Fish living in contaminated environments accumulate toxic chemicals in their tissues. Biomarkers are needed to identify the resulting health effects, particularly focusing on early changes at a subcellular level. We used a suite of complementary biomarkers to signal contaminant-induced changes in the DNA structure and cellular physiology of the livers and gills of English sole (Parophrys vetulus). These sediment-dwelling fish were obtained from the industrialized lower Duwamish River (DR) in Seattle, Washington, and from Quartermaster Harbor (QMH), a relatively clean reference site in south Puget Sound. Fourier transform–infrared (FT-IR) spectroscopy, liquid chromatography/mass spectrometry (LC/MS), and gas chromatography/mass spectrometry (GC/MS) identified potentially deleterious alterations in the DNA structure of the DR fish livers and gills, compared with the QMH fish. Expression of CYP1A (a member of the cytochrome P450 multigene family of enzymes) signaled changes in the liver associated with the oxidation of organic xenobiotics, as previously found with the gill. The FT-IR models demonstrated that the liver DNA of the DR fish had a unique structure likely arising from exposure to environmental chemicals. Analysis by LC/MS and GC/MS showed higher concentrations of DNA base lesions in the liver DNA of the DR fish, suggesting that these base modifications contributed to this discrete DNA structure. A comparable analysis by LC/MS and GC/MS of base modifications provided similar results with the gill. The biomarkers described are highly promising for identifying contaminant-induced stresses in fish populations from polluted and reference sites and, in addition, for monitoring the progress of remedial actions. 

**Key words:** cyclopurine nucleosides, cytochrome P4501A, DNA markers, DNA structure, Fourier transform–infrared spectroscopy, liquid chromatography/mass spectrometry. 

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In this article, we describe the results of a study of the same group of English sole used in the previous gill work. In addition to the two biomarkers used for identifying subcellular changes in the gill, we have now found damage to the base structure of the liver and gill DNA using liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). Although previously used with mammalian tissues (Dizdaroglu et al. 2001; Jaruga et al. 2002, 2004), to our knowledge this is the first reported use of LC/MS for evaluating differences between DNA base lesion concentrations in aquatic organisms from test and reference sites.

Materials and Methods

**English sole tissues.** The liver and gills of English sole from the lower DR (n = 18; 141 ± 70 g) and QMH (n = 18; 183 ± 79 g) in October 2000 (Malins et al. 2004c) were provided by Applied Marine Sciences (Livermore, CA). Sections of liver tissue were placed in formalin for histologic examination. The remaining liver samples were frozen (−80°C). The gill tissues were treated similarly, as previously reported (Malins et al. 2004c).

**CYP1A expression and histology.** Levels of CYP1A expression were determined using immunohistochemical techniques (Van Veld et al. 1997; Woodin et al. 1997). Histologic changes in the liver were identified as previously described (Malins et al. 1997; Moore and Myers 1994; Myers et al. 1991).

**DNA extraction.** As previously reported (Malins et al. 2004c, 2005), approximately 300 μg DNA was extracted from each tissue sample (−350 mg: DR, n = 10; QMH, n = 10) with 500/G Genomic-tips (Qiagen, Chatsworth, CA) using the recommended protocol. DNA, in a buffer solution, was passed through a 5.0 μm Cameo 30N filter (Osmonics, Minnetonka, MN) at room temperature before precipitation and then washed with ice-cold 70% ethanol. This mild extraction procedure, conducted on test and reference samples under identical conditions, essentially precludes the possibility that base oxidation would influence the reported comparative studies. Before FT-IR spectral analysis, the DNA was dissolved in 10–40 μl Optima-grade distilled water (Fisher Scientific, Hampton, NH).

**Analysis by FT-IR spectroscopy.** We analyzed the liver DNA using previously reported protocols (Malins et al. 2004c, 2005). A 0.2 μL aliquot of DNA solution was spotted on a BaF2 plate. As the spot dried, an outer ring of DNA was formed. Two separate spots were formed for each DNA sample. The rings of DNA were lyophilized to complete dryness. Using an FT-IR microscope spectrometer (System 2000; PerkinElmer, Wellesley, MA), we made 20 spectral measurements randomly around each of the two rings per sample. Resulting spectral measurements were expressed as percent transmittance, which was converted (Fourier-transformed) into absorbance. Each spectrum was baseline and normalized between 1,300 and 760 cm−1. The remaining DNA was lyophilized for subsequent base lesion analysis.

**Analysis by LC/MS.** We used LC/MS with isotope-dilution to identify and quantify 8-hydroxy-2′-deoxyguanosine (8-OH-dG), 8-hydroxy-2′-deoxyadenosine (8-OH-dA), (5′S)-8,5′-cyclo-2′-deoxyguanosine [(5′S)-cdG], and (5′S)-8,5′-cyclo-2′-deoxyadenosine [(5′S)-cdA] in the liver and gill DNA samples. A stable isotope-labeled analog of 8-OH-dG (8-OH-dG-[15N2]) purchased from Cambridge Isotope Laboratories (Cambridge, MA) was used as an internal standard. Stable isotope-labeled analogues of 8-OH-dA, (5′S)-cdG, and (5′S)-cdA-[8-OH-da-15N4, (5′S)-cdG-[15N2], and (5′S)-cdA-[15N2], respectively) were purchased as described (Jaruga et al. 2002, 2004) and used as internal standards. Aliquots (50 μg) of DNA were supplemented with aliquots of internal standards; hydrolyzed with nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase for 24 h; and then analyzed by LC/MS as described by Jaruga et al. (2004). For identification and quantification, selected ion monitoring served to monitor the characteristic ions of the modified nucleosides and their stable isotopically labeled analogues.

**Analysis by GC/MS.** 2,6-Diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine (FapyA) were identified and quantified using GC/MS with isotope-dilution after hydrolysis of DNA samples with *E. coli* Fpg protein to release FapyG and FapyA. Fpg was isolated as previously described (Reddy et al. 2004). Stable isotope-labeled analogues of FapyG and FapyA (FapyG-[13C3, 15N2] and FapyA-[13C3, 15N2]) were purchased from Cambridge Isotope Laboratories. Aliquots (50 μg) of DNA were supplemented with aliquots of the internal standards FapyG-[13C3, 15N2] and FapyA-[13C3, 15N2] and hydrolyzed with 2 μg Fpg. The hydrolysates were trimethylsilylated and then analyzed by GC/MS as described by Reddy et al. (2004). For identification and quantification, we used selected ion monitoring to monitor the characteristic ions of the trimethylsilylated FapyG and FapyA and their stable isotope-labeled analogues.

**Statistical analyses.** For FT-IR spectral data, we performed a t-test to establish statistical differences (p-values) between the mean DNA spectra for each fish group at each wavenumber. Although p-values over the range of wavenumbers were not statistically independent, spectral regions with p < 0.05 likely represent actual structural differences between groups (Malins et al. 2000).
We conducted principal components analysis (PCA) as reported previously (Malins et al. 2005). PCA involves approximately $10^6$ correlations between spectral absorbances and integrates differences in peak heights, peak locations, and various combinations thereof. This statistical procedure was undertaken on the spectrum of each sample, resulting in 10 principal component (PC) scores per sample. Using t-tests, significant differences ($p < 0.05$) in PC scores were determined between groups. PCs showing the most significant differences were used to construct a three-dimensional plot. Logistic regression analysis, using a highly significant PC, was the basis for establishing a DNA damage index (Malins et al. 2004c, 2005) that reflected differences in the unique spectral properties of the liver DNA for each fish.

For LC/MS and GC/MS data, the following statistical procedures provided comparative information on differences in base lesion concentrations in the liver and gills of fish from the contaminated (DR) and reference (QMH) sites. We used t-tests to determine statistical differences ($p$-values) between groups for each base lesion. A Levene’s test was used to identify significant differences ($p$-values) in the variance among groups for base lesion concentrations (Malins et al. 2002).

**Results and Discussion**

**Histology.** We identified several idiopathic hepatic lesions in the DR fish. The most prominent lesions were basophilia and macrophage aggregates, which were found in 10 and 6 of the samples, respectively. However, these lesions were detected in only 1 and 2 of the QMH samples, respectively (Table 1). These findings are comparable with those previously obtained in the gills of these fish groups (Malins et al. 2004c) in which all the DR fish and half the QMH fish exhibited idiopathic lesions. These changes in the liver would be expected to arise preferentially from the intake of toxic substances in food (e.g., sediment invertebrates), whereas the changes in the gills likely resulted from exposure to waterborne chemicals that are readily transferred across the gills (e.g., low-molecular-weight organic compounds, such as alkylated phenols) (Randall et al. 1996). However, these routes of exposure are not mutually exclusive in that many toxic chemicals absorbed through the gills are ultimately destined for the liver.

**CYP1A expression.** CYP1A staining in the livers of the DR fish ($9.3 \pm 5.2$) was approximately 25-fold greater than in the QMH fish ($0.4 \pm 0.9$) (Figure 1). This high degree of CYP1A expression is very likely associated with the accumulation of comparatively high concentrations of toxic chemicals, such as PAHs and planar PCB congeners, aryl hydrocarbon receptor agonists that occur in the DR environment (e.g., low-molecular-weight organic compounds, such as alkylated phenols) (Randall et al. 1996). However, these routes of exposure are not mutually exclusive in that many toxic chemicals absorbed through the gills are ultimately destined for the liver.

**Comparative data on the gill histology have been reported previously (Malins et al. 2004c).**

Table 1. Number of English sole with liver lesions.

| Condition    | DR ($n = 18$) | QMH ($n = 18$) |
|--------------|--------------|----------------|
| Basophilia   | 10           | 1              |
| Macrophage aggregates | 6           | 2              |
| Spongiosis hepatitis | 3           | 0              |
| Foci of cellular alteration | 2           | 0              |

![Figure 1](image1.png)  
**Figure 1.** Mean ± SD for CYP1A staining of the liver from each fish group. Seventeen of 19 DR samples and 3 of 16 QMH samples stained positive.

![Figure 2](image2.png)  
**Figure 2.** (A) Comparison of mean DNA spectra of liver from the DR and QMH fish between 1,050 and 1,009 cm$^{-1}$. (B) $p$-Values from a t-test showing significant differences between these mean spectra. (C) Comparison of mean DNA spectra of liver from the DR and QMH fish between 829 and 803 cm$^{-1}$. (D) $p$-Values from a t-test showing significant differences between these mean spectra.
of the spectral range (1,300–700 cm⁻¹), indicating differences in backbone structure. This percentage is about twice that expected by chance (Malins et al. 2000). No spectral evidence was found for differences in the nucleotide base structure, which is assigned to approximately 1,750–1,300 cm⁻¹. The comparison of spectral means from 1,050–1,009 cm⁻¹ and 829–803 cm⁻¹ with the corresponding p-values is shown in Figure 2. Spectral differences, such as at approximately 1,025 cm⁻¹ (Figure 2A,B) and between approximately 829 and 803 cm⁻¹ (Figure 2C,D), are attributed to ribose–phosphate main-chain vibrations (Tsuboi 1969).

PCA of the DNA spectra from the DR and QMH liver samples yielded four PCs (p ≤ 0.01), of which PC6, PC9, and PC10 were the most statistically significant (p ≤ 0.001). These PCs were used to construct a three-dimensional plot (Figure 3A). The plot demonstrates that the samples from each group clustered in different regions of three-dimensional space. This virtually complete separation establishes that each group had a unique DNA structure.

The separation of the DR and QMH liver DNA into two structurally distinct groups (Figure 3A) led us to conduct a logistic regression analysis on the spectral data. This analysis provided the basis for establishing a DNA damage index (Malins et al. 2004c) using PC9, the most significant PC (p < 0.001). Strikingly, the resulting damage index (Figure 3B) had a 90% probability for correctly identifying a DR sample and a 100% probability for correctly identifying a QMH sample.

In the present study of the liver DNA, structural differences between the DR and QMH groups were only found in the spectral region associated with the backbone (1,300–700 cm⁻¹). In contrast, in the gill study, the DNA structural differences were restricted to the spectral area associated with the nucleotide bases (1,750–1,300 cm⁻¹) (Malins et al. 2004c). The results of the two studies were quite similar with respect to the high probability of correctly differentiating between the fish exposed to toxic chemicals and those from a relatively clean environment. The highly discriminating power of the DNA damage index, first used with the gill DNA (Malins et al. 2004c) and now with the liver, suggests that such an index holds considerable promise for identifying DNA damage in different tissues from aquatic organisms exposed to environmental chemicals.

Analysis by LC/MS and GC/MS. Living cells are continually exposed to damaging ROS arising from normal cellular metabolism or from one-electron oxidations of xenobiotics. Notably, planar PCB congeners stimulate the release of ROS from induced fish liver microsomes, ostensibly by uncoupling the cytochrome P450 catalytic cycles (Schlezinger et al. 1999). The liver is a prominent site for these reactions, as demonstrated with aquatic vertebrates (Maccubbin 1994; Malins et al. 1996). The highly reactive 'OH, generated from the superoxide radical and H₂O₂ via metal ion catalysis (Imlay et al. 1988), reacts with guanine and adenine to produce redox-ambivalent 8-ÅH–adduct radicals (Candeias and Steenken 2000; Steenken 1989). These intermediate radicals are converted oxidatively to mutagenic 8-ÅH purines (i.e., 8-ÅH-G and 8-ÅH-A) and reductively to mutagenic formamidopyrimidines (i.e., FapyG and FapyA) (Gajewski et al. 1990; Steenken 1989). More than 30 of these types of base lesions have been identified in tissues from different terrestrial species (Dizdaroglu 1992) and in the livers of fish from contaminated environments (Malins et al. 1996). The 8,5´-cyclopurine-2´-deoxynucleosides are an additional class of oxidatively induced DNA lesions that have been found previously in human tissues (Dizdaroglu et al. 2001; Jaruga et al. 2002, 2004). These cyclopurine nucleosides result from abstraction of an H from C-5´ of 2´-deoxyribose by the ‘OH, followed by cyclization of the resulting sugar radical onto the C-8 position of the purine of the DNA. This process is an example of 8-ÅH–fingerprint mechanistic commonality in DNA lesions, broadly implying a need for covalent lesion recognition and repair at this site. In addition, a number of adducts (Figure 4) are also formed in liver DNA of the fish.

| Chemical Structure |
|--------------------|
| **A** 8-OH-dG |
| **B** 8-OH-dA |
| **C** (5'S)-cdG |
| **D** (5'S)-cdA |
| **E** FapyG |
| **F** FapyA |

Figure 3. (A) Three-dimensional separation of PC scores from the DNA spectra of liver from the DR and QMH fish. Dashed lines show the distance from the PC9 baseline level of 0. (B) DNA damage index derived by logistic regression analysis using PC9 scores for liver DNA from the DR and QMH fish. Overlapping points: 1, Two QMH samples; 2, two DR samples.

Figure 4. Chemical structures identified in the liver and gill DNA of the DR and QMH fish. (A) 8-OH-dG. (B) 8-OH-dA. (C) (5’S)-cdG. (D) (5’S)-cdA. (E) FapyG. (F) FapyA.
Biomarkers signal contaminant effects

same nucleoside, and ultimately oxidation of
the resulting radical (Dizdaroglu 1986;
Dizdaroglu et al. 2002). Cyclopurine nucleo-
sides are considered tandem lesions in that
concomitant damage is produced in both the
base moieties and the sugar (Dizdaroglu 1986).

Analyses by LC/MS of the liver DNA
from the DR and QMH fish revealed the
presence of 8-OH-dG and 8-OH-dA, as well
as the analogous cyclopurine nucleosides
(Figure 4). This comparative study revealed
that the DR fish had significantly \( p \leq 0.05 \)
higher DNA concentrations of \( (5\'S)-\text{cdG} \)
(Figure 5A), 8-OH-dA, and \( (5\'S)-\text{cdA} \)
(Figure 5B). GC/MS was used to measure
FapyG and FapyA (Figure 4). Concentrations
of 8-OH-dG, FapyG (Figure 5A), and FapyA
(Figure 5B) were also higher in the DR fish
than in the QMH group, but the differences
were not statistically significant \( (p > 0.05) \).
Although the DNA base lesion concentrations
of the liver were higher in the DR compared
with the QMH fish, the actual levels (parts per
million) were too low to be detected by FT-IR
spectroscopy. Four of the base lesion concen-
trations in the gill DNA were also significantly
\( (p < 0.01) \) higher in the DR fish compared
with the QMH fish (Figure 5C,D). The concen-
trations of \( (5\'S)-\text{cdG} \) and FapyA were too
low to measure.

In a previous study using GC/MS, we com-
pared concentrations of 8-OH-G and 8-OH-A
in the liver DNA of English sole obtained from
the DR in 1993 and 1995 and from QMH in
1995 (Malins et al. 1996). In 1993, the con-
centration of 8-OH-A was eight times higher
in the DR fish compared with the reference fish
\( (p < 0.001) \), and the concentration of 8-OH-G
was four times higher \( (p = 0.01) \). Further,
FT-IR spectral differences were demonstrated
in both the DNA base and backbone structures.

The present base lesion comparisons by
LC/MS and GC/MS clearly demonstrate that
about 7 years later, the liver DNA of fish from
the DR still show a considerably higher degree
of oxidative damage compared with the refer-
ence fish. Although the lower base lesion levels
presently obtained are not surprising because
of pollution controls affecting the contaminant
status of the DR (U.S. EPA 2001), any direct
comparison of the 1993 and 1995 data with the
results obtained in 2000 would be ques-
tionable in that the earlier findings involved
phenol/chloroform extraction of DNA.

Statistically higher variances for the base
lesion concentrations were found with the DR
liver DNA compared with those of the QMH
samples, ranging from approximately 5-fold
for the 8-OH-dG \( (p = 0.03) \) to 30-fold for the
\( (5\'S)-\text{cdG} \) \( (p < 0.01) \) (Table 2). The consist-
tently greater variance in the DR samples
implies more heterogeneity in the nucleotide
base structures compared with the QMH
group. With the exception of FapyG, the vari-
ance of the base lesions concentrations was
also significantly \( (p \leq 0.03) \) higher in the gill
DNA of the DR fish (Table 2). The higher
degree of variance in the DR group is most
likely attributable to reactions of xenobiotics
and/or metabolites with DNA, thus disrupt-
ing the normal architecture of this biopolymer
and creating a variety of oxidation products.
These findings suggest that variance in the
DNA base structure of fish tissues is a poten-
tially useful new biomarker for signaling
chemical-contaminant–induced alterations at
the population level.

Conclusions

FT-IR statistical models demonstrated the
ability to differentiate between the DR and
QMH fish based on the unique DNA struc-
ture of each group. These unique structural
characteristics were the means for establish-
ing a DNA damage index, which was shown to be
an effective indicator of contaminant-induced
damage to liver DNA. These results are com-
parable with those previously obtained with
the gills of these fish (Malins et al. 2004c) and
suggest that the damage index has the poten-
tial to be used with a variety of other tissues.
The changes in the liver DNA likely reflect
contamination primarily through the diet,
whereas the changes in the gill DNA probably
mostly reflect exposure to waterborne chemi-
cals. The DNA damage index can thus pro-
vide insight into routes of contamination and
their relative impacts on cellular physiology.

PC plots derived from FT-IR spectra of
liver DNA of English sole obtained from
the DR in 1993 and 1995 showed that the DNA
structures were readily distinguishable from
each other, as well as from the DNA of the
QMH reference fish (Malins et al. 1997). In
the present study, the liver DNA of the DR
fish is still structurally different from that of
the QMH fish (implying the continued pres-
ence of xenobiotic-induced DNA damage),
deeply many years of sediment cleanups and
efforts to control the input of toxic chemicals
(Lower Duwamish Waterway Group 2004;
Mickelson and McElhany 2002).

Table 2. Variance differences in base lesion
concentrations in the DNA from the DR
and QMH fish livers and gills (expressed as
base lesions/10^6 nucleosides).

| Tissue | 8-OH-dG | 8-OH-dA | (5’S)-cdG | (5’S)-cdA | FapyG | FapyA |
|--------|---------|---------|-----------|-----------|-------|-------|
| Liver  |
| DR variance | 16.88 | 1.73 | 4.43 | 0.08 | 25.12 | 0.54 |
| QMH variance | 3.39 | 0.10 | 0.15 | 0.01 | 5.01 | 0.05 |
| Levene’s test | 0.03 | 0.00 | 0.00 | 0.00 | 0.03 | 0.04 |
| Gill   |
| DR variance | 1.76 | 0.33 | ND | 0.01 | 1.63 | ND |
| QMH variance | 0.59 | 0.07 | ND | 0.00 | 0.62 | ND |
| Levene’s test | 0.03 | 0.04 | ND | 0.00 | 0.16 | ND |

ND, not determined.
The differences in base lesion concentrations found in the liver and gill DNA between the fish from the DR and QMH provide useful biomarker information on reactions resulting in base oxidations. The discovery that the cyclopurine nucleosides accumulated in relatively higher concentrations in the DNA of the DR fish suggests that variance is also an advantageous biomarker for identifying pollution effects at the population level. Moreover, the information on DNA structure obtained by FT-IR spectroscopy, complemented by LC/MS and GC/MS, has the advantage of providing an assessment of contaminant effects on various tissues in aquatic species. The differences found in DNA structure between the DR and QMH fish illustrate the potential that the biomarker systems described have for monitoring and evaluating the efficacy of environmental remediation.

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