Relations among Biochemical, Individual, and Community Indicators of Stress in Fish: Stream Degradation in the Clinch River Drainage

by

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RELATIONS AMONG BIOCHEMICAL, INDIVIDUAL, AND COMMUNITY INDICATORS OF STRESS IN FISH: STREAM DEGRADATION IN THE CLINCH RIVER DRAINAGE

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ABSTRACT

Bioindicators were used to assess degradation to fish resident to the Clinch River drainage. Species studied were rock bass (Ambloplites rupestris), northern hogsucker (Hypentelium nigricans) and striped shiner (Luxilus chrysocephalus). The data were collected in parallel with a study of the index of biotic integrity (IBI) on fish communities also in the Clinch River drainage. Sites selected for this study were identical to those used for IBI. Data obtained from fish sampled at relatively pristine sites (i.e., high IBI) weie used as references to be compared with data obtained from fish sampled at sites suspected of human impact (low IBI). Results demonstrated variable bioindicator response to degraded sites. While bioindicators were elevated at certain sites, others were not significantly different from corresponding reference values. Furthermore, results showed a number of correlations between certain bioindicators and IBI and several IBI components, implying a possible relationship between these initial individual-level responses (biondicators) and eventual longer term population- and community-level effects (i.e., IBI and its components). However, these results also varied between impacted sites. Future field application of bioindicators in the presence of such a multiplicity of potential stressors was discussed.

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bass (Ambloplites rupestris), northern hogsucker (Hypentilium nigricans) and striped shiver (Luxilus chrysocephalus) captured at selected sites in the Clinch River Basin (arranged in order of decreasing IBI values). Four to nine specimens were collected for each species, depending upon availability. The letter ‘a’ indicates sites significantly different from a pool of reference sites at the p=0.00 level taking into account experiment-wise error. Dashed lines indicate that measurements were not taken.

Table 10: Spearman’s rank correlation results: IBI against bioindicators for northern hogsuckers. Each column is a component of the total IBI score (i.e., an IBI metric or an important factor within a metric). Numbers in each entry denote correlation and significance respectively. Lithophils indicates proportion of lithophilous spawners, anomalies indicates total number of fish with anomalies, and intol indicates total number of intolerant species. Tol indicates proportion of tolerant individuals, omniv., pisciv., and inverti. indicate proportion of individuals as omnivores, piscivores and invertivores respectively. Nativ. indicates total number of native species and fin deg. indicates the total number of specimens with fin degradation.

Table 11: Spearman’s rank correlation results: IBI against bioindicators for rock bass. Each column is a component of the total IBI score (i.e., an IBI metric or an important factor within a metric). Numbers in each entry denote correlation and significance respectively. Lithophils indicates proportion of lithophilous spawners, anomalies indicates total number of fish with anomalies, and intol indicates total number of intolerant species. Tol indicates proportion of tolerant individuals, omniv., pisciv., and inverti. indicate proportion of individuals as omnivores, piscivores and invertivores respectively.

Introduction

Anthropogenic impacts are a major threat to freshwater ecosystems (Pritchard 1993; Moyle and Leidy 1992). One conservative estimate of these impacts is that 20% of the world's freshwater fishes are already extinct or are being subjected to serious threats (Moyle and Leidy 1992). Among the major threats to freshwater fish are chemical contaminants, habitat degradation and introduced species (Karr 1991). Contaminants and other stressors that have negative impacts on organisms can eventually affect entire communities (Adams, 1990). Field-based biological assessments of degradation have been emphasized as a critical step in developing appropriate remediation plans for impacted aquatic ecosystems (USEPA, 1990).

Most freshwater systems that are degraded by chemical contaminants contain a complex mixture of chemicals whose negative impacts on biota are difficult to assess (Jimenez and Stegeman, 1990). Current field assessment approaches used in documenting stress in aquatic systems include measures of changes in physiology of individual organisms in response to stress (biomarkers), and effects at the populations and community level of biological organization (Khosla et al. in press). While biomarkers are initial responses to stressors by individual organisms (Canada National Research Council, 1985), population- and community-level indicators allow long-term assessments (Heath 1990, Fausch et al. 1990). For example, stress-response to No. 2 fuel oil exposure induces enzyme ethoxyresorufin deethylase (EROD) and metallothionein in experimental rainbow trout (Oncorhynchus mykiss) in three days and two weeks respectively (Steadman et al. 1991). However, population- and community-level effects probably take at least a few years (i.e. more than one generation time) to take place in fish (Adams, 1990). In addition, certain biomarkers have the advantage of indicating exposure to specific contaminants which indicators at higher levels of biological organization lack. Finally, all biomarker measures can be made under complex field conditions, which thus complement higher level assessments. Linking these two approaches (i.e., assessments at lower and at higher levels of biological organization) could provide ideas as to the mechanisms of initial exposure resulting in longer-term effects.

However, there are numerous shortcomings to such measures of stress response. First, little is currently known about the interaction of biomarker inducers with other chemical contaminants and other stressors that are likely to be present under field conditions. Unfortunately, the presence of complex waste mixtures may confound the measures of contaminant responses. Worse still, response may not be induced in the presence of severe environmental stress (described as the "state of exhaustion" by Selve, 1976). Theoretically, biomarker responses to long-term stressors could vary in several ways (Figure 1). If fish are able to adaptively change, biomarker levels may remain elevated. However, severe stress could result in a decline in biomarker levels. Community effects of long term exposure, however, are likely to be a steady decline in community health (Figure 1).

Secondly, little is known about the effects of non-contaminant stressors, some of which have been shown to induce biomarkers in laboratory-based studies. Some of these responses (e.g., metallothionein induction in response to elevated glucocorticoid levels) have been categorized as generalized stress responses (Cousins 1985). It is a challenge to separate direct biomarker induction responses from those that take place in response to such general stress.

Field studies have used biomarkers as diagnostic tools to indicate a possible causal relationship between specific contaminant types and responses in fish (Munkittrick et al. 1991, Adams et al. 1992). While such documentations are useful measures of initial response, most of them lack ecological relevance (e.g., consequences of exposure on populations). Possible exceptions include reproductive indicators of stress (Adams et al. 1992). Reasons for linking estimates at lower and at higher levels of biological organization include (a) exploring the possible ecological relevance of biomarkers (McCarthy and Shugart, 1990) and (b) elucidating likely causes of stress (including contaminant effects) to aquatic ecosystems that might eventually be detrimental to aquatic communities. Studies have documented correlations between biomarkers of contaminant exposure and population-level responses of fish in streams (Adams et al. 1992; Munkittrick et al. 1992). For example, white sucker (Catostomus commersoni) exposed to pulp mill effluents showed increases in cytochrome P450 enzymes in conjunction with declines in gonad size and egg size (Munkittrick et al. 1991). However, no study has attempted to explore the relationship between biomarkers of stress and possible community-level effects.

The major focus of this study was to document stress to individual fish captured from the Clinch River drainage. Numerous species in this drainage (i.e. 18 species of fish and 34 mussel species) are threatened or endangered (Jenkins and Burkhead 1994). Likely nonpoint sources of degradation include widespread coal mining and agricultural practices in the area, and urbanization. Point sources of stress to biota include municipal and industrial outfalls at specific sites along the drainage (e.g. in 1970 a major acid spill killed over 5,000 fish in the Clinch River, Neves and Angermeier, 1990). A community level study conducted in 1991-92 indicated that several sites were degraded (Angermeier and Smogor, 1993). According to this study, many of these degraded sites could be the long term effect of several sources of stress (e.g., land use practices). It was hence the purpose of this study to document specific causes of stress several of these degraded sites. A suite of biomarkers were used to assess stress in fish resident to several sites in this drainage.

The first objective of this study was to document possible stress in fish resident to the Clinch River drainage using biomarkers sensitive to specific contaminant sources of stress. Fish with elevated biomarker levels were assumed to be under stress or undergoing adaptive responses to environmental stressors. Relatively undegraded sites were used as reference stations throughout the study. Secondly, based on the biomarker data and other existing data, sources of stress were evaluated at individual sites suspected of degradation. Finally, possible relationships between the biomarkers examined in this study and existing individual-, population- and community-level data for fish at the same sites were explored. Possible use of biomarkers early warning indicators of stress was also explored. Parameters measured in this study were hepatosomatic index, ethoxyresorufin deethylase activity, microsomal protein and metallothionein levels. Each of these biomarkers have been extensively used in field situations in investigating the impact of various contaminants stressors on fish.

Literature Review

Most aquatic systems today are negatively affected by anthropogenic impacts. Broadly, these impacts may be divided into those that occur through contaminant entry, habitat loss, and changes in hydrology. Ultimate consequences on resident biota include alterations in trophic relationships, and species composition changes. Surface waters have been regarded as the "ultimate sink" for many of the wastes produced by human activities (Pritchard 1993; Moyle and Leidy 1992). While direct measures of chemical contamination are important analytical tools for assessing impacts, they essentially represent "snapshot" types of sampling, which lack the power of integrating impacts over time and space (McCarthy and Shugart 1990). However, biological indicators of stress are capable of integrating effects of stressors over time and space. Thus there is a need to use responses of resident biota in aquatic systems to assess the effects of human impacts.

Selve (1976) divided response to environmental stress into three phases: (1) the alarm reactions, where changes reflect mobilization of the body's defense mechanisms to counteract the stress, (2) the resistance stage, where the internal feedback system permits the body to cope with stressors' influence and maintain a steady state and (3) state of exhaustion, where the physical deterioration results from failure to cope with high intensity or prolonged exposure to the stressor. The first two states probably correspond to induction of biochemical biomarkers in response to specific and/or general stressors which alter the physiology of the organism (Heath 1990). Stress may result in mobilization of resources by the affected organism in an effort to compensate for the physiological or biochemical alteration incurred by exposure (Giesy et al. 1988). The third stage may correspond to a state in which the organism is weakened to a point where it is not able to respond (Heath 1990). In accordance with this hypothesis, it might be expected that at this "stage" of stress, levels of biochemical biomarkers (physiological indicators of organism condition that can be specific to contaminant sources) might be significantly lower than expected of contaminated or stressed sites.

The basis for using biological assessments is that degradation can negatively impact the biota in a system and these impacts increase with the degree of degradation. Selected biomarkers have shown a dose-response relationship with the levels of certain types of contaminants present in the environment (Adams et al. 1990; Van Veld et al. 1990; Munkittrick et al. 1991; Hogstrand and Haux 1991). In order of increasing response time, field-based assessment approaches can be divided into subindividual and individual (i.e., biomarkers) level approaches, population and finally community level approaches. Biomarkers are generally short term physiological responses, of the order of a few days to weeks. For example, one study on rainbow trout (Oncorhynchus mykiss) exposed to No. 2 fuel oil showed significant increases in ethoxyresorufin deethylase

(EROD) activity and metallothionein (MT) in three days and two weeks respectively (Steadman et al. 1991). The population and community level responses are longer term of the order of seasons and even years (e.g., several biological generations long, Adams 1990).

While biomarkers have the advantage of assessing contaminant exposure directly, they lack the direct ecological relevance that population and community level indicators of stress have (McCarthy and Shugart 1990). In order to assess degradation effects on aquatic systems, it is important to measure the consequence of such stress exposure to the biota. Measurements made at higher levels of biological organization integrate effects of degradation across causes such as exposure to various stress regimes. Hence there is a need to link measurements that can elucidate causes of degradation with those that essentially measure ecologically relevant effects thereof.

A commonly used field technique to assess contaminant exposure is to examine body burdens of suspected contaminants in tissue of organisms from the field. The advantage of such exposure measurements is that they may be good estimates of bioavailability of contaminants. However, such analyses may not be amenable to field situations where complex mixtures of contaminants often occur. Such mixtures may have toxic action on organisms that take place synergistically or antagonistically with each other (Niimi 1990). Furthermore, such measures may not detect contaminants that can be transformed (e.g., be metabolized) in tissues. This problem is especially relevant to studies of organic compounds such as pulp mill effluents and oils, which aquatic organisms are able to metabolize. Biological responses of exposure to heavy metals also may not be accurately assessed using body burdens alone (Hogstrand and Haux 1990).

One method of estimating contaminant exposure in fish is direct chemical assays of bile, where organic wastes and metabolites are temporarily stored for excretion (Britvic et al. 1993; Stein et al. 1992). This technique is hence sensitive to organic contaminants that may have undergone biotransformation, and hence go undetected by chemical analyses of tissues (Varanasi and Gmur 1981, Pritchard 1993; Theodorakis et al. 1992).

Contaminant stressors first impact the organism at the biochemical level, which may cause dysfunction. Individual cells can respond as a form of adaptation to minimize toxic effects within them. Dysfunctional and adaptive responses (e.g., protein induction, Giesy et al. 1988; Saunders 1990) at subcellular level can in turn lead to impairment at higher levels of biological organization such as whole organ level. Subcellular and cellular level responses can eventually cause detrimental effects at the whole-organism level (e.g., processes such as hormonal regulation, metabolism and electrolyte balance can be affected, Adams et al. 1990). Recently, numerous individual-level assessment techniques termed biomarkers have been widely used to assess exposure and even to link it with possible consequences of exposure (McCarthy and Shugart, 1990, Saunders, 1990).

Biomarkers

Biomarkers have been broadly defined as a range of biological assessments of organism condition using biochemical, cellular (subindividual level), morphological and physiological (individual level) parameters as diagnostic screening tools in environmental monitoring (Saunders, 1990, McCarthy and Shugart, 1990). Many of them have been applied to field conditions. At the cellular level, they represent a broad range of events that are targeted towards protecting cells from environmental insults or can represent cellular dysfunction. Biomarkers have been stressed as being more potentially useful than assessments of exposure to contaminants through analysis of body burdens, which may be inaccurate if biotransformation of contaminants takes place. Many biomarkers used as tools of contaminant exposure assessment are physiologically important (e.g., cytochrome P450 enzymes are used in steroidogenesis, Higuchi et al. 1991). In addition, a levels of a number of biomarkers may change as a function of generalized stress (i.e., stress response irrespective of the source) so they may integrate a variety of natural and anthropogenic stressors.

Biomarkers applicable to field conditions have been recently categorized as tier I and tier II subdivisions (Saunders, 1990).

Broadly speaking, tier I biomarkers are those that respond to general stress, irrespective of the cause. However, some biomarkers that respond to general stress (e.g., stress proteins) may not increase significantly in response to chronic, low level stress (Saunders, 1990), and have yet to be applied to field conditions. Tier II biomarkers may be related to specific causes of stress, such as organic contaminant stress. These categories have some degree of overlap due to the complexity involved in response of individual biomarkers. For example, certain hepatic cytochrome P450 enzymes are induced by exposure to pulp mill effluents (Munkittrick et al. 1991), however the same enzymes may be induced by corticosteroids (which in turn respond to non-specific sources of stress, Mathis et al. 1986) , and by nutritional status (Lemaire et al. 1992).

Heavy metal exposure has also been documented to elicit a fairly specific biochemical response (i.e., metallothionein protein induction) in organisms (Roesijadi 1992). However, metallothionein levels may also be altered in response to physiological stress (Cousins 1985). For metallothionein, induction by metals can be significantly higher than stress induction mediated by corticosteroids (Brady 1982). Such complexities must be kept in mind in the field application of biomarkers to assess sources of stress.

The Cytochrome P450 system (Tier I biomarkers)

Use of cytochrome P450 enzyme activity (categorized as a tier I biomarker in field applications) in estimating exposure to biotransformed organic compounds is a growing field. Biotransformation as a process by which a foreign chemical substance (generally an organic compound) is subjected to change within a living organism (Sipes and Gandolfi, 1986). In general, biotransformation may render a foreign chemical less toxic or easier to excrete by making it more water soluble (Goksoyr and Forlin, 1992; Sipes and Gandolfi, 1986; Pritchard, 1993). However metabolites of contaminants may also be highly toxic (Kleinow et al. 1987). Excretion takes place via the gill, bile or urine.

Broadly, the biotransformation of organic contaminants can be divided into phase I and phase II processes Pritchard, 1993). During phase I reactions, enzymes catalyze the addition of an oxygen unit to the molecule (e.g., through hydrolysis or oxidation), thus rendering it more polar in nature. In the cell endoplasmic reticulum, the cytochrome P450 enzyme family are an example phase I enzymes. Induction by specific contaminants can cause enzyme activity to increase considerably (for example, Vindiman et al. 1991 documented an order of magnitude increase in P450 enzymes in Leuciscus cephalus exposed to organic contaminants compared with fish that were not exposed). During phase II reactions, conjugation of endogenous compounds takes place with metabolites produced in phase I. The result may be a reduction in the toxicity of the molecule through masking of their reactivity (e.g., as in glycosylation reactions). However, not all the metabolic reactions performed by these enzymes result in detoxification. Some organic compounds may be metabolized to products that are highly carcinogenic (Jimenez and Stegeman, 1990).

The cytochrome P450 enzymes play a major role in metabolism of endogenous compounds (such as steroid hormones) as well as exogenous compounds (Table 1, Nebert and Gonzalez, 1985; Goksoyr and Forlin, 1992). High levels of cortisol and dexamethazone have been found to induce activity of phase I enzymes (Schulte-Herman et al. 1988; Mathis et al. 1986). As cortisol is recognized as an indicator of general stress (Hontela et al. 1992), it is possible that stressed organisms may have higher levels of P450 enzymes than unstressed organisms. However, although well established as a response to acute stress (Mathis 1986), the possibility of elevated cortisol levels inducing cytochrome P450 enzymes in response to chronic stress exposure has not been documented in the field. It is possible that the ability of aquatic organisms to respond to stress is impaired at severely contaminated sites (Jimenez et al. 1990). For example, one study of contaminant-exposed northern pike (Esox lucius) and yellow perch (Perca flavescens) documented declines in cortisol levels compared to uncontaminated sites (Hontela et al. 1992).

Since P450 inducers are usually highly lipophilic and persist in sediments, the bottom feeding and dwelling organisms have a high risk of exposure. Studies on bottom feeders such as the Catostomus commersoni (white sucker) and barbel (Barbus barbus) have revealed high levels of P450 enzyme activities in liver tissue (Vindiman et al. 1991; Munkittrick et al. 1991; Melacon et al. 1992; Smith et al. 1991; McMaster et al. 1991). One study reported the barbel (Barbus barbus, a bottom feeder and dweller) to be twice as sensitive as other cyprinids (Leuciscus cephalus and Chondrostoma nasus) which live in the water column. Benthic species have been recommended as sentinel species for biomonitoring of pollution using ethoxyresorufin-o-deethylase induction as a contaminant indicator (Vindiman et al. 1991). Also, top predators may biomagnify persistent contaminants such as organics. This may have been the case with a study on redbreast sunfish (Adams et al. 1990).

One useful technique for isolating intake of contaminants in fish via ingestion of prey or sediments is analyses of P450 activities in the intestine (Van Veld et al. 1990). For example, high basal ethoxyresorufin-o-deethylase (EROD) activity in herbivorous fish has been attributed to the presence of natural inducers that may occur in their diet (Bradfield and Bjeeldanes 1987).

Laboratory based bioassays have documented increases in cytochrome P450 enzyme activities proportional to contaminant concentration (Pluta 1993; Jedamski-Grymlas et al. 1993). and complex mixtures of chemicals (Jimenez and Stegeman 1990). Increased P450 activities may provide useful information even if the inducing chemicals remain unidentified (Anderson et al. 1988) Among the more commonly documented inducers of cytochrome

P450 enzyme activity are polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins, and dibenzofurans (Nebert and Gonzalez 1985; Goksoyr and Forlin 1992; Lech 1982, Table 1). Most foreign organic contaminants are metabolized primarily in the liver.

Field studies of liver P450 enzymes in fish have demonstrated that they may be induced to high levels in response to specific organic contaminants (Jimenez et al. 1990). Fish caught from waters contaminated with oil (Lingstrom-Seppa 1988) or municipal wastes exhibited increased P450 activity compared to those in reference sites (Forlin et al. 1986). Another study documented elevated P450 enzyme activities for 80 km downstream of a site contaminated with organics (Vindiman et al. 1991). One study showed a good correlation between sediment contaminants loads and liver P450 enzyme activities in fish (Van Veld. et al. 1990).

There are several complicating factors in assessing exposure to organic compounds using liver cytochrome P450 enzymes as indicators of exposure and possible effects. These include diet, age, sex and reproductive state and the presence of a complex mixture of compounds (Giesy et al 1988; Jimenez and Stegeman 1990, Lemaire et al. 1992). All these factors are relevant considerations in the interpretation of field-collected data. Liver size may also play an important role in total enzyme activity. One study documented increases in total liver size in brown bullheads exposed to benzo(a)pyrenes at a contaminated site but no significant increases were detected in the activity of cytochrome P450 enzyme assayed (Fabacher et al. 1985). It is further possible that severe hepatotoxicity may result in significant declines in the ability to produce P450 enzymes in hepatocytes, owing to the degeneration of cell functional integrity (Jimenez et al. 1990; Jimenez and Stegeman 1990; Fabacher et al. 1985; Gallagher et al. 1989). Also, most measures of enzyme activity are made per microgram of microsomal protein. Hence, if total hepatic endoplasmic reticulum increase as a function of liver size, estimates of increasing enzyme activity may be masked to a certain degree (Nagyova and Ginter 1993). Such factors are important considerations in the interpretation of field collected data.

Estimates of cytochrome P450 enzyme activities are also complicated by the presence of other contaminants such as cadmium which inhibit induction (Forlin et al. 1986). Also, chlorine-induced gill damage has been found to increase uptake of polycyclicaromatic hydrocarbons and polychlorinated biphenyls in rainbow trout (Lingstrom-Seppa. 1990). (Stegeman et al. 1992). In addition, some organic contaminants, notably DDT, have been known to not induce P450 enzymes (Lech et al. 1982). Enzymes may also be inhibited by the inducer or some other chemical, resulting in reduced activities even when induction of the protein occurs (Jimenez and Stegeman 1990). Organic contaminants have different rates of metabolism in fish. Laboratory studies have found that English sole metabolize benzo(a)pyrene faster than naphthalene. Twenty four hours after fish were treated only 2% of the benzo(a)pyrene injected remained whereas 85% naphthalene remained in liver tissue (Varanasi and Gmur 1981).

During the reproductive season, males and females could have different levels of phase I enzymes in their liver, especially during prespawning and spawning periods. This effect may be linked to high levels of circulating estradiol in reproductively mature fish. For example, EROD activities in redbreast sunfish females declined during late spring through midsummer, which coincided with their spawning season (Jimenez and Stegeman 1990; Lange et al. 1993). This effect may be species specific since another study documented no significant differences in cytochrome P450 enzyme activities between males and females, but it was unclear whether these fish were collected during their reproductive season (Vindiman et al. 1991).

Two major effects must therefore be considered (a) effects of cytochrome P450 enzyme-inducible compounds on certain physiological processes (Goksoyr 1992) and (b) effects of increases in stress-induced cortisol levels on cytochrome P450 enzyme activity.

Cytochrome P450 enzymes are involved in the synthesis and biodegradation of a number of physiologically important compounds (Table 1). Studies on mammals have shown that steroidogenesis of certain steroids occurs through a series of monooxygenase enzyme reactions (Higuchi et al 1991). Also, endogenous compounds such as cortisol are metabolized in liver microsomes (Abel and Back 1993). These factors must be considered in field-based studies of cytochrome P450 enzyme induction. For example, high activities of cytochrome P450 enzymes in fish livers may cause reduction in the levels of circulating steroids which are important for reproduction. High activities might thus impair reproductive success (Lech 1982). Increased levels of these enzymes were associated with decreased levels of steroids and decreased reproductive success in white suckers in a field-based study where the fish were exposed to bleached kraft pulp mill effluents (Munkittrick et al. 1991).

Metallothioneins

Metallothionein is a low molecular weight, cysteine rich protein with a high binding affinity for heavy metals. Metallothionein-like proteins were isolated in fish (Roesijadi 1992, Krezoski et al. 1988; Kille et al. 1992). In vitro studies have shown that virtually all metal ions can bind the apo-protein form of metallothionein (Neilson et al. 1985). Assessment of metal exposure to aquatic species have been conducted using metallothionein (or metallothionein-like protein) assays (Garvey 1990; Doherty et al. 1987). However, laboratory studies have demonstrated induction only by selected metals, such as copper, zinc, mercury and cadmium. Metallothioneins bind metals in the following decreasing order of affinity: Hg > Cd > Cu > Zn although there seems to be some debate about the order of affinity of metallothionein for copper and cadmium (Eaton 1985; Petering et al. 1990; Ley et al. 1983).

The synthesis of metallothionein is initiated by transcriptional induction. Metal ions entering cells bind to a transcriptional factor which then binds to an appropriate segment of the DNA. This results in metallothionein-mRNA transcription which results in the synthesis of the apoprotein that binds the metals found in the cellular compartment (Roesijadi 1992).

Metallothioneins have been regarded as two pools (a) basally synthesized protein involved with regulation of essential metals and (b) induced proteins involved with detoxification (Roesijadi 1992). Basal metallothionein appears to function as part of a cellular compartmentalization/sequestration system that has evolved to regulate uptake and tissue distribution of essential metals such as copper and zinc via storage and displacement from their metallothionein-bound forms respectively (Ley et al. 1983; Saunders 1990). Metallothionein occurs in basal concentrations in most cells (Petering et al. 1990). As copper and zinc are essential metals required for biochemical reactions, zinc- and copperthionein are present in most cells in fish. Induced metallothionein binds with exogenous metals to render them inactive, essentially detoxifying them by sequestration (Hogstrand and Haux 1991).

Metal toxicity seems to occur after the "capacity" of the cells to produce metallothionein is exceeded (Squib et al. 1984). It has been proposed that exposure to a higher concentration of metals than metallothionein can detoxify, results in "spillover" into other cellular compartments, leading to toxic effects (Hamilton and Mehrle 1986; Brown and Parsons 1978; Brown 1990). If the spillover hypothesis is true, then elevated metallothioneins can be used as a good early warning detection system for metal stress. Field tests have found correlations between metal exposure and heavy metal levels (Hogstrand and Haux 1990). However, there are complicating factors that make interpretation not as straightforward. For example, toxic action could occur before the maximum metallothionein induction capacity is reached in an organism (Brown et al. 1990).

Induction of metallothionein above basal levels can take place by exposure to several non-metallic stressors (Table 2, Petering et al. 1990, Steadman et al. 1991) and it has even been included under the category of stress proteins (Saunders 1990). An understanding of the specific intracellular function of metallothionein and the biological and environmental factors that influence such functions is still limited (Roesijadi 1992). A number of studies have demonstrated that metallothionein induction in response to general stress is not as high as induction in response to copper and zinc exposure (Kille et al. 1992). While significant increases in metallothionein have been detected in response to cortisol or dexamethazone, 2-3 fold increases were observed in response to copper and zinc exposure. This may be a helpful factor in separating possible causes of metallothionein induction. Another study documents much greater induction in metallothionein for metals (20-50 fold) than in response to glucocorticoids (2-4 fold, Brady 1982).

Metallothionein has also been found to be induced by stressors other than heavy metals. Studies on largemouth bass have shown that restraint stress results in mobilization of endogenous zinc and copper from the plasma. The copperthionein fraction of metallothionein was found to increase in liver tissue in response to a twenty minute-long exposure to restraint stress each day for a period of three days (Petering et al. 1990). Heat-shock, nutrient deficiency, restraint and exposure to oxygen radicals are examples of factors that result in gene expression of metallothionein protein (Honda et al. 1991).

Field studies with metallothionein have revealed elevated levels in response to exposure to heavy metals (Harrison and Lam 1986; Malins and Ostrander 1991). A field study found significant correlations between hepatic copper, zinc and cadmium and hepatic metallothionein levels respectively (Hogstrand and Haux 1991; Hogstrand and Haux 1990). As the liver is a primary detoxification organ for metal contaminants, it is useful for assays of metal exposure (Harrison and Lam 1986). Induction time for metallothionein in fish appears to be quite species-specific (Hogstrand and Haux 1990).

Metallothionein has a complex biology that may be species-specific

(Engel and Brouwer 1987). It may be necessary to determine the basal levels of metallothionein to really be able to interpret field collected data, where they may be high regardless of presence or absence of metal stress to organisms. For example, stress-mediated synthesis of metallothionein may also occur through extracellular signals such as glucocorticoid-induced synthesis (Cousins 1985). One suggested solution to this problem of determining causes of high levels of metallothioneins in the field is determination of plasma glucocorticoid levels to assess the presence of acute stress (Petering et al. 1990). However, this technique may be difficult to use under field conditions as capture stress itself may confound the results obtained.

It is possible that excretion of metals may eventually occur through the breakdown of blood-borne metallothionein in the kidney tubule cells (Petering et al. 1990). In the presence of certain organic compounds, inhibition of metallothionein production may occur. Low liver metal concentration has been found in a field study on fish around municipal wastewater outfalls where sediment metals are high in concentration (Brown et al. 1987). A complex mechanism of reduced metallothionein synthesis has been proposed, through increasing demands for cysteine residues for the purpose of glutathione synthesis.

Chronic heavy metal exposure may cause natural selection for organisms which can produce high metallothionein levels likely to sequester the heavy metal contaminant (Klerks 1992). This factor may be associated with a higher resistance to heavy metal stress. This can take place through gene duplication of the metallothionein gene, resulting in ability to form higher concentrations of the protein in cells (Theodorakis et al. 1992). Another mechanism that could be involved with resistance is an enlarged pool of preexisting metallothioneins (bound to endogenous metals such as zinc, Roesijadi 1992). However, metal resistance in exposed organisms may be a function of both a reduced accumulation of metals and an increased ability to sequester metals. Also, the relationship between elevated metallothionein and resistance to metal toxicity in fish remains unclear (Roch and McCarter 1984). Recently, studies on field collected Drosophila showed an increase in metallothionein-RNA and gene duplication of the metallothionein gene occurring in copper-resistant specimens (Klerks 1992). This may imply a higher tolerance for metals that can be sequestered by metallothionein.

Hepatosomatic Index (HSI)

At the organ level, the hepatosomatic index (HSI) is a frequently used indicator of stress in fish (Goede and Barton 1990). This is the ratio of the fish liver weight to the total fish weight and may increase or decrease, depending on the stressor(s) to which the fish is exposed. Liver size can increase through an increase in the individual number of cells (hyperplasia) or through an increase in overall cell size (hypertrophy). HSI responds differently to various chemical stressors (Goede and Barton 1990). For example, HSI has been found to decline in response to acidity in fish and in rats has been found to decline in response to metal toxicity (Lee et al. 1983). There is also evidence that liver size can significantly vary as a function of starvation stress, presumably in response to depletion of glycogen supply, although the evidence here is conflicting (Barton et al. 1988). Increase in liver size as a function of stress from exposure to contaminants has also been observed (Steadman et al. 1991; Adams et al. 1990). Fuel oils and complex mixtures of contaminants have been found to increase HSI in fish.

Summary: Applications of Biomarkers

The field applications of biomarkers is a growing area of research. The majority of studies on biochemical/cellular level biomarkers have been carried out for areas where existing chemical data have documented possible sources of exposure stress to resident biota. However, most of the existing techniques for exposure identification have not been standardized, and a wide variety of techniques are currently used for exposure assessment. Establishing standard values for field use of biomarkers is critical to their application. For example, a key problem with biomarker measures is that for many species currently studied in the field, the "normal" range of the indicator under study has not been established (Niimi 1990, Huggett et al. 1992). Most field-based biomarker studies use uncontaminated site conditions a reference sampling stations, assuming that they represent the "normal" range of levels for the biomarkers used (Niimi 1990).

It might also be useful to compile a list of sentinel species for exposure identification in the field in order to standardize assessment approaches based on the use of biomarkers. Individual species respond to pollutants to varying extents, which necessitates the selection of an appropriately sensitive species for exposure studies. Studies assessing sediment contaminant effects on organisms often use bottom-dwelling fish (Munkittrick et al. 1991; Varanasi et al. 1981) which appear to be highly sensitive to sediment contamination due to their feeding habits and to more direct contact with sediment-bound contaminants (e.g., organic compounds).

An important problem is discerning between complex biochemical responses to general stress (not necessarily with a chemical as the source) and stress due to contaminant exposure (Huggett et al. 1992). Most commonly used biomarkers at the biochemical level are required for a wide range of physiological functions, and levels may vary with the physiological state of the organism (Stegeman et al. 1992). One possible solution to this second problem is the validation of biochemical-level exposure effects observed in the field with laboratory-based studies (Theodorakis et al. 1992). However, there may be inherent problems with such comparisons. Factors that may contribute to differences between laboratory and field data include chronic exposure under field conditions, and higher level biological interactions (Theodorakis et al. 1992; Adams 1992). To facilitate such comparisons, long term exposures in the laboratory might be more appropriate to mimic field conditions than short term exposure experiments .

Site selection must also be conducted with careful consideration of physicochemical conditions, to make comparisons valid. Temperature is an important factor in the field application of biomarkers so both reference and contaminated site should have similar temperatures. Poikilotherms can produce higher levels of proteins (including specific enzymes) at elevated temperatures. For example, a study in bluegill sunfish demonstrated that benzo(a) pyrene metabolism tripled with a 10 degree rise in temperature (Jimenez et al. 1987). In contrast to this study, fish acclimated to colder temperatures have been found to exhibit greater cytochrome P450 enzyme reactivity than those at higher temperatures (Kleinow et al. 1987). However, species (e.g., trout) may show compensation for enzyme activity at new temperatures, thus possibly lowering their sensitivity to temperature changes over longer periods of exposure.

The advantage of assessing stress at lower levels of biological organization is that they may serve as early warning systems to detect potential stressors before population- and community-level effects take place (Adams et al. 1990). Also, such indicators can be pooled to rank sites according to condition (Stein et al 1992). However, most studies that attempt to link responses at more than one level of biological organization have largely been conducted on systems where degradation has already affected higher levels of biological organization (Adams et al. 1990; Dickson et al. 1992, Munkittrick, et al. 1991). Hence, long term documentation of water resource condition is needed to provide evidence for the early warning hypothesis.

Biochemical and cellular-level biomarkers are now well established as techniques for confirmation of contaminant exposure (Adams et al. 1990; Huggett et al. 1992, Munkittrick et al. 1991 Payne art). However, such studies document exposure response in organisms, but do not necessarily document ultimate effects of exposure on the health of the organism (Heath, personal communication). Thus, linking biomarkers to higher levels of biological organization (which indicate effects on organisms and populations) is a criterion that would make the field use more ecologically relevant. Reproductive indicators of stress may be useful in such an approach as they involve both short term response and long term consequences in fish (Adams, 1990). Hypothetically, biochemical responses to exposure could result in allocation of energy resources to maintenance of homeostasis, thus resulting in the decline of overall health of the individual (Giesy et al. 1988).

Selected field studies have been able to document possible links between biochemical exposure responses and possible physiological effects that could result from such exposures. One study showed declines in clutch size of females in response to contaminant loads and a corresponding increase in the amount of cytochrome P450 enzyme (Adams et al. 1990). Biochemical indicators of stress were also linked to individual level responses (e.g. in aryl hydrocarbon hydroxylase activity in response to dioxin downstream matched the high incidence of fin ray anomalies and increase in liver size). Studies on the white sucker exposed to bleached kraft pulp mill effluents showed significant increases in cytochrome P450 enzyme activities and a correspondingly low reproductive performance (measured by parameters such as gonadal size and age to maturity) at the contaminated sites compared to two reference sites (Munkittrick et al. 1991). Exposed organisms showed increased age to fecundity and abnormal growth rate compared to fish at the reference site. These effluents also were shown to negatively affect reproduction by acting on the pituitary-gonadal axis at multiple points (Munkittrick, et al. 1992).

At the physiological level, biomarkers have been linked together to yield an overall health index (Adams et al. 1990). Parameters included in the development of the health index included DNA integrity, RNA/DNA ratio (a growth indicator) and cytochrome P450. Site ranking using the combined index matched with site condition (Adams et al. 1990).

Studies on sunfish in a contaminated stream found low enzyme (EROD) activities at the most contaminated site, while high values were reported in sites further downstream (Adams et al. 1990). The cause of such a trend was hypothesized to be due to hepatotoxicity. A separate laboratory study revealed hepatotoxicity in fish exposed to high doses of the contaminants found in the stream.

The Index of Biotic Integrity

The index of biotic integrity (IBI) is a community level indicator of fish health used to assess degradation to freshwater streams (Karr 1981). The basis of the IBI is that stream communities are altered in response to degradation. Furthermore, the extent of that change is proportional to the degree of degradation in the stream. The advantage of the IBI is that it detects cumulative human impacts from multiple sources over time that are detrimental to native fish communities (Karr 1991). Initially used to assess degradation to midwestern streams, the IBI has been applied to the Clinch River basin system in Virginia (Fausch et al. 1990; Angermeier and Smogor 1992).

IBI estimates the health of a fish community in a stream relative to what would be expected of it were it in an undisturbed condition. In the IBI, twelve community-, population- and individual-level attributes of stream health are used to characterize stream condition (Table 3, Fausch et al. 1990). These attributes may be broadly divided into structural (e.g., species composition related metrics), functional (e.g., metrics estimating proportion of total number of species in one trophic group) and miscellaneous attributes (e.g., total number of organisms with anomalies). Each attribute is assigned a score that reflects the extent to which it deviates from its expected value under ideal conditions (i.e., in an undegraded system). The maximum value of a score is 5 (assigned to a metric which closely resembles those under ideal conditions) and the minimum score is 1 (assigned to a metric that deviates substantially from that expected under ideal conditions). For example, if the total number of intolerant species equals or highly deviates from that expected of an undisturbed stream, the score assigned to that metric is 5 or 1 respectively. The sum of all metric scores yields an IBI score related to the health of the ecosystem as quantified by attributes of its fauna.

The IBI includes individual, population and community metrics. Structural community attributes used in the IBI include metrics such as total number of native fish species (a community level metric) and fish abundance (a population level metric). These species richness or diversity metrics can decline in response to degradation. Functional attributes that are used include proportion of individuals as top carnivores (Karr 1991). The IBI also contains metrics that reflect individual health such as counts of total number of fish with anomalies (e.g., lesions, fin degradation etc.)

In North America, the state of Ohio has incorporated the IBI into its monitoring and regulatory framework (Ohio EPA 1987), and has found them to be sensitive indicators of a wide range of degradation. The IBI has been used in a variety of contexts to detect effects of mine drainage, sewage effluents and habitat alterations (Karr et al. 1986; Miller et al. 1988; Steedman et al. 1988). The IBI has been found to be sensitive to habitat degradation and to degradation from toxic wastes entering aquatic systems. For example, IBI has been documented as is sensitive to streams degraded by high levels of sedimentation caused by deforestation and other land use modification (Crumby, 1990). Also, incidence of blackspot disease (an individual level components of the IBI) was found to be agricultural degradation (Steedman et al. 1988). The IBI has been used in conjunction with physicochemical data in order to isolate possible causes of stress in aquatic biota (Karr 1985). In the Clinch River Basin system, an additional habitat-specific metric (proportion of individuals as lithophilic spawners) has been added to increase sensitivity to habitat degradation (Angermeier and Smogor 1993).

Comparison of individual-level indicators of stress with population- and community- level indicators of stress

Biomarkers provide evidence of exposure responses in individual organisms, whereas population- and community-level effects provide clues as to the possible consequences of exposure. The hypothesis that links biomarkers to ecologically relevant effects is that effects radiate from lower to higher levels of biological organization (Adams 1990). Few studies have attempted to link short-term indicators with ecologically relevant indicators at population levels of biological organization that might provide evidence of a causal relationship between exposure responses and subsequent effects. No studies have so far been done on the relationship between community-level indicators and biomarkers. Interpretation of such data is difficult due to the complexity of aquatic systems where several factors can influence estimates of population- and community-level effects (Adams 1992; Giesy et al. 1988; Huggett et al. 1992). Population-level effects of specific contaminant exposure have been observed for sunfish and for white sucker. Mean sizes of a sunfish population were found to decline in response at a contaminated site compared to reference site (Adams et al. 1990). Studies have documented a possible relationship between individual- and population-level effects of contaminant exposure (Munkittrick et al. 1990; Munkittrick et al. 1992; McMaster et al. 1991). A comparison of laboratory studies on ambient toxicity and field-based studies on biological response (e.g., species richness measures) at the community level have shown a good relationship between the two (Dickson et al. 1992).

Levels of individual biomarkers are highly variable under field conditions. Among other factors that should be considered. Nutritional and reproductive status may also effect the levels of some proteins assayed. A well known example of this is depressed levels of P450 enzymes in females during their reproductive season. High circulating estradiol levels are thought to have some affect on the depressed activities of these enzymes (Hanssen et al. 1982; Jimenez and Stegeman, 1990; Petering et al. 1990). Nutritional status may cause elevated levels of metallothionein (Petering et al. 1990). Another problem to keep in mind with such comparisons is the high variability in field collected data, which makes it necessary to define efficient sampling strategies and statistical methods for analyses (Pluta, 1993). Among biochemical biomarkers commonly used, cytochrome P450 enzyme level variability as a function of species, size and sex and maturity and season are to be kept in mind while design a study. Hence it may be appropriate to collect high sample sizes to minimize the effects of such variables.

Methodology

Site history

Human activities that could cause degradation to the numerous sites in the Clinch River drainage include coal mining, agricultural, industrial activities and urbanization. In addition, sporadic impacts in the Clinch River in the past have been responsible for much damage (e.g., two major spills into the river, at Carbo, Virginia, that killed thousands of fish in 1967 and 1970, Neves and Angermeier 1990). Based on current land use information, observations and available data (i.e., IBI data on all sites, chemical and benthic community data on selected sites) it is possible to list likely causes of stress at impacted sites surveyed in this study. Relatively unimpacted sites were used as reference locations for this study.

Angermeier and Smogor (1993) concluded that impacts on the Clinch River Basin came predominantly from widespread sources (e.g., nonpoint sources such as surface coal mining) throughout the area linked to land use rather than to specific point-source discharges. Surface coal mining is practiced in numerous areas drained by many of the tributaries flowing southwards into the Clinch River (e.g., Toms Creek, Guest River, Swords Creek and Dumps Creek) and is likely to impact these sites. Agriculture is widespread throughout the Clinch River Basin and is likely to have degraded streams such as Elk Garden Creek. Also, streams such as the Guest River and Big Cedar

Creek are probably degraded by more than one source (e.g., coal mining and urbanization in some reaches of the Guest River; agriculture and urbanization in Big Cedar Creek). Sites such as Little Cedar Creek appear to suffer from urbanization effects, as it runs through the town of Lebanon. Overall, widespread degradation to tributaries has several possible causes.

Recent IBI data (collected in1991-93) indicate degradation to fish communities at numerous locations in the Clinch River drainage (Table 4, Angermeier and Smogor, 1993). Overall, the IBI data indicate that numerous tributaries are in poor or fair condition, while all Clinch River mainstem sites are in good or excellent condition. The US Environmental Protection Agency (EPA) has long term monitoring sites at five locations along Clinch River, and one location each at Guest River, Little River and Copper Creek. Average values of selected chemical data over a period of 12 years (1981-1992) indicate low levels of metal contaminants in the water and high hardness throughout the sites sampled (Table 5). In addition, EPA has conducted a Rapid Bioassessment Protocol (a community-level index of stress to benthic organisms in streams) on five sites in the drainage (Table 6). According to those data, both Guest River and Dumps Creek (both sites used in this study) are categorized as moderately impaired. The Clinch River (Dunganon) had an IBI score at the lower limit of the "good" range. This site was located close to a railroad switchyard, which may contribute to degradation. In addition, Dunganon is located directly downstream of the Guest River, which may contribute to its level of degradation.

Additional chemical and biological data on selected sites from the EPA STORET database were included for comparison (Tables 5 and 6). Data collected in 1992 documents polychlorinated biphenyls (PCBs) in the sediment (680 ug/g) in the Clinch River at Dunganon (2 miles upstream from the sampling sites used in this study) Rapid bioassessments (RBA) using macroinvertebrate communities were conducted in selected tributaries in 1992 at sites that were within 1.5 miles upstream or downstream from corresponding sites used in this study (Table 5). In addition, RBAs were conducted in the Clinch River sites that were 9 miles upstream from the Clinch River at St. Paul site used in this study (Table 6). These studies reported that Dumps Creek and the Guest River were moderately impaired sites, while Copper Creek and two Clinch River sites downstream of Dumps Creek were in good condition.

Site selection for biomarker measurements

Sites were selected from a wide range of land uses throughout the Clinch River drainage in Virginia, in order to assess possible sources of degradation (possibly to link degradation to land use). Sites selected included all major tributaries that were substantially degraded according to available community-level IBI data (e.g., the Big Cedar Creek,17 and the Guest River drainages, 19 and 20, were each sampled at three locations, Figure 2). Sampling location for each stream was identical to that used for IBI studies to facilitate comparisons with results from these studies. In the tributaries, most IBI sites were located close to where each drained into the Clinch River (Angermeier and Smogor, 1993) to assess effects of the streams on the mainstem of the Clinch River. Large streams were sampled at more than one location (e.g., the Guest River). In addition, selected smaller streams suspected of impacting to the water quality of larger tributaries were sampled (e.g., tributaries of Big Cedar Creek and Guest River, Table 4).

Twenty one sites were selected throughout the Clinch river drainage (Figure 2). All major tributaries draining into the Clinch River (i.e., Copper Creek, Guest River, Indian Creek, Little River and Big Cedar Creek) were sampled. Stream order of most sites was 4; however there were a few sites with stream order of 5 or 6 (e.g. all three mainstem sites were sixth order, Table 4). The three mainstem sites were in good or excellent condition according to IBI data. The remaining sites are all tributaries of the Clinch River in a wide range of site conditions ranging from good to poor as indicated by IBI data (Table 4, Angermeier and Smogor, 1993). Eight of the selected sites for this study were in poor to fair condition according to IBI data. All these sites corresponded to areas impacted by mining, agriculture and/or urbanization.

For comparative analyses of site condition, selected reference sites were assumed to be in good condition. All reference sites were in good or excellent condition according to IBI data. Data from reference sites were pooled to compare with sites suspected of impact (Angermeier and Smogor, 1993). One or more of the following criteria were used to select reference sites: (1) IBI of at least 49, (2) no known history of major contaminant sources entering the stream, (3) available EPA STORET data indicating no impairment.

Sampling

Fish species selected for the study were northern hogsucker (Hypentilium nigricans), rock bass (Ambloplites rupestris) and striped shiner (Luxilus Chrysocephalus). Northern hogsuckers are bottom dwelling insectivorous fish, striped shiners are column fish that feed on insects in the water-column and rock bass are top predators (insectivore-piscivores, Angermeier and Smogor 1993). Each was habitat-specific and/or represented one position at a unique trophic level. Each trophic group might represent a unique profile of stress exposure. For example, bottom feeders have been documented as being more sensitive to sediment-bound lipophilic organic contaminants than fish that obtain food from swimming in the water column (Vindiman et al. 1991). Also, possible biomagnification effects of contaminant exposure could be explored using fish from different trophic levels (Niimi 1990).

None of the species selected have been previously tested for bioindicator levels. All fish selected were classified as moderately tolerant (defined as fish that are present under degraded condition) to impairment (Angermeier and Smogor 1993) and were abundant at most sites selected. Capture was carried out during the months of July and August. Depending on availability, six to ten specimens from each species were captured at each site. Fish were captured by electrofishing (alternating current) using a backpack unit in smaller streams or an electric seine in the larger streams. Fish were kept in buckets for the duration of electrofishing, then transfered into a dry ice (-78°C) cooler. Frozen fish were transported to the laboratory within 48 hours, where they were stored in a freezer (-80° C) until further processed.

Sample Processing

Fish were thawed for dissection and weighed. Sample processing followed the technique used by Steadman et al (1991) with minor modifications. In order to remove excess blood from the sample, extracted liver tissue samples were rinsed in an ice-cold solution of 154 mM KCl buffered with 10mM Tris-HCl in distilled deionized water at pH 7.6, and vortexed. Rinsed tissue samples were blotted, weighed, and homogenized in 250mM sucrose, 2.5 mM dithiothreitol (to increase microsomal fraction, Lake, 1990), and 50 mM Tris-HCl using 4ml/g of tissue at pH 7.6 on ice. Owing to small size, the liver samples from striped shiners were pooled (two livers per sample) to increase the total amount of liver tissue. Tissue was homogenized in a model 985-370 Biospec tissue tearor. Homogenates were then centrifuged at low speed for 10 minutes at 4° C in a Sorval RC-5B superspeed centrifuge (10,780*g*). The resulting post mitochondrial supernatant was centrifuged at high speed (179,000*g*) for 30 minutes using a Beckman TL 100 ultracentrifuge at 4° C to separate the microsomal fraction from the cytosol (Lake, 1990). The supernatant was frozen at -20° C and set aside for further metallothionein analyses. The microsomal pellet was resusupended in solution by homogenizing in 0.1 mM Tris-HCl solution buffered at a pH of 7.6, and analyzed the same day for enzyme activity.

Analysis of 7-Ethoxyresorufin-O-deethylase (EROD) activity

EROD activity was estimated by the technique of Burke and Mayer (1974). The substrate ethoxyresorufin was dissolved in 0.1 mM Tris-HCl (pH 7.6) by sonication, in an ultrasonic water bath for one hour. Depending on the amount of microsomal suspension present, 0.2 ml or 0.5 ml of microsomal preparation was added to 10 µm ethoxyresorufin substrate in 0.1 µm tris buffer to make a total volume of 2ml in the reaction mixture. A baseline of nonenzymatic activity was recorded for one minute and then the reaction was initiated by adding 10ul of 10 mM nicotinamide adenine dinucleotide phosphate (NADPH). The formation of resorufin product was monitored flourometrically (excitation 530 nm; emission 585 nm) with a McPherson FL-750 Spectrofluorescence detector. The relative increase in the slope of the baseline (rate of fluorescence change per unit time) was recorded for 1.5 minutes. The amount of resorufin was determined using an internal standard on the reaction mixture by adding ethoxyresorufin standard to the reaction mixture in each cuvette.

Protein Assays

Microsomal protein (MP) was quantified using the bichinchoninic acid protein assay (Smith et al. 1985). 2 ml of a mixture of bicinchoninic acid solution and copper sulfate pentahydrate (4% solution) in a 50:1 ratio were added to10 ul microsomal protein in a cuvette. Five concentrations of diluted 1 mg/ml bovine serum albumin (BSA) were used as standards, and 2ml of each concentration were used for spectrophotometric analyses. The cuvettes were incubated at 37° C for 30 minutes and then readings were taken at 562 nm using a spectrophotometer. Water was used to zero the instrument. The absorbance of the blank (a solution of bicinchoninic acid solution and copper sulfate pentahydrate with no protein added) was subtracted from the absorbance of the remaining absorbance tubes to obtain the final reading for absorbance.

Metallothionein assay

Metallothionein (MT) protein was quantified indirectly using the cadmium back saturation assay following the procedure of Eaton and Toal (1982, 1983). An aliquot of the supernatant from the high speed centrifugation was boiled for 3 minutes to eliminate any protease activity that might be present. Samples were then saturated with cadmium for ten minutes to ensure its complete complexation with metallothionein. All non-metallothionein bound cadmium was then removed by adding 2% hemoglobin solution to the cadmium-saturated samples. Samples were incubated for ten minutes to ensure binding of all excess cadmium to metallothionein, and then boiled for 3 minutes to denature the hemoglobin-cadmium complex. Centrifugation was carried out at 10, 000g to remove the hemoglobin-cadmium complex and the supernatant was transfered into acid-washed glass tubes and digested in concentrated nitric acid at 70° C for 16 hours or until a clear solution of digested material was formed. Samples were assayed for cadmium by using the furnace on a Perkin Elmer atomic absorption spectrophotometer.

Analyses of liver copper were also conducted at a subset of sites. Homogenized liver samples were extracted with nitric acid and analyzed for copper by atomic absorption spectroscopy

Bile Fluorescence

Preliminary data on total bile metabolites was obtained using fish from one impacted site and one reference site. Northern hogsucker from Little River in Richlands (reference station) were compared to those collected from Little Cedar Creek (impacted station). Although equal number of specimens were collected at each site, fewer bile samples were available at the Little River site (n=3) than at the Little

Cedar Creek site (n=9) due to the fact that the gall bladder of most fish were empty in fish from the former. Fish were dissected and bile samples were collected by injecting a syringe into the gall bladder and collecting bile fluid.

Fluorescence in the bile of northern hogsucker was estimated by diluting each sample in distilled deionized water in a ratio 1:200. Fluorescence was read at the following excitation and emission wavelength pairs respectively: 290, 335; 256, 380; 340; 380 and 380, 430 nm using a McPherson FL-750 Spectrofluorescence detector. A comparative study of bile metabolites detected using fluorescence detection and HPLC found a .99 correlation between the levels detected by each technique, thus reflecting the accuracy of the fluorescence detection technique (Lin and Courmier, personal communication). Fluorescence detected was expressed simply in fluorescence units (Britvic et al. 1993), normalized to a value of 5 units for each wavelength at the reference site.

Site comparisons

All comparisons between impacted and non-impacted sites were made assuming that high IBI score indicated little or no stress to the community. However, there were sites in the drainage where land use observations and existing chemical data were also used to explain observed trends in bioindicators.

Statistical analyses

Comparisons between sites suspected of impact and non-impacted sites were made all the by pooling all the data from pooled reference sites. It was assumed that values of bioindicators at reference sites expressed the "normal" range of these indicators in fish in relatively good condition (Niimi 1990). The reference pool was selected on the basis of (a) IBI score (>49 qualified as a possible site) and (b) EPA STORET data and (c) land use observations/history that might indicate that the site was relatively undisturbed. Two mainstem sites and two Little River sites were primarily used as reference sites, depending on the availability of data for each test conducted (Table 7).

Most tests of field data apply stringent standards for committing type I errors, which increases the likelihood of committing type II errors and of overlooking biologically significant data (Toft and Shea 1988; Gelwick and Mathews 1992). In such experiments, it is possible to fail to reject a false null hypothesis and make erroneous conclusions as a result (Peterman 1990). For example, if at p=0.05 the power of a test is (1-ß)= 0.85, at p=0.01, the power goes down to (1-ß)= 0.66 (Toft and Shea 1988).

Type I error is the chance of rejecting a true null hypothesis and Type II error is the chance of failing to reject a false null hypothesis (Toft and Shea 1983). When the power of an experiment is likely to be low (as is often the case in field-based ecological experiments) a value larger than the traditional p value has been recommended as appropriate for increasing statistical power and balancing type I and type II errors (Gelwick and Mathews, 1992; Threlkeld and Drenner 1987). In these experiments, an alpha value of 0.1 was selected as the significance level to balance the type I and type II errors (Gelwick and Mathews 1992).

When there are numerous ANOVAs reported within one experiment, there is an increased risk of experiment-wise error. As there were multiple site comparisons made, the significance was selected at the p/n level, where n was the number of between-sites comparisons made. In order to add statistical power to the comparisons made, the sequential Bonferroni test instead of the standard Bonferroni test was used to determine significance (Rice 1989). If the smallest p value was $< 0.1/n$ (where n was the total number of comparisons being made), the test was considered significant. For example, if the total number of between-site comparisons made was 18, the significance levels was selected at $p= 0.1/18$ ($p= 0.0055$). For the next most significant difference a significance level of $p= 0.1/17$ ($p=0.0059$) would be selected, as the criterion for comparison and so on (Rice, 1989).

Spearmans rank correlations were run for IBI and several of its metrics and other components against bioindicators measured for rock bass and northern hogsucker. Striped shiners were not included in this analysis because data for striped shiners were pooled within sites for the same stream (e.g., data from both Little River sites was pooled to yield higher sample size). Both stream order-dependent, and stream order-independent metrics and components of IBI were tested for possible relationships with bioindicators examined. In the case of stream order-dependent values (such as number of intolerant species, total number of native species and species with fin degradation) only fourth order streams were compared.

Results

Hepatosomatic index (HSI)

Comparisons between sites primarily impacted by urbanization revealed significantly higher values of HSI at Big Cedar Creek, and the two Indian Creek sites than at reference sites (Table 8). HSI was also significantly higher at Elk Garden Creek and Guest River at Wise than at reference sites when the Bonferroni correction was not applied (Table 8). Spearmans correlations between HSI and individual IBI components (including individual metrics and total IBI) revealed a positive correlation with number of intolerant species and a negative correlation with proportion of omnivores (Table 11). No other IBI components examined were correlated with HSI.

Metallothionein (MT)

Concentrations of MT (as cadmium) in fish liver tissue were significantly higher in rock bass than in northern hogsucker across all reference sites (Table 9). For rock bass, significantly high values of MT were observed in Clinch River at Dunganon, Guest River at Wise, Stony Creek and Dumps Creek compared to reference sites used (Table 9). In addition, MT values were significantly high at Little Cedar Creek, and at Swords Creek than those of reference sites. Big Cedar Creek and Lick Creek had an elevated MT values compared to reference sites without taking the Bonferroni correction into account.

For northern hogsucker the levels of MT were significantly higher at Stony Creek than those of reference sites and at the North Fork of the Clinch River site at Duffield (Table 9). Levels were also slightly higher for northern hogsucker at Dumps Creek if the Bonferroni correction was not used. There were no significant differences in MT values for striped shiners between any impacted and reference sites (Table 9).

Spearmans rank correlations between MT and IBI components for northern hogsuckers showed significant correlations with total number of fish with anomalies, total number of invertivores, total number of piscivores and total IBI score (Table 10). Spearman's rank correlation also indicated significant correlations between IBI components and MT in rock bass for total number of fish with anomalies, proportion of piscivores, total number of native species and total number of fish with fin degradation (Table 11). Correlations between MT in rock bass, northern hogsucker and all other IBI components were insignificant (Table 10 and 11).

Copper in hepatic tissue

Hepatic copper concentrations were significantly higher for northern hogsucker than for rock bass at two sites (comparing rock bass and northern hogsucker at Dumps Creek and Guest River respectively). Dumps Creek was significantly higher in copper in rock bass liver tissue than the reference sites (Table 12).

Ethoxyresorufin-O-deethylase (EROD) Activity

Specific EROD activity (as defined by pmol/min/mg protein resorufin product) were calculated for all three species and total EROD (as defined by pmol/min/g liver resorufin product) was calculated for northern hogsucker and rock bass (Tables 13-14). EROD levels for rock bass were also similar to those of northern hogsucker across all reference sites.

For Rock bass, specific EROD was significantly higher than reference sites at Clinch River, Dunganon. The specific EROD level in northern hogsucker was significantly higher at Little Cedar Creek than at pooled reference sites. All EROD values estimated for the striped shiner were similar (Table 15). Also, specific EROD levels at Indian Creek (Richlands) were significantly lower for both northern hogsucker and rock bass than at reference sites, not taking into account the Bonferroni correction.

Total EROD levels in northern hogsucker were higher than reference sites at Toms Creek, Little Cedar Creek and at Big Cedar Creek. Total EROD levels in rock bass were higher than reference sites at Clinch River at Dunganon when the Bonferroni correction was not applied.

Spearman's correlations for both rock bass and northern hogsuckers were conducted between total IBI scores for all sites, several components of IBI (including individual IBI metrics), specific and total EROD (Tables 10 and 11). Specific EROD did not correlate with IBI. However, a correlation was found between specific EROD and several IBI components namely, proportion of tolerant individuals, proportion of omnivores and for the total number of native species (Table 10). Several correlations were inconsistent with what was expected. For example, proportion of tolerant individuals correlated negatively with with EROD, which would imply that as the proportion of tolerant individuals increased (and therefore stream quality decreased), the EROD levels in fish declined.

A significant correlation was found between total IBI and the total EROD score in northern hogsuckers (Table 10). Comparisons between several IBI components and specific EROD activity across all sites using Spearmans rank correlation revealed significant correlations for total number of intolerant species, proportion of tolerant individuals, proportion of omnivores and total number of native species for northern hogsuckers. Similar comparisons indicated significant correlations between EROD, proportion of piscivores and fin degradation for rock bass.

Total EROD in northern hogsuckers correlated with proportion of simple lithophilous spawners, total number of intolerant species , proportion of invertivores total number of native species (Table 11). However, in rock bass, no IBI components correlated significantly with total EROD. Also, there were negative correlations between total number of intolerant species and EROD.

Microsomal proteins (MP)

Total microsomal protein was calculated for the northern hogsucker and for the rock bass. Overall values of total microsomal protein were similar for the northern hogsucker and for rock bass. For northern hogsucker, microsomal protein levels at the Guest River at Coeburn, Dumps Creek and at Big Cedar Creek were significantly higher than reference levels. Levels compared without using the Bonferroni correction were also higher at Lick Creek. None of the sites tested for the rock bass microsomal protein levels were significantly higher than reference sites.

Spearmans rank correlation revealed that IBI correlated with northern hogsucker microsomal protein across all sites (Table 10). Similar comparisons of microsomal protein with individual IBI components revealed significant relations between microsomal protein and proportion of simple lithophilous spawners , proportion of tolerant individuals, proportion of invertivores and for the total number of tolerant species, and total number of fish with fin degradation across all sites. In rock bass microsomal protein levels, correlations were found with total number of fish with anomalies, proportion of piscivores and total number of species with fin degradation. Assuming that increased microsomal protein reflects stress in fish, there were some inconsistent Spearman's correlations between MP and certain IBI components. For example, total number of fish with fin degradation correlated negatively with MP, which was inconsistent with expected levels.

Bile Metabolites

Fluorescence levels of bile metabolites in northern hogsucker were significantly elevated at Little Cedar Creek compared to one reference station (Little River, Richlands). Increases in bile metabolites were detected at each of four wavelength pairs. Napthalene- (excitation=290 nm), phenanthrene- (excitation=256 nm), pyrenol- (excitation= 340 nm) and benzo(a)pyrene-type (excitation=380 nm) metabolites were detected (Figure 3).

Discussion

In this investigation, a suite of biochemical and organ level biomarkers were used to assess stress in fish resident to several sites in the Clinch River drainage. All species selected were abundant at the impacted sites (i.e., those subjected to numerous sources of stress) and at reference or relatively undisturbed sites. As in previous studies by others, fish with biomarker levels significantly higher than those at reference sites were assumed to be under stress or undergoing adaptive responses to human-induced environmental changes (Munkittrick et al. 1991; Saunders 1990). The diagnostic ability of each biomarker was evaluated in the context of suspected multiple sources of stress. In addition, interspecies comparisons were made in order to explore the use of each species used as indicators of anthropogenic stress Finally, comparisons were made between biomarker data across sites and species, and coexisting individual-population-, and community-level data (i.e., IBI data and its components) across all sites. These comparisons were used to explore the possibility that human-impact related responses at lower levels of biological organization could be linked to effects at higher levels of organization.

Overall biomarker assessments

Sites with high IBI scores (i.e., IBI > 49) were categorized as reference sites (e.g., Little River at Honaker, and at Richlands). Other sites in this study were termed "fair" or "poor" according to the IBI score and observations of human activities in each area and were also considered to be impacted from one or more sources of stress (Angermeier and Smogor 1993). High levels of biomarkers in fish were present at several "fair" and "poor" locations compared to those at reference sites (Table 16). With few exceptions, these were at sites that were degraded according to (a) IBI score, (b) available chemical data at the site (c) macroinvertebrate community data and/or (d) land-use information for the site (Table 16). For example in Tom's Creek, (a site categorized as poor by IBI, Table 4) there were high levels of total EROD in northern hogsucker hepatic tissue (Table 13). Also in Dumps Creek, located in close proximity to a coal mining area, high MT was found in rock bass (Table 9). This site was categorized as "moderately impaired" in an EPA rapid bioassessment survey of macroinvertebrates. It was also in the lower limit of the "good range" for IBI. However, other biomarker levels at these impacted sites were not significantly different from reference levels (Tables 13 and 14). Finally, there were several significant Spearmans correlations between biomarkers and community-level effects across all sites, thereby indicating that such initial biomarker responses to stress may be important among factors that ultimately determine community condition. Numerous correlations were showed trends that were the reverese of what was expected. For example, the correlation between total biomarker levels in fish at several sites documented as impacted according to IBI data (i.e., in poor or fair condition) showed relatively little or no response according to these data (e.g. northern hogsucker metallothionein levels in Toms Creek, Table 9). One example was at Guest River (poor condition) which was sampled at two sites. At the Guest River at Coeburn, rock bass MT levels were not significantly different from reference sites. However, MT values in Guest River at Wise site were significantly higher than those of reference sites (Table 9). Also, microsomal protein levels for Northern hogsucker were significantly higher than reference sites at Coeburn ($p<0.0001$) but not at Guest River, Wise (both sites are categorized as poor according to IBI data, Table 4). It is possible that poor condition of fish at some of these sites results in hepatotoxicity and inability to respond to external stressors.

Certain sites that showed significantly high levels of biomarkers appeared to be in good condition according to the IBI. For example, the HSI values at both Indian Creek sites were significantly higher than at reference sites. Rock bass MT at Clinch River (Dunganon, categorized as "good" by IBI) was also high. In a third example, northern hogsucker at the North Fork Clinch River site (Duffield, categorized as good by IBI) had high levels of metallothionein. Independent information for selected sites may explain some of these apparent discrepancies. For example, EPA studies document high PCB levels in sediments in the Clinch River site (at Dunganon), which is located adjacent to a railway switchyard (USEPA storet data). Such levels may have contributed to stress observed in fish. Also, a diesel spill occurred in 1992 at the North Fork of the Clinch River site. Fuel oils can induce higher metallothoinein levels in exposed fish (Steadman et al. 1991). There may not have been enugh time to observe changes in the IBI at this site.

Biomarker data for each site were highly variable. Variation can be attributed to influence of factors such as seasonal effects, age, size and sex, each of which could be species specific (Vindiman et al. 1991; Jimenez and Stegeman 1990). Other factors that any contribute to variability are alterations in water flow and local absences in contamination. Also, low biomarker levels may be due to absence of sensitivity to contaminants that are detailed in discussions on each biomarker. Many sites designated "impaired" from previous IBI data had slightly higher biomarker levels than those at reference sites. However, it is likely that due to small sample size and high variability, few were significantly higher than corresponding reference values.

Although the chemical data needed to confirm this was largely unavailable, contaminant stressors may be a cause of induction of high biomarker levels in this system. Previous field studies in other aquatic systems have documented high biomarker levels in fish from areas with metal and organic contamination respectively (Vindiman et al. 1991; Hogstrand and Haux 1990). These studies concluded that the contaminants were likely to induce a response in fish. For example, metal-induced high metallothionein levels is ubiquitous in fish (Roesijadi 1990), and it is likely that metal contamination would have induced a response in the species in this study. There is some evidence for metal contamination (e.g., copper) in the Clinch River drainage (Farris et al. 1988).

Existing land-use information on the Clinch River drainage implies that the impacted sites may be suffering from a variety of contaminant and other stressors including habitat degradation. Sites such as Guest River for example are impacted primarily by coal mining and urbanization (Table 4). Previous studies of aquatic systems exposed to a complex mixture of contaminants indicate that at the sites with the highest concentration of contaminants the cytochrome P450 levels were low (Gallagher et al. 1989; Fabacher et al. 1985), and one study attributed such responses to possible hepatotoxicity or response to a complex mixture of contaminants (Adams et al. 1990). If severe hepatotoxicity were occurring in fish from poor sites (e.g. Guest River which is heavily impacted by coal mining and urbanization) then a high response would not be expected for these fish.

Induced levels observed in biomarkers may not be attributed to contaminant stress alone. Evidence to support this hypothesis exist in the chemical data available for selected sites which indicated no detectable levels of chemical stress (EPA data). A second observation that may support this hypothesis is that responses seemed to vary with species. A third argument to support this hypothesis is that observed increases in biomarker levels in these sites were less dramatic (e.g., few elevations in biomarker levels by a factor of 2-5) compared to other investigations of such contaminant-induced biomarker elevation. For example, measures of the cytochrome P450 activity in fish from contaminated sites have been 3-5 times higher than reference values in redbreast sunfish (Jimenez et al. 1990). Also, field studies of metallothionein levels revealed that they increased by a factor of 2-4 in metal contaminated areas (Hogstrand and Haux 1990). Elevations in glucocorticoids appear to mediate increases in both metallothoinein levels and in cytochrome P450 enzyme activity (Brady 1982; Schulte et al. 1988). Thus, such elevations can occur from stressors other than contaminants Also, microsomal protein increases have been observed in response to general stress in laboratory studies (Steadman et al. 1991).

Although there were few elevated biomarker responses across sites, they appeared at sites that were categorized as stressed by IBI and other data. Overall, results not significantly differernt from those in reference sites may be due to (a) absence of or variable levels in contaminants or other stressors in fish surveyed at these sites, (b) undetectable low level contaminant responses in biomarkers (i.e., biologically significant effects that are not statistically significant), (c) toxic effects that render the organisms unable to synthesize proteins in response to stressed conditions as seen in a previous study (Jimenez et al. 1990, Gallagher et al. 1989), and (d) trends that appear to be the reverse of what is predicted for biomarker responses to stress (e.g., decline in biomarker levels as a function of stress).

Significant Spearmans correlations between IBI data and biomarker data were observed for several biomarkers of stress measured in northern hogsuckers and rock bass. Such results imply a possible link between lower and higher levels of biological organization. Specific causes of stress are unknown, and in most sites there are multiple possible sources of stress to resident fish. However, initial responses to stress could eventually radiate up to higher levels of biological organization (further discussed in a section on possible links between biomarkers and higher level effects).

Hepatosomatic Index (HSI)

Slightly elevated HSI levels were observed in both Indian Creek sites, Big Cedar Creek, Elk Garden Creek and Guest River at Wise (Table 8). These sites are probably impacted by land use activities (e.g., mining or agriculture) or by urbanization (Table 4). As HSI is known to rise in response to stress, it is possible that rock bass at these sites were stressed. With the exception of the Indian Creek sites, these streams are all currently in fair or poor condition according to IBI data. Fair or poor condition implies there is a potential for stressors to affect fish at these sites.

There is an absence of responses in HSI levels in all other sites suspected of being impacted by human activities. However, as stress can cause HSI to either rise or decline in value, this absence in response does not necessarily exclude the possibility of stress in resident fish. Unpredictable changes in HSI as a function of stress makes it unreliable as an indicator of stress in a field situation. Previous studies have shown that HSI changes depend on the sources of stress (Goede and Barton 1990). Increases or decreases in HSI in response to stress have been observed in white sucker, bullhead catfish and rainbow trout in response to multiple sources of contaminant stress (McMaster et al. 1991, Gallagher et al. 1989, Steadman et al. 1991, and Fabacher et al. 1985). Most impacted sites in the Clinch river drainage are exposed to multiple sources of stress, so it is not necessary that only HSI increase would be observed in these. Hence it is still unclear from this lack of increase in HSI whether these sites are under stress or not.

Metallothionein (MT)

Overall, elevations in rock bass hepatic MT relative to reference values occurred across several sites. Fewer responses were observed in northern hogsucker, possibly due to lack of sensitivity, of this species, undetectable MT components, or metal bound in other forms in that species. For example, copper present in hepatic tissues (probably as copperthionein) in northern hogsucker was generally higher than in rock bass (e.g., in Dumps and Stony Creek, discussed below).

There is a wide range of potential stressors (e.g., from contaminant sources such as coal mining, urban, and agricultural runoff) that could induce MT in fish in the Clinch River drainage. High MT values documented for rock bass in sites near coal mining activity (Guest River at Wise, Clinch River at Dunganon and Swords Creek) are possibly due to (a) low levels of metals that are present in runoff from these areas or (b) other stressors present that cause elevated corticosteroid levels in fish (Brady 1982). However, MT induction would probably occur in both species at sites where metal contamination was a problem, as it is a common response to metal stress (Roesijadi 1992). This is only true for Stony Creek, where there is a possibility that metal contamination may be causing high MT levels.

The relatively low degree of induction observed may indicate low levels of metals in the water and sediment. Low levels of heavy metals have been documented in sites in the Clinch River drainage both in the sediment and the water column (Table 5, EPA STORET data). One possible source for metals that might induce metallothionein levels observed is industrial activity (e.g., copper has been documented as a problem coming from the Carbo plant situated close to the Clinch River (Farris et al. 1988).

Tissue metal burdens have been correlated with metals present in the water column (Munkittrick et al. 1992). However, high levels of calcium ions serve to decrease gill membrane permeability to metal contaminants (Heath 1987; Spry and Weiner 1991). High levels of water hardness throughout the basin (Table 5), may be contributing to a low level of heavy metal uptake by fish. Runoff from surface coal mining can contain trace levels of heavy metals (Dick et al. 1986). Metal toxicity increases with the acidity of the water and with declines in hardness (Anderson et al. 1991; Spry and Weiner 1991). For example, a laboratory study on rainbow trout documented decreases in metal toxicity with increase in water hardness. A three-fold increase in the lethal concentration of zinc in tissues was observed for an order of magnitude increase in hardness Bradley and Sprague 1985). Uptake of heavy metals can occur through food (Klerks 1990) or through the water, as may be important for metals such as zinc (Niimi 1990). In general, it appears from the metallothionein data in this study that metal exposure stress at the sites selected is not severe.

However, tributaries with severe mining problems also do not appear to have high metallothionein values in the species studied. Localized effects of specific contaminants may cause higher levels of biomarkers at certain sites. For example, on the mainstem of the Clinch River, the Dunganon site showed higher levels of metallothionein in Rock bass than at other Clinch River sites. This site is located beside a railroad switchyard, which could be an intermittent source of heavy metals (Anderson et al. 1991).

MT increases in response to non-metal stress have only been reported in a few cases. For example, Steadman et al. (1991) reported induction in rainbow trout MT as a function of exposure to

No. 2 fuel oil over time in a laboratory-based study. They attributed the observed response to general stress. A possible mechanism for MT elevations as a function of general stress is through elevated glucocorticoids (Cousins 1985; Brady 1982). Glucocorticoids could increase MT levels two- to four-fold, as opposed to twenty- to fifty-fold increases resulting from metal exposure (Hamilton and Mehrle 1986). Also, studies on rats have revealed that MT can respond to a wide range of stressors other than metals, such as nutritional status and restraint (Garvey 1990; Hidalgo et al. 1986). In this study, increase in metallothionein were less than two-fold, and hence the stress in fish surveyed at these sites could possibly be from sources other than metal exposure.

Copper

Low levels of copper found in sediments throughout the Clinch River Basin are likely to accumulate in hepatic tissue of fish such as northern hogsuckers which are bottom dwellers and feeders (Petering et al. 1990). From the observed copperthionein levels in this study, it is clear that a detectable level of copperthionein occurs in northern hogsucker. This metal fraction is undetectable by the MT detection technique used in this study. Hence a possible explanation for low MT in northern hogsucker is the presence of a large fraction of it in the form of copperthionein. Estimates of metallothionein using the cadmium saturation assay is likely to underestimate the amount of copperthionein present due to competition with cadmium. Copper has been reported to bind more strongly to metallothionein than cadmium (Ley et al. 1983). If a higher fraction of hepatic metallothionein in northern hogsucker is present as copper, the assays conducted may underestimate total metallothionein.

EROD activities

Levels of EROD in rock bass at reference sites were similar to those found in female redbreast sunfish (Lepomis auritus), from reference sites used in another study (Jimenez et al. 1990). Numerous studies report high hepatic P450-dependent activities in fish exposed to organic contaminants such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls (e.g., Stein et al. 1992; McMaster et al. 1991; Adams et al. 1992). Benthic fish have been shown to be particularly susceptible to organic contaminants present in the sediment (Vindiman et al. 1991; Smith et al. 1991; Stein et al. 1992). The northern hogsucker is a benthic invertivore (Jenkins and Burkhead 1994) which may result in possible exposure to high amounts of sediment-bound contaminants. In this study, high specific EROD activity was observed at Little Cedar Creek, Toms Creek and at Big Cedar Creek. It is likely that contaminants are inducing high enzyme activities in these sites. For example, bile metabolite levels were high at Little Cedar Creek compared to Little River (Richlands). Studies have shown that some of these metabolites may originate from crude oils (Britvic et al. 1993). IBI data reveals that community condition is poor or fair at these above sites (Table 4).

The collection site at Little Cedar Creek was located directly upstream of a wastewater treatment facility in the town of Lebanon, which could possibly contribute to an increased chlorinated organic contaminant loading downstream of the plant. In addition, Little Cedar Creek is in the town of Lebanon, whose urban runoff could be a source of PCBs and crude oils from industrial wastes in that town (Little Cedar Creek drains directly into Big Cedar Creek, which may in part be responsible for degraded conditions in the latter).

Toms Creek has a high amount of mining activity and high total EROD activity. However, EROD levels in the Guest River, also with a high amount of mining activity were only slightly higher than reference sites, and these levels were not significantly higher than reference values. Polycyclic aromatic hydrocarbons (strong inducers of P450 activity) have been documented in river systems draining coal bearing strata (Barric and Prahl 1987). IBI data indicate that Toms Creek is in the poorest condition of all sites sampled (IBI=32). Rock bass were not available at this site, which may also indicate its degree of degradation.

Previous studies on sunfish have shown a response to cytochrome P450 enzyme inducible compounds (Adams et al. 1990; Theodorakis et al. 1992). It was therefore surprising that the rock bass in this study did not seem to respond to contaminants with the same sensitivity as the northern hogsucker. These results may have to do with the reproductive status of the fish at the time of capture (i.e., many fish were still gravid). Although gravid rock bass females were excluded from this study, both sexes were mixed, which is likely to have contributed to low EROD values. Several studies have documented as much as a fourfold differences in hepatic enzyme activities between prespawning to postspawning season in fish (see Jimenez and Stegeman 1990).

An important factor contributing to differences in EROD activity between rock bass and northern hogsucker in this study may be their respective reproductive seasons. Estimates of redbreast sunfish cytochrome P450 activity revealed declines in enzyme levels through their reproductive season, presumably down-regulated by reproductive hormones (Jimenez and Stegeman 1990). Rock bass spawn from April until July, depending on temperature (Jenkins and Burkhead 1994). It is likely that EROD activities had not recovered to postspawning values at the time of capture of rock bass used in this study.

Northern hogsucker, which spawn in midspring (sometimes extending to late May in Virginia, Jenkins and Burkhead, 1994), were likely to have recovered their post-spawning EROD activities. Other factors responsible for the relative lack of response in rock bass EROD levels may include differences in feeding behavior, presence of localized areas of high contamination (Stein et al. 1992), low sensitivity to contaminants present, hepatotoxicity (Gallagher et al. 1989; Adams et al. 1992) and nutritional status (Yamauchi et al

1975). Rock bass are top predators that may not be exposed to sediment contamination as much as northern hogsucker, which are bottom feeders (bottom dwellers). Also, possible species specificity to chemical contaminants was shown by Gallagher et al. (1989). They reported actual declines in cytochrome P450 enzymes in brown bullhead catfish (Ictalurus nebulosus) exposed to a mixture of organic and metal wastes compared to a reference stream.

As with the metallothionein data, significant increases in cytochrome P450 enzyme activity may be occurring in fish in this study in response to non-contaminant stressors. For example, these enzymes are important in both synthesis and metabolism of steroids, which can change as a function of stress (McMaster et al. 1991). Studies on human fetal hepatocytes have found increases in cytochrome P450 enzymes as a function of glucocorticoids and polyaromatic hydrocarbons, which appear to have acted synergistically (Mathis et al. 1986). Stress in rats was shown to increase cytochrome P450 enzyme activity (Blohm et al. 1985). Furthermore, increases in hepatic cytochrome P450 enzyme activities have also been documented in rats responding to cortisol injections (Schulte-Herman et al. 1988). However, declines in cortisol levels have also been observed in field studies of highly stressed fish (Hontela et al. 1992). Documentation of glucocorticoid-mediated responses in EROD levels would also be useful but challenging in the field, where there is much variability in stress exposure. However, there is a possibility that elevated cortisol levels could have contributed to the observed cytochrome P450 induction in this study.

Microsomal proteins

In this study, levels of microsomal protein increased at degraded sites, possibly in response to stress in Northern Hogsuckers. Increases in microsomal protein in response to stress have been observed in laboratory studies. For example, increases in levels of microsomal protein were observed in a laboratory study of rainbow trout exposed to fuel oils (Steadman et al. 1991). In addition, rats exposed to acute immobilization stress or noise pollution exhibited significantly higher microsomal protein levels than did the controls (Nagyova and Ginter 1993; Blohm et al. 1985). However, Fabacher et al (1985) documented microsomal protein levels that were relatively low in comparison to reference values in brown bullhead catfish collected in a site heavily polluted with organic contaminants compared to a reference site. They attributed this trend to metabolic degeneration in hepatic tissue of the fish.

Although not previously observed in field studies, it is possible that microsomal proteins could increase as a function of stress, contaminant exposure, or combinations thereof. High levels of microsomal proteins in northern hogsucker at sites such as Guest River (Coeburn) and Big Cedar Creek may reflect stressed conditions in fish at these sites, both of which are in poor condition according to IBI data (Table 10). High microsomal protein levels were also observed in Dumps Creek, an area of extensive mining activity, where impairment was detected in macroinvertebrate communities (Table 6) although IBI indicates Dumps Creek to be in good condition (Table 4). Rock bass exhibited similar values in microsomal proteins at all sites.

In summary, both the biomarkers EROD and MP appear to be highly inducible in northern hogsucker relative to rock bass. Hepatic EROD may be induced in fish by several organic contaminants (Munkittrick et al. 1991; Steadman et al. 1991). Benthic fish have been shown to be particularly sensitive to organic contaminants present in the sediment (Vindiman et al. 1991; Smith et al. 1991; Stein et al. 1992) and to show high EROD levels in response to such stressors. Thus organic contaminant exposure is a possible cause of high EROD levels in northern hogsucker across several sites in this study. However, additional chemical data is needed for validating the cause of stress.

Bile Metabolites

High levels of bile metabolites were found in northern hogsucker at Little Cedar Creek compared to those in Little River in Richlands (Figure 3). Lin and Courmier (personal communication) found high levels of these metabolites in white sucker exposed to organic contaminants. It is possible that the contaminants present in Little Cedar Creek belong to polycyclic aromatic hydrocarbons found in oils, such as naphthalene, phenanthrene, pyrenol and benzo(a)pyrene groups of organic compounds.

Other workers have shown a 5.5-fold increase in bile fluorescence compared to controls (excitation wavelength = 365 nm; emission wavelength = 520 nm) corresponding to a 5.1=fold increase in P450 enzyme activity in a laboratory-based study of crude oil exposure (Britvic et al. 1993). However, field studies have detected 2-3-fold increases in bile fluorescence with a corresponding 3-7-fold increases in benzo(a)pyrene enzyme activity in livers of the same fish (Britvic et al. 1993). In this study, a 2-3.5-fold increase in bile metabolites (depending on the wavelength pair) was found between Little Cedar Creek and Little River, compared to a 11.5-fold increase in EROD activity (Figure 3). This effect may be species-specific (Britvic et al. 1993), an effect of long-term exposure conditions, feeding status or exposure to complex waste mixtures, some of which are not detected by bile fluorescence technique (e.g., PCBs).

Possible links between biomarker responses and higher level effects

While biomarkers often are able to indicate possible causes of stress or exposure to contaminants, they lack ecological relevance provided by higher level indicators in fish (Adams 1990). Data on biomarkers and on population- and community-level indices taken together in a system can theoretically be used to link stress exposure in individual organisms to a larger, ecologically relevant context (McCarthy and Shugart 1990). In this study, Spearmans correlations between existing IBI data (and its individual-population-, and community-level components) with 4 biomarker categories (i.e., EROD, total EROD, MP and MT) revealed several significant correlations between trends in biomarkers and IBI components across sites in both northern hogsucker and rock bass. Spearmans correlations were observed between biomarkers and a wide range of IBI components, including total IBI score (Table 10). However both positive and negative Spearmans correlations were observed across IBI components in northern hogsuckers and in rock bass, which indicate opposing trends.

Of a total of ten IBI components examined, there were 4-6 significant correlations in each biomarker category for northern hogsuckers indicating a possible relationship between initial responses to stress (at the biochemical level) and ultimate higher level effects (at population and community levels). There were fewer correlations (0-4) between rock bass biomarkers and IBI components. For each species studied, there were a number of correlations which were inconsistent with expectations. The higher number of correlations for northern hogsucker suggests that it may be a better indicator species than rock bass for (a) assessing individual level responses to stress and (b) comparing biochemical and higher level effects. This may be due to its increased sensitivity as a function of its habitat and feeding habits (bottom dwelling and feeding) compared to rock bass (a top predator). Species from the sucker family have been previously used and recommended as sensitive indicators of contaminant stress (Vindiman et al. 1991). However, to our knowledge, no study has previously documented use of such species to link biochemical responses to field conditions with community-level effects. One other study has demonstrated a possible link between laboratory-based toxicity studies and several community-level attributes, especially fish species richness (Dickson et al.1992). In this study, declines in fish species richness were consistent with increase in toxicity of waters as measured by laboratory-based toxicity studies on the same systems. These correlations were consistent with those observed between native species richness and northern hogsucker EROD values in this study.

Prolonged elevations in cytochrome P450 activities may have negative effects on reproduction in fish, thereby possibly affecting whole populations and communities (McMaster et al. 1991). A suggested mechanism for reproductive impairment as a consequence of elevated P450 enzyme activities is the subsequent decline in circulating steroids that might result (McMaster et al. 1991). The correlations obtained in this study may imply that physiological responses to stress radiate to higher levels of biological organization.

There were several correlations between external anomalies (e.g., fin degradation, lesions etc..) in fish and biomarkers measured in rock bass and northern hogsucker. For example, both fin degradation and proportion of individuals with anomalies were significantly correlated with hepatic MT in rock bass (Table 11). Other studies have documented, high incidence of lesions in contaminated aquatic systems (Collier et al. 1992; Munkittrick et al. 1991). Gallagher et al (1989) observed fatty livers and lip and jaw lesions at a polluted site. A high incidence of lateral lesions were observed in bleached kraft pulp mill effluent exposed lake whitefish which had high hepatic cytochrome P450 activities (Munkittrick et al. 1992). These correlations imply that initial biochemical-level responses to stress result in whole-organism effects such as lesions over longer periods of exposure.

In addition, trophic and habitat-specific components of the IBI were correlated with selected biomarkers. For example, three out of four northern hogsucker biomarkers were correlated with the IBI metric for proportion of invertivorous individuals, and three out of five rock bass biomarkers were correlated with the IBI metric for proportion of piscivorous individuals. Northern hogsucker and rock bass are invertivores and piscivores respectively. These results suggest that individual stress response to a member of a certain trophic group can eventually lead to community-level effects in species from the same trophic group. Also, proportion of lithophilous spawning individuals correlated negatively with several biomarkers. These fish, and their eggs and larvae are likely to interact with the sediment to a high degree during their reproductive season and hence this metric could be sensitive to contaminants present in the substrate.

In this study, it was assumed that increasing levels of biomarkers indicated increase in stress. There are certain IBI components that increase (e.g., total number of tolerant species) or decrease (e.g., proportion of invertivores) in value as a function of stream quality Examples of IBI components that are expected to decrease with a decline in stream quality include total number of native species and proportion of invertivores. Examples of IBI components that are expected to increase in response to increasing stress are total number of omnivores and total number of tolerant species (Tables 10 and 11). Accordingly, correlations between biomarkers and the IBI components which decrease with increase in stress should be positive, while those between biomarkers and IBI components that increase with increase in stress should be negative. Examples of correlations that occurred as predicted include negative correlations between total EROD in northern hogsucker and number of intolerant species, total number of native species and total IBI score. In all of the above cases, as biomarker values rose (in response to stress) the community-level indicator declined as predicted. However, it is difficult to assess the exact nature of the cause and effect relationship between these metrics and the biomarkers (Adams et al. 1992; Niimi 1990).

However, not all trends in correlations were as predicted. In northern hogsuckers, negative correlations were observed between MT and proportion of individuals with anomalies, proportion of piscivorous individuals, and total number of individuals with fin degradation, while positive correlations were observed between MT and total number of invertivores (Table 10). These results were contrary to predictions. For example, increases in proportion of fish with anomalies, total number of individuals with fin degradation and proportion of piscivorous individuals suggests declines in stream quality. If their correlation with MT is negative, this implies that MT values decrease as a function of individual stress, which is the opposite of predictions. Several other correlations were also contrary to predictions. Thus biomarkers may not always be increasing as a function of stress. It is possible that selected biomarkers levels actually decrease in response to stress. In addition, northern hogsucker MT values did not increase predictably with stress across sites studied (except at Stony Creek, Table 9). With the possible exception of Stony Creek, it is doubtful that MT-inducible metal stress is occurring at the sites examined in the Clinch River drainage. In contrast to corresponding northern hogsucker data, Spearmans correlation between rock bass MT and proportion of individuals with anomalies, total number of individuals with fin degradation and total number of piscivorous individuals are all positive and significant. These trends are in accordance with expectations that increased hepatic MT implies increased individual level stress.. In what appears to be a absence of metal contamination stress it is possible that changes in MT values are entirely species-specific. Northern hogsuckers appear to respond to stress in ways that are different compared to rock bass. However, relatively elevated copper-thionein values in northern hogsucker could contribute to apparently low levels of MT from rock bass.

Biomarker levels for several sites were not in agreement with IBI data in predicting stress and several insignificant correlations between IBI data and biomarkers levels across sites. These were tests where biomarker levels (a) indicated stress/adaptation response in individual fish where IBI data indicated no stress effects on fish communities, (b) indicated no individual-level stress/adaptation response although those sites were categorized as impaired (i.e., poor or fair) by IBI data, (c) showed species specific response that varied within the same site and, (d) showed responses that varied in two sites within the same stream and (e) Spearman's correlations with IBI that showed insignificant relationship with the IBI data. In general, these inconsistencies may be attributed to two possibilities. First, fish may not be subjected to a stress regime that causes induction of proteins measured in this study. Second, fish under extreme stress may not be able to respond by producing these adaptive responses to stressed conditions. Inconsistencies under all categories outlined above will be discussed in further detail.

Diagnostic ability of biomarkers

Based on this study, EROD, MT and MP are capable of estimating responses to stress over a wide range of land use conditions. However, given the complex nature of biomarker responses to various stress regimes, isolating possible causes is a challenging task. EROD and MT are generally known to be indicators of organic contamination and metals respectively. However, both may also be elevated by increases in circulating corticosteroids. Microsomal protein is apparently not related to a specific source of stress because it seems to respond to a wide range of stressors, making it likely to be a general stress indicator. Biomarkers are more useful when more than one of them are used together, as single biomarkers may yield misleading information. Future uses of biomarkers need to keep such complexities in perspective.

Future work

Biomarkers have been used as early warning indicators of contaminant stress to aquatic organisms in disturbed conditions (Adams et al. 1992; McCarthy and Shugart 1990; Petering et al. 1990). However, previous studies have not concentrated efforts to (a) explore induction levels in reponse to stressors other than contaminants, (b) investigate the effect of complex waste mixtures on biomarker responses and (c) explore species-specific physiology of responses to stressors. Gallagher et al. (1989) stated that a serious obstacle to field application of EROD and other cytochrome P450 enzymes is pollution scenarios involving complex mixtures that alter the kinetics of metabolism. Future research efforts on field-applications of biomarkers should include such efforts. Complex contaminant (and other stressors) mixtures may interfere with normal responses by acting synrergistically, or by inhibiting induction. Both are possible mechanisms for responses observed in fish resident to the Clinch River drainage. For example, Little Cedar Creek induced high levels of EROD in northern hogsuckers but EROD levels in rock bass were not significantly different from those at reference sites. Such species- specific effects might be explained if the mechanism of exsposures response (e.g., hepatotoxicity) was known.

Most field based biomarker studies demonstrating stress in fish use sites with documented elevated levels of contaminants (Munkittrick et al. 1991; Adams et al. 1992; Stein et al. 1992). Trends in biomarkers levels across sites studied suggest that contaminant stress from sources such as organic contaminants or heavy metals (which can induce cytochrome P450 activity and metallothionein respectively) may be low at several locations. Also, biomarker responses to stress occurred over a wide range of land use impacts that were likly to cause multiple sources of stress. Several correlations between IBI components and biomarkers across all sites were significant despite the low level biomarker responses to suspected sources of impact (e.g., few biomarker levels increasesed by a factor of 2-3).

Since it is known that these biomarkers may also respond to other stressors in the field, it would be useful to explore the extent to which such elevations could reflect stress in fish from sources other than contaminant stressors. For such a task, field sample size would have to be higher than those used in this study. Low level effects possibly observed in this study may be masked by the "noise" associated with low sample size. Also, it is important to isolate sites that are subjected predominantly to one specific stressor.

Among biomarkers used in this study, both MT and EROD are capable of being induced in the presence of high glucocorticoid levels. As elevations in cortisol levels occur in response to general stress, it would be useful to explore the relationship between stress-induced cortisol levels and biomarker levels. Such laboratory-based tests could use water from impacted sites to isolate possible contaminant stress responses. Future studies should also focus on using biomarkers that respond to non-specific sources of stress (e.g., habitat degradation).

In this study, biomarker levels at several sites suspected of severe impairment were not significaltny elevated above reference values. Severe stress could induce an opposite effect of decreasing protein levels, as has been observed in the case of cytochrome P450 enzyme activities (Adams et al. 1990; Jimenez and Stegeman 1990). This aspect of exposure response in fish sampled in the field needs further exploration, possibly using a laboratory-based design.

Conclusions

All elevated biomarkers levels were found at sites where impairment from some source was previously detected. However, the two species studied in streams throughout the Clinch River drainage respond to stressors present in the system with different sensitivities. It is likely that these differences correspond to differences in feeding habits and other species specific effects that expose them to different stress regimes (e.g. northern hogsucker is a benthic feeder). However, trends across sites seem to indicate responses that may not be confined to contaminant stress alone. On the whole, northern hogsucker responded to biomarkers that might be sensitive to organic contamination, while the rock bass appears to respond primarily to metal contamination and other possible metallothoinein inducers. Response of northern hogsuckers to conditions at Little Cedar Creek was a particularly interesting illustration of the potential impact of urbanization on a small stream.

Discrepancies between community-levels effects and biomarkers studied (e.g., insignificant changes in biomarkers at sites suspected of being impacted compared to reference sites) were probably due to (a) individual-level effects that have not impacted streams long enough to impact the community (b) low-level stress responses that do not negatively affect reproduction and (c) highly degraded sites where fish are unable to respond to stressors (e.g., in the case where hepatotoxicity occurs).

A significant correlation was observed between several biomarker responses and community-level effects (e.g., for the northern hogsucker). These patterns suggest that initial responses to stress may be an important factor affecting fish community health. Prolonged exposure to stress in fish from the Clinch River drainage may be having negative impacts on the community. However, some correlations are the reverse of what was expected. Reverse trends (e.g., a positive correlation where negative correlation was expected) may indicate that certain biomarker levels may in fact decrease as a function of increased exposure to stress. Such trends therefore need to be explored in more detail. Overall, Spearmans correlations indicate that individual-level effects observed in this study may result in effects at population- and community levels of biological organization.

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Figure 1: A schematic of long term trends in biomarker, population- and community-level effects in response to stress

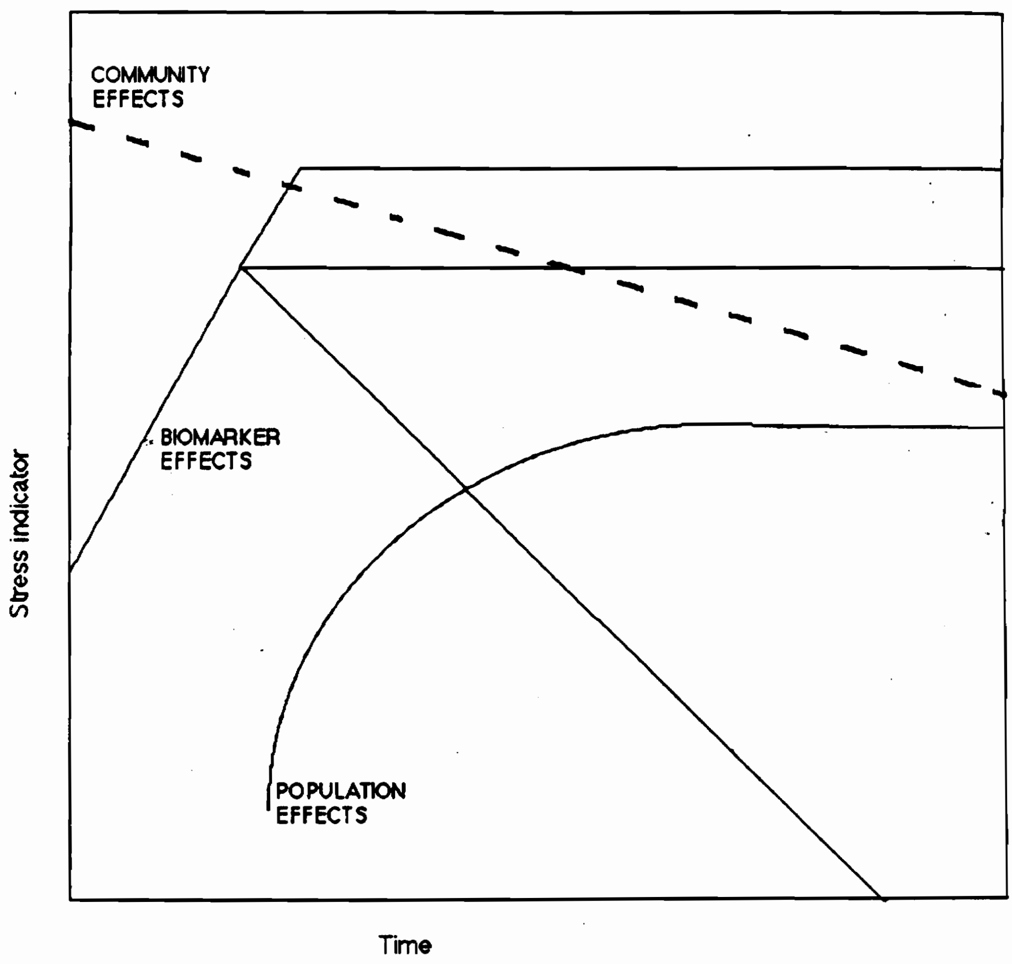

Figure 2: Location of study sites in the Clinch River drainage. Numbers correspond to those in Table 4.

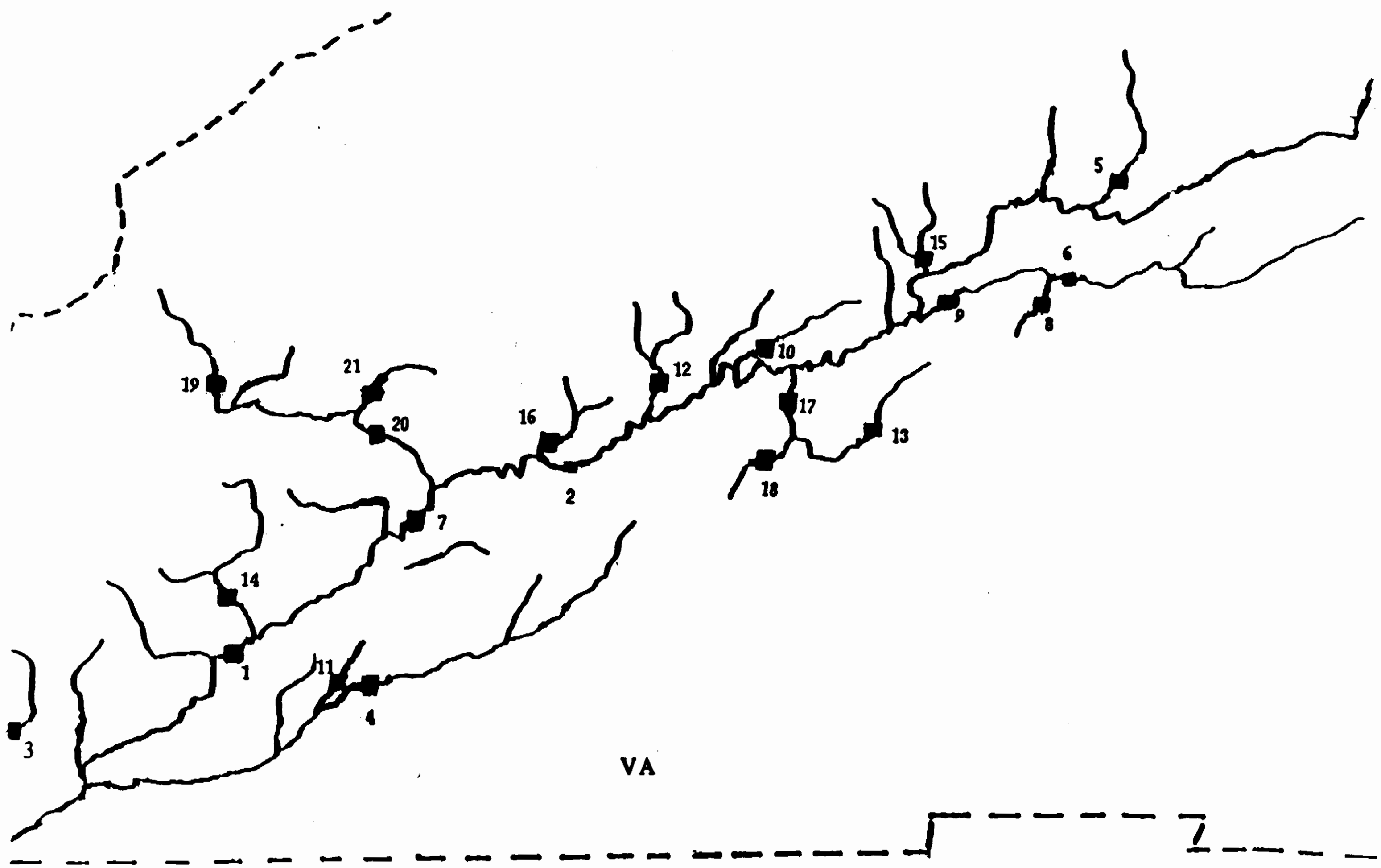

Figure 3: Bile fluorescence data comparing northern hogsucker from Little Cedar Creek with those from Little River (richlands) at four emission wavelengths. Shaded bars indicate Little Cedar Creek and solid bars indicate Little River specimens. Data have been normalized to a value of 5 fluorescence units for the Little River sites in each case. Emissions at all wavelengths are higher for fish at Little Cedar Creek.

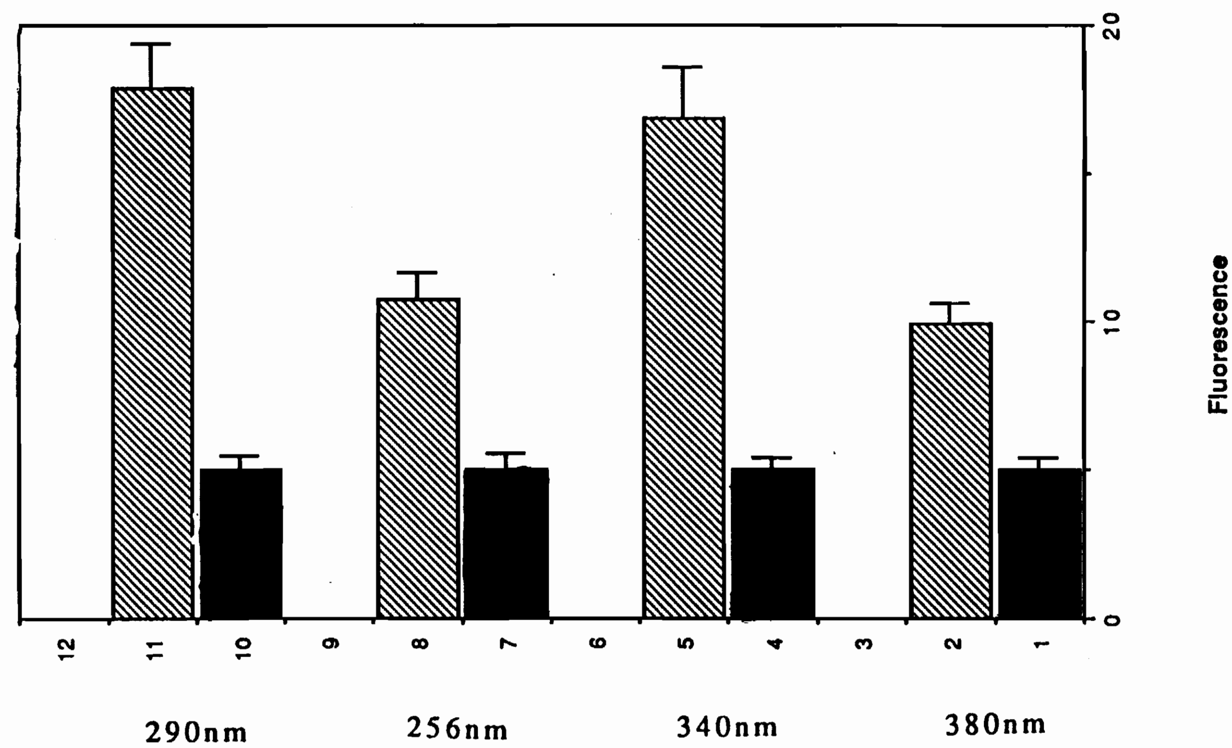

Table 1: List of classes of compounds transformed or synthesized by the action of cytochrome p450 enzymes (from Guengerich, 1993; Goksoyr, 1992; McMaster at al, 1991)

Inducers of Cytochrome p450 Enzymes	
Endogenous Compounds	Exogenous Compounds
Fatty acids	Polyaromatic hyrocarbons (e.g., benzo(a)pyrene)
eicosanoids	Polychlorinated biphenyls
alkaloids	chlorinated aromatic compounds
fat soluble vitamins	
steroids	
prostaglanins	

Table 2 Compounds that cause induction of metallothionein (from Petering et al, 1990)

Direct Induction	Indirect induction
Heavy Metals: Cd, Zn, Cu, Hg, Co, Ni, Bi, Ag	Carbon tetrachloride
Glucocorticoids	Ethanol
Catecholamines	Urethan
Progesterone	Lead
Glucagon	Manganese
Interleukin	
Interferon	
Phorbol esters; Iodoacetate	

Table 3: A list of the categories and metrics used in IBI for community-level assessment in the Clinch River Basin (Taken from Angermeier and Smogor, 1993)

Category	Metric
Species Richness and composition	Total number of native species
	Number and identity of native sucker species
	number and identity of native sunfish species
	number and identity of native darter species
	number and identity intolerant species
	Proportion of tolerant individuals
Trophic Composition	Proportion of omnivorous individuals
	Proportion of invertivorous individuals
	Proportion of piscivorous individuals
Miscellaneous	Catch per unit effort
	Proportion of individuals with anomalies
	Proportion of individuals as simple lithiphilous spawners

Table 4: IBI scores (two year averages) for selected sites in the Clinch River Basin in decreasing order.

River Basin in decreasing

Site name	IBI score	Category
Clinch R. 1	57	Excellent
Clinch R. 2	55	Good
North Fork Clinch R.	54	Good
Copper Cr.	53	Good
Indian Cr. 1	52	Good
Little R. 1	52	Good
Clinch R. 3	51	Good
Indian Cr. 2	50	Good
Little R. 2	49	Good
Tomsom Cr.	48	Good
Obeys Cr.	48	Good
Dumps Cr.	47	Good
Elk Garden Cr.	42	Fair
Stony Cr.	42	Fair
Swords Cr.	42	Fair
Lick Cr.	39	Fair
Big Cedar Cr.	36	Poor
Little Cedar Cr.	36	Poor
Guest R. 1	33	Poor
Guest R 2	33	Poor
Toms Cr.	32	Poor

Key: Clinch River1: Fort Blackmore site; Clinch River 2: St. Paul site; Clinch River 3: Dunganon site
Indian Creek 1: site at Pounding Mill; Indian Creek 2: site at Richlands
Little River 1: Richlands site; Little River 2: Honaker site
Guest River 1: Wise site; Guest River 2: Coeburn site

Table 5 : Average concentration of selected chemicals in the Clinch River Basin over the period 1981-1992). Data were obtained from EPA STORET files.

Site name	Mercury (sediment) mg/kg	Copper (sediment) mg/kg	Copper (water)ug/l	Zinc (sediment) mg/kg	Zinc (water) ug/l	Hardness (mg/l as CaCo3)
Clinch River*	0.059+0.027	20.41+2.27	9.09+4.56	75.52+9.74	37.50+25.4	147.07+2.38
Guest River	0.280+0.256	15.10+6.83	2.50+1.79	59.20+18.1	12.00+4.05	178.10+10.6
Little River	0.014+0.14	33.90+12.10	2.73+1.95	58.72+7.36	47.9+28.90	136.64+1.64
Copper Creek	T	12.00	T	40.00	5.33+5. 33	174.29+5.02

*Sites sampled throughout the Clinch River Basin
T indicates amounts of metals present were lower than detection limits

Table 6: Summary of results of EPA Rapid Bioassessment protocol conducted in 1991 on sites in the Clinch River Basin. Corresponding IBI values are given for comparison. Distance from the sites used in this study are indicated below.

Site Name	EPA rapid Bioassessment category	IBI data (category)
Big Cedar Creek: 1.5 miles upstream	Good Condition	36 (fair)
Dumps Creek 0.4 miles downstream	Moderately Impaired	47 (good)
Guest River (Coeburn) same site	Moderately Impaired	33 (poor)
Copper Creek (Clinchport) same site	Good condition	53 (good)
Clinch River* 9 miles upstream	Good condition	55

*The Clinch River site used in this study was closest to the Clinch River site at St. Paul.

Table 7 : Data at the following reference sites were pooled for comparisons with individual sites suspected of impact in the Clinch River basin. HSI is hepatosomatic index, MT is metallothionein, EROD is ethoxyresorufin-o-deethylase and MP is microsomal protein.

HSI (bass)	Copper Cr., Clinch R. sites*, Little R. sites**
MT (rock bass)	Copper Cr., Clinch R. sites*
MT (hogsucker)	Little R. sites**
MT (shiner)	Little R. sites**
EROD pm/min/g liver (bass)	Clinch R. (Ft. Blackmore), Little R. sites**
EROD pm/min/g liver (hogsucker)	Little R. sites**
EROD pm/min/mg protein (bass)	Copper Cr., Clinch R. sites*, Little R. sites**
EROD pm/min/mg protein (hogsucker)	Little R. sites**
EROD pm/min/mg protein (shiner)	Little R. sites**
MP (bass)	Clinch R. sites*, Little R. sites**
MP (hogsucker)	Little R. sites**

* Clinch River at Ft. Blackmore and at St. Paul
** Little River at Honaker and at Richlands

Table 8: HSI data were collected in 1993. IBI scores have been averaged over two years.

Site Name	Index of biotic integrity	Hepatosomatic index X 10-2
Clinch River 1	57	6.1 ± 0.3
Clinch River 2	55	5.3 ± 0.5
Copper Creek	53	5.5 + 0.2
Little River 1	52	5.8 ± 0.5
Indian Creek 1	52	8.1± 0.5*
Clinch River 3	51	5.5± 0.5
Indian Creek	50	8.1± 0.5*
Little River 2	49	5.9± 0.2
Obeys Creek	48	6.6± 0.7
Dumps Creek	47	5.6± 0.9
Swords Creek	42	5.7± 0.5
Elk Garden Creek	42	6.9± 0.7
Stony Creek	42	5.7± 0.8
Lick Creek	39	4.7± 0.7
Little Cedar Creek	36	5.9± 0.6
Big Cedar Creek	36	9.1± 1.0*
Guest River (Wise)	33	7.5± 1.2

* indicates significant difference from a pool of reference sites
Clinch River 1: Fort Blackmore; Clinch River 2: St. Paul; Clinch River 3: Dunganon
Little River 1: Richlands; Little River 2: Honaker
Indian Creek 1: Pounding Mill; Indian Creek 2: Richlands

Table 9: Metallothionein (MT) values for Rock bass (Ambloplites rupestris), Northern hogsucker (Hypentilium nigricans) and Striped shiner (Luxiilus chrysocephalus) captured at selected sites in the Clinch River Basin (arranged in order of decreasing IBI values). 4-9 specimens were collected for each species, depending upon availability (MT values and standard errors given). 'a' indicates those sites that were significantly different from the pool of reference sites at the p=0.100 level taking into account comparison-wise error. Dashed lines indicate negligible levels and blank spaces indicate that measurements were not taken.

Site Name	rock bass MT (ug/g liver)	northern hogsucker MT (ug/g liver)	striped shiner MT (ug/g liver)
Little River (Richlands)	19.32 + 3.22	2.97 + 1.13	2.78 + 0.805
Little River (Honaker)	20.83 + 2.00	11.58 + 3.17	
Indian Creek (P. Mill)	26.06 + 4.08	13.61 + 6.80	
Clinch River (Dunganon)	47.50 + 4.06 a	9.52 + 2.68	----
Clinch River (Ft. Blackmore)	21.58 + 4.99	----	----
Clinch River (St. Paul)	19.89 + 2.98	----	----
N. Fork Clinch River		23.36 + 5.03 a	
Indian Creek (Richlands)	13.18 + 3.00	9.39 + 3.51	5.69 + 1.72*
Tomson Creek	17.44 + 3.39	----	1.43 + 0.40
Dumps Creek	39.13 + 0.59	17.88 + 5.92	5.99 + 2.55
Swords Creek	34.50 + 6.15 a	6.21 + 3.09	8.16 + 4.37
Stony Creek	41.76 + 7.81 a	24.40 + 6.89 a	----
Copper Creek	16.70 + 4.88		
Obeys Creek	14.21 + 1.16	9.01 + 2.21	----
Lick Creek	12.00 + 3.02	8.58 + 1.29	----
Little Cedar Creek	35.60 + 8.65 a	9.96 + 2.49	2.87 + 1.04
Big Cedar Creek	13.41 + 1.51	3.96 + 0.92	----
Guest River (Coeburn)	14.94 + 3.25	----	11.69 + 6.40*
Guest River (Wise)	41.78 + 4.33 a	11.97 + 3.91	
Toms Creek	----	7.86 + 1.17	6.43 + 0.97 a

* pooled data for both sites in these streams.

Table 10: Spearman's rank correlation results: IBI against bioindicators for northern hogsuckers. Each column is a component of the total IBI score (i.e., an IBI metric or an important factor within a metric). Numbers in each entry denote correlation and significance respectively. Lithophils indicates proportion of lithophilous spawners, anomalies indicates total number of fish with anomalies, and intol indicates total number of intolerant species. Tol indicates proportion of tolerant individuals, omniv., housekeeping pisciv., and inverti. indicate proportion of individuals as omnivores, piscivores and invertivores respectively. Nativ. indicates total number of native species and fin deg. indicates the total number of specimens with fin degradation.

Param.	Litho-phils	Anoma-lies	Intol.	Toler.	Omniv.	Inverti.	Pisciv.	Nativ	Fin Deg	IBI
MT	0.0801 .5067	-.3426 0.0034	0.0888 0.5355	0.1253 0.2976	0.1336 0.2667	0.3177 0.0069	-0.2014 0.0921	0.1691 0.2309	-0.2162 0.1274	0.2186 0.0613
EROD	-0.0702 0.4508	-0.0078 0.9356	-0.1979 0.0865	-0.3097 0.0010	0.2465 0.0098	0.0789 0.4149	-0.0208 0.8319	-0.2302 0.044	0.115 0.3468	0.0825 0.3629
Total EROD	-0.3289 0.0012	0.1241 0.2330	-0.3207 0.0077	-0.1455 0.1610	-0.1600 0.1236	-0.3057 0.0027	0.0808 0.4391	-0.3119 0.0124	-0.1304 0.2522	-0.4350 0.0001
MP	-0.228 0.0247	0.0838 0.4141	-0.1472 0.2241	0.2908 0.0039	0.1095 0.3017	-0.1923 0.0592	0.1722 0.0916	-0.0180 0.8834	-0.3505 0.0052	0.3542 0.0003

Table 11: Spearman's rank correlation results: IBI against bioindicators for rock bass. Each column is a component of the total IBI score (i.e., an IBI metric or an important factor within a metric). Numbers in each entry denote correlation and significance respectively. Lithophils indicates proportion of lithophilous spawners, anomalies indicates total number of fish with anomalies, and intol indicates total number of intolerant species. Tol indicates proportion of tolerant individuals, omniv., pisciv., and inverti. indicate proportion of individuals as omnivores, piscivores and invertivores respectively. Nativ. indicates total number of native species and fin deg. indicates the total number of specimens with fin degradation.

Param.	Litho-phils	Anoma-lies	Intol	Toler.	Omniv.	Inverti.	Pisciv.	Nativ	Fin Deg	IBI
MT	-0.1329 0.1662	0.2263 0.0190	-0.1716 0.1587	0.1397 0.1440	0.0434 0.1440	-0.0168 0.8362	0.1607 0.0982	-0.2933 0.0112	0.3331 0.0031	0.053 0.8294
EROD	-0.1267 0.1494	0.0540 0.5405	0.1226 0.2881	-0.0705 0.4236	-0.0965 0.2730	-0.1120 0.2028	0.2185 0.0122	0.0878 0.4448	0.2036 0.0757	-0.0804 0.3432
Total EROD	-0.1092 0.2391	-0.1220 0.1882	-0.0532 0.6639	-0.0811 0.3826	-0.1229 0.1849	-0.0829 0.3724	0.0003 0.9978	0.0364 0.7648	-.1465 .2298	0.0639 0.4665
MP	-0.0044 0.9583	-0.1930 0.0204	-0.1465 0.1708	-0.1132 0.1769	-0.0438 0.6019	0.0997 0.2343	-0.2215 0.0076	-0.0080 0.9407	-0.1991 0.0614	0.0325 0.6886
HSI	-0.0043 0.9686	0.0406 0.7073	0.2345 0.0873	-0.1312 0.2232	-0.2182 0.0411	-0.0091 0.9332	0.0330 0.7599	0.0028 0.9830	0.1459 0.2580	-0.1298 0.1912

Table 12: Hepatic copper estimated in rock bass and northern hogsucker at selected sites in the Clinch River Basin. Blank spaces are meaures that were not made and dashed lines indicate that the values were negligible.

Site name	Cu (ug/g liver) rock bass	Cu (ug/g liver) northern hogsucker
Copper Creek	2.14 + 0.62	5.71 + 0.68*
Little River (Honaker)	1.66 + 0.15	----
Indian Creek (Richlands)	1.23 + 0.19	----
Dumps Creek	2.03 + 0.58	7.75 + 1.30
Elk Garden Creek	1.78 + 0.11	----
Swords Creek	1.17 + 0.11	----
Lick Creek	2.03 + 0.58	3.46 + 0.76
Big Cedar Creek	1.63 + 0.33	----
Little Cedar Creek	1.18 + 0.41*	----
Guest River (Coeburn)	0.82 + 0.11*	6.22 + 1.49

*n=2 for these values

Table 13: Summary of cytochrome p450 enzyme activities and microsomal protein levels for northern hogsucker across sites in the Clinch River Basin (in order of decreasing IBI values) EROD is ethoxyresorufin-o-deethylase; blank spaces aremeaures that were not made and dashed lines indicate that the values were negligible.

Site Name	EROD (pm/min/mg protein)	Microsomal Protein	EROD (pm/min/g liver)
North Fork Clinch River	4.76 + 2.02	12.04 + 2.18	36.09 + 7.35
Indian Creek 1	3.12 + 0.71	14.70 + 1.36	48.10 + 5.74
Little River 1	3.76 + 1.00	14.33 + 1.38	48.70 + 11.00
Clinch River a	3.94 + 1.78	16.07 + 1.07	37.30 + 14.00
Indian Creek 2	1.44 + 0.39	14.41 + 2.92	26.98 + 7.83
Little River 2	3.82 + 0.38	11.93 + 0.96	45.40 + 5.71
Thompson Creek	----	----	56.40 + 21.90
Dumps Creek	2.80 + 0.45	17.87 + 1.12*	63.60 + 14.30
Obeys Creek	4.34 + 0.56	----	65.11 + 6.44
Stony Creek	7.61 + 5.26	16.82 + 1.49	36.34 + 4.15
Swords Creek	4.38 + 1.41	14.00 + 0.75	75.89 + 9.52
Lick Creek	4.03 + 0.81	17.34 + 1.36	64.29 + 9.23
Big Cedar Creek	5.80 + 1.02	19.71 + 1.95*	117.40 + 27.30*
Little Cedar Creek	43.20 + 21.30*	13.94 + 1.39	472.00 + 183.00*
Guest River 1	3.05 + 0.82	15.77 + 2.33	48.70 + 11.00
Guest River 2	3.05 + 0.52	22.61 + 1.17*	52.20 + 21.10
Toms Creek	7.22 + 3.64	17.67 + 2.77	140.40 + 35.10*

Key: a Clinch River at Dunganon

Indian Creek 1: site at Pounding Mill; Indian Creek 2: site at Richlands

Little River 1: Richlands site; Little River 2: Honaker site

Guest River 1: Wise site; Guest River 2: Coeburn site

* sites significantly different from pooled reference sites

Table 14: Summary of cytochrome p450 enzyme activities and microsomal protein levels for rock bass across sites in the Clinch River Basin (in order of decreasing IBI values)
EROD is ethoxyresorufin-o-deethylase; blank spaces are meaures that were not made and dashed lines indicate that the values were negligible.

Site Name	EROD (pm/min/mg protein)	Microsomal Protein	EROD (pm/min/g liver)
Clinch River 1	3.79 + 0.36	11.70 + 0.76	42.97 + 2.56
Clinch River 2	1.58 + 0.33	13.41 + 1.91	29.22 + 9.90
Copper Creek	5.14 + 1.16	15.44 + 1.54	85.00 + 28.70
Indian Creek 1	4.21 + 0.80	12.27 + 0.98	52.90 + 10.4
Little River 1	4.02 + 0.59	13.54 + 2.62	54.00 + m11.70
Clinch River 3	6.62 + 0.61	12.52 + 0.49	82.72 + 7.48
Indian Creek 2	2.59 + 0.45	15.57 + 0.99	41.21 + 6.71
Little River 2	4.61 + 0.36	12.99 + 0.89	61.79 + 7.49
Tomson Creek	4.50 + 0.65	15.21 + 1.47	76.42 + 8.68
Dumps Creek	4.42 + 0.54	17.28 + 2.60	82.16 + 9.47
Obeys Creek	2.90 + 0.29	16.94 + 1.98	57.30 + 6.64
Stony Creek	3.62 + 0.36	14.96 + 0.99	57.60 + 6.99
Swords Creek	5.23 + 0.65	12.22 + 0.46	65.61 + 8.74
Lick Creek	3.68 + 0.61	12.99 + 1.24	60.20 + 12.7
Big Cedar Creek	4.73 + 0.62	10.57 + 0.87	55.50 + 10.30
Little Cedar Creek	4.27 + 0.71*	16.15 + 1.40	63.00 + 13.70
Guest River 1	2.86 + 0.91	12.01 + 2.89	46.01 + 7.87
Guest River 2	4.07 + 1.08	13.60 + 1.19	----

Key: Clinch River1: Fort Blackmore site; Clinch River 2: St. Paul site; Clinch River 3: Dunganon site
Indian Creek 1: site at Pounding Mill; Indian Creek 2: site at Richlands
Little River 1: Richlands site; Little River 2: Honaker site
Guest River 1: Wise site; Guest River 2: Coeburn site
* sites significantly different from pooled reference sites

Table 15: Specific EROD for the Striped Shiner across selected sites in the Clinch River Basin. EROD is ethoxyresorufin-o-deethylase

Site name	EROD (pm/min/mg protein)
Indian Creek 1	3.48 + 1.90
Little River 1	3.78 + 1.63
Indian Creek 2	0.26 + 0.26
Little River 2	2.70 + 0.69
Thompson Creek	0.46 + 0.31
Dumps Creek	2.06 + 0.76
Swords Creek	6.38 + 1.28
Little Cedar Creek	6.77 + 1.79
Guest River (Coeburn)	1.95 + 1.00
Toms Creek	2.63 + 1.86

Indian Creek 1: site at Pounding Mill; Indian Creek 2: site at Richlands
Little River 1: Richlands site; Little River 2: Honaker site

Table 16: A summary of results of bioindicator study for three species of fish from the Clinch River Basin. A value higher than those at reference sites is shown by +; +* indicates higher value not taking the Bonferroni correction into account. Dashed lines (---) show values that were not significantly different from reference values. Reference sites are not included in this summary. Key: RB=rock bass; NH= northern hogsucker; SS= striped shiner; MP= microsomal protein; E= specific EROD and E'= total EROD. Unfilled spaces indicate that the measurement was not made for that particular bioindicator.

Site	RB HSI	RB MT	RB E	RB E	RB MP	NH MT	NH E	NH E	NH MP
N.Fork Clinch R.	---	---				+	---	---	---
Copper Cr.	---		---	---	---				
Indian Cr. (P. Mill)	+	---	---	---	---	---	---	---	---
Clinch R. Dunganon	---	+	---	---	---	---	---	---	---
Indian Cr. Richlands	+	---	---	---	---	---	---	---	---
Thompson Cr.	---	---	---	---	---			---	
Dumps Cr.	---	+	---	---	---	+*	---	---	+
Swords Cr.	---	+	---	---	---	---	---	---	---
Stony Cr.	---	+	---	---	---	+	---	---	---
Copper Cr.	---	---	---	---	---		---	---	---
Obeys Cr.	---	---	---	---	---	---	---	---	---
Lick Cr.	---	---	---	---	---	---	---	---	---
L. Cedar Cr.	---	+	+	---	---	---	+	+	---
Big Cedar Cr.	+	+*	---	---	---	---	---	+	+
Guest R. Coeburn	---	---	---	---	---		---	---	+
Guest R. Wise	+*	+	---		---	---	---	---	---
Toms Cr.						---	---	+	---