Shenandoah over a 3-year period showed that rock bass, RBS, and bluegills all accumulated mercury in muscle tissue with age. Concentrations ranged from <0.1 to approximately 0.4 mg/kg in age-0 fish and from 0.48 to 1.3 mg/kg in age-3 fish. Rock bass and RBS had higher concentrations than bluegills, and in general females had higher concentrations than males. Mercury concentration did not affect condition factor (Nicoletto and Hendricks 1988). More recently, sexual and seasonal variations in muscle total mercury concentrations were assessed in SMB collected in the South Fork. In this study only sexually mature fish (mean age, 4.3 years) were analyzed, and mercury concentration ranged from 0.30 to 1.38 ppm. Concentrations were higher in females and 14–21% higher (although not significantly so) in the spring than in the summer or fall (Murphy et al. 2007). Mercury has also been shown to modulate the immune system of exposed fish (reviewed by Sweet and Zelikoff 2001), and hence it could contribute to greater disease susceptibility.

Arsenic is found naturally in many soils and can enter aquatic systems from natural geological sources (Duker et al. 2005). It can also occur due to anthropogenic inputs. Arsenic is extensively used in pesticides and fungicides (Merwin et al. 1994; Campos 2003) and in lawn and turf herbicides. The organoarsenical compound roxarsone was added to poultry feed at concentrations of 20.6–41.3 mg/kg, originally to control coccidiosis but also for growth stimulation, improved feed conversion, better feathering, increased egg production, and pigmentation (Anderson 1983). Total arsenic concentrations of 10–50 mg/kg are reported in poultry litter (Garbarino et al. 2003). Arsenic can build up in soils and so may be present as a result of historic use. Interestingly, exposure of humans to arsenic in drinking water increases the prevalence of skin lesions (Kitchin 2001; Kazi et al. 2009). Arsenic is an endocrine disruptor that affects estrogen receptor–mediated gene expression (Davey et al. 2007), other steroid hormone receptors (Bodwell et al. 2006), and thyroid receptors (Davey et al. 2008) at low, environmentally relevant concentrations. It also modulates factors involved in the innate immune response of fishes (Hermann and Kim 2005; Lage et al. 2006). Exposure to arsenic increased the susceptibility of striped bass to *F. columnare* (MacFarlane et al. 1986). A series of studies evaluating the influence of arsenic on the immune responses of the walking catfish *Clarias batrarchus* indicated a number of effects, including increased susceptibility to *A. hydrophila* (Ghosh et al. 2006, 2007; Datta et al. 2009). Exposure to a nonlethal concentration (1/10 LC50) resulted in tissue-specific alterations in T and B cell function, suppressed antibody and cytokine production, reduced bacterial phagocytosis, and an increased the degree of tissue damage, ulceration, and mortality when fish were exposed to *A. hydrophila* by intramuscular injection (Ghosh et al. 2006, 2007). A subsequent study used lower, more environmentally relevant, micromolar concentrations. Chronic (30-d) exposures to 0.50-μM (37.5 μg/kg) concentrations resulted in reduced anterior kidney macrophage number and viability. In addition, the ability of *A. hydrophila* to colonize and disseminate was enhanced in exposed fish (Datta et al. 2009). Similarly, aqueous exposure of zebra danio *Danio rerio* to 2 or 10 ppb (considered safe for drinking water) inhibited the host's ability to clear both viral and bacterial infections, reduced the respiratory burst response, and delayed or inhibited the induction of antiviral and antibacterial cytokines compared with unexposed controls (Nayak et al. 2007).

Levels up to 0.784 ppb arsenic were measured in filtered water in the Shenandoah; however, in a storm runoff event suspended solids contained up to 2.48 mg/kg or ppm arsenic (Downey et al. 2009). The effects of suspended solids and sediment on fish health have been demonstrated (Newcombe and MacDonald 1991; Scheurer et al. 2009) and include damage to gill and skin epithelia, hyperplasia, and hypertrophy of the gill epithelium. However, little attention has been directed toward contaminants present in pulses of sediment and suspended solids entering the water during storm events and how they may directly or indirectly influence fish health. Newcombe and Jensen (1996) suggested that fine particles and any adsorbed toxicants may be phagocytized by macrophages and other phagocytic cells present in the gill, gut, and skin and

transported to specific sites (the spleen) within the body. Since macrophages containing carbon particles and other particulate material have been shown to migrate to macrophage aggregates in the spleen and anterior kidney, where the immune response is initiated (Wolke 1992; Aguis and Roberts 2003), this is not an unlikely scenario. The increasing arsenic concentrations noted in the skin and hemopoietic tissue between March and May (Figure 7) support this hypothesis and raise the question as to what other contaminants are associated with suspended solids, increase during runoff events, and enter the fish's body. For instance, numerous current-use pesticides have been detected in association with suspended sediments (Hladik et al. 2009).

Contaminants of Emerging Concern

During the comprehensive histopathological assessments conducted on SMB from the South Branch Potomac River in 2003, intersex or the presence of testicular oocytes was noted. Further sampling also showed a high prevalence in SMB collected in the Shenandoah and much lower prevalence in SMB collected at out-of-basin sites (Blazer et al. 2007). The presence of testicular oocytes in other fish species has been used as an indicator of exposure to estrogens or chemicals with estrogenic or antiandrogenic activity (Jobling et al. 2006, 2009), most often associated with human wastewater treatment plant (WWTP) effluent (Aerni et al. 2004; Thorpe et al. 2006). Many of these chemicals have biological effects at very low (parts per billion) concentrations and are difficult to detect using grab water samples. Hence, a number of studies were conducted using passive sampler devices. Two types of device, semipermeable membrane devices and polar organic chemical integrative samplers, have been deployed at numerous sites within the Potomac drainage (Alvarez et al. 2008b, 2008c, 2009). These devices provide a means of integratively sampling a variety of bioavailable waterborne nonpolar and polar organic contaminants. They are deployed for weeks to months and so can be used for chemicals at low concentrations or those that are present episodically (Huckins et al. 2006; Alvarez et al. 2008a). In addition, the extracts obtained from the samplers can be used in in vitro assays such as the YES (yeast estrogen screen) or BLYES (bioluminescent yeast estrogen screen) to test for the total estrogenicity of the complex mixtures (Sanseverino et al. 2005).

Atrazine

A reconnaissance of contaminants, using the passive samplers deployed for 42–49 d, was conducted in the Shenandoah (11 sites, including the North and South Forks) and the James (2 sites, including the Cowpasture) rivers in spring 2007. Few chemicals indicative of WWTP effluent or septic tank discharge were detected at any sites. Rather, chemicals characteristic of a largely agricultural area were measured. Atrazine was detected at the greatest concentrations, while chlorpyrifos, endosulfan, and lindane were the most commonly detected chlorinated pesticides (Alvarez et al. 2008b, 2008c). Atrazine was also found at sites on the Monocacy River, and the concentrations were considerably higher in the spring than in the fall (Alvarez et al. 2009).

Atrazine is a commonly used triazine herbicide with indirect effects in vertebrates, one of which is the disruption of estrogen signaling (although reports on the estrogenicity of atrazine are inconclusive; Eldridge et al. 2008). Perhaps one of the best examples of the complex interactions of water quality, chemicals, parasites, and animal health is demonstrated in recent research on trematode infections in amphibians. Johnson et al. (2007) demonstrated that eutrophication promoted growth of periphytic algae that leads to increases in population biomass and enhanced egg production in herbivorous snails. The snails *Planorbella* spp., an intermediate host of the amphibian trematode parasite *Ribeiroia* sp., in turn produced significantly greater cercariae in high-nutrient treatments than in low-nutrient treatments. Hence, there was a two- to five-fold increase in the infection of larval amphibians in the high-nutrient treatments. More recently, field studies evaluated trematode abundance in larval frogs and quantified 240 potential predictors of trematode infections, including the abundance of intermediate hosts, water quality, and pollutants that might suppress the host's immunity or be lethal to hosts or the cercarial stage and landscape attributes, in 18 wetlands. Atrazine was found to be the best predictor of total trematode abundance (Rohr et al. 2008). The proposed mechanisms were (1) a net increase in the periphytic algae that is a food source for the snail intermediate hosts caused by atrazine and other herbicides (Brock et al. 2000; Johnson et al. 2007) and (2) immunosuppressive effects of low atrazine concentrations on ranid frogs (Kiesecker 2002; Brodkin et al. 2007). Interestingly, the highest atrazine concentration measured in these field studies was 0.59 μg/L (Rohr et al. 2008; supplementary information). Similar concentrations were measured in the North Fork (up to 0.43 μg/L) and Monocacy (0.69 μg/L) in spring 2007 and much higher concentrations (2.10 μg/L) in the Monocacy in spring 2006 (Table 5). Since nutrients are a major concern throughout the Chesapeake Bay drainage, similar mechanisms may be occurring with SMB. Atrazine

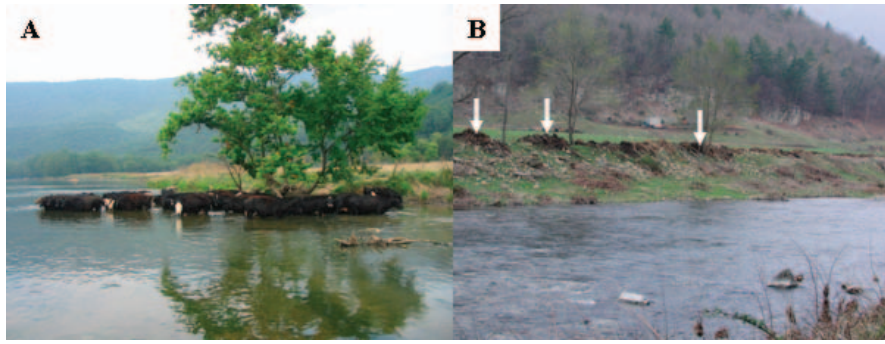

FIGURE 9.—Agricultural practices that add nutrients, hormones, and potentially other contaminants of emerging concern to water in the Potomac River watershed: **(A)** cattle with free access to the rivers and streams in the Shenandoah drainage and (B) piles of chicken litter and manure (arrows) along the South Branch of the Potomac.

and herbicide mixtures were shown to modulate disease resistance in goldfish (Fatima et al. 2007), suggesting the role of atrazine in fish health.

Estrogenic Compounds

The co-occurrence of intersex and fish kills and lesions focused our attention on estrogens and estrogenic compounds as possible contributing factors since they can be potent modulators of the immune response and disease resistance (reviewed by Iwanowicz and Ottinger 2009). Hence, a study was conducted evaluating reproductive health upstream and downstream of two WWTPs on tributaries of the Potomac River. A significantly higher prevalence or severity of intersex was not detected at the downstream sites (Iwanowicz et al. 2009), nor was there a higher level of total estrogenicity of the passive sampler extracts as measured using the in vitro yeast estrogen screens at downstream sites (Alvarez et al. 2009). The passive sampler results emphasize the need for measuring the effects, sometimes additive, of complex mixtures. Although 17β-estradiol, 17α-ethynylestradiol, estrone, and estriol were all at levels below the method quantification level in the passive sampler extracts from the North Fork sites in spring 2007, the yeast estrogen screen indicated estimated estrogen equivalents ranging from 14 to 79 ng estradiol/sample (Alvarez et al. 2008b). Similarly, on the Monocacy none of the above hormones were at detectable levels in the sampler extracts despite measurable total estrogenicity from the yeast estrogen screen (Alvarez et al. 2009).

The lack of correlation between testicular oocyte prevalence of severity and WWTPs in the Potomac drainage suggests that, while WWTPs are certainly contributors of nutrients and other potentially harmful chemicals, agriculture is also an important contributor in this ecosystem. The headwaters of the Potomac, Shenandoah, and James rivers have high densities of concentrated animal feeding operations (CAFOs). Poultry (primarily turkeys and broilers) and livestock (mainly dairy and beef) predominate in the area. Pasture is the dominant land use in the Shenandoah watershed, followed by hay and row crops (corn and soybeans). Cattle with access to the river (Figure 9A) and confined and nonconfined feeding areas in the headwaters and small streams contribute direct and indirect deposits of waste to the river. Waste from CAFOs may add excess nutrients and heavy metals (Mallin 2000; Mallin and Cahoon 2003; Burkholder et al. 2007), infectious disease agents and antibiotics (Gilchrist et al. 2007), and endocrine disrupting compounds (Orlando et al. 2004) to streams and rivers. In the Shenandoah Valley approximately 330,000,000 kg of poultry litter (a mixture of poultry manure and bedding material) are produced each year, and most of it is being applied as fertilizer on cropland (Brown et al. 2005). The locations of CAFOs relative to receiving waters may be an important determinant of how much poultry litter and by-products end up in streams. This is especially true where poultry litter is stored in the open or along rivers (Figure 9B) or where litter is applied over karst features, right up to the edge of the river or stream bank and applied at high rates to meet the nitrogen needs of crops. In either case, strong rainstorms can wash poultry litter into streams, potentially exposing resident fish populations to contaminants.

Tissue Contaminants

Although halogenated organic contaminants were only found in the tissues of a few fish from affected

and reference sites, the results indicate that a wide variety of chemicals are present and that their levels vary among tissues (Tables 5, 6). Many of the chemicals measured can have immunosuppressive as well as endocrine-modulating effects, and hence further research should be directed toward analyzing tissues of dying fish or fish with lesions versus apparently healthy individuals. It would be helpful to determine whether, as with arsenic, these compounds tend to increase in specific tissues over the months leading up to mortalities and whether these concentrations correlate with immune system indicators. Pesticides (Blakley et al. 1999; Galloway and Handy 2003), PCBs (Duffy et al. 2003; Duffy and Zelikoff 2006), and brominated flame retardants (Birchmeier et al. 2005) have all been shown to have adverse effects on the immune system and disease resistance. The fact that primarily adult fishes are affected may be the result of accumulation of certain chemicals with age and/or association with reproductive physiology or behavior. In addition, most of the information available on contaminant concentrations in fish is based on whole bodies or fillets. While this information is appropriate for issuing human consumption advisories, it may not be the most informative in terms of fish health. The preliminary tissue contaminant analyses presented here indicated that muscle had the lowest concentrations of all contaminant groups. The considerably higher concentrations (Table 5), particularly in the brain, spleen, and ovary (just prior to spawning) may be associated with the reduced health of affected species.

Understanding of the effects of the complex mixtures of chemicals to which fish are exposed in our rivers is seriously lacking. Species and life stage are important factors even in controlled laboratory experiments. For instance, in bluegills there was no significant difference in the acute toxicity of chlorpyrifos alone relative to that of a combination of chlorpyrifos and atrazine, while in fathead minnow *Pimephalus promelas* there was a synergistic (greater than additive) interaction of the two pesticides (Mehler et al. 2008). Prolarvae of walleyes *Sander vitreus* were less sensitive to chlorpyrifos than juvenile walleyes (Phillips et al. 2002), and in Japanese medaka *Oryzias latipes* sensitivity to diazinon increased with age (Hamm et al. 2001). Hence, assuming that individual chemicals are not important because their levels are below reported lethal concentrations (or even benchmark criteria) without taking into account species differences, life stage, degree of parasitism, other pathological conditions, and the additive or synergistic effects is not appropriate in studies of the health of wild populations.

Summary

In conclusion, there is recognition that environmental change (including climate change, land use change, and pollution) is often closely associated with infectious diseases. However, the evidence for ecosystem drivers of many wildlife diseases is lacking due to the complexity of pathogen–host–environment interactions. The results presented here emphasize the need for comprehensive studies that integrate pathogen identification, pathological responses, and immune function with water quality analyses and tissue contaminant concentrations to truly understand the causes of and factors contributing to skin lesions and mortality events. Many physiological functions, including the immune responses of fish, vary temporally (i.e., seasonally). Contaminant exposures (as evidenced by the arsenic findings) may also vary temporally, and the concentrations of potential stressors during critical periods of development need to be better understood. There is much circumstantial evidence that selected species within the Chesapeake Bay drainage, and the Potomac River watershed in particular, are stressed. These fish have reduced disease resistance, as evidenced by skin and sometimes internal infections with a variety of bacterial pathogens. Other organisms, such as LMBV and those causing secondary fungal infections, may also play a role in mortality. The stressors include high helminth and myxozoan parasite loads, possibly as a consequence of high nutrient loads and/or immunosuppression; contaminant exposure, particularly to estrogenic compounds and complex mixtures; and other water quality factors, such as high pH and increased mean temperature. A gradient in the prevalence and severity of testicular oocytes, with a high prevalence in SMB collected in the Shenandoah River, a moderate prevalence in the South Branch Potomac River, and a low prevalence at a number of out-of-basin sites, was previously noted (Blazer et al. 2007). A similar gradient is evident in ulcerative lesion/mortality prevalence and severity, with more extensive and severe lesions or mortality occurring in the Shenandoah, moderate levels in the South Branch, and no similar mortalities to date in the out-of-basin sites with a low prevalence of intersex and lower concentrations of a variety of water and tissue contaminant concentrations (Table 6). Integrative, multidisciplinary research that takes into account water quality, tissue contaminant concentrations, and land use and that incorporates an understanding of the spatially and temporally varying effects of pollution sources and hydrological and climatic influences on biological endpoints is needed to truly understand fish health—and ultimately ecosystem health—and to

determine the management actions needed to reduce the various stressors.

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