Structural Changes in Gill DNA Reveal the Effects of Contaminants on Puget Sound Fish

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Structural differences were identified in gill DNA from two groups of English sole collected from Puget Sound, Washington, in October 2000. One group was from the industrialized Duwamish River (DR) in Seattle and the other from relatively clean Quartermaster Harbor (QMH). Chemical markers of sediment contamination [e.g., polynuclear aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs)] established that the DR was substantially more contaminated than QMH. The levels of these chemicals in the sediments of both sites were consistent with levels of cytochrome P450 1A (CYP1A) expression in the gills of English sole from the same sites. Structural differences in gill DNA between the groups were evinced via statistical models of Fourier transform-infrared (FT-IR) spectra. Marked structural damage was found in the gill DNA of the DR fish as reflected in differences in base functional groups (e.g., C–O and NH_2) and conformational properties (e.g., arising from perturbations in vertical base stacking interactions). These DNA differences were used to discriminate between the two fish groups through principal components analysis of mean FT-IR spectra. In addition, logistic regression analysis allowed for the development of a "DNA damage index" to assess the effects of contaminants on the gill. The evidence implies that environmental chemicals contribute to the DNA changes in the gill. The damaged DNA is a promising marker for identifying, through gill biopsies, contaminant effects on fish. *Key words:* contaminant effects, cytochrome P450 1A, DNA markers, DNA structure, fish gills, Fourier transform-infrared spectroscopy, Puget Sound. *Environ Health Perspect* 112:511–515 (2004). doi:10.1289/ehp.6719 available via *http://dx.doi.org/* [Online 18 December 2003]

The substantial surface area of the gills in fish serves as an interface between the environment and blood, notably for the continuous diffusion of oxygen and the maintenance of acid–base and ion balance (Claiborne et al. 2002; Randall et al. 1996). Fish that inhabit polluted environments are particularly susceptible to contaminants (soluble and suspended) that can damage gill structure and physiology (Lichtenfels et al. 1996; Randall et al. 1996). Metals (e.g., Cu^{2+}) and relatively low-molecular-weight organic compounds (e.g., alkylated phenols) are readily absorbed across the gill (Randall et al. 1996). These contaminants cause deleterious changes in cellular structures, including the epithelium and pillar cells (Laurent and Perry 1991; Randall et al. 1996; Van Veld et al. 1997). For example: bis(tri-*n*-butyltin)oxide produces gill lesions in flounder (*Platichthys flesus*) (Grinwis et al. 1998) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induces branchial neoplasms in medaka (*Oryzias latipes*) (Brittelli et al. 1985). Additional changes in the gill have been attributed to aromatic hydrocarbons (AHs) (Gagnon and Holdway 1999) and various other contaminants (Abel 1974; Teh et al. 1997).

Effects of foreign chemicals frequently involve oxidative metabolism by members of the cytochrome P450 (CYP) multigene family of enzymes. Induction of CYP1A has been identified in the gill (Miller et al. 1989; Van Veld et al. 1997). CYP1A, in particular, is critical for the oxidation of a variety of xenobiotics in aquatic environments, especially polynuclear aromatic hydrocarbons (PAHs), and oxidation of benzo[*a*]pyrene by gill P450 has been described (Stegeman et al. 1984). Fish CYP1A also catalyzes oxidation of planar polychlorinated biphenyl (PCB) congeners, albeit slowly (White et al. 1997). The degree of CYP1A expression in fish tissues is frequently used as an indicator of exposure to these types of contaminants (Miller et al. 2004; Stegeman et al. 2001; Woodin et al. 1997).

Many of the contaminant-induced changes in the gill are likely preceded by, or associated with, subcellular changes such as those related to oxidative and other modifications of DNA. For example, induced CYP1A bound with coplanar PCBs can be a source of H_2O_2 and O_2^- (Schlezinger et al. 1999). In vertebrates, the hydroxyl radical (•OH), which arises from the metal-catalyzed (e.g., Fe^{2+}) decomposition of H_2O_2, reacts with DNA bases to form adducts, such as the redox-ambivalent 8-oxylpurines (Steenken 1989). These are converted either to mutagenic 8-hydroxypurines or to ring-opened, putatively nonmutagenic formamidopyrimidines (Steenken 1989). As reviewed by Maccubbin (1994), other changes may arise from "bulky" aromatic structures bound to the DNA that are derived metabolically from the oxidation of PAHs. Both the

8-hydroxypurine and bulky aromatic adducts are believed to be associated with various cellular transformations (e.g., hyperplasia and neoplasia) in a variety of fish tissues (Maccubbin 1994; Moore and Myers 1994).

Our objective was to test the hypothesis that contaminants in polluted coastal environments react with gill DNA to produce detectable structural changes that serve as markers of contaminant exposure and have the potential to alter gill physiology. Contaminant-induced disruptions in gill DNA structure were determined using previously established statistical models of Fourier transform-infrared (FT-IR) spectra (Malins et al. 1997c). An advantage of using these DNA structural markers in environmental assessments would be that tissues could be obtained through nonlethal biopsies of the gill.

We chose to use English sole (*Parophrys vetulus*) from two sites in Puget Sound, Washington: the lower Duwamish River (DR) and Quartermaster Harbor (QMH) (Figure 1). The DR, which flows through a heavily industrialized area of south Seattle, was added to the National Priorities List by the U.S. Environmental Protection Agency (U.S. EPA) in September 2001 due to sediment contamination (U.S. EPA 2001b). An advisory

issued in June 2002 by the Washington State Department of Health (WA State DOH) warned of a possible adverse health risk from eating English sole and other bottom fish from the lower DR (WA State DOH 2002). This advisory was based primarily on sediment contamination by PCBs, PAHs, and other pollutants. In the 1980s and 1990s, QMH was used as a reference site in studies of the effects of contaminants on fish (Malins et al. 1986, 1997a). Subsequent studies have shown that sediments in QMH are less contaminated than are sediments in the lower DR (Long et al. 2000, 2002). For example, cell-based bioassays to detect compounds binding to the aryl hydrocarbon receptor [PAHs, coplanar PCBs, polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs)] (Anderson et al. 1995, 1999a, 1999b; Jones and Anderson 1999), expressed as benzo[a]pyrene equivalents (B[a]PEq), gave results averaging 16.9 μg/g for sediments from the three sites sampled in QMH in June 1999 (Long et al. 2002), whereas three sites sampled in the lower DR in June 1998 averaged 73.6 μg/g (Long et al. 2000).

In the present study, we used FT-IR spectroscopy to identify a broad array of subtle structural changes in gill DNA, and we used CYP1A expression to measure exposure of the gill to a variety of contaminants that are AH receptor agonists (PAHs and planar halogenated aromatic hydrocarbons). The highly sensitive FT-IR technology was previously used to identify changes in the structure of liver DNA of fish obtained from Puget Sound in 1993 and 1995 (Malins et al. 1997a). It has also been used extensively to elucidate changes in DNA structure associated with the

transformation of healthy human tissues to cancer, for example, the prostate (Malins et al. 1997b) and breast (Malins et al. 1996b, 1997c). The striking ability of the FT-IR technology to discriminate between similar DNA structures was demonstrated using oligonucleotides. The introduction of a single oxygen atom, centrally located in a 25-base oligonucleotide strand, was found to significantly alter the spectral/structural properties of the nucleotide bases compared with the parent structure. Moreover, the 8-oxo substituent also induced readily detectable changes in the spectral/structural properties of the phosphodiesterdeoxyribose backbone (Malins et al. 2000). Most recently, the FT-IR technology was able to discriminate between the DNA of primary prostate tumors and metastasizing primary prostate tumors with a sensitivity of 91% and a specificity of 89% (Malins et al. 2003).

Materials and Methods

Sediment collection and analyses. Sediments were obtained with a sediment grab at three locations in both the DR and QMH on the same days as the fish trawls and in the same immediate vicinity. The QMH sediments, however, were acquired somewhat nearer shore in shallower water. For each site, the three sediment grabs were combined, and 10 aliquots were prepared for subsequent analyses. Following standard U.S. EPA analytical methods (U.S. EPA 2001a), sediments were analyzed for PAHs (Method 8270C, selected ion monitoring), PCBs (Method 8082), PCDDs

and PCDFs (Method 8290), and B[a]PEq (Method 4425). The toxic equivalency quotient (TEQ) values for the 17 PCDDs and PCDFs were calculated, as required in the method, using U.S. EPA 1989 toxic equivalency factors. After solvent extraction, the organic fraction was analyzed for PAHs and PCBs, and TEQs were determined for dioxins and furans.

English sole acquisition. English sole were obtained from the lower DR (*n* = 11; weight, 240 ± 34 g) and QMH (*n* = 11; weight, 258 ± 49 g) in October 2000 (Figure 1). Fish were caught by trawls towed for 10–15 min at depths from 5 to 15.5 m. Fish were kept alive until sacrificed onboard the vessel. Before dissection, the length and weight of each fish were noted. Sections of gill tissue (5–10 filaments and the attached gill arch) were placed in formalin for subsequent histologic examination. The remaining gill samples were rapidly frozen on dry ice.

Histology and CYP1A expression. Light microscopy for identifying histologic changes in gill tissue was performed as described previously (Van Veld et al. 1997). Levels of CYP1A expression in the gill were determined using immunohistochemical techniques as applied previously to gill and other fish tissues (Smolowitz et al. 1991; Woodin et al. 1997).

DNA extraction. About 50 μg of DNA was extracted from each gill tissue sample (~ 250 mg) with Qiagen 100/G Genomic-tips (Qiagen, Chatsworth, CA) using the procedure described by the manufacturer. The DNA was
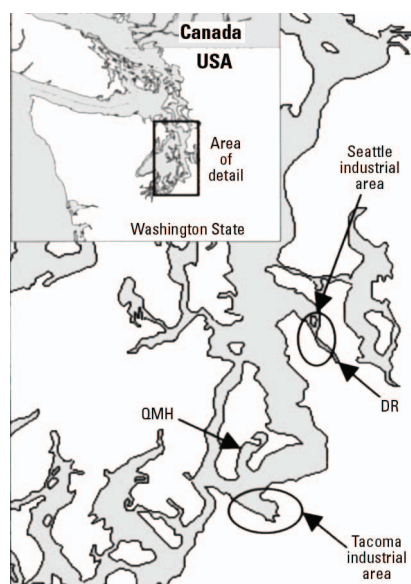
Figure 1. Map of Puget Sound, Washington, showing the DR and QMH where English sole were collected for this study.

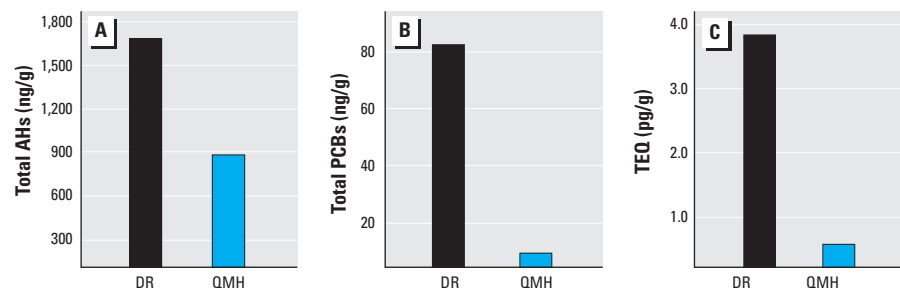**Figure 2.** Sediment data from the DR and QMH. Concentrations of (*A*) 24 AHs and (*B*) 35 PCB congeners. (*C*) TEQ values for 17 dioxins and furans.
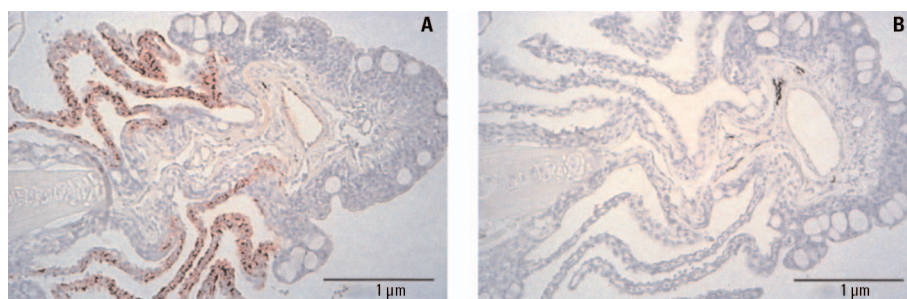
Figure 3. Gill tissue from (*A*) DR fish showing prominently stained epithelial cells reflecting CYP1A expression and from (*B*) QMH fish showing virtually no staining, thus no evidence for CYP1A expression. Specific CYP1A values are shown in Table 1.

then passed through a 5.0 μm Cameo 30N filter (Osmonics, Minnetonka, MN) before precipitation and washed three times with ice-cold 70% ethanol. The Qiagen procedure is an ion-exchange system and thus does not constitute a significant source for artifactual oxidation of base structure during extraction. In preparation for FT-IR spectral analysis, the DNA was dissolved in 10–40 μL optima grade distilled water (Fisher Scientific, Hampton, NH).

FT-IR spectroscopy. FT-IR spectral analysis of DNA was conducted essentially as previously reported (Malins et al. 2003). A 0.2 μL aliquot of DNA solution was spotted on a BaF$_2$ plate. As the spot spread and dried, an outer ring of DNA was formed. Two separate spots were created for each DNA sample. Spotting was repeated until the width of the DNA ring was at least 100 μm wide. Using a microscope spectrometer (System 2000, PerkinElmer, Wellesley, MA), we established a background energy reading from a blank area of the BaF$_2$ plate. For each sample, 10 spectral measurements were made at various points around each of the two rings. Energy readings were then obtained for each of those points. The energy readings were maintained at 15–25% below the background energy. The resulting spectral measurements were expressed as percent transmittance, which was converted (Fourier-transformed) into absorbance. Each spectrum was baselined as previously described (Malins et al. 2002).

Table 1. Mean ± SD for CYP1A staining in different cell types.

Cell type	DR (n = 11)	QMH (n = 11)
Epithelium	7.7 ± 2.0 (11)	0
Vascular endothelium	0.7 ± 1.3 (3)	0
Pillar cells	3.9 ± 4.2 (6)	0.2 ± 0.6 (1)

Values in parentheses indicate number of samples showing staining.

Statistical analyses. Representative FT-IR spectra for each gill DNA were obtained by determining the mean of 20 spectral measurements (Malins et al. 2003). A *t*-test was performed to establish statistical differences (*p*-values) at each wavenumber between the mean spectra for each fish group. Although the *p*-values across approximately 1,000 wavenumbers are not statistically independent, spectral regions with $p \leq 0.05$ are likely to represent real structural differences between groups (Malins et al. 2000).

As recently reported (Malins et al. 2003), model development is accomplished by first conducting principal components analysis (PCA) on the mean spectrum of each isolated DNA sample, resulting in 10 principal component (PC) scores per sample. Significant differences ($p \leq 0.05$) in PC scores between groups were determined using *t*-tests. The PC scores showing the most significant difference between the groups were used to construct two- or three-dimensional PC plots. Subsequent logistic regression analysis (SPSS statistical package; SPSS, Inc., Chicago, IL) was performed using a significant PC score to establish a "DNA damage index" based upon the different spectral properties of gill DNA from each fish group.

Results and Discussion

Sediment data: implications. The analyses of sediments from the DR and QMH for AH, PCB, and TEQ values showed that these were substantially higher in the DR sediments (Figure 2). The PCB values and the TEQ values for dioxins and furans were 8–10 times higher in the DR, and the AH levels were about two times higher. The means ± SDs for DR and QMH sediment analyses (10 aliquots each) for B[a]PEq were 118.2 ± 23.2 and 78.5 ± 8.6 μg/g dry weight, respectively. These diverse measures of contaminants were

selected because they represent compounds known to undergo one- and two-electron oxidations in biologic systems yielding metabolites that react with DNA (Maccubbin 1994). For example, AHs undergo oxidation from free radicals, such as the •OH produced via redox cycling, and two-electron oxidation to produce bulky aromatic metabolites. Both of these types of reactive oxidation products are known to damage DNA (Maccubbin 1994).

Gill histology. We found histologic differences between the gills of the DR and QMH fish. All the gills of the DR fish (*n* = 9), but only about half of the gills from the QMH fish (*n* = 11), exhibited minor cellular changes of unknown etiology (e.g., small foci of necrosis), as well as some degree of epithelial hyperplasia of the lamellae. One of the DR fish had an apparent carcinoma of the gill epithelium, similar to other gill carcinomas that have been described (Spitsbergen et al. 2000).

CYP1A expression. No evidence was found for CYP1A expression in the epithelium and vascular endothelium of the gills from the QMH fish; however, pillar cells in gills from 1 of 11 fish showed slightly positive staining for CYP1A (Figure 3, Table 1). In contrast, strong CYP1A expression was evident in the gill lamellar epithelium and pillar cells of the DR fish. There was also staining in the endothelium of vessels in the gill arches of the DR fish. The degree of CYP1A expression in various fish tissues has been shown to correlate with exposure to environmental chemicals that are AH receptor agonists (Stegeman et al. 2001; Woodin et al. 1997). Thus, these findings support the concept that QMH is relatively free of these contaminants, consistent with its use as a reference site in 1993 and 1995 (Malins et al. 1996a, 1997a) and in the present study.

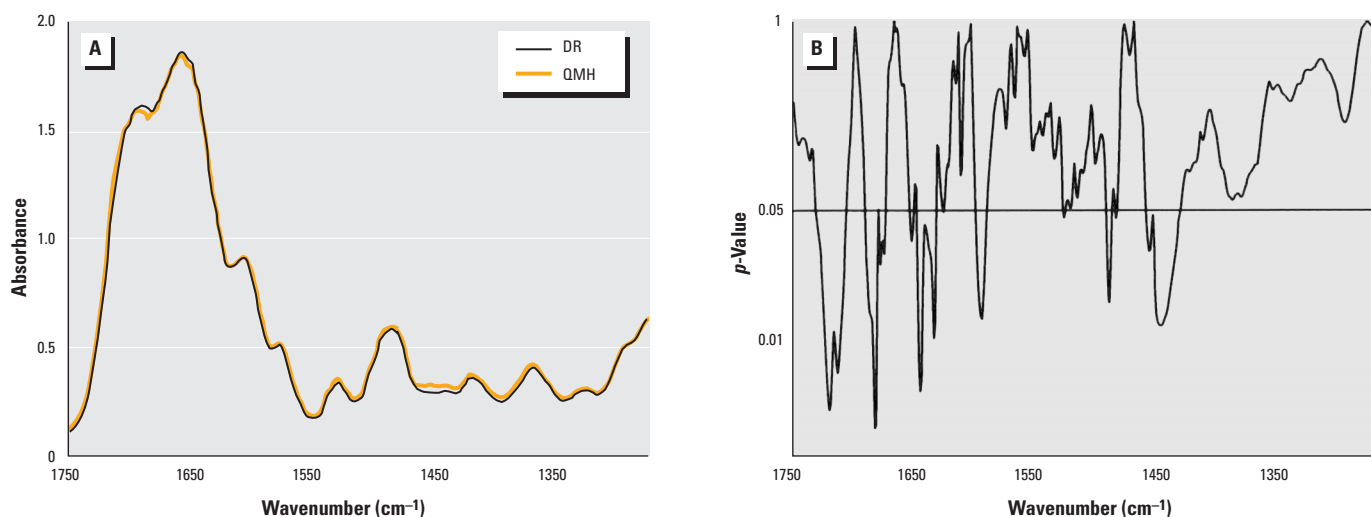*FT-IR spectral studies.* It is well recognized that vibrational and rotational frequencies of a

Figure 4. Comparison of mean FT-IR spectra of gill DNA from DR and QMH (*A*) and *p*-values from a *t*-test between mean spectra at each wavenumber (*B*).

variety of DNA functional groups can be identified by FT-IR spectroscopy (Parker 1983; Tsuboi 1969). Thus, FT-IR spectral analysis has the ability to reveal information on the structures of a variety of molecules, as well as changes occurring in these molecules when exposed to various chemical and biologic stresses.

A major advance in the broad application of FT-IR spectroscopy was the development of a commercially available FT-IR microscope spectrometer, coupled with advanced computer software (Perkins 1987). This achievement facilitated the use of FT-IR spectroscopy for determining structural changes in DNA, such as those associated with the transformation of normal prostate (Malins et al. 2003) and breast (Garcia-Closas et al. 2000) tissues to the cancer state, as well as for identifying contaminant-induced structural changes in the livers of wild fish populations (Malins et al. 1997a). Statistical analyses of spectral changes related to the base and backbone structures of DNA have resulted in the identification of damaged DNA structures and the development of models for predicting the occurrence of cancer (Garcia-Closas et al. 2000; Malins et al. 1997c, 2003).

Although differences between the mean DNA spectra of the DR and QMH fish groups in Figure 4 may appear to be almost imperceptible, PCA is a powerful statistical technique capable of revealing significant differences between relatively similar DNA groups. In previous studies (Garcia-Closas et al. 2000; Malins et al. 1997c, 2003), model development was accomplished by first conducting PCA on each spectrum of isolated DNA and then plotting the PC scores. PCA involves nearly 1 million correlations between ~1,000 independent variables relating to absorbance, wavenumbers, and other properties of the spectra. PC scores from groups of DNA samples representing, for example, fish from contaminated and reference sites will cluster in different areas of the PC plots by virtue of their different mean spectral properties. These separated groups of PC scores are the basis for the development of disease probability models (often having a sensitivity and specificity $\geq$ 80%)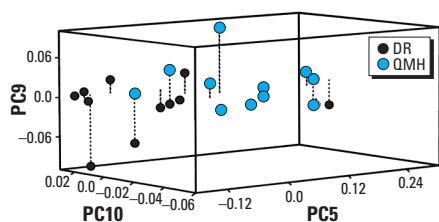 derived from logistic regression analysis using one or more PC scores (Garcia-Closas et al. 2000; Malins et al. 1997c, 2003).

In the present study, FT-IR spectroscopy of gill DNA from the DR and QMH fish groups resulted in mean spectra exhibiting significant differences ($p \leq 0.05$) across 26.5% of the spectral range, far greater than the 5% expected by chance (Figure 4A,B). We attribute these differences, expressed as p-values next to the comparisons of spectral means (Figure 4A,B), to the type and degree of exposure of the gills to the unique suites of xenobiotics at each site. For example, the significant differences found between approximately 1,725 and 1,350 cm^{-1} are attributed to perturbations of C–O-stretching and NH$_2$-bending vibrations (e.g., of adenine and guanine), some of which are coupled to vibrations of the sugar moiety ($\approx$ 1,500–1,300 cm^{-1}) (Taillandier and Liquier 1992). The significant difference ($p < 0.05$) in the peak at $\approx$ 1,530 cm^{-1} (Figure 4) is attributed to alterations in N7–C8 stretching vibrations of imidazole rings (Taillandier et al. 1992). These and other spectral differences (Figure 4B) represent perturbations in normal planar base configurations, thus triggering a "ripple effect" creating conformational disruptions farther afield capable of affecting critical biologic functions, such as transcription and replication (Maccubbin 1994; Malins et al. 2000). The DNA polymerase recognizes the base sequence to which it must bind and align the ribonucleoside triphosphates with the complementary bases. Xenobiotic-introduced structural alterations in the base structure (e.g., inducing alterations in vertical base stacking properties) are likely to subvert this process and lead to aberrant cell development, such as found to a relatively high degree in the DR fish (Figure 2).

PCA used to compare the DNA spectra of the DR and QMH fish resulted in three PC scores (5, 9, and 10) having the most significant differences ($p \leq 0.05$) between the groups. A three-dimensional plot (Figure 5) of these three
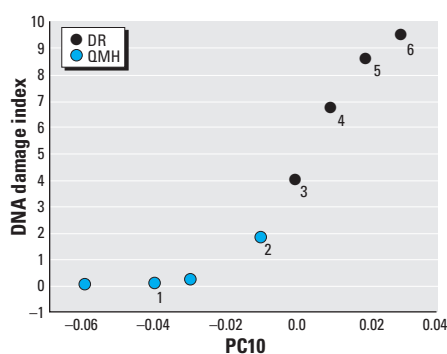
PCs revealed a distinct clustering of the two groups, demonstrating that the DNA from each group has unique spectral/structural properties. This distinct separation led us to conduct a logistic regression analysis (Figure 6) based on PC10, which was the most significant ($p < 0.01$). Based on the logistic regression plot there is an 82% probability of correctly identifying a DR sample and a 92% probability of correctly identifying a QMH sample. The logistic regression plot (Figure 6) is expressed as a DNA damage index, using a scale of 1–10. In this context, 9 of 11 DR scores fall above $\approx$ 5.0 on the index and 10 of 11 of the QMH scores are located below this value, indicating that the DR fish have relatively more damage to their gill DNA than those of QMH fish.

Conclusions

Clearly, a number of factors may contribute to the structural differences in DNA found between the DR and QMH fish. For example, CYP1A bound with planar PCBs is known to generate free radicals (Schlezinger et al. 1999). Thus, these radicals may contribute to the observed structural differences in DNA between the fish groups. However, reactions of PAH metabolites may also contribute to these differences (Maccubbin 1994). The application of the FT-IR statistical models to DNA obtained via nonlethal biopsies of the gill (McCormick 1993) would not involve sacrificing the fish being studied. Moreover, the DNA damage index is a biomarker, which could be linked to changes measured by complementary markers (Hinton 1994), such as CYP1A expression. A DNA damage index, based on reference and test data, similar to the one described, could be readily constructed to quantify effects of contaminants on fish and other aquatic life forms. We believe that this initial study may well stimulate future interest in the relationship between the structural changes observed with gill DNA and histologic abnormalities, using larger numbers of samples. Similarly, we would hope that future studies would relate the FT-IR data to a number of other biologic indices (Ostrander 1996). Overall, the DNA damage index is a promising tool for evaluating biologic effects of contaminants, as well as the effectiveness of remediation.

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Figure 5. Three-dimensional separation of PC scores derived from FT-IR spectra of gill DNA from the DR ($n = 11$) and QMH ($n = 11$). Dotted lines represent the distance from the PC9 baseline level of 0.

Figure 6. DNA damage index derived by logistic regression analysis using PC10 scores for gill DNA from the DR ($n = 11$) and QMH ($n = 11$). Overlapping points: 1, two QMH; 2, one QMH and one DR; 3, five QMH and one DR; 4, four DR; 5, one DR and one QMH; 6, four DR.

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