Application of Multi-stable Isotope ($^{13}$C, $^{15}$N, $^{34}$S, $^{37}$Cl) Assays to Assess Spatial Separation of Fish (Longnose Sucker *Catostomus catostomus*) in an Area Receiving Complex Effluents

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Incorporation of stable isotope analysis (SIA) into routine environmental effects monitoring programs of receiving waters may enable determination of the spatial extent of biotic exposure and discrimination among sources of complex effluents. To evaluate this hypothesis, longnose sucker (*Catostomus catostomus*) were collected from four sites along the Athabasca River, Alberta (upstream reference site, two sites downstream of effluents from two pulp and paper mills, and a site downstream of effluent from a municipal sewage treatment plant). Stable isotopes of carbon, nitrogen, sulfur and chlorine were analyzed in bone, gonad, liver and white muscle tissues of the fish. In general, most sites and tissues differed according to $\delta^{13}$C, $\delta^{15}$N and $\delta^{34}$S values. Also, an interaction between site and tissue was observed for $\delta^{15}$N values. A better insight into the usefulness of stable isotopes was obtained through the use of multivariate discriminant function analysis. $\delta^{15}$N and $\delta^{34}$S signatures of gonad and liver tissues of males were most effective at classifying fish according to site (~70% for both tissues). For all tissues except bone, fish from the upstream reference site were most separable from all others, especially females. $\delta^{37}$Cl values for female gonads and male livers were related to sites downstream of the pulp and paper mills. Future research should routinely include SIA of fish tissues, but also of effluents, receiving waters and food web components to better resolve links between specific effluents and fish exposure.

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Key words: effluents, fish exposure, multi-isotopic assays, pulp and paper mills, river ecology, sewage treatment plants

Introduction

Rivers and streams commonly serve as receiving waters for a wide variety of treated effluents. Among the more common sources of effluent are pulp and paper mills and municipal sewage treatment facilities. When effluents from single sources are considered in isolation, it is often straightforward to link the impacts of these effluents to communities, populations, organisms and tissues of aquatic biota. However, in many situations, the mixing of effluents from multiple sources and the dilution of effluents in hydraulically dynamic receiving waters complicates establishing specific links between effluents and effects. This is problematic because characterization and quantification of effluent exposure on aquatic biota is essential to shape policies intended to encourage mitigation and restorative efforts.

With the advent of the Environmental Effects Monitoring (EEM) Program associated with revised *Pulp and Paper Effluent Regulations* in Canada, a formal frame-work was established to assess the status of aquatic systems subjected to pulp mill effluents (PMEs) (Walker et al. 2002). Although not explicitly included in the EEM Program, when effluents from other sources, such as municipal sewage treatment plants, are also present in receiving waters, their effects on aquatic biota need to be assessed insofar as they may confound interpretation of any effects due to PMEs. A key component of this framework is that mills are required to conduct adult fish surveys (Munkittrick et al. 2002). Due to their longevity and trophic position, adult fish are hypothesized to be indicators of exposure levels to effluents in aquatic environments (Gibbons and Munkittrick 1994). Comparisons of fish populations upstream and downstream of effluent discharges are made to determine if effluent is affecting population age structure, energy use or energy storage. However, fish are also mobile, so unless fish populations are isolated within specific effluent plumes, supplemental analytical tools are needed to assess exposure (Dubé 2004).

The incorporation of stable isotope assays of fish tissues into routine EEM programs offers the potential to quantify the spatial extent of exposure and to trace the
source of effluents (Gannes et al. 1998; Dubé 2004). For
the stable isotopes of carbon ($^{13}$C/$^{12}$C), nitrogen ($^{15}$N/$^{14}$N) and sulfur ($^{34}$S/$^{32}$S), fish acquire isotopic signatures through food web interactions that reflect the diets of prey resources. Isotopic values of carbon and sulfur are considered to be indicative of food source as biological fractionation (or discrimination) associated with the ingestion of prey is minimal (~0–1‰; Fry and Sherr 1984; Fry 1991; McCutchan et al. 2003). By contrast, isotopic enrichment of $^{15}$N is positively correlated with trophic position (~3–3.5‰ per trophic level; Minagawa and Wada 1984; Vander Zanden and Rasmussen 1999). Another element, chlorine ($^{37}$Cl/$^{35}$Cl), also holds promise to be an informative isotopic indicator of fish exposure. Unlike the stable isotopes of $^{13}$C, $^{15}$N and $^{34}$S, uptake of $^{37}$Cl appears to be via the gills (Dubé and Wassenaar 2002). The implication of this pathway of absorption is that $^{37}$Cl values may be unaffected by variation in food source and could, therefore, act as another conservative tracer of effluent source. Given the distinct characteristics of each of these stable isotopes, this multi-isotopic approach may provide detailed information on the exposure of fish to complex effluents.

The premise behind the approach is that naturally occurring stable isotope ratios in organisms from reference, or unpolluted sites, are distinct from ratios derived from pollution or nutrient inputs (Van Dover et al. 1992; Wassenaar and Culp 1996). Stable isotopes of carbon and sulfur can track movement of PME through aquatic food webs. $^{13}$C values are a function of the relative contribution of terrestrial or exogenous carbon (i.e., wood pulp) to the river food web (Rounick and Winterbourn 1986; France 1995a) and $^{34}$S signatures indicate loadings of sodium sulfate used in the digestion (kraft mills) or softening (chemi-thermomechanical mills) of pulp (Wassenaar and Culp 1996). While stable isotopes of nitrogen have also been used as a measure of allochthony (France 1995b), nitrogen more ordinarily has been used as an indicator of municipal sewage effluent that is enriched in ammonia and nitrate (Van Dover et al. 1992; Lake et al. 2001), as well as a measure of trophic position (Vander Zanden and Rasmussen 1999; Post 2002).

The use of $^{37}$Cl values in fish to assess exposure to PME has not been sufficiently tested in a natural setting. However, van Warmerdam et al. (1995) showed that the same chlorinated organic solvents produced by different manufacturers had distinctive $^{37}$Cl and $^{13}$C values, suggesting there is potential for isotopic fingerprinting. Dubé and Wassenaar (2002) tested this hypothesis in a laboratory experiment and found that effluents from a variety of pulp mill processes could be reliably identified using all four stable isotopes. They further found that fish (rainbow trout [Onchorhynchus mykiss]) and a benthic invertebrate (Chironomus tentans) exposed to 10% PME were significantly depleted in $^{37}$Cl relative to controls.

The largest industrial source of chlorinated compounds in Sweden is from the paper pulp industry, which produces 10,000 tonnes per year (Gribble 1994). Although the use of elemental chlorine as a bleaching agent has been discontinued at most mills in Canada and abroad, many other sources of chlorination can exist within the mill process including use of alternate bleaching agents such as chlorine dioxide and use of chlorine-containing additives (Dubé et al. In press). In Canada in 2005, from the 123 mills examined, 35% report using chlorine dioxide as a bleaching agent suggesting that use of $^{37}$Cl as a tracer of exposure in fish has relevance for further investigation (Environment Canada, National Environmental Effects Monitoring Office, Unpublished data). In addition, the significance of chlorine as a natural component of trees should also not be overlooked. As a fundamental chemical element, chlorine is not only abundant in the Earth’s crust (ranking 18th in the list of elements) but it is also ubiquitous in soil, rivers, lakes, trees, plants and, of course, oceans (Winterton 2000). In terrestrial plants and trees, natural chlorine-based compounds are involved in photosynthesis and organochlorines often act as anti-feedants to protect plants from attack by insects or herbivores.

We hypothesized that longnose sucker (Catostomus catostomus Forster 1773) exposed to effluents from two different pulp mills and a sewage treatment plant along the Athabasca River, Alberta, would have altered isotope compositions due to the input of terrestrially derived and processed organic matter, chemical additions such as sodium sulphate and chlorine-bearing compounds, and nitrogen enrichment from sewage. Longnose sucker was designated as an appropriate sentinel species for the EEM Program (Munkittrick et al. 2002). We further hypothesized that relationships between exposure and individual isotope signatures would be at least partially dependent on the type of fish tissue analyzed. For example, compared to all other tissues, white muscle in rainbow trout was found to be the least variable in $^{13}$C and $^{15}$N ratios (Pinnegar and Polunin 1999).

There were two objectives of this study: (1) to assess whether fish collected downstream from PME and sewage treatment plant effluents in the Athabasca River, Alberta, could be distinguished from fish collected from reference sites using an independent multi-isotope classification approach, and (2) to rank sex and type of fish tissue according to their efficacy as indicators of exposure to either PME or sewage treatment plant effluents. Our overall goal was to assess the applicability of a multi-stable isotope analysis as a tracer methodology to link the presence of effluents to exposure in wild fish populations.

Materials and Methods

Study Sites

The Athabasca River is in the boreal plains ecozone of Alberta. Its headwaters originate in the Columbia Ice
Fields in Jasper National Park and the river discharges 1300 km downstream into Lake Athabasca (Fig. 1). During September 2000, samples of wild longnose sucker were collected from four sites along a 70-km reach of the Upper Athabasca River. There are no barriers to fish movement along this reach of the Athabasca River. The reference site (REF) is located near the confluence of the Athabasca River and a tributary, Windfall Creek, approximately 31 km upstream of the Town of Whitecourt, Alberta. The first pulp and paper mill (Alberta Newsprint Company—ANC) is 22 km downstream of the REF site. ANC operates an integrated thermomechanical, de-inked pulp and paper mill, which produces newsprint and rotogravure newsprint (Shelast et al. 2000). ANC produces 600 t day⁻¹ of softwood pulp and paper and discharges 16,000 m³ day⁻¹ of effluent. The second pulp and paper mill is located 10 km downstream from the first mill (Millar Western—MW). MW operates a bleached chemi-thermomechanical mill, which produces high-yield pulp for tissue, towel, and printing and writing papers (Shelast et al. 2000). MW produces 800 t day⁻¹ of softwood and hardwood pulp and discharges 11,000 m³ day⁻¹ of effluent. Neither of the mills uses elemental chlorine or chlorine derivatives during the bleaching process but whiten pulp using hydrogen peroxide. Both mills have secondary wastewater treatment and discharge treated effluent through a diffuser structure. Finally, the sewage treatment plant (STP) for the Town of Whitecourt, Alberta, is located another 4 km downstream from the MW pulp and paper mill. The STP also employs secondary wastewater treatment, primarily to reduce biochemical oxygen demand before effluent release. Sampling sites for ANC, MW and STP were all immediately downstream of their respective outfalls.

Fish Sampling and Processing
Twelve male and twelve female longnose sucker were sampled using electrofishing (Smith-Root SR-20 electrofishing workboat, provided by Alberta Environment) from each of the four locations, for a total of 96 fish. Fish were removed from the river by dip nets and a pole seine held downstream of the electrofisher and then transported live to the on-site processing laboratory. Each fish was rendered unconscious by concussion and basic morphometric measurements were recorded including fork length (±1 cm) and body weight (±0.01 g). Fish were dissected in the field and samples of bone, gonad, liver and white muscle tissues were collected for a total of 384 tissue samples. Tissues were stored at -40°C, air-dried and then ground into powder using mortar and pestle. All tissues were stored in glass scintillation vials.

Stable Isotope Analysis of Fish Tissues
Stable isotope analyses were performed on freeze-dried tissues. Tissue samples for δ¹³C and δ¹⁵N analysis were lipid-extracted by soaking the samples overnight in a 50:50 (V/V) mixture of methanol and chloroform and air-dried prior to analysis. Samples for δ³⁴S and δ³⁷Cl analyses were not lipid-extracted. However, due to insufficient mass of tissue following allocations for δ¹³C, δ¹⁵N and δ³⁴S analyses, samples had to be pooled among several fish at each site for each sex to attain the minimum mass of material needed for δ³⁷Cl analysis. δ³⁷Cl analyses on pooled samples were conducted for gonad, liver and muscle tissues. Insufficient material was available to conduct δ³⁷Cl analyses on bone tissue.

Stable carbon and nitrogen isotope ratios were quantified by conventional elemental analyzer flash combustion to CO₂ and N₂, and measured by continuous flow isotope ratio mass spectrometry using a GV Instruments Isoprime and Eurovector EA at the National Hydrology Research Centre (NHRC), Saskatoon, Saskatchewan. Sulfur isotope analyses of non-lipid-extracted tissues were conducted at the Isotope Science Laboratory, Department of Physics, University of Calgary, also by continuous flow isotope ratio mass spectrometry. For δ³⁴S assays of fish tissues, non-lipid-extracted samples were combusted using a Parr oxygen combustion bomb procedure. This high-pressure oxidation-combustion technique converts all tissue Cl forms to NaCl. This NaCl was then redissolved in distilled water, filtered and a 100% AgCl precipitate was obtained. AgCl was then converted to MeCl for measurement of δ³⁷Cl at NHRC (Wassenaar and Koehler In press).

Isotopic results were normalized to the ratio of the sample to a reference standard (¹³C—Vienna Pee Dee Belemnite; ¹⁵N—Air; ³⁴S—Canyon Diablo Meteorite; and ³⁷Cl—Standard Mean Ocean Chloride). All values are expressed using the conventional delta notation (δ), which refers to values in parts per thousand (%). Replicate measurements of internal homogenized working standards were better than 0.1‰ for carbon, 0.2‰ for nitrogen, 0.4‰ for sulfur and 0.08 % for chlorine.

Statistical Analysis
The stable isotope data were analyzed using the SAS (SAS Institute 1990) statistical software package. Data were screened to identify outliers and assess normality prior to analysis. Analysis of variance (ANOVA), multivariate ANOVA (MANOVA) and canonical discriminant function analysis (DISCRIM) were employed to identify significant relationships among isotopes, sites and tissues, and to estimate the reliability of matching specific isotopes to specific effluents. δ³⁷Cl values were only analyzed using ANOVA; pooling of tissues among fish to achieve sufficient mass for isotopic analysis reduced the “sample size” by 75%. For the ANOVA analyses, post hoc Bonferroni adjusted pairwise comparisons, Tukey Studentized Range (HSD) tests, were conducted to detect significant differences among sites and tissue types. For
Fig. 1. Map of the Athabasca River, Alberta, study area, indicating sampling sites. REF (Windfall Reference Site); ANC (Alberta Newsprint Company); MW (Millar Western Industries); STP (Sewage Treatment Plant at Whitecourt, AB).
the MANOVA analyses, Wilks’ Lambda (λ) was used as the statistic to test for patterns between sites and tissues. For the canonical discriminant function analyses, Kappa (κ) likelihood tests were used to calculate the improvement in predictive power of classifying sites by discriminant function over chance alone. Because there were four study sites, there was a 25% chance that isotopically distinct fish would be correctly matched to one of the sites. Significance was assessed at α = 0.05.

Results

Fish Morphometry

Across all four sites, female fish were almost 10% longer and almost 25% heavier than male fish (Table 1). Female lengths and weights did not differ among sites (one-way ANOVA for length: \( F_{3, 44} = 0.14, P = 0.93 \); one-way ANOVA for weight: \( F_{3, 44} = 0.15, P = 0.93 \)). By contrast, there were significant differences in male lengths and weights between some of the sites (one-way ANOVA for length: \( F_{3, 44} = 3.48, P = 0.024 \); one-way ANOVA for weight: \( F_{3, 44} = 2.97, P = 0.042 \)). No significant interactions between sex and site were detected for either females or males. For male lengths, fish differed between the REF and ANC sites (Tukey HSD test), and for male weights, fish differed between ANC and STP sites (Tukey HSD test).

Stable Isotope Ratios in Fish Tissues – Univariate ANOVAs

In general, \( \delta^{13}C \), \( \delta^{15}N \) and \( \delta^{34}S \) values varied with both site and tissue type (Table 2; Fig. 2). Further, for \( \delta^{15}N \), the interaction term between site and tissue type was also significant. Overall patterns were similar between males and females.

Among tissues, for both males and females, \( \delta^{13}C \) was most positive in bone (males: \( F_{3, 188} = 98.97 \), \( P = <0.0001 \); females: \( F_{3, 188} = 111.01 \), \( P = <0.0001 \); Tukey HSD tests) and \( \delta^{15}N \) was most positive in muscle (males: \( F_{3, 188} = 54.92 \), \( P = <0.0001 \); females: \( F_{3, 188} = 29.21 \), \( P = <0.0001 \); Tukey HSD tests). Variation among sites in \( \delta^{13}C \) values was less for females than for males. In males, \( \delta^{13}C \) was most negative in liver (Tukey HSD test), and in females, \( \delta^{15}N \) was most negative in liver (Tukey HSD test). Both \( \delta^{13}C \) and \( \delta^{15}N \) discriminated better among male tissues than female tissues. For males and, to a lesser degree for females, \( \delta^{34}S \) values were more positive in gonad and liver than muscle and bone (males: \( F_{3, 188} = 5.26 \), \( P = <0.0017 \); females: \( F_{3, 188} = 4.35 \), \( P = <0.0055 \); Tukey HSD tests).

Among sites, male \( \delta^{13}C \) values were generally more positive across all tissues at the ANC mill downstream from the REF site, and then gradually declined to levels similar to REF by the STP site (\( F_{3, 188} = 4.40 \), \( P = 0.0051 \); Tukey HSD test). No patterns were detected for female \( \delta^{13}C \) values among sites (\( F_{3, 188} = 0.87 \), \( P = 0.46 \); Tukey HSD test).

Overall, for both males and females, \( \delta^{15}N \) values were most negative at the upstream REF and ANC sites; progressive \( ^{15}N \) enrichment occurred downstream through the following mill sites until reaching a maximum below the STP site (males: \( F_{3, 188} = 17.47 \), \( P = <0.0001 \); females: \( F_{3, 188} = 11.04 \), \( P = <0.0001 \); Tukey HSD tests). This was evident in all male tissues, although this trend was only statistically significant in muscle and liver tissues of females (Fig. 2) and not in gonad or bone.

In contrast, for both males and females, \( \delta^{34}S \) values were most positive at the upstream REF site, then gradually declining further downstream to the MW and STP sites (males: \( F_{3, 188} = 29.84 \), \( P = <0.0001 \); females: \( F_{3, 188} = 22.59 \), \( P = <0.0001 \); Tukey HSD tests). Sites with the highest tissue \( \delta^{34}S \) values generally had the lowest \( \delta^{15}N \) values. For \( \delta^{34}S \), females were a more reliable indicator of reference condition as all four tissues were significantly enriched in \( ^{34}S \) compared to any of the exposed sites (Tukey HSD test).

One-way ANOVAs of \( \delta^{37}Cl \) were less consistent than the other isotopes between sexes and among tissues. \( \delta^{37}Cl \) values of female gonads showed that ANC was significantly different than all other sites (\( F_{3, 7} = 8.71 \), \( P = 0.0092 \); Tukey HSD test). Significant differences between sites MW and ANC using \( \delta^{37}Cl \) from male livers were also observed (\( F_{3, 7} = 6.82 \), \( P = 0.0135 \); Tukey HSD test). These \( \delta^{37}Cl \) results suggest unique iso-

### Table 1. Summary statistics for longnose sucker sampled from the Athabasca River (mean and standard error)*

<table>
<thead>
<tr>
<th>Site</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (cm)</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>Windfall (REF)</td>
<td>41.5 (1.0)</td>
<td>817.4 (38.8)</td>
</tr>
<tr>
<td>Alberta Newsprint Company (ANC)</td>
<td>41.6 (0.9)</td>
<td>801.3 (87.9)</td>
</tr>
<tr>
<td>Millar Western Industries (MW)</td>
<td>42.0 (0.6)</td>
<td>787.2 (75.6)</td>
</tr>
<tr>
<td>Sewage treatment plant (STP)</td>
<td>41.4 (0.4)</td>
<td>844.2 (23.0)</td>
</tr>
<tr>
<td>All sites combined</td>
<td>41.6 (0.4)</td>
<td>812.3 (30.3)</td>
</tr>
</tbody>
</table>

*Significant differences (\( P < 0.05 \)) observed between sites for male lengths and weights are indicated by values in bold (Tukey HSD tests). Twelve males and twelve females were sampled from each site.
tope patterns occur in $\delta^{37}$Cl in the PME from ANC and MW; however, they must be interpreted cautiously due to our small sample sizes.

Stable Isotope Ratios in Fish Tissues – Multivariate Analyses

With the exception of female bone tissue, all MANOVAs (i.e., DF1) were significant, indicating that isotopic values of tissues differed among sites when analyzed all together (Fig. 3). Further, four of seven MANOVAs had significant second discriminant functions (i.e., DF2). For all male tissues, DF1, the function that accounted for the most variation in the data, was composed of $\delta^{15}$N and $\delta^{34}$S. For the female tissues, $\delta^{34}$S was present in DF1 for all tissues and $\delta^{15}$N was present in all tissues but bone. No consistent pattern among tissues was observed for the isotopic composition of DF2 for either males or females.

$\delta^{15}$N and $\delta^{34}$S were more useful than $\delta^{13}$C for identifying specific sites (Fig. 3). The REF site (Windfall upstream) was the most clearly separable, compared to the other sites, especially for the females. Male gonad and liver tissues had the best classification rate or success rate, over chance alone, at distinguishing between sites (61.1 and 58.3%, respectively), followed by muscle tissue (50.0%). Liver tissue had the best classification rate for females (40.4%), but it was much lower than that for males. Bone generally fared poorly as a tissue to discriminate among sites for both females and males.

**Discussion**

The use of stable isotopic forensics as indicators of pollutant exposure is still at an early stage. Stable isotopes hold promise as forensic tools to provide empirical evidence of links between effluents and fish exposure (Dubé 2004). However, the natural range of variability of isotopic val-

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**TABLE 2. Summary statistics of longnose sucker stable isotope values by sex, site and tissue (mean and standard error) a**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Site</th>
<th>Tissue type</th>
<th>$\delta^{13}$C ‰</th>
<th>$\delta^{15}$N ‰</th>
<th>$\delta^{34}$S ‰</th>
<th>$\delta^{37}$Cl ‰</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>REF</td>
<td>Bone</td>
<td>-24.2 (0.3)</td>
<td>7.1 (0.2)</td>
<td>8.4 (0.5)</td>
<td>—</td>
</tr>
<tr>
<td>F</td>
<td>REF</td>
<td>Gonad</td>
<td>-26.3 (0.2)</td>
<td>6.3 (0.2)</td>
<td>8.2 (0.2)</td>
<td>-0.3 (0.4)</td>
</tr>
<tr>
<td>F</td>
<td>REF</td>
<td>Liver</td>
<td>-26.5 (0.2)</td>
<td>5.3 (0.3)</td>
<td>9.3 (0.4)</td>
<td>-2.8 (0.7)</td>
</tr>
<tr>
<td>F</td>
<td>REF</td>
<td>White muscle</td>
<td>-26.1 (0.2)</td>
<td>8.1 (0.1)</td>
<td>8.5 (0.4)</td>
<td>-2.7 (0.8)</td>
</tr>
<tr>
<td>F</td>
<td>ANC</td>
<td>Bone</td>
<td>-24.4 (0.2)</td>
<td>7.8 (0.3)</td>
<td>6.8 (0.6)</td>
<td>—</td>
</tr>
<tr>
<td>F</td>
<td>ANC</td>
<td>Gonad</td>
<td>-26.5 (0.2)</td>
<td>6.8 (0.3)</td>
<td>7.3 (0.3)</td>
<td>-4.4 (0.6)</td>
</tr>
<tr>
<td>F</td>
<td>ANC</td>
<td>Liver</td>
<td>-26.6 (0.2)</td>
<td>6.0 (0.2)</td>
<td>7.5 (0.4)</td>
<td>-4.2 (0.1)</td>
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<tr>
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<td>ANC</td>
<td>White muscle</td>
<td>-26.1 (0.1)</td>
<td>8.3 (0.2)</td>
<td>6.6 (0.5)</td>
<td>-1.8 (1.2)</td>
</tr>
<tr>
<td>F</td>
<td>MW</td>
<td>Bone</td>
<td>-24.5 (0.1)</td>
<td>7.6 (0.2)</td>
<td>5.9 (0.6)</td>
<td>—</td>
</tr>
<tr>
<td>F</td>
<td>MW</td>
<td>Gonad</td>
<td>-26.8 (0.3)</td>
<td>7.9 (0.3)</td>
<td>6.5 (0.2)</td>
<td>-0.8 (0.9)</td>
</tr>
<tr>
<td>F</td>
<td>MW</td>
<td>Liver</td>
<td>-26.9 (0.2)</td>
<td>7.1 (0.2)</td>
<td>7.2 (0.3)</td>
<td>-2.0 (1.3)</td>
</tr>
<tr>
<td>F</td>
<td>MW</td>
<td>White muscle</td>
<td>-26.3 (0.1)</td>
<td>8.8 (0.2)</td>
<td>5.2 (0.5)</td>
<td>-3.7 (0.7)</td>
</tr>
<tr>
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<td>STP</td>
<td>Bone</td>
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<td>—</td>
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<tr>
<td>F</td>
<td>STP</td>
<td>Gonad</td>
<td>-26.2 (0.2)</td>
<td>7.6 (0.4)</td>
<td>6.4 (0.4)</td>
<td>-1.1 (0.6)c</td>
</tr>
<tr>
<td>F</td>
<td>STP</td>
<td>Liver</td>
<td>-26.2 (0.2)</td>
<td>7.8 (0.4)</td>
<td>7.2 (0.4)</td>
<td>-1.5 (0.4)</td>
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<td>STP</td>
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<td>8.7 (0.2)</td>
<td>5.5 (0.7)</td>
<td>-5.6 (0.2)c</td>
</tr>
<tr>
<td>M</td>
<td>REF</td>
<td>Bone</td>
<td>-25.2 (0.2)</td>
<td>5.9 (0.2)</td>
<td>8.4 (0.2)</td>
<td>—</td>
</tr>
<tr>
<td>M</td>
<td>REF</td>
<td>Gonad</td>
<td>-25.5 (0.2)</td>
<td>5.1 (0.2)</td>
<td>8.2 (0.1)</td>
<td>-4.4 (0.6)</td>
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<td>M</td>
<td>REF</td>
<td>Liver</td>
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<td>6.3 (0.2)</td>
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<tr>
<td>M</td>
<td>REF</td>
<td>White muscle</td>
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<td>7.4 (0.3)</td>
<td>8.2 (0.2)</td>
<td>-2.9 (0.4)c</td>
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<tr>
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<td>ANC</td>
<td>Bone</td>
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<td>6.7 (0.2)</td>
<td>7.7 (0.5)c</td>
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<tr>
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<td>ANC</td>
<td>Gonad</td>
<td>-25.2 (0.2)</td>
<td>5.1 (0.2)</td>
<td>9.5 (0.3)</td>
<td>-3.0 (0.1)</td>
</tr>
<tr>
<td>M</td>
<td>ANC</td>
<td>Liver</td>
<td>-26.8 (0.2)</td>
<td>6.5 (0.2)</td>
<td>8.6 (0.3)</td>
<td>-4.4 (0.5)</td>
</tr>
<tr>
<td>M</td>
<td>ANC</td>
<td>White muscle</td>
<td>-25.8 (0.1)</td>
<td>8.2 (0.4)</td>
<td>7.3 (0.4)</td>
<td>-2.3 (0.3)</td>
</tr>
<tr>
<td>M</td>
<td>MW</td>
<td>Bone</td>
<td>-25.0 (0.2)</td>
<td>7.4 (0.1)</td>
<td>6.4 (0.3)c</td>
<td>—</td>
</tr>
<tr>
<td>M</td>
<td>MW</td>
<td>Gonad</td>
<td>-26.0 (0.2)</td>
<td>5.7 (0.2)</td>
<td>6.9 (0.3)</td>
<td>-2.5 (0.0)</td>
</tr>
<tr>
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<td>7.0 (0.3)</td>
<td>-1.8 (0.1)</td>
</tr>
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<td>MW</td>
<td>White muscle</td>
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<td>8.9 (0.3)</td>
<td>6.1 (0.5)</td>
<td>-3.2 (0.7)</td>
</tr>
<tr>
<td>M</td>
<td>STP</td>
<td>Bone</td>
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<td>7.4 (0.2)</td>
<td>6.5 (0.5)c</td>
<td>—</td>
</tr>
<tr>
<td>M</td>
<td>STP</td>
<td>Gonad</td>
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<td>6.6 (0.2)</td>
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<td>-2.8 (0.5)</td>
</tr>
<tr>
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<td>Liver</td>
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<td>8.8 (0.2)</td>
<td>7.0 (0.3)</td>
<td>-2.5 (0.1)</td>
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<tr>
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<td>STP</td>
<td>White muscle</td>
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<td>8.8 (0.2)</td>
<td>6.0 (0.6)</td>
<td>-2.5 (0.6)</td>
</tr>
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</table>

aFor isotopes of carbon, nitrogen and sulfur, the sample sizes were 12, and for Cl, the sample size was 3, unless noted otherwise. F (females); M (males); REF (Windfall Reference Site); ANC (Alberta Newsprint Company); MW (Millar Western Industries); STP (Sewage Treatment Plant at Whitecourt, Alta.). Statistics results are summarized in Fig. 2 and 3. Insufficient sample was available for $\delta^{37}$Cl assays of bone.

bSample size = 11.

cSample size = 2.
Fig. 2. Stable isotope values for tissues (bone, gonad, liver, muscle) taken from male and female long-nose sucker sampled from sites along the Athabasca River (means and standard errors). Note that for all clusters of bars, the sites are listed sequentially from upstream to downstream sites. REF (Windfall Reference Site); ANC (Alberta Newsprint Company); MW (Millar Western Industries); STP (Sewage Treatment Plant at Whitecourt, Alberta). Where applicable, significance between sites for each tissue is indicated by letter combinations.
Fig. 3. Plots of results of canonical discriminant function analyses (means and standard errors). Note that the x- and y-axes (DF1 and DF2) have standardized scales between females and males for each tissue type. REF (Windfall Reference Site); ANC (Alberta Newsprint Company); MW (Millar Western Industries); STP (Sewage Treatment Plant at Whitecourt, Alberta).
ues may weaken the efficacy of this approach. Stable isotopes of carbon and nitrogen are especially vulnerable to distortion due to natural source variability, biochemical processes and food web interactions. The utility of these isotopes is predicated on exacting isotopic signatures of effluents, receiving waters and food web components that are sufficiently distinct as to permit causal links to be made between effluents and fish exposure. A natural range of δ13C and δ15N values in aquatic ecosystems also raises the possibility that no level of isotopic resolution will permit such causal links to be made. Therefore, additional isotopes have been considered that should be less affected by ecological processes, thereby retaining signatures that more accurately reflect exposure.

Stable isotopes of sulfur and chlorine may provide such information, except that the reliability of these isotopes is also limited by how well effluents from multiple sources can be assigned unique isotopic signatures. Taken altogether, these potential complications argue for a multi-isotopic approach because there is a natural complementarity among individual isotopes. The technique works best when the relationships between effluents and biota are orthogonal (i.e., specific) to their isotopic signatures. However, such delineations are likely to be rare or unique in natural environments. Thus, a multi-isotopic approach to the analysis of fish tissues as an index of exposure increases the likelihood that specific anthropogenic sources are correctly identified.

Both univariate and multivariate statistical analyses of stable isotope ratios in tissues of longnose sucker showed that males were, in general, more effective at distinguishing differences between sites than females. The exception to this generalization was the clear distinction obtained between the REF site and all other sites, regardless of tissue, when comparing stable isotopes of 34S in females alone. However, the emphasis on particular isotopes differed between the two types of statistical analysis. Univariate ANOVAs indicated that signatures of all three stable isotopes, 13C, 15N and 34S, were variously useful in distinguishing between sites. In contrast, the exclusion of 13C from the first discriminant function in all of the multivariate analyses suggested that 15N and 34S, either separately or together, were relatively more effective at tracing sources of effluent. Only through multivariate analysis of multi-isotopic data was this pattern revealed. Further, the inclusion of discriminant function analysis enabled the calculation of benchmarks by which to gauge confidence that effluents from particular mills resulted in exposure in specific fish populations.

Enrichment of 13C in receiving waters due to PME has been shown in other river systems (i.e., Thompson River, B.C.; Wassenaar and Culp 1996), and to reflect δ13C values in the pulp mill effluent itself (Farwell et al. 1995a; Farwell 2000). In both cases, altered carbon isotope signatures were assumed to be a function of subsidization of autochthonous within-river production by processed, terrestrially derived dissolved and particulate organic carbon. Because δ13C values tend to be conserved regardless of trophic transfers (Post 2002), identification of source material should translate into fish populations. No such pattern was observed in the Athabasca River, suggesting exogenous sources of carbon may have also subsidized the food web at the REF site. Observed δ34S at the REF site ranged from -25.0 to -27.5‰ which falls within the natural range of terrestrially derived organic carbon (-25 to -30‰; France 1995a). In addition, physical and biological factors that contribute to the variability of δ13C values in aquatic biota may render them uninterpretable, even if the initial δ13C of receiving waters and effluents differ. Differences in turbulence among sites can also affect δ13C signatures in algae and bacteria (Rounick and Winterbourn 1986; France 1995a). Finally, upper-level consumers, such as fish in river ecosystems, can exhibit a variety of foraging habits and dietary preferences, further complicating resolution of patterns in δ13C signatures.

Similar to δ13C, δ34S should also be conserved through food webs. Discrimination of sites using δ34S values generally showed that upstream sites were enriched in 34S compared to downstream sites, regardless of tissue. This pattern is opposite to that observed in other rivers of the boreal plains ecozone (Wapiti River near Grande Prairie, Alta., and North Saskatchewan River near Prince Albert, Sask.; Wayland and Hobson 2001), which were consistent with expectations that δ34S in aquatic biota should become enriched with greater exposure to pulp mill effluents. On average, δ34S values were higher across all sites along the Athabasca River than several other rivers (Wapiti River, North Saskatchewan River, Thompson River, B.C.; Wassenaar and Culp [1996]), suggesting that sulphate sources upstream persisted downstream into our study area. This hypothesis is supported by analyses of water quality data on the Athabasca River from the headwaters upstream of Hinton to the Town of Athabasca more than 600 km downstream. The median dissolved sulphate concentration in the Athabasca River headwaters above Hinton from 1973 to 2002 was approximately 20 mg/L (n > 318 samples) (Glozier et al. 2004). Concentrations increased to 90 mg/L downstream of a pulp mill and municipal sewage effluent at Hinton and then continued to decrease with distance downstream where concentrations of 50 mg/L were reported at the Town of Athabasca (Noton and Saffran 1995). These results support our hypothesis that sulphate loadings from upstream of our study area could have saturated our ability to detect differences between our reference site at Windfall and our pulp mill sites. Although Noton and Saffran (1995) show that pulp mill loadings from the ANC and MW mills (total load of 6000 kg/d) slightly increase sulphate concentration in the Athabasca River near Whitecourt (approximately 5 mg/L), this increase is
insignificant compared to the downward decrease in concentration from the Town of Hinton to our reference site. These results suggest that although significant differences in tissue $\delta^{34}$S were measured between our reference site and downstream sites, this difference was not due to the discharge of effluents within our study reach but more likely a consequence of our reference site being enriched in $^{34}$S due to upstream sources. We are not concluding that $\delta^{34}$S is inadequate as an isotopic tracer of fish exposure but recognize that our examination of sources assumed that our reference site at Windfall was outside the exposure zone of the pulp mill at Hinton (100 km upstream) which may not be the case. Further research is required to quantify the spatial profiles in dissolved sulphate concentration and $\delta^{34}$S from the headwaters of the Athabasca River to the Town of Athabasca.

$\delta^{15}$N values were increasingly more positive in fish tissues from upstream to downstream sites. This pattern was generally consistent for both sexes across most tissue types. Liver tissues in both males and females had the most obvious stepwise increase in $^{15}$N enrichment from site to site. Unlike C and S isotopes, N isotopes can be fractionated through food web interactions, meaning that enrichment in $^{15}$N can also occur independent of location along a river. Assuming that the aquatic food web structure (i.e., algal primary producers, macroinvertebrate taxa) was similar at each of the sampling sites, increased enrichment of $^{15}$N downstream may reflect increased loading of ammonia and nitrate at the pulp and paper mill effluents, but especially at the municipal sewage treatment effluent. In an earlier study, chlorophyll a concentrations from periphyton samples along another set of sites upstream and downstream (near and far) of ANC and MW indicated almost a doubling of algal production immediately downstream of both pulp mill discharges (Shelast et al. 2000). Concentrations of phosphorus (i.e., total P, dissolved P) and nitrogen (i.e., TN-Kjeldahl, NO$_3^-$ and NO$_2^-$, NH$_4^+$) in the water column reflected the same pattern between sites upstream and downstream of the discharges. This suggests that a cumulative increase in the net loading of nutrients (i.e., nitrogen) exists along successive sites in the Athabasca River. Similar patterns were observed for $^{15}$N enrichment downstream from reference sites in food webs along the Wapiti and North Saskatchewan rivers (Wayland and Hobson 2001). Evidence of $^{14}$N enrichment was greatest at lower levels of the food web (i.e., algae, macroinvertebrates) and weakest at the top level. $\delta^{15}$N of tree swallow (Tachycineta bicolor) breast muscle tissues were only significantly enriched downstream of the Grande Prairie municipal sewage treatment plant along the Wapiti River and the Prince Albert pulp mill along the North Saskatchewan River.

Weak links between isotope signatures of effluents and tissues of top consumers have been shown to be a function of diet complexity (Spies et al. 1989; Gearing et al. 1991; Vander Zanden and Rasmussen 1999; Pearson et al. 2003) and variable trophic fractionation (Vander Zanden and Rasmussen 2001; Post 2002). Omnivory in benthic-feeding longnose sucker (Scott and Crossman 1973) may have contributed to the variation surrounding $\delta^{15}$N values observed among sites. Despite this additional source of variation, progressive enrichment in $^{15}$N downstream from the REF site was observed. Further resolution of longnose sucker $\delta^{15}$N isotopic values would require supplemental sampling of food web components at each site to establish baseline conditions.

Few data exist from earlier studies on the isotopic composition of fish tissues from the Athabasca River. However, as part of the Northern River Basins Study (NRBS), muscle tissues from longnose sucker sampled from the reference site (Windfall Bridge) in 1992 were analyzed for the stable isotopes of carbon, sulfur and nitrogen (Hesslein and Ramal 1992). While $\delta^{13}$C and $\delta^{34}$S values were very similar between 1992 and 2000, the $\delta^{15}$N values increased from +5.7‰ in 1992 to +7.8‰ in 2000, meaning that longnose sucker had, on average, increased their trophic position by approximately 35%, or the equivalent of almost two-thirds of a trophic level. One possible explanation for this isotopic shift is that the overall productivity of the Athabasca River increased between sampling occasions, due to increased loadings of limiting macronutrients, such as nitrogen and phosphorus, due to development and population increases over the past decade. This may also have been accompanied by alteration of the food web structure such that longnose sucker shifted their diets towards prey communities of relatively higher trophic position.

Univariate statistical analyses showed that for both males and females, $\delta^{37}$Cl values were only distinguishable for the ANC fish. In female gonad tissues, $\delta^{37}$Cl at ANC differed from all other sites, while in male liver tissues, $\delta^{37}$Cl differed between ANC and MW sites. Examination of spatial profiles of dissolved chloride in the Athabasca River showed that median concentrations in the headwaters at the boundary of Jasper National Park measured 0.7 mg/L from 1973 to 2002 ($n = 318$) (Glozier et al. 2004). Noton and Saffran (1995) report that in 1993 the pulp mill at Hinton loaded 34,400 kg/d of dissolved chloride into the Athabasca River resulting in a downstream concentration of 13 mg/L; a 19-fold increase from upstream. Chloride concentrations decrease slightly with distance downstream and measured approximately 9 mg/L at Whitecourt. Noton and Saffran (1995) also reported that the spatial patterns in dissolved chloride remained consistent for each year from 1990 to 1993.

Since 1993, the bleaching process at the kraft pulp mill in Hinton includes oxygen delignification, peroxyde and 100% chlorine dioxide substitution (Shelast et al. 2000). Despite the apparent influence of the pulp mill at Hinton on downstream chloride concentrations in our study area, differences in $\delta^{37}$Cl could still be detected in
some tissues collected downstream of the ANC mill. It is also possible that the separation between ANC and MW is due to discharge of effluents containing different chlorinated compounds. However, before conclusions can be reached regarding the influence of the ANC and MW effluents on $\delta^{37}$Cl in fish tissue, further research is required to spatially delineate dissolved chloride concentrations and $\delta^{37}$Cl in the Athabasca River from the headwaters to below our study area. The significance of any spatial differences requires evaluation in a broader watershed context and relative to significant sources of chloride upstream.

Resolution of patterns using $\delta^{37}$Cl values may have been compromised by the availability of fish tissues, resulting in low sample sizes ($n = 3$ for gonad, liver and muscle, per sex, per site). Insufficient bone material was available to measure $\delta^{37}$Cl. In theory, Cl isotopes should function as a conservative tracer of chlorine-bearing compounds in food web analyses, as uptake of $^{37}$Cl is via the gills, directly from the water. van Warmerdam et al. (1995) found that a variety of organic chlorinated solvents from different manufacturers could be identified when isotopic signatures of $\delta^{37}$Cl and $\delta^{13}$C were analyzed in combination. In a 45-day laboratory experiment using rainbow trout, Dubé and Wassenaar (2002) found that of the four stable isotopes analyzed (C, N, S, Cl), $\delta^{37}$Cl values most reliably traced 10% PME to fish exposure. On average, trout exposed to PME had $\delta^{37}$Cl values three times that of control fish. All of the male and most of the female longnose sucker tissues sampled from the Athabasca River had $\delta^{37}$Cl values at least as negative as the trout exposed to PME in the laboratory (-2.3‰).

In addition to the discharge of effluent from the pulp mill, municipal sewage from the Town of Hinton is also discharged with the pulp mill effluent. In a related study to this one, Oakes et al. (2004) found that from a suite of biochemical parameters only FAO (fatty-acyl-CoA oxidase) activity, an indicator of oxidative stress, was a consistent indicator of longnose sucker exposure to effluents of pulp and paper mills and an infrequent indicator of exposure to effluents from sewage treatment plants. Exposure effects were more pronounced in fish from the Wapiti River than from the Athabasca River. Oakes et al. (2004) reasoned that fish sampled from the REF, ANC, MW and STP sites had condition factors ($K$—an index of overall health and somatic fitness) near their maximum because of upstream nutrient inputs to the river near Hinton. Nutrient saturation along the study reach may have reduced the likelihood that differences could be detected in additional biochemical parameters. These results provide some independent evidence as to why greater differences in stable isotope ratios were not observed between the REF site and the other sites immediately downstream of effluent discharges.

Although some interpretable links were obtained between stable isotope ratios in longnose sucker and different sites along the Athabasca River, improvements in predictive relationships between river water chemistry, effluents and fish physiology are necessary before this technology can be used as a routine diagnostic tool to quantify effluent exposure of wild fish in the EEM Program. This study, like previous studies (Wassenaar and Culp 1996; Wayland and Hobson 2001), found that the interpretability of isotopic signatures was compromised by factors that are common to river ecosystems: variable hydraulic regimes, confluence with tributaries, multiple effluents and food web interactions. Among the stable isotopes of fish tissues that were analyzed in this study, some distinct patterns were observed. Links between isotopic signatures of effluents and fish tissues were generally better for males than females, and within males and females, gonad and liver tissues were more useful than muscle tissues. Isotopes of nitrogen and sulfur were, overall, more effective at classifying fish tissue among sites than isotopes of carbon. If effluents are combined (i.e., combine all PME and STP sites), even greater site discrimination is obtained in comparison to the REF site. This suggests that for these particular anthropogenic discharge sources, effluents may be more complex than assumed possibly due to mixing from one site to the next, which prevented identification of individual effluents based on the stable isotopes of carbon, nitrogen and sulfur. Finally, there is still the possibility that stable isotopes of chlorine may effectively distinguish between sites. However, pooled samples of each type of tissue greatly reduced the level of replication at each site, which may have obscured any underlying differences in $\delta^{37}$Cl values. Improvements in the methodology for analyzing tissues for stable isotopes of chlorine include requirements of smaller sample amounts (Wassenaar and Koehler In press), suggesting that a repeat of this fish survey or the use of $\delta^{37}$Cl in other studies merits consideration.

Future studies intended to test the utility of stable isotopes as tracers of effluent in exposed fish populations should, at a minimum, simultaneously sample effluents, receiving waters, less mobile forage fish and associated aquatic biota from all sites to enable improved characterization of contributing influences on isotopic signatures and to better establish the natural range of isotopic variance in the local ecosystem. Due to mixing and dilution in hydraulically dynamic river environments, measurement of isotope ratios from effluents is essential to enable baseline signatures to be established at each site, thereby “correcting” the observed signatures in sampled fish.

**Conclusions**

The detection and quantification of biotic exposure to specific effluents is challenging when there are multiple effluents that are discharged into hydraulically dynamic receiving waters. By analyzing tissues of sentinel fish populations for carbon, nitrogen, sulfur and chlorine stable
isotope values, links may be made between specific discharge sources and downstream effects. With the exception of bone, stable isotope values of all other sampled longnose sucker tissues (i.e., gonad, liver, white muscle) were correlated with one or more study sites along the Athabasca River. While univariate analyses of isotopic values indicated that three isotopes (i.e., carbon, nitrogen and sulfur) were similarly useful for linking effluents to fish exposure, multivariate analyses revealed that nitrogen and sulfur better discriminated among sites than carbon. Multivariate analyses further revealed that
\[ \delta^{15}N \] and
\[ \delta^{34}S \] values of gonad and liver tissues from males and females could effectively discriminate between the REF site and all other sites. This finding satisfies requirements of EEM to identify analytical tools that can compare reference and exposed sites, though more research is necessary to fully tease apart the relative contribution of multiple effluents from pulp and paper mills and sewage treatment plants to cumulative fish exposure. The pattern of isotope values of
\[ \delta^{34}S \] and
\[ \delta^{37}Cl \] suggest fish sampled from all sites along the Athabasca River were likely exposed to pulp mill effluents. The usefulness of the multi-isotopic approach to tracing effluents through river food webs is dependent on the distinctiveness of the isotopic signatures of the effluents versus the natural background values of the system. Further research is needed to resolve isotopic relationships between effluents and exposure, and to thereby further improve the likelihood of successful isotopic fingerprinting in EEM.

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