EVALUATION OF STABLE ISOTOPES FOR THE IDENTIFICATION OF AQUACULTURE WASTE IN THE AQUATIC ENVIRONMENT

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FERNANDO SALAZAR-HERMOSO

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ABSTRACT

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Fernando Salazar-Hermoso
University of Guelph, 2007

Advisor:
Professor Richard D. Moccia

Stable isotope signatures are used as tracers of foreign materials in ecosystems. Their applicability in aquaculture was evaluated in this study. The signatures of eight feed ingredients showed $\delta^{13}C$ values between -12.92 to -25.85‰ and $\delta^{15}N$ between 0.47 to 12.43‰. Three diets with different proportions of the above ingredients had $\delta^{13}C$ values between -19.55 and -20.78‰ and $\delta^{15}N$ values between 5.27 and 9.34‰. The signatures were identified in the feces and muscle of rainbow trout fed each diet, demonstrating the potential for isotope ratios to be used as tracers. Sediment from a commercial fish farm was sampled to determine if it had an isotope signature related to the feeds used ($\delta^{13}C$ -20.12‰; $\delta^{15}N$ 6.51‰). Results revealed isotope signatures more related to the control sites of $\delta^{13}C$ -25.24‰ and $\delta^{15}N$ 3.93‰ indicating that for this particular site, the feed isotopic signatures couldn't be used as tracers of aquaculture-derived organic matter.

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Declaration of Work Performed

All work reported in this thesis was performed by me with the exception of the following:

Isotopic analysis of the samples was carried out by William Mark and staff of the Environmental Isotopes Laboratory, University of Waterloo.

Formulation and determination of the chemical analysis of the experimental diets was performed by Dr. Dominique Bureau, UG/OMNR Fish Nutrition Research Laboratory, Dept. of Animal and Poultry Science, University of Guelph.

Chapter 1: Literature Review

1.1. Introduction

The growth of the Canadian aquaculture industry is constrained by the need to better manage and sometimes mitigate, the environmental impact generated by a farm's operations. The environmental management of an aquaculture operation can become the most important limiting factor when licensing and regulating new or expanding aquaculture farms, particularly for open water cage fish farms.

The fecal waste produced by the fish and the uneaten feed particles are probably the main sources of organic matter loadings from the farm to the aquatic environment. The quantification and identification of the dispersal of such loadings is a crucial step towards the successful development of an environmental management plan.

The present chapter reviews the techniques currently used for the identification and/or quantification of aquaculture originated organic matter and gives an insight into how the use of stable isotopes of carbon and nitrogen may be a promising tool for the identification of aquaculture waste in freshwater environments.

1.2. Aquaculture's Importance and Recent Developments

The aquaculture industry is a rapidly growing sector of agricultural food production worldwide and within Canada. It still represents a relatively small segment of Canadian agricultural production, but during the past 5 years it has grown substantially, and despite a slow down in the growth rate, it still shows good potential for expansion (Statistics Canada, 2006). The aquaculture sector accounts for more than 33% of the total landed value of the Canadian fisheries sector (Statistics Canada, 2006). In 2005, when the most recent data was recorded, aquaculture production in Canada reached 154,083 tonnes, more than twice the amount produced 10 years ago. The value of Canadian aquaculture production reached $715 million in 2005, with finfish accounting for nearly $640 million (Statistics Canada, 2006). However the contribution of Canadian aquaculture to world production was a mere 1.1% of the total value (Alain, 2005).

Of the 73 species licensed for aquaculture in Canada, 51 are finfish, with salmonids being the primary fish group cultivated. Aquaculture in Ontario is dominated by salmonids produced through land-based intensive culture and open water cage farms (Moccia *et al.*, 1997). In 2005, 4,000 tons of trout were produced in Ontario, representing 3.5% of the national finfish production (Statistics Canada, 2006). Ontario is an ideal province in which to promote further growth in Canada's aquaculture sector because of its high quality water, geographic location, access to domestic and international markets and abundance of technological infrastructure and qualified people (Moccia *et al.*,

1997). To ensure strong and steady development and to avoid future issues of conflict that could undermine the industry, growth in Ontario's aquaculture sector has to be oriented towards sustainable environmental practices.

1.3. Current Issues in Aquaculture

The challenges facing sustainability are various, and include environmental management, social conflicts, welfare issues, market stability and the international competitiveness in our domestic marketplace (Moccia and Hynes, 1998). In addition, the reduction of production costs, perceived food safety issues and consumer confidence are three more important challenges that affect aquaculture sustainability (Bureau, 2006). From all these challenges, environmental management is the one addressed in the present thesis, particularly in reference to the identification of solid waste fish farm effluents in the aquatic environment.

The pressure on the aquaculture industry from environmental groups and regulatory agencies has constrained its growth, but also accelerated scientific developments to overcome these obstacles. The complexity of the legislation regulating aquaculture in Ontario relies on how provincial and federal jurisdiction affects property rights (Moccia and Bevan, 2000). Agencies such as Fisheries and Oceans, Environment Canada, and the Ministry of Natural Resources on both the federal and provincial levels, as well as other local regulatory agencies, play a role in aquaculture licensing. Each of these agencies uses a different

definition of sustainability depending on their point of view, making the licensing process tedious, prolonged and sometimes unsuccessful. An in-depth review of aquaculture legislation in Ontario can be found in Moccia and Bevan (2000). When it comes to environmental management of waste in land based operations, the Ontario Ministry of the Environment is responsible for administering several Acts that regulate the use, treatment, management and disposal of water and wastewater. However, the application of these regulations to cage aquaculture can be confused and complicated. The Ministry of Natural Resources is responsible for issuing an Aquaculture License that sets a limit on the number of fish to be held in the cages based on recommendations and waste production estimates from the Ministry of the Environment. It is done this way instead of setting effluent discharge limits, as is done with land-based operations, because appropriate and cost-effective treatment of wastewater is difficult, or impossible, in an environment where the dispersal of wastes does not have a single point of discharge (Moccia and Bevan, 2000).

Cage aquaculture in Ontario is currently facing a delicate and complicated evolution due to licensing constraints. The lack of precise knowledge about the fate of organic matter and nutrients discharged to an open lake environment by cage aquaculture is an important issue for the various agencies involved in licensing. It is also hard to calculate the amount of previously existing naturally occurring materials and organic matter inputs produced by other sources. The need for knowledge on organic matter loads put into the aquatic environment is a

decisive factor when it comes to establishing rights and responsibilities for ecosystem management and protection, and not having such knowledge has led to an effective moratorium on the granting of farm expansion permits and new licenses. Accordingly, techniques and methodologies for estimating the quantity of waste production from aquaculture operations have been developed by the Fish Nutrition Research Laboratory at the University of Guelph (Bureau and Cho, 1999; Bureau *et al.,* 2003; Cho and Bureau, 1998; Cho and Bureau, 2001; Cho *et al.,* 1994). Along with models to estimate the quantity of waste, techniques for the identification of waste in the natural environment, which is the main scope of this thesis, will generate information that will aid the regulatory agencies in making better informed decisions when licensing, thereby benefiting both the environment and the aquaculture industry.

1.4. Environmental Management in Aquaculture

As the aquaculture industry grows, demands for enhanced management grows, with the reduction of environmental impact being a primary goal (Naylor *et al.*, 1999). In an effort to address better management and sustainability of aquaculture, the Minister of Fisheries and Oceans announced in August 2000 a $75 million Program for Sustainable Aquaculture (PSA) with the goal of enhancing public confidence in the sector and improving the industry's global competitiveness (Fisheries and Oceans Canada, 2003). A State of Knowledge (SOK) Initiative was developed with this program to review and recommend scientific studies relevant to Canadian aquaculture. SOK addressed the

environmental impacts of aquaculture under three main themes: the impacts of wastes (including nutrients and organic matter); chemicals used by the industry (including pesticides, drugs and anti-foulants); and interactions between farmed fish and wild species (including disease transfer and genetic and ecological interactions). Regarding environmental impacts of aquaculture, although the SOK's review summarizes available knowledge, it does not focus on site-specific issues and it does not examine the possible effects on the environment of aquaculture production (Fisheries and Oceans Canada, 2003). In this sense, the studies on environmental management in aquaculture focus on addressing the following recognized effects:

- eutrophication (nutrient and organic matter enrichment leading to increased dissolved inorganic nutrient concentrations and decreased dissolved oxygen);
- sedimentation (direct settling of feed pellets, feces, increased organic matter flocculation leading to higher deposition rates of small particles, changes in turbidity);
- food web structure and function (changes in planktonic, fish and benthic community composition, enhanced/decreased productivity, behavioral avoidance, stimulation of harmful algal blooms, intertidal/macroalgal community effects).

The results from these investigations will help minimize gaps in our knowledge of the quantity and quality of sediments originated from finfish farms, as well as our

capacity to distinguish aquaculture sediments from those originating elsewhere (both natural and anthropogenic). So far, the need for knowledge concerning environmental effects of aquaculture has been addressed by many researchers in many different ways. For example, it is common for unconsumed feed pellets and feces originating from finfish cages to increase the amount of suspended and settleable particulate matter in the water column. Much of the waste material is supposed to settle in close proximity to the cage, but hydrological characteristics of the receiving water may influence dispersal, causing it to settle several meters away. McGhie *et al.* (2000) showed that sediment samples taken near a cage aquaculture site in Australia have an isotopic profile that can be related to waste originated from the cages to a distance of up to 30 m in the currents' direction. Similarly, by characterizing the particulate organic matter around salmonid cages, Sutherland *et al.* (2001) demonstrated that the waste from cage aquaculture can disperse to distances as far as 500 m. The accumulation of organic matter from net-pen culture either directly underneath, or at a distance from the culture site, may have negative effects on both the ecosystem and the farm itself. The impacts of organic matter accumulation include changes in the dissolved oxygen levels and other physicochemical properties of surrounding waters (Boaventura *et al.*, 1997); eutrophication and enrichment of the water body containing the fish farm (Penczak *et al.*, 1982); damage to populations of other organisms within the ecosystem (Ruiz *et al.*, 2001) and self-pollution of the farm itself (Lumb, 1989).

Measurement of physical, chemical and biological (benthic fauna) parameters to evaluate and monitor aquaculture impacts in a given ecosystem, is common practice in some countries. Cole (2002) recognized that finfish aquaculture in New Zealand is growing and research studies in environmental management should include measurements of all the parameters mentioned, particularly for coastal aquaculture. In a similar report Crawford *et al.* (2002) evaluated the techniques for environmental monitoring of Atlantic salmon (*Salmo salar*) farms in Tasmania. They found that the impact of salmon farms cannot be monitored based on only one parameter, and recommended that a successful monitoring program should include measurements of physical and chemical variables (particle size, redox and stable isotopes); benthic faunal community structures and video footage. Modeling nitrogen cycling is another approach to asses and monitor the outflow of land based aquaculture facilities. For example, Lefebvre *et al.* (2001) used nitrogen modeling in a sea bass (*Dicentrarchus labrax*) farm in the French Atlantic. In this study, the nitrogen cycling model was composed of 3 sub-models: (1) hydrological, (2) fish growth and excretion bioenergetics and (3) nitrogen transformation and loss. The model was validated with two years of water quality data and fish growth sampling. Its application showed that nitrogen contributions from the farm can be calculated with enough precision so to use the model as a monitoring tool for similar aquaculture facilities.

Other studies concentrate on estimating the waste output based on a nutritional or mass-balance approach (Azevedo *et al.*, 1998; Bureau and Cho, 1999; Bureau *et al.*, 2003; Cho *et al.*, 1994; Cho and Bureau, 1998). This approach is based on

the application of a mathematical model that accounts for most of the endogenous (biological) and exogenous (feed, environmental variables) factors that influence waste production. The calculation of waste from metabolism (dissolved) and undigested feed (solid) is crucial for the development of environmental monitoring models. The information generated from the model further motivates nutrition studies directed at improving feed formulae, better feeding practices and feed manufacturing techniques to achieve waste reduction at the source (Bureau and Hua, 2006a; Bureau and Hua, 2006b).

Techniques that focus on analyzing specific compounds associated with organic matter originating from aquaculture are gaining ground, especially when it comes to identifying the origin and dispersal of the farm-derived anthropogenic materials. Fatty acids, digestible proteins, sterols, elemental sulfur and trace elements have all been used successfully to differentiate organic matter originating in aquaculture from organic matter originating from other natural sources (McGhie *et al.*, 2000; Jones, *et al*., 2001; Vizzini and Mazzola, 2004). Measurement of abnormal quantities of trace elements in sediments, such as zinc found in fish pellets, have also been used as tracers of uneaten feed particles and feces (Yeats, 2002). However, one of the more recent and promising tools to determine the origin of organic materials in the natural environment is the use of techniques employing stable isotope analysis (Grey *et al*., 2004). The following sections provide a description of stable isotope analysis, current trends, studies and their relevance and applicability to the present thesis.

1.5. Stable Isotopes as Ecological and Environmental Management Tools

Stable isotopes are widely distributed in the inorganic and organic compounds of the entire planet. The most commonly used isotopes in ecological and environmental studies are those of carbon (C), nitrogen (N), sulfur (S), hydrogen (H) and oxygen (O) with the following average abundances:

Table 1. Natural abundance of the most commonly used stable isotopes in ecological and environmental studies (Fry, 2006).

Element	Isotope	Abundance (%)
H	^{1}H	99.94
	^{2}H	0.016
C	^{12}C	98.89
	^{13}C	1.11
N	^{14}N	99.64
	^{15}N	0.36
O	^{16}O	99.76
	^{18}O	0.20
S	^{32}S	95.02
	^{34}S	4.21

The ratios at which these stable isotopes present themselves in any given material depend on the degree to which the isotopes are fractionated in comparison to a designated international standard as a result of physical and

chemical alterations. Fractionation refers to preferential chemical or physical reactions that discriminate against heavy isotopes; and enzymatic discrimination and differences in kinetic characteristics and equilibria can result in reaction products that are isotopically heavier (enriched) or lighter (depleted) than their precursor materials (Lajtha and Michener, 1994). Therefore, the value of each isotopic ratio (named delta = δ) is usually distinguishable and characteristic of its source material. When a source material is considered isotopically distinct and the isotope's δ does not change or it changes in a predictable way that particular ratio is designated as a signature (Lajtha and Michener, 1994). Other, unidentified materials that have such signatures, therefore, can potentially be traced in the environment through analysis of their isotope ratios.

The fractionation of the isotopes is an important factor to keep in mind when evaluating the potential of signatures to give accurate indications of the source of the material being examined. Descriptions of the fractionation of the most commonly studied isotopes (C and N) in biological systems have been published for many terrestrial organisms (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981; Nier and Gulbransen, 1939) and for several aquatic food webs (Fry, 1991; Minagawa and Wada, 1984; Vander Zanden and Rasmussen, 2001; Post, 2002). In most cases the fractionations follow a similar pattern, with average changes with each trophic level of 3-4‰ for nitrogen and of 0-1‰ for carbon. However, in some cases, the fractionation may not follow the typical averages. According to Post (2002) the level of fractionation of the elements is influenced by the type

and quality of the available source. Some sources are isotopically lighter than others causing a preferential utilization that is reflected in smaller fractionation values and vice versa. In those cases it is better to conduct fractionation experiments on the materials being studied for better accuracy (Gannes *et al.*, 1997).

The use of stable isotope analysis is becoming widespread in many disciplines. They are applicable to an extensive array of situations which allows for their use in diverse fields of investigations such as, archaeology, biomedical sciences, sports, biosynthesis, environment studies, extraterrestrial chemistry, food science, forensic science, microbiology, organic geochemistry and soil science (Lichtfouse, 2000).

In fisheries ecology studies for example, the use of stable isotopes has become useful in describing migration patterns of brown trout (*Salmo trutta*) (McCarthy and Waldron, 2000) and some species of herring (*Alosa* spp.) (Limburg, 1998) by identifying marine isotope signatures in the bodies of anadromous fish versus non-anadromous (year-round freshwater resident) individuals for each species. The use of resources by mallard ducklings (*Anas platyrhynchos*) waterfowl was elucidated by analyzing the isotopic signatures of their feathers, and their relation to a particular ecosystem and diet (Hebert and Wassenaar, 2001). Variations in oceanographic conditions, including the characterization and comparison of sinking and suspended particles, and their relation to the seasonal productivity of

the surface waters (Altabet, 1988; Altabet *et al.*, 1999; Nakatsuka *et al.*, 1992; Saino and Hattori, 1987; Wada and Hattori, 1976) and seasonal and/or bathymetric distribution (Altabet *et al.*, 1991; Holmes *et al.*, 2002), have been studied using stable isotopes.

Most ecological studies to date that have used stable isotopes, were designed to determine carbon and nitrogen flows in food webs. In the aquatic environment alone, more than 50 papers have relied on stable isotopes to elucidate trophic interactions within a biotic community (Jennings *et al.*, 2002) or at an ecosystem scale (Kwak and Zedler, 1997; Yoshii *et al.*, 1999; Yoshioka *et al.*, 1994). Stable isotopes have also be used to investigate the dietary habits of opossum shrimp (*Mysis relicta*) demonstrating that the population studied exhibited a strong omnivorous behavior (Branstrator *et al.*, 2000). Stable isotopes have also been helpful in describing the isotopic signatures of carbon and nitrogen of several aquatic organisms and the fractionation that occurred after pelagic consumers fed on them (Focken and Becker, 1998; Grey, 2000) and to study overlaps in the diets of various fish species (Gu *et al.*, 1996b; Hesslein *et al.*, 1993). Other studies have used stable isotopes to explain the trophic dynamics of economically important fish such as Nile tilapias (*Oreochromis niloticus*) (Gaye-Siessegger *et al.*, 2003; Gaye-Siessegger *et al.*, 2004a; Lochmann, 2002), carp (*Cyprinus carpio*) (Gaye-Siessegger *et al.*, 2004b), red drum (*Sciaenops ocellatus*) (Herzka and Holt, 2000) and salmon (*Oncorhynchus* spp.) (Kline and Willette, 2002; Sakano *et al.*, 2005; Satterfield and Finney, 2002).

In environmental studies, it is now a common practice to determine the source of pollutants or foreign materials in an ecosystem by measuring their isotopic signatures (Lajtha and Michener, 1994; Macko and Ostrom, 1994; Philp, 2002). Typical examples of stable isotope analysis in environment research include the monitoring and evaluation of air quality (Heaton, 1986), assessments of soil and plant tissues from forest ecosystems (Nadelhoffer and Fry, 1994) and environmental forensics to determine sources of contaminant chemicals, such as, hydrocarbons and chlorinated compounds on land (Philp, 2002). In aquatic environments, stable isotopes and their ratios have been used successfully in the identification of organic matter and sludge from anthropogenic sources. For example, isotopes have been used in the determination of the nitrogenous plumes from domestic septic systems in ground water in a Canadian rural area watershed (Aravena *et al.*, 1993), tracing sewage inputs in Boston Harbor and Massachusetts Bay (Tucker *et al.*, 1999), determining oxygen isotope compositions from water treatment plant discharges and their relation to fertilizers (Gruau *et al.*, 2005); and examining the origin of sulfur in the sulfates from precipitation, inflows and outflows of the basin and streams of Lake 239 at the Experimental Lakes Area in northwestern Ontario (Hesslein *et al.*, 1988).

Other environmental studies in aquatic ecosystems which have used stable isotopes have been oriented towards quantifying the presence and/or effects of allochtonous materials on the biota. Examples of this include monitoring of the recovery of marine biota polluted with sewage in New Zealand (Rogers, 2003)

and determining the origin and destination of particulate organic matter in estuarine zones (Andrews *et al.*, 1998; Thornton and McManus, 1994) and salt-marshes (Peterson *et al.*, 1986; Peterson and Howarth, 1987), as well as elucidating the sources of organic matter being deposited in rivers and lagoons (Machás and Santos, 1999; Maren and Struck, 1997; Voβ and Struck, 1997).

1.6. Stable Isotopes in Aquaculture

The use of stable isotopes is emerging as a possible approach to target some of the current issues faced by the aquaculture industry. For example, the problem with escaped fish has been a concern shared by both environmentalists and the farmers themselves. Research has investigated the utility of applying stable isotope measurements to identify escaped fish in the wild (Dempson and Power, 2004).

Isotopic analysis has also been applied in nutritional studies. The utilization and fate of different nitrogen sources in shrimp aquaculture have been determined by measuring stable isotope ratios from formulated feeds, detritus and plankton in comparison to the values of shrimp tissues (Burford *et al.*, 2002; Preston *et al.*, 1996). A similar approach has been applied to trace the deposition of particular sources of carbon in tilapia (*Tilapia aurea*) and paeneid shrimp (unknown species) culture, and to discern whether the principal contributor of the deposited nutrients was natural food or formulated feeds (Schroeder, 1983). A further study investigating the effects of diet shifts on isotopic signatures in cultured juvenile

Japanese flounder (*Paralichtys olivaceus*) (Tominaga *et al.*, 2003), showed that the isotopic signatures began changing immediately after the diet switch and gradually approaching the signature of the diet during the 14 days that the experiment lasted.

Along with this research in nutrition, growth and nutrients flow, stable isotopes have been used in studies examining the environmental fate of organic matter originating from aquaculture practices. The main objectives of these studies have been to identify and trace material contributions to the receiving environment. Identifying the source of organic matter is important to determine if it has an anthropogenic origin, namely household waste and sewage or waste run offs from typical agricultural practices. For example, measuring the stable isotope signatures in the tissues of bivalves in proximity to a fish farm in the Gulf of Gaeta (Mediterranean), showed that mussels and clams cultivated around the fish cages actively filter and utilize the available organic matter from the cages, which may help to reduce the environmental impact of the farms (Mazzola and Sarà, 2001). In another important study conducted in Moreton Bay, Australia, sewage effluent was compared to the loadings from a commercial shrimp farm, and it was demonstrated that sewage was more widely dispersed in the receiving environment (Jones *et al.*, 2001). These two studies highlight the potential of using stable isotopes to identify exogenous sources of organic matter in the aquatic environment.

In the case of finfish aquaculture, one of the first studies that relied on the use of stable isotopes as an environmental tracer was conducted by Ye *et al*. (1991). The study used stable carbon isotopes measured in samples of sediments collected with plastic jars and traps to locate and describe the influence of organic waste sediments derived from a salmonid farm. The results showed that the waste composed up to 75% of the organic matter under the cages and it decreased gradually up to 30 meters away from the cages, where it could still be positively identified.

In an investigation of the degradation of organic matter derived from fish cages in Tasmania. Samples of sediment were taken with cores by SCUBA equipped divers at 10 meter intervals from the centre of the cage following the direction of the currents and compared to the isotopic values from samples of feces and feed. The isotopic analysis showed that even during a 12 month fallowing period, feces and uneaten feed could be detected in surface sediments in close proximity (30 m) to where the cages were positioned (McGhie *et al.*, 2000). Similarly, in a study to characterize the organic matter surrounding a cage aquaculture operation, Sutherland *et al.* (2001) collected water samples and suspended particulate matter during feeding cycles at a salmonid fish farm located in the Broughton Archipelago, British Columbia. The sediment traps and water samples were taken at a 5 meter depth every 5 meters up to 30 meters from the cage. In addition a sediment trap was positioned at a 20m depth beside the central cage of the farm. The organic matter was visually characterized and

analyzed for chemical composition and carbon isotope signature. Results showed that in conjunction with the C:N ratios, the isotopic signatures of the organic matter were useful indicators of farm derived effluent. However, the identification of the carbon signature was not detected with the 20 m deep sediment trap. The authors attributed the absence of the signature to isotopic fractionation occurred by fish digestion, a lack of critical mass of feed pellets for detection of pellets and/or dilution of the feed signature by additional sources of organic matter.

Another study analyzed the historical feeding behaviors applied in some fish farms in Japan by analyzing the isotopic signatures of core samples of sediments in proximity to the cages. Shifts in the vertical profile of the sample's isotopic values that were observed corresponded to shifts in the types of feed used since the farm began its operations (Yamada *et al.*, 2003).

Finally, isotopic examination of organic matter sources and the organisms that feed on it has also been used to asses the impact and dispersal of wastewater from a land based fish farm in the western Mediterranean. The study site was located in proximity to a land based fish farm rearing European sea bass (*Dicentrarchus labrax*), Gilthead seabream (*Sparus aurata*) and sharpsnout seabream (*Diplodus puntazzo*). Samples of sediment, macrophytes (*Posidonia oceanica, Sphaerococcus coronopifoulis* and *Pavina pavonica*), benthic invertebrates (*Arbacia lixula, Holothuria tubulosa* and *Paracentrotus lividus*) and

fish (*Chromis chromis, Diplodus annularis, Sarpa salpa* and *Scorpaena porcus*) were taken at 3 distances 0.5, 1 and 2 km away from the outflow of the farm. Carbon and nitrogen isotopes were measured in the sediment and in samples of tissue from each organism. The results showed that the aquaculture waste entered the food web of the receiving waters at distances of up to 500 m from the point of discharge, with primary producers being most influenced by the assimilation of the aquaculture-derived nutrients (Vizzini and Mazzola, 2004).

Besides having been used in closed systems, and land based flow through aquaculture systems, isotopic signatures have also been used to describe the dispersion of particulate and sedimentary organic matter from open water fish farming activities in the western Mediterranean (Sarà *et al.*, 2004). The study results showed that organic matter could be traced confidently and depending on the hydrological conditions and dynamics of wild fish populations, the sediments can be dispersed up to 1,000 m from the cages.

To date, the majority of studies using stable isotopes to investigate aquaculture wastes have been conducted in marine ecosystems. Comparatively little research has been done using stable isotopes in freshwater systems, especially as it relates to cage aquaculture systems in large lake ecosystems. In one freshwater study, the utility of carbon and nitrogen stable isotopes was evaluated for its ability to help trace loadings from fish farms in small freshwater ecosystems (Esthwaite Water, UK) (Grey *et al.*, 2004). In the study, results

suggested that isotopic signatures were a useful tool that could be used to follow aquaculture-derived material through the food web.

The successful application of stable isotope techniques to identify aquaculture waste in a freshwater ecosystem relies on the premise that the stable isotopes of the fish feeds are different and distinct when compared to other sources found in the ecosystem itself. It is known that in addition to various types of ingredients of terrestrial origin, the fish feed commonly used in trout cage culture operations contains various types of marine fish meal. These types of fish meal are composed of planktivorous fish, such as, herring (*Clupea* spp.), menhaden (*Brevortia* spp., *Ethmidium* spp.) or sardines (*Sardinops* spp., *Sardinella* spp., *Sardina* spp.) that have isotopic signatures distinct from other terrestrial and aquatic biota found in freshwater lakes, therefore the fish incorporate the isotopic signature of the plankton which is typically different from that of terrestrial primary producers and consumers. Thus, the fish meals may have isotopic values which are distinct from the terrestrial ingredients, giving the feed a unique signature that could be used as a tracer in freshwater environments.

The present thesis is an attempt to broaden our knowledge of the utility of stable isotopes as tracers of farm-derived organic waste from freshwater aquaculture operations. It is important to investigate if a unique signature can be associated with fish feeds and how the signature is affected by differences in the proportion of ingredients used in feeds. Also, it is necessary to determine the possible

changes that the signature may experience after the feed is consumed by the fish and excreted as feces. To accomplish the above objectives, the research project was divided in two parts:

1) An experimental trial to test if the isotopic signature of feeds formulated with different proportions of fish meals could be identified from the feces and muscle of cultured fish.
2) A field sampling study at an operational trout farm in Lake Wolsey, Manitoulin Island, Lake Huron to corroborate the experimental findings.

Chapter 2: Determination of the Isotopic Signatures of Feeds, Feces and Muscle. Feeding Trial.

2.1. Introduction

Every one of the ingredients commonly used to formulate fish feeds is associated with a unique carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic signature. Such uniqueness is particularly evident in the nitrogen signature of the fish meals of marine origin compared to the rest of the ingredients. When all the ingredients are combined to form a diet, the resulting isotopic signature varies in relation to the type and amounts of ingredients mixed. Because the isotopic signatures of the fish meals are considered to be the most distinctive of all the ingredients, it was assumed that varying the concentration of fish meal in the diets would result in feeds with different signatures that were distinguishable from the signatures of other materials commonly found in freshwater environments. Nevertheless, for the present thesis it was considered that even with small changes in the proportions of fish meal, the feeds would still have distinct and unique isotopic signatures that might be used as tracers for aquaculture-derived wastes. Given the above mentioned assumptions three treatment diets were formulated with fish meal concentrations of 15, 30 and 45% and fed to rainbow trout (*Oncorhynchus mykiss*). The isotopic signatures of the feeds were measured and compared to those of muscle and feces from the fish used to determine if the isotopic signatures could be considered as tracers. The results showed a strong correspondence between the signatures of each diet and the muscle and feces

produced from them. This correspondence suggests that isotopic signatures of feeds have the potential to be used as aquaculture waste tracers. In addition, the isotopic signatures of the feeds, feces and muscle were demonstrated to be different and unique when compared with values of other biota and organic materials typically found in lakes.

2.2. Hypothesis and Objectives

The following hypothesis was formulated for the present chapter:

The isotopic signatures of fish feeds formulated with varying proportions of fish meal will be recognizable in the feces and muscle of cultivated fish during an experimental feeding trial.

The objectives set to test the hypothesis were:

1. Identify the isotopic signatures of carbon and nitrogen in common marine and terrestrial ingredients used in the production of salmonid fish feeds.
2. Measure and compare the isotopic signatures of feeds that have been formulated with different proportions of ingredients.
3. Determine whether the isotopic signature of the formulated feeds is detectable in the feces and muscle of rainbow trout fed the formulated diets.
4. Determine the ability of the discriminant statistical analysis of the isotopic data to distinguish between diets of different origins.

2.3. Methodology

2.3.1. Diet Formulation

Samples of several common fish feed ingredients were obtained from Martin Feed Mills (Elmira, Ontario, Canada). The samples obtained were: soybean meal, menhaden meal, herring meal, corn gluten meal, poultry meal, feather meal, wheat middlings, and fish oil.

Three isoproteic and isoenergetic (38% digestible protein (DP) and 19 MJ digestible energy (DE)) diets were formulated with decreasing proportions of fish meal (marine ingredients) and increasing levels of terrestrial ingredients (Table 1). The commercial formula (Martin Mills Profishent Classic 3 mm, Martin Mills, Elmira, Ontario, Canada) used to feed the fish before the experiment started was also included in the study to serve as a baseline for comparison purposes. The exact formulation is proprietary information but the available information printed on the label is presented in Table 2.

The ingredients were blended in a Foss Tecator 2096 homogenizer (FOSS Inc., USA) and refrigerated for 2 days. The mash was then pelleted using a steam pellet mill (California Pellet Mill Co., San Francisco, CA, USA) with a 3 mm die. Pellets were dried under a forced-air oven at room temperature for 24 hours and stored afterwards at 4º C

Table 2. Composition of the experimental diets.

INGREDIENTS	DIET 1	DIET 2	DIET 3	Commercial
		%		
Fish meal, herring	45	30	15	
Poultry by-products meal	4	12	20	
Feather meal	4	7	10	
Corn gluten meal	6	12	18	
Wheat middlings	19	17	15	
Fish oil	17	17	17	
Vitamin premix	1	1	1	
L-Lysine HCL	1.5	1.5	1.5	
Mineral premix	2	2	2	
Celite	0.5	0.5	0.5	
Proximate Composition (measured)				
Dry matter (DM) basis (%):				
CP	46.16	46.30	46.44	42
Lipid	24.79	24.25	23.71	16
Ash	9.41	9.17	8.93	
Gross Energy (GE) MJ/kg	23.61	23.78	23.94	
Digestible Nutrient Composition (calculated)				
Digestible dry matter (DDM), %	70.73	70.65	70.58	
Digestible protein (DP), %	38.76	38.44	38.12	>37
Digestible energy (DE), MJ/kg	18.59	18.57	18.55	17
DP/DE g/MJ	20.85	20.70	20.55	22
ADC Dry matter, %	75.32	75.27	75.21	
ADC Nitrogen, %	89.42	88.45	87.49	
ADC Energy, %	83.87	83.22	82.58	

2.3.2. Experimental Design

Using the diets formulated above, a feeding trial was conducted at the Alma Aquaculture Research Station, University of Guelph, for 56 days. The duration of the trial was determined by calculating the time it would take for the fish to achieve a twofold increase in live weight. Eight kilograms of feed per diet was manufactured as it was calculated to be enough for the 56 days of the trial. The feeding trials were carried out according to Animal Care Committee policies and Canadian Council on Animal Care requirements (CCAC, 2005), under Animal Utilization Protocol # 05R027.

The photoperiod was adjusted to a 12h light: 12 h dark cycle.

The experimental design consisted of nine (9) tanks, each with 70-75 liters of water and 12 liters per minute flow rate. Each tank was fitted with a PVC upright elbow at the drain, to which was attached a 400 micron mesh for feces collection. The mesh was submerged in a bucket overfilled with water flowing from the tanks to capture the feces floating in the water that could be carried out of the tank in the outflow. The collectors were used only during sampling (Fig. 1).

Figure 1. Tank display showing the feces collectors and automatic feeders

2.3.3. Feeding and care of the animals

The diets were randomly assigned to each tank using a Random Block Design with 3 replicates per diet. Each tank had an average of 42 fish totaling 2 kg of biomass. During the first week, the fish were fed a ration of 1.5% of their bodyweight according to the typical feeding rations proposed by the Aquaculture Centre personnel. Once acclimated, the fish were hand fed to near-satiety every Monday and Thursday twice per day and the ration for the following days was set based on the amount of feed consumed by the fish in each tank during the hand feeding sessions.

2.3.4. Sampling

A sample of the commercial and experimental diets, feces and muscle tissues were taken at the beginning of the trial to analyze the initial isotopic signature of all materials.

Feces Collection:

Samples of feces were collected at Days 40 and 56. Prior to sampling, the tanks were thoroughly flushed and the feces and any uneaten feed from previous days were washed out of the pipes. Feces collectors were then put in place to collect a sample that comprised the total amount of solids trapped over a 24 hour period. The mesh was removed and put at the bottom of the drain and the plug was pulled to collect the solids that had settled there. The sample was gently washed off the mesh and filtered in a vacuum flask with Whatman filter paper, pore size >25μm. The solids were then dried for 24 to 30 hours at 60°C in a temperature controlled oven.

Muscle Sample Collection:

Five fish per tank were sampled at day 56. The portion of muscle removed from each fish was taken from the dorsal left side, starting below the first ray of the dorsal fin and moving towards the posterior of the flank up to the adipose fin (Figure 2). The skin was then carefully removed from the underlying musculature and the sample was washed in distilled water and dried at 60°C for 24 hours.

Figure 2. Anatomical location of dorsal musculature used for isotopic analysis

2.3.4. Isotopic Analyses

The dried, solid samples of feces and muscle materials were ground to a fine powder using a mortar and pestle as recommended by Lajtha and Michener (1994). A sample of one gram of each material was submitted to the Environmental Isotope Laboratory at the University of Waterloo in Ontario from which a sub-sample of 1mg was taken for isotope analyses. Solid samples were analyzed for nitrogen and carbon isotope ratios on an Isochrom Continuous Flow Stable Isotope Mass Spectrometer (Micromass) coupled to a Carlo Erba Elemental Analyzer (CHNS-O EA1108).

Stable isotope ratios are expressed as delta values (δ) and are measures of the per thousand (‰) differences between the isotope ratio of a sample and that of a known international standard material for that same isotope:

$$\delta X = [(R_{sample} - R_{standard}) / R_{standard}] \times 1000$$

where $\delta X = \delta^{13}C$ or $\delta^{15}N$ and $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$.

All international standards are set at 0‰ by convention. Carbonate rock from the Pee Dee Belemnite formation (Craig, 1957) and nitrogen gas in the atmosphere (Mariotti, 1983) were used, respectively, as the carbon and nitrogen standards. Machine analytical precision (±0.2‰ for C, ±0.3‰ for N) was determined by repeat analysis (n=25) of International Atomic Energy Agency working standards CH6 for $\delta^{13}C$ and N1 and N2 for $\delta^{15}N$. As analytical error may increase depending on sample homogeneity and the type and amount of sample used in the analysis, working standards were placed throughout each run of samples at a range of weights to facilitate linear correction of analytical drift during the analysis of a sequence sample.

2.3.5. Statistical Methods

The isotopic values of both carbon and nitrogen from the ingredients of the diets, the diets, the feces and the muscles were analyzed using a one-way ANOVA. A Discriminant Function Analysis was also performed on the values of all materials tested. This analysis uses both variables (carbon and nitrogen) of each material as parameters and determines groups (i.e. feces from Diet 1, feces from Diet 2

and so on) based on the interval of values that each pair of variables has. Once the groups are formed, the analysis calculates the probability of every single observation belonging to each group. The results are expressed as “probability of posterior membership” which indicates the likelihood of each observation belonging to a group other than the one it is already in (Losos *et al.,* 1982). For example, if the group “feces from Diet 1” (group 1) shows a posterior membership of 50% to the group “feces from Diet 2” (group 2) it means that half of the observations in group 1 could be grouped in group 2. The analysis further indicates which group 1 observations specifically are considered as “possibly misinterpreted”.

The materials analyzed by discriminant analysis were all the diets, feces and muscle of each treatment. In addition, the diets, feces and muscle were also compared to published $\delta\,^{13}C$ and $\delta\,^{15}N$ values of biota, sediments and organic matter from Lake Ontario, Lake Superior and the Experimental Lakes Area to further test the strength of the analysis when classifying and grouping the isotopic signatures from the materials obtained from the feeding trial against other aquatic materials. All statistical analyses were completed using the Statistical Analysis Software (SAS) version 9.1. (SAS Institute Inc., Cary, NC, USA)

2.4. Results

2.4.1. Feed Ingredients

The isotopic analysis of the various feed ingredients showed that each one was associated with a unique carbon and nitrogen signature (Table 3). The fish meals utilized had the highest nitrogen signatures with mean $\delta^{15}N$ values of 12.17‰ for herring meal and 12.43‰ for menhaden meal. In contrast, soybean meal had the lowest nitrogen signature, with a mean $\delta^{15}N$ value of 0.48‰, followed by corn gluten meal (3.27‰), poultry meal (3.55‰), wheat middlings (3.77‰) and feather meal (4.9‰). The carbon signatures of each ingredient were also statistically different, although the differences were not as evident as those of nitrogen. Nevertheless, both isotopes were statistically different in all ingredients ($P<0.001$, $n=21$), with a marked difference between materials of terrestrial and marine origin (Fig. 3).

The comparison of the isotopic signatures of the complete diets formulated from the tested ingredients showed no significant difference in the $\delta^{13}C$ signatures between Diets 2 and 3 ($P>0.05$, $n=12$), or between Diet 1 and the commercial diet ($P>0.05$, $n=9$). In the case of $\delta^{15}N$, only Diet 3 and the commercial formula were not significantly different ($P>0.05$, $n=9$).

Table 3. Isotopic signatures of the ingredients and the diets formulated with them.

INGREDIENT	n	$\delta^{13}C \pm$ (SD) ‰	$\delta^{15}N \pm$ (SD) ‰
Poultry meal	3	-16.45 (0.18)	3.55 (0.07)
Feather meal	3	-18.11 (0.06)	4.9 (0.17)
Corn gluten meal	3	-12.92 (0.14)	3.27 (0.07)
Herring meal	3	-20.42 (0.11)	12.17 (0.14)
Menhaden meal	3	-19.55 (0.06)	12.43 (0.13)
Wheat middlings	3	-23.95 (0.26)	3.77 (0.16)
Soybean meal	3	-25.85 (0.28)	0.48 (0.61)
Diet 1	6	-20.78 (0.31)^{b}	9.35 (0.52)
Diet 2	6	-19.55 (0.77)^{a}	7.09 (1.01)
Diet 3	6	-19.86 (0.56)^{a}	5.27 (0.28)^{c}
Commercial diet	3	-21.08 (0.40)^{b}	6.12 (0.21)^{c}

SD = standard deviation; n = sample size; values with common superscripts in each vertical column indicate diet signatures that were not significantly different (P>0.05) from one another. Absence of superscript indicates significantly different (P<0.05) values.

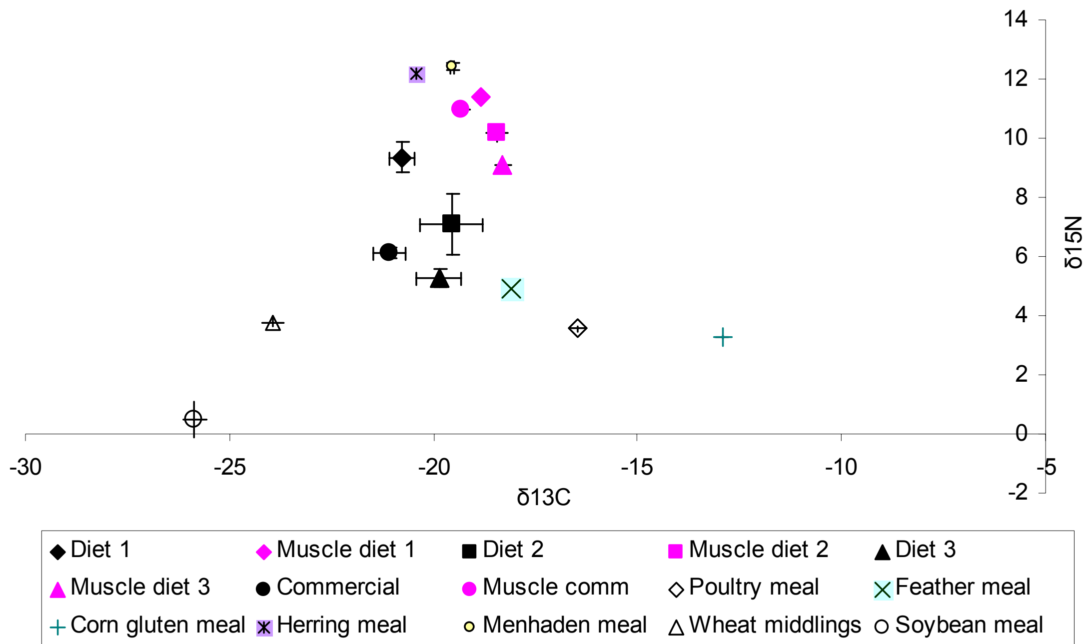


Figure 3. Mean isotopic signatures of the ingredients, feeds and dorsal musculature analyzed.

2.4.2. Feces

The $\delta^{13}C$ and $\delta^{15}N$ signatures of the feces from Diets 1, 2 and 3 showed no statistical differences between days 40 and 56, except for the $\delta^{13}C$ signature of the feces from Diet 3 (Table 4).

The $\delta^{13}C$ signatures of the feces from all diet treatments were significantly different from each other at day 56. The fecal $\delta^{15}N$ signatures from Diet 3 differed statistically from Diets 1, 2 and the commercial diet but no statistical difference was observed between Diets 1, 2 and the commercial diet (Table 4).

Table 4. Biomass and weight gain and isotopic signatures of feces.

Diet	n	Initial Mean Weight (g) (Total Biomass in Kg)	Final Mean Weight (g) (Total Biomass in Kg)	Weight gain (g) (Biomass in Kg)	Feed consumed (Kg)	Feed efficiency	Isotopic signatures between treatments at day 56[1]			Isotopic signatures within each treatment between sample days[2]				
							n	$\delta^{13}C$ ± (SD) ‰	$\delta^{15}N$ ± (SD) ‰	n	$\delta^{13}C$ ± (SD) ‰ Day 40	$\delta^{13}C$ ± (SD) ‰ Day 56	$\delta^{15}N$ ± (SD) ‰ Day 40	$\delta^{15}N$ ± (SD) ‰ Day 56
1	15	48.7 (6.0)	213.2 (13.0)	164.5 (7)	6.3	1.11	6	-21.71 (0.19)[a]	9.28 (0.62)[a]	6	-21.76 (0.22)[c]	-21.67 (0.20)[c]	8.84 (0.61)[d]	9.72 (0.15)[d]
2	15	48.4 (6.1)	213.9 (12.9)	165.5 (6.8)	6.3	1.08	6	-20.98 (0.19)[b]	8.08 (1.08)[b]	6	-21.08 (0.14)[e]	-20.87 (0.21)[e]	7.29 (0.27)[f]	8.87 (0.98)[f]
3	15	48 (6.1)	201.8 (12.1)	153.8 (6)	5.2	1.15	6	-20.64 (0.21)[c]	6.37 (0.44)[c]	6	-20.80 (0.10)[k]	-20.48 (0.15)[m]	6.25 (0.37)[g]	6.48 (0.55)[g]
Com	n/a	n/a	n/a	n/a	n/a	n/a	3	-23.22 (0.16)[d]	8.74 (0.50)[a,b]	n/a	n/a	n/a	n/a	n/a

Com= commercial diet; Feed efficiency (Biomass gain/Feed consumed); n= sample size; C, N = carbon and nitrogen respectively; SD= standard deviation; n/a = not applicable, no trial was conducted with the commercial diet, the feces analyzed for the commercial diet were collected from the tanks holding the fish before the experiments started and their isotopic signatures were measured as a baseline.

[1]Comparisons of signatures were performed between treatments (diets) for each separate isotope and the values should be read as vertical columns. Values sharing the same superscript within the same column were not significantly different (P>0.05)

[2]Comparisons between days were performed for each isotope separately and the values should be read as horizontal rows. Values sharing the same superscript within a row were not significantly different (P>0.05).

2.4.3. Muscle

The fish in all three treatment groups showed at least a two fold increase in live body weight (Table 5). The $\delta^{13}C$ and $\delta^{15}N$ signatures of the muscle samples were statistically different between all treatments with exception of the $\delta^{13}C$ signature between diets 2 and 3. The isotopic signatures of the muscle from every treatment showed a significant fractionation with values above 1‰ for the $\delta^{13}C$ isotope and above 2‰ for the $\delta^{15}N$ isotope (Table 5).

The samples tested were those of fish taken from each experimental unit at Day 56.

Table 5. Biomass and individual mean weights, isotopic signatures of the muscle and fractionation of the diets' isotopes in the muscle.

Diet	n	Initial Mean Weight (g) (Total Biomass in Kg)	Final Mean Weight (g) (Total Biomass in Kg)	Isotopic signatures of the muscle between treatments[1]			Fractionation of each diet's isotopic signature as shown in the analysis of muscle[2]						
								$\delta^{13}C$ ± (SD) ‰			$\delta^{15}N$ ± (SD) ‰		
				n	$\delta^{13}C$ ± (SD) ‰	$\delta^{15}N$ ± (SD) ‰	n	Diet signature	Muscle signature	Frac	Diet signature	Muscle signature	Frac
1	15	48.7 (6.0)	213.2 (13.0)	9	-18.85 (0.16)[a]	11.39 (0.19)[a]	15	-20.78 (0.31)[e]	-18.85 (0.16)[f]	1.93	9.35 (0.52)[p]	11.40 (0.20)[q]	2.05
2	15	48.4 (6.1)	213.9 (12.9)	9	-18.45 (0.26)[b]	10.15 (0.28)[b]	15	-19.55 (0.77)[g]	-18.45 (0.26)[h]	1.10	7.09 (1.01)[s]	10.16 (0.27)[t]	3.06
3	15	48 (6.1)	201.8 (12.1)	9	-18.29 (0.20)[b]	9.09 (0.29)[c]	15	-19.86 (0.56)[k]	-18.30 (0.20)[l]	1.57	5.27 (0.28)[w]	9.09 (0.29)[x]	3.82
Com	n/a	n/a	n/a	3	-19.32 (0.22)[c]	10.98 (0.17)[d]	6	-21.08 (0.39)[m]	-19.33 (0.22)[n]	1.76	6.11 (0.20)[y]	10.99 (0.16)[z]	4.87

Com = commercial; Frac = fractionation; n = number of observations; n/a = not applicable, no trial was conducted with the commercial diet, the muscle analyzed for the commercial diet came from fish sampled before the experiments started and their isotopic signatures were measured as a baseline.
[1]Comparisons of signatures were performed between treatments (diets) for each separate isotope and the values should be read as vertical columns. Values sharing the same superscript within the same column were not significantly different (P>0.05)
[2]Comparisons between diet and muscle were performed for each isotope separately within each treatment and the values should be read as horizontal rows. Values sharing the same superscript within a row were not significantly different (P>0.05).

2.4.4. Discriminant analysis

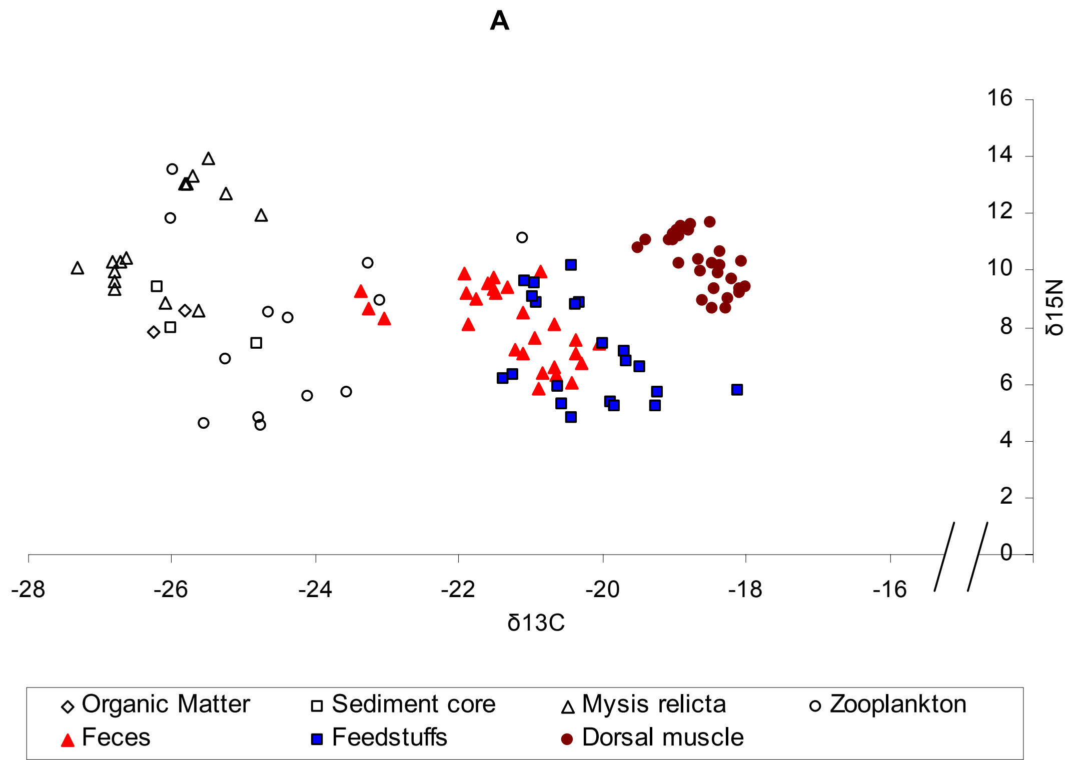
The discriminant analysis grouped the signatures of all materials successfully, with only 7 of 78 (9.0%) observations that could be classified under a different group, hereafter referred to as misclassified. The probability of those values belonging to another group, hereafter called posterior membership or misclassification, is summarized in Table 6. The comparisons were made with data belonging to values of various other biota, sediments and organic matter commonly found in Lake Ontario (Hodell and Schelske, 1998; Johannsson *et al.*, 2001), Lake Superior (Harvey and Kitchell, 2000; Sierszen *et al.*, 2004) and Lakes 110 and 227 from the Northern Ontario Experimental Lakes area (Kidd *et al.*, 1999). For graphic purposes, the comparison was performed by just grouping the data according to the type of material, without making a distinction between our experimental diets. It can be noted that the clusters formed by our data points are set apart and clearly distinguishable from the other materials in all comparisons, such as zooplankton, sediments, aquatic insects and organic matter (Figs. 4 and 5). The analysis revealed that a few signatures of zooplankton from Lake Ontario could be classified as belonging to the feces produced by fish fed the commercial diet (Table 6). The proportion of these misclassifications was very small (3.6%) and it is not considered significant.

The comparison with the signatures of the biota and organic matter from Lake Superior showed that the misclassification occurred only within the Lake's materials but not between our materials and the lakes'. This means that the

range of values between our data and the Lake's are different enough as not to share similarities that could create and overlapping of signatures. Some of our values were thus reclassified as belonging to either a different diet or a different type of material, but not as belonging to any of the lake's materials.

For Lake 227, only one of 40 values measured from aquatic insects was misclassified, with an 80% chance of belonging to the commercial feces group. One misclassified value represents a proportion of misclassification of only 0.8%, which is not considered significant.

Finally, all the signatures from Lake 110 were different from our data and no misclassification occurred.



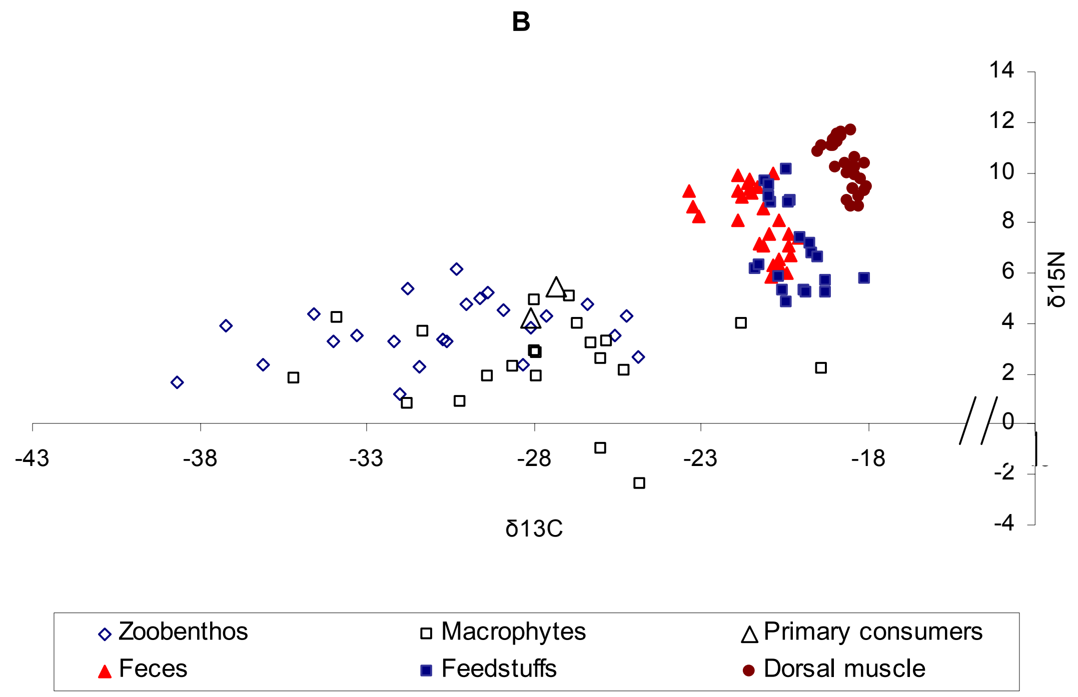
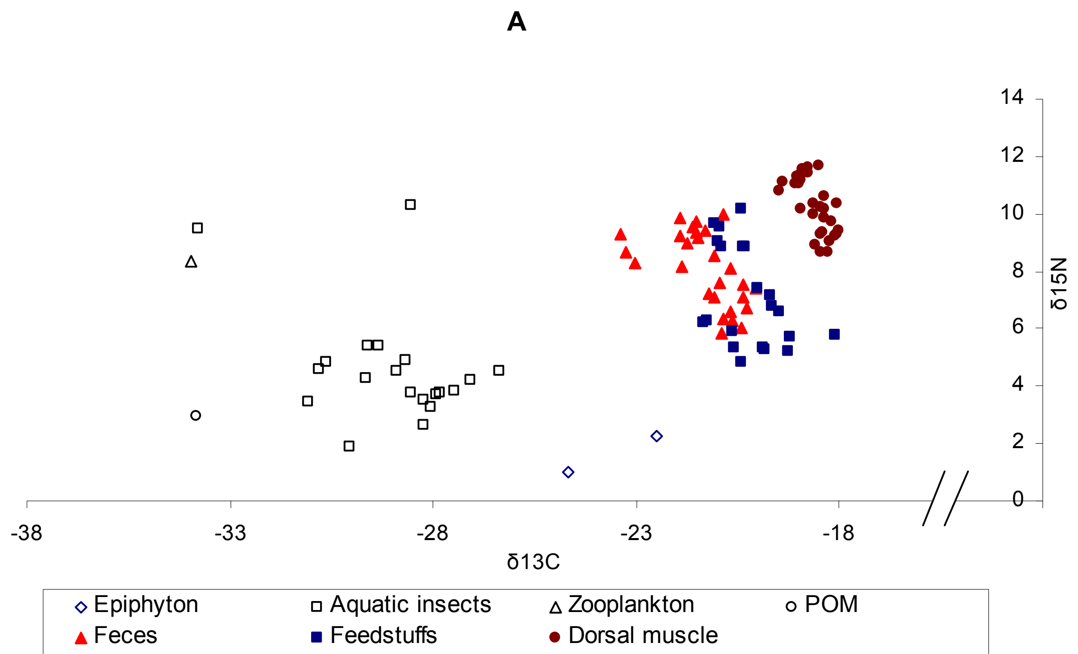


Figure 4. Comparison of some typical isotopic signatures from: Lake Ontario (A) and Lake Superior (B) (open symbols), with our data (solid symbols).



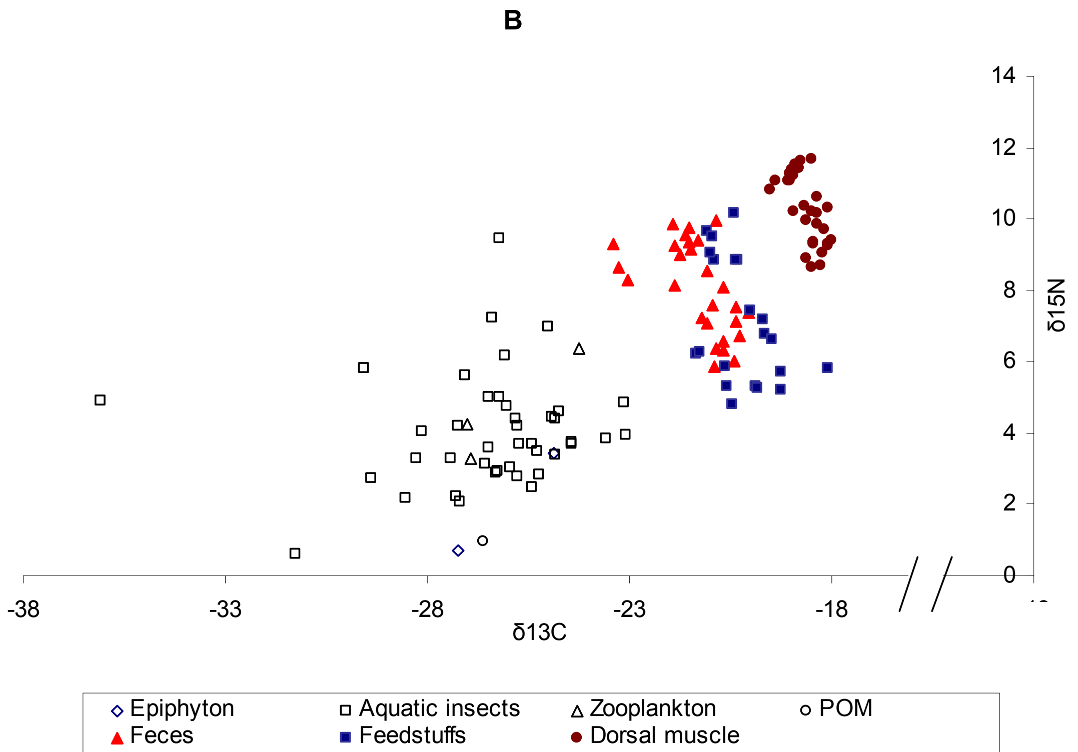


Figure 5. Comparison of some typical isotopic signatures from oligotrophic Lake 110, (A) and eutrophic Lake 227, (B) (open sybols), with our data (solid symbols).

Table 6. Probability of posterior membership of our materials and the ones labeled as misclassified from the Lakes comparisons, according to the discriminant analysis. . "From group" indicates the origin of the samples. "To group" indicates the category where the samples could belong based on their paired $\delta^{13}C$ and $\delta^{15}N$ values.

From group	To group m1	mc	f1	f2	f3	fc	d1	d2	dc	mr	zp
mc	33.3 (1)	66.6 (2)	x	x	x	x	x	x	x	x	x
f2	x	x	x	66.6 (4)	16.6 (1)	x	16.6 (1)	x	x	x	x
f3	x	x	x	x	77.7 (7)	x	x	11.1 (1)	11.1 (1)	x	x
fc	x	x	x	x	x	66.6 (2)	x	x	x	x	33.3 (1)
d2	x	x	x	x	x	x	16.6 (1)	83.3 (5)	x	x	x
dc	x	x	x	x	33.3 (1)	x	x	x	66.6 (2)	x	x
zp	x	x	7.69 (1)	x	x	15.3 (2)	x	x	x	15.3 (2)	61.5 (8)

Only the groups considered as misclassified are shown. Values expressed as percentages. Number between parenthesis indicates observations. m, f, d = muscle, fecal, and feed; c,1,2,3 = feed formulations: commercial, diet 1, 2 and 3, respectively; mr = *Mysis relicta*; zp = zooplankton

2.5. Discussion

2.5.1. Feed Ingredients

The $\delta^{13}C$ values of the plant-based ingredients examined fell within the typical ranges expected for C3 and C4 plants (corn gluten meal (C4), mean = 13.2‰; wheat middlings and soybean meal (C3), mean = -24 and -25.9‰, respectively) (Peterson and Fry, 1987). Primary producers' $\delta^{13}C$ depend on the photosynthetic pathway used: C3 plants' values range from -20 to -35 ‰, C4 plants, from -17 to -9 ‰ (Peterson and Fry, 1987) and in Crassulacean Acid Metabolism (CAM) plants, from -14 to -34‰ (Bender *et al.*, 1973). The terrestrial animal by-products (poultry by-products meal, feather) also had $\delta^{13}C$ values within the range of C3 plants after accounting for the average fractionation for carbon of 1 ‰ every trophic level (DeNiro and Epstein, 1978). This may be a reflection of the fact that diets fed to these animals are predominantly based on ingredients of terrestrial plant origin.

On the other hand, the $\delta^{13}C$ of the feed ingredients from marine origins yielded intermediate values in relation to the rest of the ingredients tested. Plankton is likely the source of carbon in both the menhaden and herring processed into fish meals that were used in the present study. Marine phytoplankton exhibits a narrow range of $\delta^{13}C$, from -23.8 to -19.3‰ (Rounick and Winterbourn, 1986). Nevertheless, the plankton isotopic values could be strongly affected by variables such as the photosynthetic pathway, temperature (Power *et al.,* 2003), and carbon source (atmospheric CO2, dissolved CO2 or bicarbonates). Values of

$\delta^{13}C$ up to -35‰ have been reported for some marine phytoplankton species (Wong and Sacket, 1978), and in freshwater ecosystems, algal $\delta^{13}C$ can even be as depleted as -45‰ (Peterson and Fry, 1987). The wide range in the carbon isotopic composition of phytoplankton is therefore an important source of variability that could influence the carbon signatures of zooplankton which is the main food source for herring and menhaden. In zooplankton, for example, their isotopic ratios are determined by the isotopic variability of the phytoplankton being consumed as well as feeding behaviors, which may result in selective feeding on some species of phytoplankton or on other sources of carbon such as particulate and dissolved organic matter (Grey and Jones, 1999; Grey *et al.*, 2000).

The above explains why carbon alone can't be an unreliable tracer, particularly when the material it is intended to track is composed of a wide selection of carbon sources in which an overlap in isotopic values would not be uncommon. Nevertheless, when $\delta^{13}C$ are used in conjunction with $\delta^{15}N$, a unique signature for a particular material is more likely to be distinguished (Peterson and Fry, 1987).

The natural distribution of nitrogen ratios depends on the rates of its supply and the amount that can be used as a metabolic substrate (Peterson and Fry, 1987), and how nitrogen is fractionated in a stepwise enrichment according to trophic position (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002).

Atmospheric nitrogen is the isotopic standard, thus every nitrogenous compound originated from the fixation of atmospheric nitrogen either by bacteria, plants or artificially created (fertilizers) is usually not heavily enriched. Therefore, primary producers have low $\delta^{15}N$ because the form of nitrogen available for plants is present in nitrites or nitrates from either one of the sources mentioned above. This statement was supported by the isotopic signatures shown by our terrestrial ingredients, whose values were characteristic of typical soil nitrogenous compounds or atmospheric nitrogen-fixing legumes (e.g., soybean) (Peterson and Fry, 1987).

On the other hand, the fish meals examined had the most enriched $\delta^{15}N$ of any ingredients in the experiment. Fish like herring and menhaden obtain nitrogen from the plankton on which they feed. The plankton itself can have $\delta^{15}N$ values as high as 14 ‰ depending on their uptake source (Minagawa and Wada, 1984) and the geographic and temporal variations (Mullin *et al.*, 1984).
The particularly high $\delta^{15}N$ in the ingredients of marine origin were important in determining the isotopic signature when all the ingredients were mixed. The resulting diets had $\delta^{15}N$ values directly related to their ratios of marine and terrestrial components (Table 3 and Figure 3). Furthermore, the diet formulations yielded unique isotopic signatures for each diet, even Diet 3, which contained only 15% fish.

2.5.2. Feces

Since feces may be the biggest source of organic material originating and dispersing from a cage aquaculture farm, special attention was given to their analysis.

The fecal material produced at different stages of the experiment was analyzed in order to evaluate if the isotopic signatures of the diets could be identified in the first set of samples. The comparison of the fecal samples from days 40 and 56 revealed that the $\delta^{13}C$ and $\delta^{15}N$ of the diets were already present in the feces when the first sample was taken at day 40, with little or no change observed by day 56. The lack of change in the isotopic signatures of the feces from days 40 and 56 suggests that the signatures were clearly identified from day 40 and were not exhibiting any variations between sampling days. Limited or no variation in the values of an isotopic signature is an important condition if it is to be considered a useful tracer (Lajtha and Michener, 1994). The presence of the isotopic signature from each treatment in the feces showed that, in some cases, the fractionation of the isotopes (Table 7) did not meet the reported typical average enrichment intervals, corresponding to changes of 1‰ for carbon and 3‰ for nitrogen with each trophic level (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981; Minagawa and Wada, 1984) although it was within the lower limits of the ranges reported by Post (2002). However, the observed slight enrichment (for nitrogen) and depletion (for carbon) of the feces is not surprising since the feces do not represent an increase in trophic position *per se*. Fish feces are

composed mostly of undigested materials so it is possible that the isotopes' fractionation was not as thorough as it would be with nutrients that are deposited in the muscle. Nevertheless, even with the relatively low enrichments observed, most of the fecal values were considered statistically different between them, and they could be associated with their source, which is an important feature if the signatures are to be used as tracers.

The comparisons between the isotopic signature of each diet and its corresponding feces showed that $\delta^{13}C$ had a significant depletion in all treatments (Table 7). For $\delta^{15}N$, on the other hand, only Diet 3 and the commercial diet had a significant enrichment (Table 7). This suggests that some of the carbon and nitrogen sources in each diet are susceptible of fractionation during their pass through the digestive tract of fish even if they are not digested. However, with the scope of the present study it is not possible to tell which ingredients exactly were the ones used (thus fractionated) preferentially.

The $\delta^{15}N$ signatures in the feces were statistically similar between Diet 1 and the commercial diet and between Diet 2 and the commercial diet (Table 7). This suggests that the $\delta^{15}N$ is not affected significantly when the proportions of fish meal incorporated in the diets range between 30 and 45%. Similarly it can be noted that the $\delta^{15}N$ of the commercial diet lies somewhere in the middle of diets 1 and 2, suggesting that the fish meal proportion in the commercial formulation may be within the same range. This is a particularly important condition that may

happen on other commercial formulations if the feed manufacturers use similar proportions of marine ingredients because the $\delta^{15}N$ will have values within a delimited range that could be characteristic of fish feeds.

On the contrary, the feces originating from diet 3 and the commercial diet showed a significant difference in the $\delta^{15}N$ even when the $\delta^{15}N$ of the diets themselves were not statistically different (Table 7). The commercial diet exhibited the highest enrichment (Table 7), which resulted in a fecal $\delta^{15}N$ similar to those in diets 1 and 2, even when the $\delta^{15}N$ value in the feed was small. One plausible explanation for why the feces produced by fish on the commercial diet showed the highest enrichment is that this fecal sample came from the tank that was holding the fish that eventually would be used for the other three treatments; the sample that was taken from this tank was therefore composed of feces deposited over a longer period of time than the 24 hours used in the experimental diets. A longer sitting time of the fecal material facilitates longer microbial degradation that could be accountable for such high enrichment of the $\delta^{15}N$ in the feces. It has been demonstrated that the $\delta^{13}C$ and $\delta^{15}N$ of organic matter change within days in both aerobic and anoxic conditions, due to microbial and chemical degradation (Lehmann *et al.*, 2002). Another explanation of the high enrichment of the $\delta^{15}N$ in the feces from fish fed the commercial diet is the differential utilization of the diet components. It has been demonstrated that some ingredients used in fish feed formulations are preferentially utilized and digested by fish (Cho and Bureau, 2001; Azevedo *et al.,* 1998). The types of ingredients

composing the commercial diet are unknown, but it is possible that the commercial diet was formulated with ingredients that were preferentially utilized compared to the ones composing the treatment diets.

Table 7. Mean fractionation of diets as shown in the analysis of feces

		$\delta^{13}C$ ± (SD) ‰			$\delta^{15}N$ ± (SD) ‰		
Diet	n	Diet signature	Feces signature	Frac	Diet signature	Feces signature	Frac
1	12	-20.78 (0.31)[a]	-21.71 (0.20)[b]	-0.93	9.34 (0.52)[k]	9.28 (0.62)[l,k]	-0.06
2	12	-19.55 (0.77)[c]	-20.98 (0.19)[d]	-1.43	7.09 (1.01)[m]	8.08 (1.08)[o,m]	0.99
3	12	-19.86 (0.56)[e]	-20.64 (0.21)[f]	-0.78	5.27 (0.28)[p]	6.37 (0.44)[q]	1.10
Com	6	-21.08 (0.39)[g]	-23.22 (0.16)[h]	-2.14	6.11 (0.20)[r]	8.74 (0.50)[l,o]	2.63

Frac = fractionation; Com = commercial diet; SD = standard deviation; n = sample size. Values in a same column or row sharing the same superscript were not significantly different ($P>0.05$).

2.5.3. Muscle

Over the course of the experiment the fish doubled their live weight. The amount of muscular tissue synthesized by a fish with this kind of growth is considered to be enough to reflect the isotopic signature of a new feed. In a study of tissue turnover rate of 3 age groups of sockeye salmon (*Oncorhynchus nerka*), Sakano *et al*. (2005) demonstrated that when the fish are subject to a diet shift, the $\delta^{15}N$ changes proportionally to their growth rates. Their results showed, that the isotopic shift in the muscle of the fish was more influenced by actual growth than metabolic turnover alone, understanding as metabolic turnover: “the portion of

the existing body weight that is replaced regularly with or without growth" (Hesslein *et al*., 1993). This study's conclusions are in concordance with previous investigations on other species. For example, MacAvoy *et al*. (2001) calculated the isotopic turnover in several tissues of channel catfish (*Ictalurus punctatus*) in a series of experiments that accounted for growth and metabolic turnover as defined above. Their results showed that although there is a difference in the speed at which different tissues are replaced, the isotopic turnover following a diet shift is more related to actual growth of the body or body part analyzed. Similarly, in red drum (*Sciaenops ocellatus*) larvae, isotopic turnover occurred in 2 days, during the time when biomass doubled, but the isotopic composition of the body stabilized in 10 days (Herzka and Holt, 2000).

The $\delta^{13}C$ and $\delta^{15}N$ signatures in the muscle tissue of the three treatment groups were clearly related to their corresponding diets. The lack of statistical difference between the $\delta^{13}C$ of the muscle from fish fed Diets 2 and 3 is not surprising since the $\delta^{13}C$ of the diets themselves varied very little, accordingly it would appear that carbon was utilized quite uniformly in all treatments (Table 5).

Isotopic turnover in fish muscle has been under recent investigation and discussion (Gannes *et al.*, 1997). It has been suggested that isotopic turnover is influenced by preferential utilization and retention of lighter isotopes of both carbon and nitrogen depending on the nature of the compound (carbohydrates, lipids, proteins, etc.) (Focken and Becker, 1998; Hesslein *et al.*, 1993). This could

explain the differences in fractionation found in our experiments, particularly for nitrogen. Although it is not possible to tell which ingredients were the preferred sources of deposited nitrogenous compounds, the chemical/enzymatic reactions involved influence the fractionation of nitrogen in different ways, since metabolic reactions show a preference for the lighter isotopes (Focken and Becker, 1998; Macko *et al.*, 1986). All isotopic muscle tissue values showed a signature which was both unique to the treatment and proportional to the signatures of the diets on which the fish were fed (Table 5). This suggests that the incorporation of the diet's isotopic signature is rapid in young trout, occurring within 56 days of the start of the treatment diets.

2.5.4. Discriminant analysis

The isotopic values of the diets, feces and muscle showed significant differences between most pairs of treatments. This information suggests that certain diets could be used as isotopic tracers of fecal material produced by aquaculture operations. However, there were also some similarities in the signatures of the diets, feces, and muscle that could result in a misclassification when compared with other materials. The discriminant analysis (DA) is considered a reliable statistical method in circumstances where the combination of two or more variables is needed to identify a trend, pattern or group in the results (Losos *et al.*, 1982).

The discriminant analysis showed that the groups “muscle commercial”, “Diet 2 feces”, “feces commercial” and “commercial diet” had the smallest probability (66.7%) of correct classification (Table 6), which means that those groups were the ones with the higher chance to be misinterpreted as belonging to another group. However, the majority of the samples in those groups were still classified correctly. The rest of the groups obtained from the experimental trial had higher values of correct classification or were not misclassified at all, which makes them unique and not likely to be misinterpreted as belonging to other group. As with any other statistical procedure, the sample size is a crucial factor, and in the DA the probabilities of classification would be more precise with more observations per group.

The published information on the isotopic signatures of organic materials from freshwater ecosystems is limited, and it proved difficult to find proper data to make comparisons with the signatures of the materials obtained in the experimental trial; however, we used whatever was available in the literature. The limited information on sediment stable isotopes from lakes constrained our comparisons to available data from Lake Ontario, Lake Superior and two of the Experimental Lakes Research Area, Ontario. Even with limited data, the discriminant analysis had sufficient power to segregate groups of materials and qualify the similarities with the materials from the experiments ($P = 0.05$). A signature composed of both $\delta^{13}C$ and $\delta^{15}N$ proved to be a reliable method for identifying aquaculture originated materials versus the organic materials from the

lakes compared. Our data suggest that using only one isotope may be unreliable, because some materials may share the same signature for a given isotope. This was especially true for $\delta^{15}N$. For instance, the reported $\delta^{15}N$ of zooplankton in Lakes Michigan and Huron have mean values of 5 and 5.31‰ (Branstrator *et al.*, 2000) which is well within the values found in our experiments. Due to the lack of information on their $\delta^{13}C$, a discriminant analysis could not be performed. A similar situation would have been the case if both isotopes have not been given for the materials we compared from Lake Ontario (Hodell and Schelske, 1998; Johannsson *et al.*, 2001). A detailed explanation of the influence of nitrogenous sources on the signature of zooplankton in Lake Ontario is given in Leggett *et al.* (2000).

Isotopic nitrogen values from Lake Superior (Harvey and Kitchell, 2000; Keough *et al.*, 1996; Sierszen *et al.*, 2004) and the experimental lakes (Kidd *et al.*, 1999) were generally more depleted than the signatures in the materials in our experiments, although there was still some overlap in the ranges.
In order to avoid possible overlaps which might result in the misidentification of materials, $\delta^{13}C$ and $\delta^{15}N$ values should be coupled, particularly in comparisons where the $\delta^{13}C$ itself is not evidently set apart in the test materials. Examining our comparisons, it appears that the $\delta^{13}C$ alone is enough to distinguish between materials. However, care should be taken when making such an assumption, since it is well known that the carbon cycle in freshwater ecosystems is highly

variable (Leggett *et al.*, 1999; Rounick and Winterbourn, 1986) and relying on $\delta^{13}C$ alone may not be useful in certain environments or seasons.

2.6. Conclusions and Recommendations

The $\delta^{13}C$ and $\delta^{15}N$ signatures of feeds can be positively identified in trout feces within 40 days after the fish were switched to the treatments diets. The fecal $\delta^{13}C$ and $\delta^{15}N$ will be slightly enriched from their source, undergoing fractionations within the typical ranges of 0-1.5 ‰ for carbon and 3-4‰ for nitrogen. Our results suggest that the isotopic signatures of feeds can be used to trace fecal organic matter produced by aquaculture in fresh water, and they can be particularly robust when analyzed with a discriminant analysis approach.

The experimental trials showed that $\delta^{13}C$ and $\delta^{15}N$ values can be used as tracers of fecal organic materials derived from cultured trout. Their applicability on commercial cage farming operations has yet to be confirmed. A field trial is thus necessary to determine whether the isotopic signature of the feeds being used in a given farm could be identified in the sediments below and around the cages.

Chapter 3: Evaluation of the Utility of the Isotopic Signature of Fish Feeds in a Cage Fish Farm Operation. Field Sampling and Validation Study

3.1. Introduction

The commercial and formulated diets used in the previous experiments and the feces produced from the consumption of such feeds showed unique isotopic signatures. These results confirmed the hypothesis that fish feeds with different proportions of fish meal are associated with a distinct isotopic signature that can be identified in the feces of the fish fed those diets. The feces collected during the experiment were those produced during 24 hours which limited the action of physical, chemical or biological factors that could have influenced a change in their isotopic composition. Therefore, it was necessary to test if the laboratory results were consistent and applicable in a real life scenario, where variables like temperature, photoperiod, water flow and residence time of the feces in the environment were not controlled. To properly validate the applicability of using isotopic signatures of fish feeds as tracers of solid waste it was decided to sample sediments from the vicinity of an operational trout farm and compare them with isotopic values of sediments from control sites within the same water body. It was expected that the sediments around the fish cages would be rich in settled solid organic matter and waste from the farm and their isotopic signatures different from those of sediments that do not have contributions from the fish farm. It was assumed that the isotopic carbon and nitrogen signatures of the feeds being used at the farm would be found in the feces and that the isotopic

signatures of both the feces and uneaten feed particles settled in the bottom could be identified and related to the feeds used at the farm. The positive identification of an isotopic signature from aquaculture-derived wastes in the natural environment would strengthen the laboratory results and give evidence that isotopic signatures of fish feeds can be used as an aquaculture waste tracer. Furthermore an aquaculture waste isotopic signature could provide enough information to map out the dispersal patterns of the wastes which was the main goal of the thesis.

3.3. Hypothesis and Objectives

The following hypothesis was formulated for the present chapter:

> *The isotopic signature of aquaculture-derived wastes will be clearly related to the source feed and it will be unique, distinctive and easily identified when compared to the isotopic values of undisturbed sediments.*

The objectives set to test the hypothesis were:

1. Identify the isotopic signatures of the feeds being used at an operational trout cage aquaculture facility.
2. Measure the isotopic signatures of sediment samples from the vicinity of the trout cage farm and from control sites as far away as possible.
3. Compare the isotopic values of the sediment samples from both control and farm sites with the discriminant analysis statistic to determine if the waste isotopic signatures are different enough to be used as an environmental tracer.

3.5. Methodology

3.4.1. Study area

The field studies were conducted at MTM Aquaculture, located in Lake Wolsey, Manitoulin Island (Fig. 6). This farm site was chosen for reasons of sample accessibility, with both aquaculture and control samples available from the same lake.

The morphometry of the Lake defines it as a restricted embayment with one entrance channel to the Campbell Bay and, via this bay, to the North Channel area. The land surrounding Lake Wolsey consists of limestone bedrock and is generally agricultural. The basin has a maximum depth of 25m and average water exchange of 14.3 m^3/sec. These conditions result in a vigorous water exchange and short term residence time of around 215 days (Hamblin and Gale, 2002).

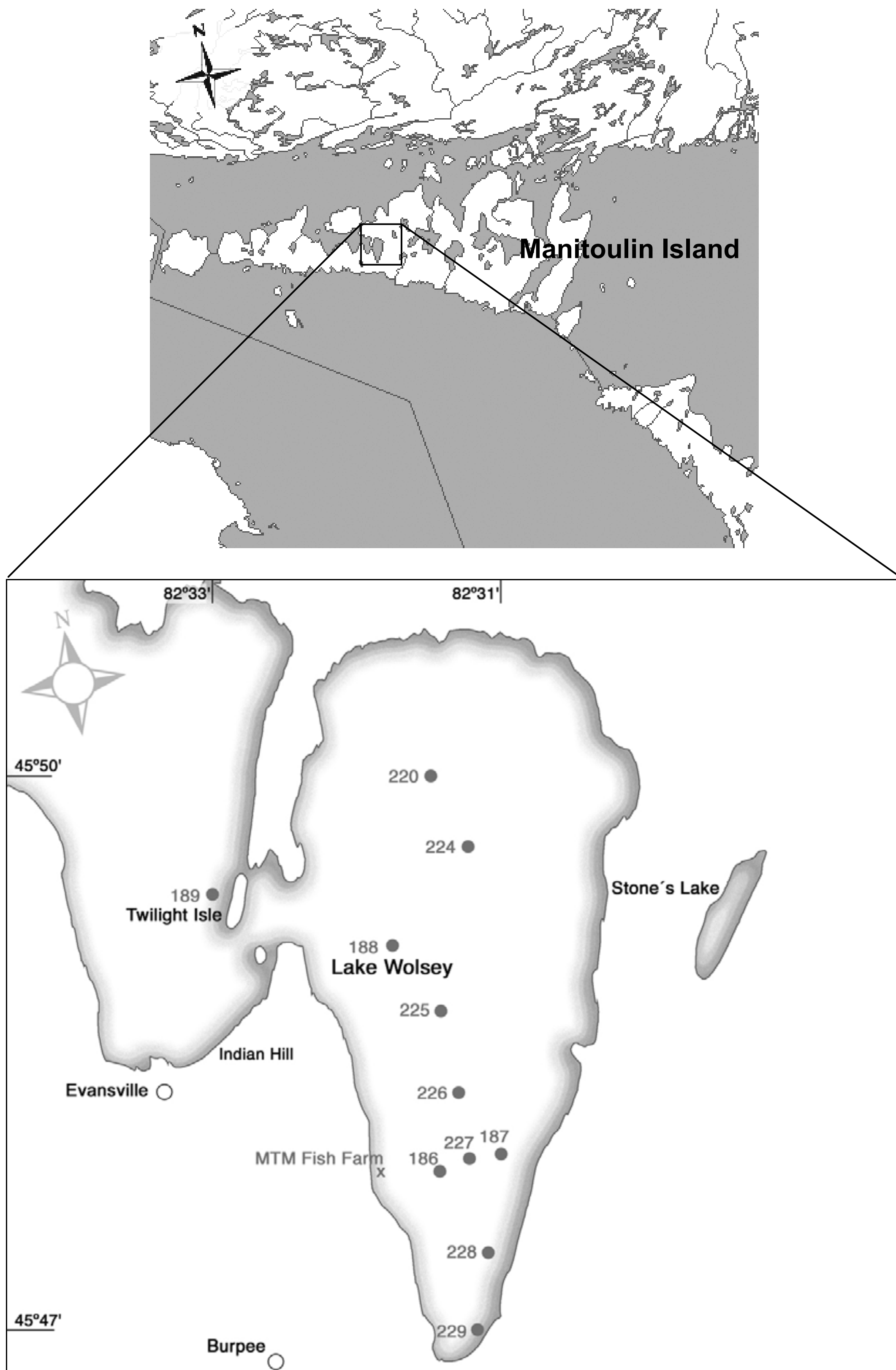


Figure 6. Geographic location of the study area. Each dot represents a sampling station according to Environment Canada's coding. For precise coordinates refer to Table 8.

3.5.1. Sample Collection

A sample of the feed used at the farm was taken directly from the bulk feed bags located on the farm property on July 2006. The feed was a 4PT high energy feed for salmonids formula (45% crude protein, 22% crude fat, 1.5% crude fibre, 1.4% calcium, 1.15% phosphate) manufactured by Martin Mills Inc. (Elmira, Ontario, Canada).

The sediment samples were taken in conjunction with Environment Canada researchers aboard an Environment Canada vessel using a Lightweight Ponar Grab Sampler (Halltech Environmental Inc., Guelph, Ontario, Canada). This collection device is formed of two self-closing scoops that resemble a half sphere when closed and jaws when open. The Ponar is attached to a line or cable. When the scoops strike the bottom, a center pivot closing action closes the scoops and their tapered cutting edges penetrate well with very little sample disturbance. An attached under-lip wipes the scoop clean of pebbles and cobble that would interfere with closing. Removable side plates prevent the lateral loss of sample as scoops close. The control sites samples were taken from stations previously assigned by Environment Canada researchers (Fig. 6), and located with the aid of a Magellan Meridian GPS that supports WASS (Wide Area Augmentation System) with an accuracy within three meters (Magellan Navigation Inc, USA). Exact coordinates are shown in Table 8. The sampling stations surrounding the farm were taken at 5, 15, 25, 55 and 85 m from the edge of the cages in north, east and south directions. Due to a mistake when

measuring the distances an extra station at 105 m was taken north of the cages (Fig. 9). At each station the Ponar was lowered from the sides of the vessel by hand on a vertical line. The Ponar was pulled inboard and opened on top of a plastic tray to hold the sediment sampled. For the samples taken from underneath the aquaculture cages, the Ponar was dropped directly from the walkway between the net pens.

From the whole surface sediment sample, a layer of approximately 3 cm thick was scooped into plastic bags and kept on ice for 2 days until they were transferred to a freezer at the Aquaculture Centre laboratory, University of Guelph.

Table 8. Coordinates of control stations on Lake Wolsey, Manitoulin Island

Station	Latitude	Longitude	Approximate distance from the farm (Km)
186	N45°48.000'	W82°32.300'	0.8
187	N45°48.066'	W82°31.742'	1.5
188	N45°49.480'	W82°32.330'	2.8
189	N45°50.022'	W82°33.835'	4*
220	N45°50.496'	W82°31.687'	5
224	N45°49.997'	W82°31.502'	4.1
225	N45°49.010'	W82°32.003'	2.1
226	N45°48.511'	W82°31.989'	1.5
227	N45°48.079'	W82°32.025'	1.2
228	N45°47.473'	W82°32.025'	1.6
229	N45°47.022'	W82°32.254'	2.1

* Distance measured as straight line between the farm and the station

3.5.2. Sample Preparation

The samples were kept frozen at -4ºC until preparation since freezing is the best sample preservation method for isotopic analysis purposes (Feuchtmayr and Grey, 2003). Preparation consisted of sifting the sediment to remove the large organic and inorganic materials, such as pebbles, shells, insect larvae and worms or other unidentified debris that could influence the isotopic signature of the sediments. Half of the sample was then washed with 1N HCl to remove inorganic carbon as suggested by Lajtha and Michener (1994), the other half was not acid treated. This was done to compare if the isotopic signatures from acidified and non acidified samples were different since carbonate isotopic values are heavy and might skew the results. The excess liquid from the sample was filtered and the solids dried at 60ºC for 24 hours in a temperature controlled oven. Once dried, the samples were ground to a fine powder with a mortar and pestle.

3.5.3. Isotopic Analyses

The methods for this analysis were performed following the recommendations of Lajtha and Michener (1994) and are the same as those presented in Chapter 2. A sample of 3 g of the powdered sediments was submitted to the Environmental Isotope Laboratory of the University of Waterloo. From the sample submitted, a sub-sample of 1 mg was taken for isotopic analysis.

3.5.4. Statistical Methods

The isotopic carbon and nitrogen values of the sediments from the control stations and the farm were analyzed in a one-way ANOVA. A discriminant function analysis was also performed on these values to group the data with carbon and nitrogen as parameters. All statistical analyses were completed using Statistical Analysis Software (SAS) version 9.1 (SAS Institute Inc., Cary, NC, USA).

3.6. Results

3.6.1. Lake Sediments

The isotopic signatures of Lake Wolsey's sediments covered a broad range of values for $\delta^{13}C$ (-27.72 to -22.91‰) and a narrow range for $\delta^{15}N$ (3.30 to 4.49‰) (Figure 7).

Figure 7. Isotopic signatures of sediments according to their origin and preparation treatment. Control = stations, Test = farm, MTMF = Feed used in the farm.

The removal of carbonates by acidification of the samples resulted in statistical differences between their $\delta^{13}C$ (Table 9). Since the carbon intended for measure was of organic origin (protein, carbohydrates and lipids from the fish feed) only the isotope values from acidified samples were considered for further interpretation. No significant differences were observed in either the $\delta^{13}C$ or $\delta^{15}N$ signatures between sediments from the control sites and the vicinity of the cages.

Table 9. Mean values of the isotopic signatures of sediments from Lake Wolsey sampling stations and MTM Aquaculture Trout Farm

Material	n	$\delta^{13}C \pm$ (SD) ‰	$\delta^{15}N \pm$ (SD) ‰
Control	10	-23.94 (2.15)[a]	4.05 (0.30)[c]
Control/Acid	10	-26.34 (1.43)[b]	3.93 (0.31)[c]
Farm	18	-23.35 (0.77)[a]	4.06 (0.45)[c]
Farm/Acid	18	-25.24 (1.47)[b]	3.93 (0.22)[c]

SD = standard deviation; n=sample size. Values in the same row or column sharing the same superscript were not significantly different ($P>0.05$)

The isotopic signatures corresponding to every sampling station are mapped in Figure 8.

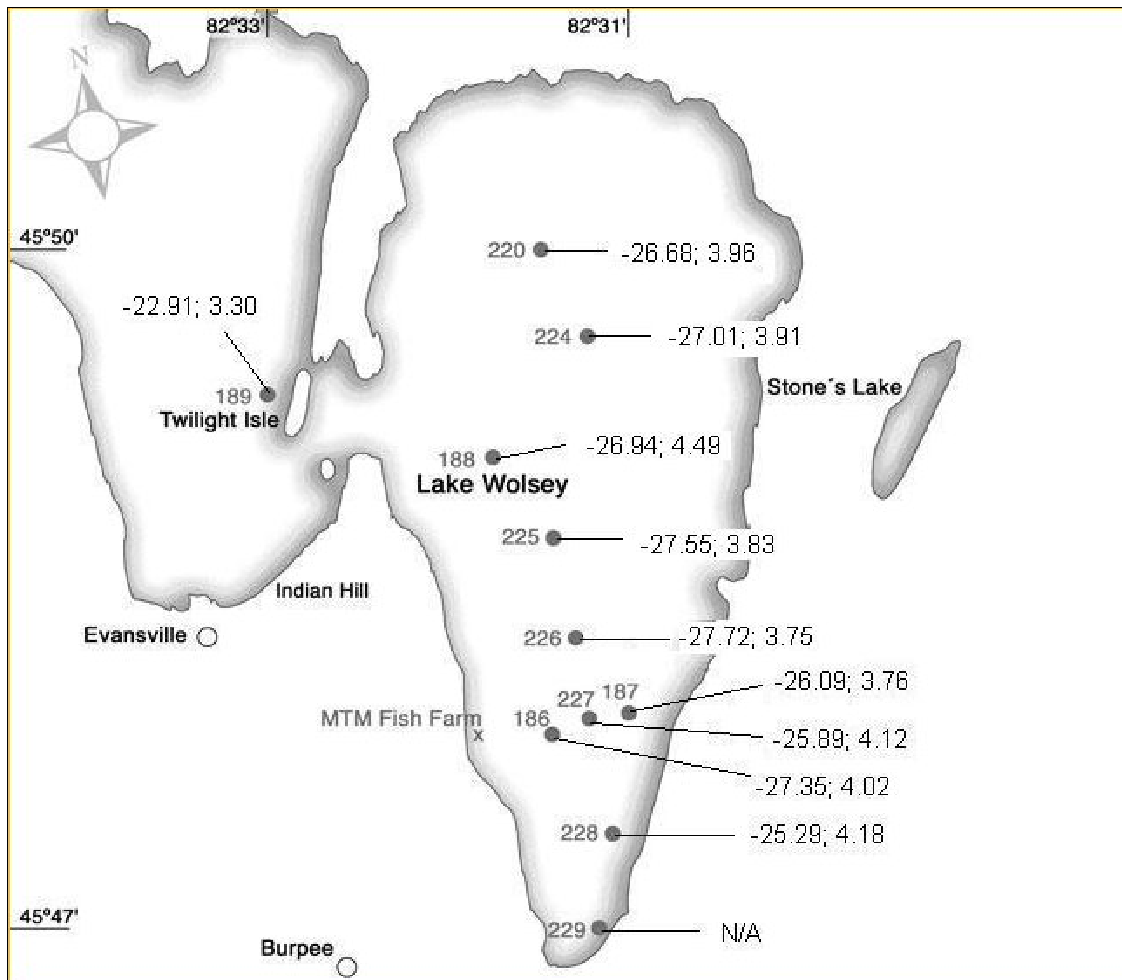


Figure 8. Map of the isotopic signatures at each sampled station. The first value corresponds to $\delta^{13}C$ followed by $\delta^{15}N$. N/A = not sampled

The isotopic signatures from the sediments near the trout cages did not show any particular pattern. It was expected that the sediments would reflect a signature similar to the feed's and gradually resemble that of the control sites with increasing distance from the cages. The values are mapped in figure 9.

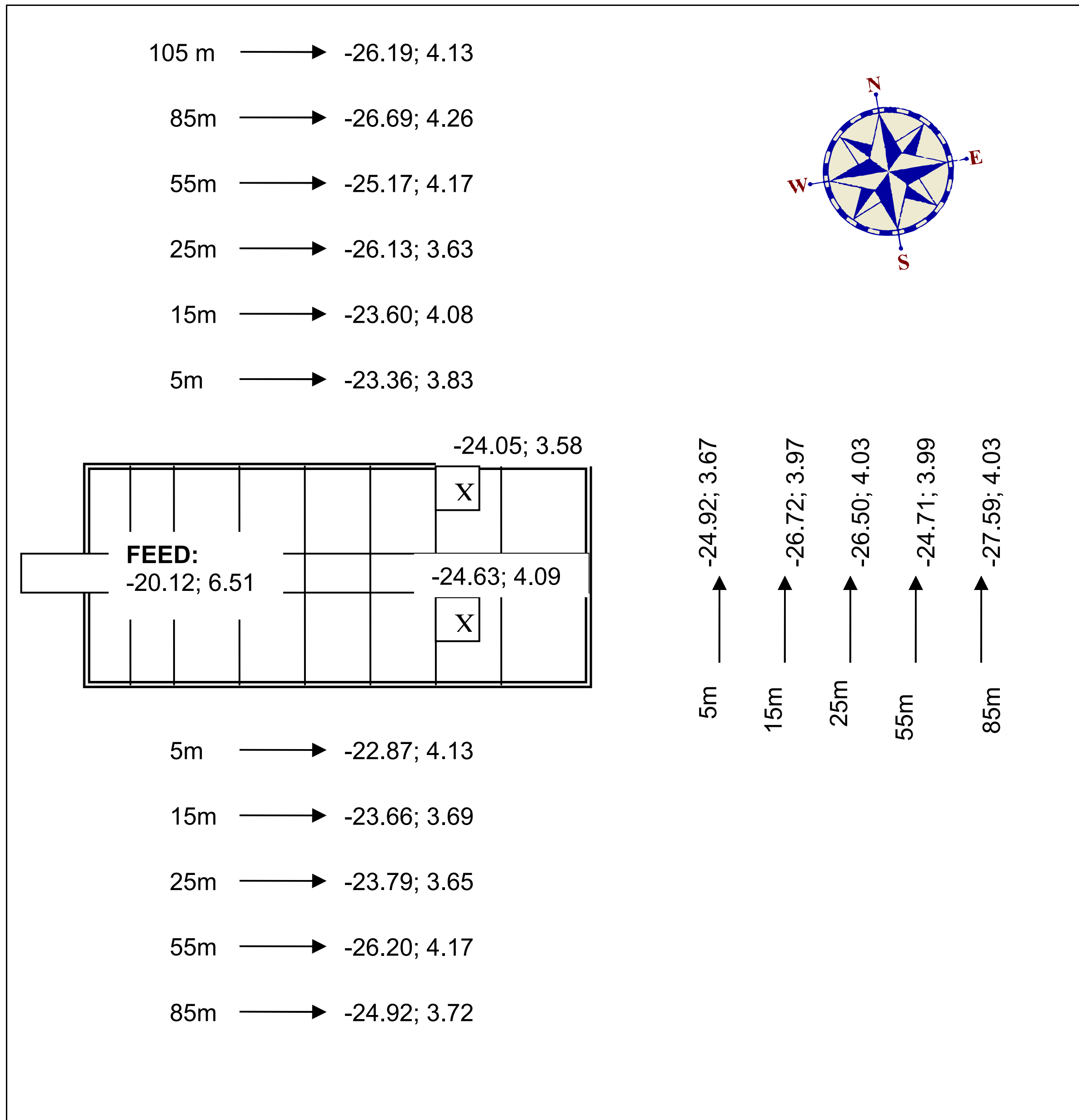


Figure 9. Diagram of the layout of the trout cages and the isotopic signatures of the surrounding sediments. The first value corresponds to $\delta^{13}C$ followed by $\delta^{15}N$. The isotopic value of the feed is given as a reference point.

3.6.2. Discriminant Analysis

The close similarity of the isotopic signatures at the farm and the control sites is statistically supported by both the ANOVA and the discriminant analysis. The probability of posterior membership for each one of the sample types is summarized in Table 10.

Table 10. Probability of posterior membership of sediments from Lake Wolsey and MTM Aquaculture. "From group" indicates the origin of the samples. "To group" indicates the category where the samples could belong based on their paired $\delta^{13}C$ and $\delta^{15}N$ values.

	To group				
From group	Control	Control Acidified	Farm	Farm Acidified	TOTAL
Control	0	0	50 (5)	50 (5)	100 (10)
Control Acidified	0	40 (4)	0	60 (6)	100 (10)
Farm	0	0	83.33 (15)	16.67 (3)	100 (18)
Farm Acidified	0	11.11 (2)	16.67 (3)	72.22 (13)	100 (18)

Values expressed as percentages. Number between parentheses indicates number of observations.

3.7. Discussion

3.7.1. All Sediments

Contrary to what was expected, the sediments collected from areas in close proximity to the fish cages were not significantly different from those collected at the far-field location control sites ($P>0.05$, $n= 56$). The only statistical difference occurred in the $\delta^{13}C$ signatures of acidified sediments of the same origin ($P<0.05$, $n=20$ for control sites and $P<0.05$, $n=36$ for farm sites). It can be noted (Table 9) that the acidified signatures for both control and farm sediments are more isotopically depleted than the non-acidified ones. This is understandable since the acidification removes inorganic carbonates commonly found in shells of many organisms which alter the $^{12}C/^{13}C$ ratios (DeNiro and Epstein, 1978). In the case of $\delta^{15}N$ a slight depletion was observed in the mean signatures for control and farm sediments after the acidification, but with no statistical difference between acidified and non-acidified signatures ($P>0.05$, $n=20$ for control sites and $P>0.05$, $n=36$ for farm sites), even though it has been reported that treatment with hydrochloric acid adversely affects the nitrogen signatures of shrimp (*Metapenaeus* spp) and sea grass (*Enhalus acoroides*) (Bunn *et al.*, 1995) and fish tissues (Pinnegar and Polunin, 1999). Due to the observed differences in acidified versus non-acidified isotopic signatures it was decided to use the values of the acidified samples hereafter since the carbon and nitrogen intended to track was of organic origin.

3.7.2. Control Site Sediment Samples

The range of $\delta^{13}C$ obtained from the control sites -26.34±1.43 ‰ corresponds to the typical reported ranges for freshwater particulate organic matter (POM), sediments and/or plankton in various freshwater watersheds: In coastal wetlands on the south shore of Lake Superior, for example, POM values measured in the summer had a signature of -26.7±0.64 ‰ and -29.9±0.5 ‰ (Sierszen *et al.*, 2004). Similarly, primary producers and invertebrates that are usually present in the sediments showed $\delta^{13}C$ ranges from -26.3 to -27.9‰ and -26.2 to -28.8‰, respectively (Keough *et al.*, 1996). For south eastern Lake Ontario the signatures follow a consistent and distinct seasonal pattern, where the weighted average over a 3 year sampling period indicated $\delta^{13}C$ values of -26.25‰ for shallow sediment traps, -25.83‰ for deep traps and -26.2‰ and -26.8‰ for the top part of cores (Hodell and Schelske, 1998). Similarly, POM samples from central and eastern locations showed an overall mean of -25.55‰ and 26.3‰, respectively for particles 1-44µm in size (Leggett *et al.*, 1999).

In the experimental lakes from northwestern Ontario, oligotrophic Lake 110 showed a $\delta^{13}C$ for POM and zooplankton of -33.81±4.57 ‰ and -33.94±2.92 ‰ respectively. In contrast, eutrophic Lake 227's values were -26.63±2.08 ‰ for POM and -24.25±4.61 ‰, -26.95 and -27 for zooplankton of various sizes (Kidd *et al.*, 1999). Most of the studies mentioned above suggest that the isotopic signatures from material deposited on the bottom are closely related to the signatures of planktonic organisms and/or other sources of organic matter related

to productivity. Such relationships have been well documented in a study of 83 lakes from Florida (Gu *et al.*, 1996a) The similarity of the signatures from Lake Wolsey and the eutrophic Lake 227 may suggest analogous productivity between the two, as it has been established that $\delta^{13}C$ enrichment is a sign of increased productivity (Schelske and Hodell, 1991).

The mean $\delta^{15}N$ of the sediments from the control sites was 3.93±0.31‰, which is different from the typical values for lake sediments. For example, the reported $\delta^{15}N$ of POM and zooplankton from wetlands in southern Lake Superior (Sierszen *et al.*, 2004) is 1.5 ±0.45 ‰ and 3.2±0.52 ‰, respectively, for West Fish Creek, and 0.42 ±0.45 ‰ and 3.0 ±0.72 ‰, respectively, for Lost Creek. Lake Wolsey's $\delta^{15}N$ are more similar to Sierszen *et al.*'s (2004) values for benthos, which had overall means of 3.91‰ and 3.1‰. In central and eastern Lake Ontario, values reported by Leggett *et al.* (1999) for POM particles ranging in size 1-44μm were ≈ 4.5‰ for the summer months. In contrast, the southeastern end of the lake was characterized by a 3 year weighted average for $\delta^{15}N$ of 7.8 and 8.6‰ for shallow and deep sediment traps, respectively, and 7.4 and 9.4‰ at the top of samples taken with sediment cores (Hodell and Schelske, 1998). Finally, in the case of the experimental lakes, the $\delta^{15}N$ of POM and zooplankton was 2.94±1.84‰ and 8.36±1.81‰, respectively, in oligotrophic Lake 110, and 0.94±1.92‰ for POM and 4.25, 3.31 and 6.36±1.46‰ for zooplankton in eutrophic Lake 227 (Kidd *et al.*, 1999). Unlike Lake Wolsey's carbon signatures, which were more similar to the eutrophic lake, its nitrogen signatures

appear to be more related to the POM values from the oligotrophic lake. The comparisons of our values with the ones from other lakes may give an insight into plausible explanations for the results presented herein. However samples of primary producers or any other primary source of nutrients was not sampled, thus a properly identified baseline was not available. An isotopic baseline is an important factor to better explain and compare isotopic signatures of other materials within an ecosystem (Post, 2002)

It is evident that Lake Wolsey's nitrogen signatures are different from those seen in any previous study. It is well known that nitrogen is an indicator of the trophic positions of organisms (DeNiro and Epstein, 1981; Minagawa and Wada, 1984), so when analyzing unknown materials like in the present case it is difficult to identify its origin particularly if the values of a reference source (baseline) or any other comparison point are not available. It is possible that the samples from the control stations were composed of bulk organic matter from a variety of sources that positioned the signature in a range of values intermediate to those of POM, plankton and benthic detritus.

3.7.3. Farm Site Sediment Samples

The isotopic signatures of the sediments collected in the near-field farm area did not show a relationship with the signature of the feeds used at the farm in any way. It was expected that if the sediments were rich in fecal material, their signature would be related to the feed's signature with a fractionation similar to

the one observed in the feces from the experimental trials (Table 4) or the ones reported in the literature (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002). According to the fractionation values obtained in the experimental trials for feces derived from all diet types, the $\delta^{13}C$ values of sediment samples taken close to the cages should have been between -20.9 to -22.26‰ , and these values should have progressively increased towards the mean value of the control stations (-26.34‰) with increasing distance from the cages . The most enriched samples, and hence those with values most similar to the ones expected (feed), were located 5m south of the cages. This may suggest that feces were being deposited there in higher amounts than in other places, but this speculation has to be confirmed with current direction measurements, on-site observations of where exactly the fecal material is being deposited and more detailed sampling. Of all the control sites, Station 189 which was the farthest sampling point from the cages exhibited the lowest $\delta^{13}C$ signature, next to the one taken 5m south of the cages thus similar to the feed's $\delta^{13}C$. However, such similarity may be just coincidental and not influenced by the farm loadings.

For nitrogen, the observed scenario is even more unexpected. Based on what was observed in the feeding experiments, the $\delta^{15}N$ values near the cages should have been between 6.5 to 9.1‰ when in fact the observed mean was 3.9‰. During the experimental trials it was concluded that the $\delta^{15}N$ signature of the marine ingredients seemed to be a better tracer of aquaculture wastes because

of its high values compared to the terrestrial ingredients. Unfortunately the field samples failed to display a $\delta^{15}N$ signature consistent with the laboratory findings. Not even the $\delta^{15}N$ signature from 5m south of the cages was close to the expected range, although the $\delta^{13}C$ values from this site and the experimental trials were similar. The absence of such similarities in the signatures is not an effect of the acidification of the sample because even with the non acidified samples the isotopic signatures were far from the expected values.

It is evident, then, that the use of stable isotopes in the study area sampled does not provide clear information about the dispersion of the aquaculture wastes. The explanation for the absence of the expected signatures for both elements implies the action of one or more of the following factors: the feces and other organic matter originating from the farm are not being deposited on the sampled areas; the consumption and/or chemical degradation of the farm effluent organic matter occurred at volumes and rates high enough to change its isotopic ratios; or the sample was not representative of the bottom conditions. A more detailed explanation of each one of these possibilities follows.

It is quite unlikely that the feces and other organic matter originating from the farm were not being deposited on the sampled areas. Even though the sedimentary rate of the uneaten feed particles and feces as well as the direction and speed of the currents was unknown at the time of the sampling, it has been demonstrated that organic matter derived from cage aquaculture typically

disperses within 50m of its point of origin in marine cage farms (Gowen and Bradbury, 1987; Holmer *et al.*, 2007; Johnsen *et al.*, 1993; McGhie *et al.*, 2000; Sutherland *et al.*, 2001; Ye *et al.*, 1991), and equally far in freshwater operations (Boaventura *et al.*, 1997; Cornel and Whoriskey, 1993; Penczak *et al.*, 1982). A detailed bibliographic review of other dispersal studies can be found at (Fisheries and Oceans Canada, 2003). These investigations were carried out in places with a wide range of hydrological conditions, covering zones of fast and slow currents, and in every case the dispersal distances were similar.

It is possible that the consumption of feces and organic matter derived from the farm by other organisms and/or microbial/chemical degradation of the farm effluent organic matter occurred at volumes and rates high enough to change the isotopic ratios of the material. This assumption may be the one that better explains our results. There is evidence both in support of, and in contradiction to, isotopic shifts due to transformations of particulate and sediment organic matter by microorganisms or invertebrates. For example, an investigation into the fate of organic matter in Lake Michigan Meyers and Eadie (1993) demonstrated that the particulate organic matter underwent significant physical transformations during sedimentation, but with no significant effect on the organic matter's isotopic signatures. In a similar way Bernasconi *et al.* (1997) found that the isotopic signatures of organic matter in Lake Lugano, Switzerland did not change significantly with depth during a given season. However, they identified a strong seasonal variation in the isotopic signatures of organic matter influenced by a

change on the nitrogen and carbon sources (terrestrial vs. aquatic) and a subsequent bacterial degradation of the settled organic matter. These two studies recognized that the isotopic signatures of particulate organic matter experienced little or no change in their vertical distribution. However, they both acknowledge that bacterial degradation may still influence isotopic shifts particularly in settled organic matter.

Evidence of isotopic shifts due to microbial degradation has been presented for many different situations. For example, in a series of experiments performed by Rysgaard *et al.* (1993) it was found that bacteria and microphytes living in lake and estuarine sediments can alter the isotopic composition of nitrogenous sources in a matter of hours. The results showed that depending on the incubation conditions (oxic or anoxic) and presence or absence of light, nitrification, denitrification and N mineralization were the most influential reactions in the isotopic shifts. Similarly, (Kellman and Hillaire-Marcel, 1998) showed that nitrates in a stream receiving agricultural discharges become heavily enriched every 100 meters downstream from the discharge point. The final values (600 m away) were enriched up to a maximum of 10‰. The main cause of their observations was attributable to denitrification by bacteria.

Another study on macrophyte-derived organic matter in salt-marshes demonstrated that aerobic and anaerobic microbial degradation was responsible for the fractionation of the $\delta^{13}C$. The experiments showed that although some

samples showed a slight enrichment in anaerobic conditions, the rest of the samples analyzed were depleted, with values ranging from 4 to 6‰ (Boschker *et al.,* 1999).

In the same manner, Lehmann *et al.* (2002) showed that microbial degradation of particulate organic matter in aerobic and anoxic conditions caused carbon isotopes to undergo a depletion of 1.6‰ after 49 days, attributable to the preferential removal of the enriched fraction (carbohydrates and proteins, rather than lipids) by microbes, and to a gain of components depleted in $\delta^{13}C$. For nitrogen, enrichment was observed during the first 24 hours in oxic conditions followed by a decrease of 3‰ by day 49. In the anoxic environment no enrichment was observed, and the $\delta^{15}N$ decreased by 5‰. The possible mechanisms for such changes in the isotopic nitrogen values include preferential removal, kinetic fractionation and bacterial growth.

It can be seen then that the above studies coincide in concluding that microbial degradation is highly responsible for chemical transformations of the source organic matter that result in an overall enrichment for $\delta^{15}N$ and depletion of $\delta^{13}C$. In the case of carbon, the depletion schemes described by the studies cited fit the results presented here. However the isotopic shifts described for nitrogen, particularly denitrification, do not explain the results of the present thesis since the $\delta^{15}N$ values of the farm samples were depleted compared to the feed source, and not heavily enriched as the literature suggests.

In either case, the isotopic values of the sediments from the farm should have been more closely related to the values of the feed if in fact the samples contained cage effluents. Although it is possible that the majority of the organic matter from the farm was consumed by other organisms and/or chemically or biologically degraded in ways that depleted the $\delta^{15}N$ instead of enriching it as it was expected. Situations have been reported where aquaculture waste was not evident or present in the sediments next to cage farms. For example, Mac Dougall and Black (1999) used an acoustic bottom discrimination system (RoxAnn^{TM}) to identify waste from a European sea bass (*Dicentrarchus labrax*) and gilt head sea bream (*Sparus aurata*) farm in Selonda Bay, Saronik Gulf, Greece. The results showed that the sediments under and around the cages were composed of pebbles and coarse gravel with no evidence of organic matter, which is presumably consumed by marine organisms, mainly fish.

Regarding studies that have used stable isotopes, in a characterization of suspended particulate matter surrounding net-pens in British Columbia, the $\delta^{13}C$ of the feed was not evident in the sediment traps deployed next to the pens (Sutherland *et al.*, 2001). Horizontal distribution and transport of the particles and biological activity were considered as the main reasons for not finding aquaculture waste in the traps. In an intensive monitoring plan of salmon farms in Tasmania (Crawford *et al.*, 2002), it was concluded that the use of stable isotope of carbon and nitrogen as indicators was reliable only in places with very high levels of organic enrichment. In the same manner, sediment $\delta^{13}C$ and $\delta^{15}N$

varied little with distance from the cages and control sites in an analysis of freshwater communities receiving fish farm organic contributions (Grey *et al.*, 2004), suggesting that the farm had a negligible impact on the sediment even when measurements of other indicators suggested otherwise. A mixed case was documented in a study of particulate and sediment organic matter from cages in the Mediterranean (Sarà *et al.*, 2004), where the $\delta^{13}C$ signatures failed to represent the feed signature and the $\delta^{15}N$ signatures were related to the feed signatures only in samples taken underneath the cages.

The studies cited above attribute the absence of a relationship between the signatures of the feed and those of the sediment organic matter to one or more of the following factors: unaccounted fractionations, a lack of critical mass of uneaten feed pellets, heavy feeding activity by wild fish and other organisms within the cages' surroundings, or dilution of the feed signature by additional sources of organic matter.

These factors may in turn be responsible for the results presented here. However, it is also possible that the samples collected were not an accurate representation of the bottom conditions because they were compromised in some way. Misrepresentation of the site due to a small amount of material would not have altered the results, because a generous volume of sediment was taken from each site. In every site, the top 3 cm of the sediment taken by the Ponar was the fraction submitted for analysis, which in most samples was distinguishable just by

color. However, it is possible that the chosen portion was so thick that it contained a high proportion of other materials that confounded the isotopic signatures of the farm effluents. It is not uncommon to find relatively large differences in isotopic signatures at different depths in a core, and these differences can be particularly large in the top layers (Hodell and Schelske, 1998; Ostrom *et al.*, 1998; Yamada *et al.*, 2003). Even though the portion taken for analysis was only 3 cm thick, the Ponar took a very thick sample of sediments (more than 10 cm) which may represent several years of sedimentation. If during the sampling, the sediments were excessively disturbed, the overlying organic matter could have been mixed with the deeper sediments, thus altering the signature of the sample to represent a pattern more typical of the natural, underlying materials than of the fecal solids in the top layers which originated from the farm itself.

3.7.4. Discriminant Analysis

As with the experimental values, the discriminant analysis proved itself useful in classifying and fitting the observational values into the best group, even though the ANOVAs indicated a lack of statistical difference. Of the samples treated with acid, those from the farm showed a very small probability of membership in the control acid group (11.11%). However, when comparing the data in the opposite direction, the control group showed a large probability of being classified as belonging to the farm. This suggests that the comparisons between the two groups have utility only when the farm group is used as the standard because the

signatures of the farm are less variable, producing a smaller interval between the limits that define the group.

Nonetheless, although the discriminant analysis may have successfully segregated the two groups, the farm values were still not related to the feed values, contrary to what was expected. Had a relationship existed, it in turn would have given the farm group a most distinctive signature when compared to the control sites. An additional approach to consider for comparing samples where multiple sources are involved would be the model IsoSource (Phillips and Gregg, 2003). In this Visual Basic program, the user supplies the isotopic signatures of the sources and the mixture along with the desired level of unit increment and statistical tolerance. The output includes all the feasible source combinations with histograms and descriptive statistics of the distributions for each source. Unfortunately, the model couldn't be used because the present study had only one possible source (feed).

3.8. Conclusions and Recommendations

The use of the isotopic signature of fish feeds as a tracer for aquaculture originated material is not as straightforward as the experimental results suggest. In the natural environment numerous chemical and biological processes may be responsible for changes to the organic matter signatures in a relatively short time, thus complicating their identification confidently. For a more precise interpretation of the bottom conditions, the hydrology, biological activity in the vicinity of the cages and microbial degradation of the organic matter are important factors to consider. The possible disturbance of the sediments during sampling is another factor that has to be considered. The use of instruments such as cores or Eckman Grabs may offer different results if they do not mix the sample as much as a Ponar may do. The discriminant analysis is a powerful tool to identify groups even if the differences are especially small. For cases where the signatures in question are positively related to the suspected source, the use of this statistic will be exceptionally useful.

Chapter 4: General Conclusions and Recommendations

The main goal of this thesis project was to evaluate the utility of stable isotopes to identify aquaculture originated wastes. The basis for considering stable isotopes as tracers of waste relied on the fact that stable isotope signatures can be unique. In the case of aquaculture waste isotopic signatures, the isotopic uniqueness was attributable to the feeds' ingredients, particularly the $\delta^{15}N$ isotope in fish meals of marine origin. It was demonstrated that varying the amount of fish meal in a feed formula yields different values in the isotopic signatures, but the differences are still within a range that corresponds to typical signatures for fish feeds. Furthermore, it was also demonstrated that the isotopic signature from the feeds can be positively identified in the feces of cultured trout and that it is also possible to link the signature in the feces to the source feed. Another interesting finding was that the isotopic signature of the feeds was incorporated in the feces within a matter of days, which is an important feature considering that if the feeds are switched, the signature of the new feed can be identified within a few days.

The laboratory evaluation suggested that the isotopic signatures of feeds could be used confidently as tracers. However a thorough evaluation had to include an examination of their applicability in a real aquaculture setting. Experiments on isotopic shifts of aquaculture waste due to microbial degradation should be conducted to account for microbial fractionation when sampling waste from a farm. The field results obtained from the farm MTM Aquaculture located in Lake

Wolsey failed to corroborate the laboratory findings. It was concluded that a combination of ecological and methodological effects could have been responsible for not finding the isotopic signature of aquaculture waste in the bottom sediments nearby the farm. The fact that the results obtained were a product of ecological or biological effects is understandable and acceptable, but it is important to exclude the methodological effects as a source of erroneous results for future work. It is recommended that further studies are conducted at this and other sites with different sampling techniques. For example, the use of cores instead of Ponars for sampling the sediment could be more appropriate if it can be demonstrated that the cores do not disturb or mix the sediment as much as a Ponar will do. Use of sediment traps as opposed to grabs is another option as long as the placement and recovery of such devices is feasible in the area of interest. Also, better care should be taken when separating the uppermost layer of the sediment. Thin layers of top sediment should be taken in samples where fecal or organic material is not clearly evident to avoid an excessive mixing that could confound the waste isotopic signatures with the underlying sediments.

Determination of the correct methodology for field samplings is the most important thing to do if an “isotopic waste tracking technique” is to be established. The creation of such a technique could be an essential tool for monitoring the dispersal of aquaculture waste in the environment and this in turn will provide some of the necessary information for licensing and regulation purposes.

It can be seen so far, that the use of isotopic signatures to identify aquaculture waste could be promising given that the possible factors of fractionation due to biological activities and methodological issues recognized in the present thesis are addressed and solved. In addition to improving the methodologies the following facts have to be taken into account:

The main assumption of using stable isotopes of feeds as waste tracers was based on the uniqueness of the isotopic signature from the marine ingredients, namely the fish meals. Even the feeds with small amounts of marine ingredients were distinguishable from other materials found in freshwater environments. In the same manner it was observed that a good way to strengthen the uniqueness of the signature was achieved by using the values of both $\delta^{13}C$ and $\delta^{15}N$ combined as opposed to relying on only one isotope. The use of a combined signature reduces the possibility of confusing aquaculture originated waste with some other material that may have a similar $\delta^{13}C$ or $\delta^{15}N$ signature alone. This is particularly important considering the tendency of many feed manufacturers to reduce, or even eliminate, the marine components in their feed formulations. To support these assumptions the isotopic values of a number of commercial and experimental feeds is provided in Appendix B. It can be seen that when eliminating the marine components, the $\delta^{15}N$ values fall within the range of typical lake sediment values. However, if the $\delta^{13}C$ values are considered in conjunction with the $\delta^{15}N$, the signature becomes unique and distinguishable

again, since the typical $\delta^{13}C$ values for lake sediments are much more depleted than those of the feeds.

It is recommended then, that when applying isotopic techniques to identify and trace aquaculture originated waste, the feeds used at the farm have to be properly sampled and analyzed for both $\delta^{13}C$ and $\delta^{15}N$ and the comparisons between samples and feeds have to include both isotopes as the signature. Moreover, it is encouraged to use statistical analyses that use the $\delta^{13}C$ and $\delta^{15}N$ values in conjunction or pairs to define parameters, like the discriminant analysis used in the present study.

Finally, it can be said that the evaluation of the use of stable isotopes ratios for the identification of aquaculture waste presented here gave a good insight into the possibility of using stable isotope ratios as a tracer. It inspired questions to be addressed in terms defining sampling techniques and into investigating the influence of microbial degradation in isotopic shifts. The results of these future studies will be crucial to assess stable isotope's applicability in the identification of aquaculture waste in the natural environment. This thesis lays the basis for future research focused on refining and perfecting the techniques that may one day be used as a standard monitoring tool for aquaculture waste management programs.

References

Alain, B. (2005) Taking stock: A profile of Canada's aquaculture industry. 21-004-XIE, 1-6. VISTA On the Agrifood Industry and the Farm Community. Statistics Canada

Altabet, M.A. (1988) Variations in nitrogen isotopic composition between sinking and suspended particles: Implications for nitrogen cycling and particle transformation in the open ocean. *Deep Sea Res.,* 35(4), 535-554.

Altabet, M.A., Deuser, W. G., Honjo, S. and Stienen, C. (1991) Seasonal and depth-related changes in the source of sinking particles in the North Atlantic. *Nature,* 354, 136-139.

Altabet, M.A., Pilskaln, C., Thunell, R., Pride, C., Sigman, D., Chavez, F. and Francois, R. (1999) The nitrogen isotope biogeochemistry of sinking particles from the margin of the eastern north pacific. *Deep Sea Res.,* 46, 655-679.

Andrews, J.E., Greenaway, A. M. and Dennis, P. F. (1998) Combined carbon isotope and C/N ratios as indicators of source and fate of organic matter in a poorly flushed, tropical estuary: Hunts bay, Kingston Harbour, Jamaica. *Est. Coast. Shelf Sci.,* 46, 743-756.

Aravena, R., Evans, M. L. and Cherry, J. A. (1993) Stable isotopes of oxygen and nitrogen in source identification of nitrate from septic systems. *Ground Water,* 31(2), 180-186.

Azevedo, P.A., Cho, C. Y., Leeson, S. and Bureau, D. P. (1998) Effects of feeding level and water temperature on growth, nutrient and energy utilization and waste outputs of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Living Resour.,* 11(4), 227-238.

Bender, M.M., Rouhani, I., Vines, H. M. and Black, C. C. (1973) $^{13}C/^{12}C$ ratio changes in crassulacean acid metabolism plants. *Plant Physiol.,* 52, 427-430.

Bernasconi, S.M., Barbieri, A. and Simona, M. (1997) Carbon and nitrogen isotope variations in sedimenting organic matter in Lake Lugano. *Limnol. Oceanogr.,* 42(8), 1755-1765.

Boaventura, R., Pedro, A. M., Coimbra, J. and Lencastre, E. (1997) Trout farm effluents: Characterization and impact on the receiving streams. *Environ. Pollut.,* 95(3), 379-387.

Boschker, H. T. S., de Brouwer, J. F. C. and Cappenberg, T. E. (1999) The contribution of macrophyte-derived organic matter to microbial biomass in

salt-marshes: stable carbon isotope analysis of microbial biomarkers. *Limnol. Oceanogr.,* 44(2): 303-319

Branstrator, D.K., Cabana, G., Mazumder, A. and Rasmussen, J. B. (2000) Measuring life-history omnivory in the opossum shrimp, *Mysis relicta*, with stable nitrogen isotopes. *Limnol. Oceanogr.,* 45(2), 463-467.

Bunn, S.E., Loneragan, N. R. and Kempster, M. A. (1995) Effects of acid washing on stable isotope ratios of C and N in penaeid shrimp and seagrass: Implications for food-web studies using multiple stable isotopes. *Limnol. Oceanogr.,* 40(3), 622-625.

Bureau, D. P. (2006) Feed issues in the aquaculture industry. Proceedings of the 27th Western Nutrition Conference: 181-196 Winnipeg, Manitoba 19-20 September 2006

Bureau, D.P. and Cho, C. Y. (1999) Phosphorus utilization by rainbow trout (*Oncorhynchus mykiss*): Estimation of dissolved phosphorous waste output. *Aquaculture,* 179, 127-140.

Bureau, D.P., Gunther, S. J. and Cho, C. Y. (2003) Chemical composition and preliminary theoretical estimates of waste outputs of rainbow trout reared in commercial cage culture operations in Ontario. *N. Am. J. Aquac.,* 65, 33-38.

Bureau, D. P. and Hua, K. (2006a) Mass balance and nutrient flows in freshwater aquaculture. Proceedings of the national freshwater aquaculture symposium-Aquaculture Canada 2004, Quebec City, QC, 17-20 October 2004 Aquaculture Association of Canada Special Publications No. 11, 98-102 pp

Bureau, D. P. and Hua, K. (2006b) Future directions in feed formulation for waste reduction. Proceedings of the Canadian Freshwater Aquaculture Symposium, Quebec City, QC, 17-20 October 2004 Aquaculture Association of Canada Special Publications No. 11, 48-52 pp

Burford, M.A., Preston, N. P., Glibert, P. M. and Dennison, W. C. (2002) Tracing the fate of ^{15}N-enriched feed in an intensive shrimp system. *Aquaculture,* 206, 199-216.

Canadian Council on Animal Care (2005). Guideliness on: the care and use of fish in research, teaching and testing. CCAC, Ottawa.

Cho, C.Y. and Bureau, D. P. (1998) Development of bioenergetic models and the fish-PrFEQ software to estimate production, feeding ration and waste output in aquaculture. *Aquat. Living Resour.,* 11(4), 199-210.

Cho, C. Y. and Bureau, D. P. (2001) A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. *Aquac. Res.,* 32((Suppl. 1)): 349-360

Cho, C.Y., Hynes, J. D., Wood, K. R. and Yoshida, H. K. (1994) Development of high-nutrient-dense, low-pollution diets and prediction of aquaculture wastes using biological approaches. *Aquaculture,* 124, 293-305.

Cole, R. (2002) Impacts of marine farming on wild fish populations. ENV2000/08, 1-51. National Institute of Water and Atmospheric Research. New Zealand.

Cornel, G.E. and Whoriskey, F. G. (1993) The effects of rainbow trout (*Oncorhynchus mykiss*) cage culture on the water quality, zooplankton, benthos and sediments of Lac du Passage, Quebec. *Aquaculture,* 109, 101-117.

Craig, C.R. (1957) Isotopic standards for carbon and oxygen and correction factors for mass spectrometric analysis of carbon dioxide. *Geochim. Cosmochim. Acta,* 12, 133-149.

Crawford, C., Macleod, C. and Mitchell, I. (2002) Evaluation of techniques for environmental monitoring of salmon farms in Tasmania. 8, 1-140. [In] Gardner, C. (ed) The Tasmanian Aquaculture and Fisheries Institute Report, University of Tasmania

Dempson, J.B. and Power, M. (2004) Use of stable isotopes to distinguish farmed from wild Atlantic salmon, S*almo salar*. *Ecol. Freshw. Fish,* 13, 176-184.

DeNiro, M.J. and Epstein, S. (1981) Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta,* 45, 341-351.

DeNiro, M.J. and Epstein, S. (1978) Influence of diet on the distribution of carbon isotopes in animals. *Geochim. Cosmochim. Acta,* 42, 495-506.

Feuchtmayr, H. and Grey, J. (2003) Effect of preparation and preservation procedures on carbon and nitrogen stable isotopes determinations from zooplankton. *Rapid Commun. Mass Spectrom*. 17: 2605-2610

Fisheries and Oceans Canada (2003) A scientific review of the potential environmental effects of aquaculture in aquatic ecosystems. volume I: Far-field environmental effects of marine finfish aquaculture (B.T. Hargrave); ecosystem level effects of marine bivalve aquaculture (P. Cranford, M. Dowd, J. grant, B. Hargrave and S.McGladdery); chemical use in marine finfish aquaculture in Canada: A review of current practices and posssible environmental effects (L.E. Burridge). *Can. Tech. Rep. Fish Aquat. Sci.,* 2450, 1-140.

Focken, U. and Becker, K. (1998) Metabolic fractionation of stable carbon isotopes: Implications of different proximate compositions for studies of the aquatic food webs using d13C data. *Oecologia,* 115:337-343.

Fry, B. (1991) Stable isotope diagrams of freshwater food webs. *Ecology,* 72(6), 2293-2297.

Fry, B. (2006) Stable Isotope Ecology. Springer Science and Business Media LLC. New York, NY 308 p.

Gannes, L.Z., O'Brien, D. M. and Martinez del Rio, C. (1997) Stable isotopes in animal ecology: Assumptions, caveats, and a call for more laboratory experiments. *Ecology,* 78(4), 1271-1276.

Gaye-Siessegger, J., Focken, U., Abel, H. and Becker, K. (2004a) Individual protein balance strongly influences $\delta^{15}N$ and $\delta^{13}C$ values in Nile tilapia, O*reochromis niloticus*. *Naturwissenschaften,* 91, 90-93.

Gaye-Siessegger, J., Focken, U., Abel, H. J. and Becker, K. (2003) Feeding level and diet quality influence trophic shift of C and N isotopes in Nile tilapia (O*reochromis niloticus* (L.)). *Isotopes Environ. Health Stud.,* 39(2), 125-134.

Gaye-Siessegger, J., Focken, U., Muetzel, S., Abel, H. and Becker, K. (2004b) Feeding level and individual metabolic rate affect $\delta^{13}C$ and $\delta^{15}N$ values in carp: Implications for food web studies. *Oecologia,* 138, 175-183.

Gowen, R.J. and Bradbury, N. B. (1987) The ecological impact of salmonid farming in coastal waters: A review. *Oceanogr. Mar. Biol. Annu. Rev.,* 25, 563-575.

Grey, J. (2000) Trophic fractionation and the effects of diet switch on the carbon stable isotopic 'signatures' of pelagic consumers. *Verh. Internat. Verein. Limnol.,* 27, 3187-3191.

Grey, J. and Jones, R. I. (1999) Carbon stable isotopes reveal complex trophic interactions in lake plankton. *Rapid Commun. Mass Spectrom.,* 13, 1311-1314.

Grey, J., Waldron, S. and Hutchinson, R. (2004) The utility of carbon and nitrogen isotope analyses to trace contributions from fish farms to the receiving communities of freshwater lakes: A pilot study in Esthwaite Water, UK. *Hydrobiologia,* 524, 253-262.

Grey, J., Jones, R. I. and Sleep, D. (2000) Stable isotope analysis of the origins of zooplankton carbon in lakes of different trophic state. *Oecologia,* 123, 232-240.

Gruau, G., Legeas, M., Riou, C., Gallacier, E., Martineau, F. and Henin, O. (2005) The oxygen isotope composition of dissolved anthropogenic phosphates: A new tool for eutrophication research? *Wat. Res.,* 39, 232-238.

Gu, B., Schelske, C. L. and Brenner, M. (1996a) Relationship between sediment and plankton isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) and primary productivity in Florida lakes. *Can. J. Fish. Aquat. Sci.,* 53, 875-883.

Gu, B., Schell, D. M., Huang, X. and Yie, F. (1996b) Stable isotope evidence for dietary overlap between two planktivorous fishes in aquaculture ponds *Can. J. Fish. Aquat. Sci.,* 53, 2814-2818.

Hamblin, P.F. and Gale, P. (2002) Water quality modeling of caged aquaculture impacts in Lake Wolsey, North Channel of Lake Huron. *J. Great Lakes Res.,* 28(1), 32-43.

Harvey, C.J. and Kitchell, J. F. (2000) A stable isotope evaluation of the structure and spatial heterogeneity of a Lake Superior food web. *Can. J. Fish. Aquat. Sci.,* 57, 1395-1403.

Heaton, T.H.E. (1986) Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: A review. *Chem. Geol.,* 59, 87-102.

Hebert, C.E. and Wassenaar, L. I. (2001) Stable nitrogen isotopes in waterfowl feathers reflect agricultural land use in western Canada. *Environ. Sci. Technol.,* 35(17), 3482-3487.

Herzka, S.Z. and Holt, G. J. (2000) Changes in isotopic composition of red drum (*Sciaenops ocellatus*) larvae in response to dietary shifts: Potential applications to settlement studies. *Can. J. Fish Aquat. Sci.,* 57, 137-147.

Hesslein, R.H., Hallard, K. A. and Ramlal, P. (1993) Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$. *Can. J. Aquat. Sci.,* 50, 2071-2076.

Hesslein, R.H., Capel, M. J. and Fox, D. E. (1988) Sulfur isotopes in sulfate in the inputs and outputs of a Canadian Shield watershed. *Biogeochem.,* 5, 263-273.

Hodell, D.A. and Schelske, C. L. (1998) Production, sedimentation and isotopic composition of organic matter in Lake Ontario. *Limnol. Oceanogr.,* 43(2), 200-214.

Holmer M., Marba N., Diaz-Almela E., Duarte C.M., Tsapakis M. and Danovaro R. (2007) Sedimentation of organic matter from fish farms in oligotrophic Mediterranean assessed through bulk and stable isotope ($\delta^{13}C$ and $\delta^{15}N$) analyses. *Aquaculture,* 262: 268-280

Holmes, E., Lavik, G., Fischer, G., Segl, M., Ruhland, G. and Wefer, G. (2002) Seasonal variability of $\delta^{15}N$ in sinking particles in the Benguela upwelling region. *Deep Sea Res. I,* 49, 377-394.

Jennings, S., Greenstreet, S. P. R., Hill, L., Piet, G. J., Pinnegar, J. K. and Warr, K. J. (2002) Long-term trends in the trophic structure of the north sea fish community: Evidence from stable-isotope analysis, size-spectra and community metrics *Mar. Biol.,* 141, 1085-1097.

Johannsson, O.E., Leggett, M. F., Rudstam, L. G., Servos, M. R., Mohammadian, M. A., Gal, G., Dermott, R. M. and Hesslein, R. H. (2001) Diet of M*ysis relicta* in Lake Ontario as revealed by stable isotope and gut content analysis. *Can. J. Fish. Aquat. Sci.,* 58, 1975-1986.

Johnsen, R.I., Grahl-Nielsen, O. and Lunestad, B. T. (1993) Environmental distribution of organic waste from a marine fish farm. *Aquaculture,* 118, 229-244.

Jones, A.B., O'Donohue, M. J., Udy, J. and Dennison, W. C. (2001) Assessing ecological impacts of shrimp and sewage effluent: Biological indicators with standard water quality analyses. *Est. Coast. Shelf Sci.,* 52, 91-109.

Kellman, L. and Hillaire-Marcel, C. (1998) Nitrate cycling in streams: using natural abundances of NO^{-3} -$\delta^{15}N$ to measure *in-situ* denitrification. *Biogeochem.,* 43: 273-292

Keough, J.R., Sierszen, M. E. and Hagley, C. A. (1996) Analysis of a Lake Superior coastal food web with stable isotope techniques. *Limnol. Oceanogr.,* 41(1), 136-146.

Kidd, K.A., Paterson, M. J., Hesslein, R. H., Muir, D. C. G. and Hecky, R. E. (1999) Effects of northern pike (E*sox lucius*) additions on pollutant accumulation and food web structure, as determined by $\delta^{13}C$ and $\delta^{15}N$, in a eutrophic and oligotrophic lake. *Can. J. Fish. Aquat. Sci.,* 56, 2193-2202.

Kline, T.C., Jr. and Willette, T. M. (2002) Pacific salmon (O*ncorhynchus* spp.) early marine feeding patterns based on $^{15N}/^{14}N$ and $^{13}C/^{12}C$ in Prince William Sound, Alaska. *Can. J. Fish. Aquat. Sci.,* 59, 1626-1638.

Kwak, T.J. and Zedler, J. B. (1997) Food web analysis of southern California coastal wetlands using multiple stable isotopes. *Oecologia,* 110, 262-277.

Lajtha, K. and Michener, R. H. (1994) *Stable isotopes in ecology and environmental science* London: Blackwell Scientific Publications.

Lefebvre, S., Bacher, C., Meuret, A. and Hussenot, J. (2001) Modelling nitrogen cycling in a mariculture ecosystem as a tool to evaluate its outflow. *Est. Coast. Shelf Sci.,* 52, 305-325.

Leggett, M.F., Johannsson, O., Hesslein, R., Dixon, D. G., Taylor, W. D. and Servos, M. R. (2000) Influence of inorganic nitrogen cycling on the $\delta^{15}N$ of

Lake Ontario biota. *Can. J. Fish. Aquat. Sci.,* 57, 1489-1496.

Leggett, M.F., Servos, M. R., Hesslein, R., Johannsson, O., Millard, E. S. and Dixon, D. G. (1999) Biogeochemical influences on the carbon isotope signatures of Lake Ontario biota. *Can. J. Fish. Aquat. Sci.,* 56, 2211-2218.

Lehmann, M.F., Bernasconi, S. M., Barbieri, A. and McKenzie, J. A. (2002) Preservation of organic matter and alteration of its carbon and nitrogen isotope composition during simulated and in situ early sedimentary diagenesis. *Geochim. Cosmochim. Acta,* 66(20), 3573-3584.

Lichtfouse, E. (2000) Compound-specific isotope analysis. application to archaelogy, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. *Rapid Commun. Mass Spectrom.,* 14, 1337-1344.

Limburg, K.E. (1998) Anomalous migrations of anadromous herrings revealed with natural chemical tracers. *Can. J. Fish. Aquat. Sci.,* 55, 431-437.

Lochmann, R. (2002) Fish yields and economic benefits of Tilapia/*clarias* polyculture in fertilized ponds receiving commercial feeds or pelleted agricultural by-products. 19 Annual Technical Report, 1-4.

Losos, G.J., McMillan, I., Minder, C. E. and Soulsby, K. (1982) Quantitative methods in the study of the pathogenesis of haemoprotozoal diseases. *Parasitology,* 84, 537-565.

Lumb, C.M. (1989) Self-pollution by Scottish salmon farms? *Mar Pollut. Bull.,* 20(8), 375-379.

Mac Dougall, N. and Black, K. D. (1999) Determining sediment properties around a marine cage farm using acoustic ground discrimination: RoxAnn^{TM}. *Aquacult. Res.,* 30, 451-458.

MacAvoy, S.E., Macko, S. A. and Garman, G. C. (2001) Isotopic turnover in aquatic predators: Quantifying the exploitation of migratory prey. *Can. J. Fish. Aquat. Sci.,* 58, 923-932.

Machás, R. and Santos, R. (1999) Sources of organic matter in Ria Formosa revealed by stable isotope analysis. *Acta Oecol.,* 20(4), 463-469.

Macko, S.A. and Ostrom, N. E. (1994) *Pollution studies using stable isotopes* 45-62 pp. [In] Lajtha, K. and Michener, R.H. (eds.) Stable Isotopes in Ecology and Environmental Science. London: Blackwell Scientific.

Macko, S.A., Fogel-Estep, M. L., Engel, M. H. and Hare, P. E. (1986) Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochim. Cosmochim. Acta,* 50, 2143-2146.

Maren, V. and Struck, U. (1997) Stable nitrogen and carbon stable isotopes as indicator of eutrophication of the Oder River (Baltic Sea). *Mar. Chem.,* 59, 35-49.

Mariotti, A. (1983) Atmospheric nitrogen is a reliable standard for natural ^{15}N abundance measurements. *Nature*, 303: 685-687

Mazzola, A. and Sarà, G. (2001) The effect of fish farming organic waste on food availability for bivalve molluscs (Gaeta gulf, central Thyrrenian, MED): Stable carbon isotopic analysis. *Aquaculture,* 192, 361-379.

McCarthy, I.D. and Waldron, S. (2000) Identifying migratory *Salmo trutta* using carbon and nitrogen stable isotope ratios. *Rapid Commun. Mass Spectrom.,* 14, 1325-1331.

McGhie, T.K., Crawford, C. M., Mitchell, I. M. and O'Brien, D. (2000) The degradation of fish-cage waste in sediments during fallowing. *Aquaculture,* 187, 351-366.

Meyers, P.A. and Eadie, B. J. (1993) Sources, degradation and recycling of organic matter associated with sinking particles in Lake Michigan. *Org. Geochem.,* 20(1), 47-56.

Minagawa, M. and Wada, E. (1984) Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. *Geochim. Cosmochim. Acta,* 48, 1135-1140.

Moccia, R.D. and Bevan, D. J. (2000) Aquaculture legislation in Ontario. AGDEX 485/872, 1-8. University of Guelph

Moccia, R.D. and Hynes, J. (1998) Sustainability of Ontario's aquaculture industry: 1998 & beyond. 98-001, 1-2. University of Guelph

Moccia, R.D., Naylor, S. and Reid, G. (1997) An overview of aquaculture in Ontario. 96-003, 1-4. University of Guelph

Mullin, M.M., Rau, G. H. and Eppley, R. W. (1984) Stable nitrogen isotopes in zooplankton: Some geographical and temporal variations in the north pacific. *Limnol. Oceanogr.,* 29(6), 1267-1273.

Nadelhoffer, K.J. and Fry, B. (1994) *Nitrogen isotope studies in forest ecosystems* London: Blackwell Scientific Publications.

Nakatsuka, T., Handa, N., Wada, E. and Wong, C. S. (1992) The dynamic changes of stable isotopic ratios of carbon and nitrogen in suspended and sedimented particulate organic matter during a phytoplankton bloom. *J. Mar. Res.,* 50, 267-296.

Naylor, S.J., Moccia, R. D. and Durant, G. M. (1999) The chemical composition of settleable solid fish waste (manure) from commercial rainbow trout farms in Ontario, Canada. *North Am. J. Aquac.,* 61, 21-26.

Nier, A.O. and Gulbransen, E. A. (1939) Variations in the relative abundance of the carbon isotopes. *J. Am. Chem. Soc.,* 61, 697-698.

Ostrom, N.E., Long, D. T., Bell, E. M. and Beals, T. (1998) The origin and cycling of particulate and sedimentary organic matter and nitrate in Lake Superior. *Chem. Geol.,* 152, 13-28.

Penczak, T., Galicka, W., Molinski, M., Kusto, E. and Zalewski, M. (1982) The enrichment of a mesotrophic lake by carbon, phosphorous and nitrogen from the cage aquaculture of rainbow trout, *Salmo gairdneri*. *J. Applied Ecol.,* 19, 371-393.

Peterson, B.J. and Fry, B. (1987) Stable isotopes in ecosystem studies. *Annu Rev Ecol Syst,* 18, 293-320.

Peterson, B.J. and Howarth, R. W. (1987) Sulfur, carbon, and nitrogen isotopes used to trace organic matter flow in the salt-marsh estuaries of Sapelo Island, Georgia. *Limnol. Oceanogr.,* 32(6), 1195-1213.

Peterson, B.J., Howarth, R. W. and Garritt, R. H. (1986) Sulfur and carbon isotopes as tracers of salt-marsh organic matter flow. *Ecology,* 67(4), 865-874.

Phillips, D.L. and Gregg, J. W. (2003) Source partitioning using stable isotopes: Coping with too many sources. *Oecologia,* 136, 261-269.

Philp, R.P. (2002) Application of stable isotopes and radioisotopes in environmental forensics. In *Introduction to Environmental Forensics* (B.L. Murphy and R.D. Morrison, eds) London: Academic Press.

Pinnegar, J.K. and Polunin, N. V. C. (1999) Differential fractionation of $\delta^{13}C$ and $\delta^{15}N$ among fish tissues: Implications for the study of trophic interactions. *Funct. Ecol.,* 13(2), 225-231.

Post, D. M. (2002) Using stable isotopes to estimate trophic position: models, methods and assumptions. *Ecology,* 83(3): 703-718

Power, M., Guiguer, K. R. R. A. and Barton, D. R. (2003) Effects of temperature on isotopic enrichment in *Daphnia magna*: implications for aquatic food-web studies. *Rapid Commun. Mass Spectrom.,* 17: 1619-1625

Preston, N.P., Smith, D. M., Kellaway, D. M. and Bunn, S. E. (1996) The use of enriched ^{15}N as an indicator of the assimilation of individual protein sources from compound diets for juvenile *Peneaus monodon*. *Aquaculture,* 147, 249-259.

Rogers, K.M. (2003) Stable carbon and nitrogen isotope signatures indicate recovery of marine biota from sewage pollution at Moa Point, New Zealand. *Mar. Pol. Bul.,* 46, 821-827.

Rounick, J.S. and Winterbourn, M. J. (1986) Stable carbon isotopes and carbon flow in ecosystems. *BioScience,* 36(3), 171-177.

Ruiz, J.M., Pérez, M. and Romero, J. (2001) Effects of fish farm loadings on seagrass (P*osidonia oceanica*) distribution, growth and photosynthesis. *Mar Pollut. Bull.,* 42(9), 749-760.

Rysgaard, S., Risgaard-Petersen, N., Nielsen, L. P. and Revsbech, N. P. (1993) Nitrification and denitrification in lake and estuarine sediments measured by the ^{15}N dilution technique and isotope pairing. *Appl. Environ. Microbiol.,* 59(7), 2093-2098.

Saino, T. and Hattori, A. (1987) Geographical variation of the water column distribution of suspended particulate organic nitrogen and its ^{15}N natural abundance in the pacific and its marginal seas. *Deep-Sea Research,* 34(5/6), 807-827.

Sakano, H., Fujiwara, E., Nohara, S. and Ueda, H. (2005) Estimation of nitrogen stable isotope turnover rate of *Oncorhynchus nerka*. *Env. Biol. Fish.,* 72, 13-18.

Sarà, G., Scilipoti, D., Mazzola, A. and Modica, A. (2004) Effects of fish farming waste to sedimentary and particulate organic matter in a southern Mediterranean area (Gulf of Castellamare, Sicily): A multiple stable isotope study ($\delta^{13}C$ and $\delta^{15}N$). *Aquaculture,* 234, 199-213.

Satterfield IV, F.R. and Finney, B. P. (2002) Stable isotope analysis of Pacific salmon: Insight into trophic status and oceanographic conditions over the last 30 years. *Prog Oceanogr,* 53, 231-246.

Schelske, C.L. and Hodell, D. A. (1991) Recent changes in productivity and climate of Lake Ontario detected by isotopic analysis of sediments. *Limnol. Oceanogr.,* 36(5), 961-975.

Schroeder, G.L. (1983) Stable isotope ratios as naturally occurring tracers in the aquaculture food web. *Aquaculture,* 30, 203-210.

Sierszen, M.E., Morrice, J. A., Moffett, M. F. and West, C. W. (2004) Benthic versus planktonic foundations of three Lake Superior coastal wetland food webs. *J. Great Lakes Res.,* 30(1), 31-43.

Statistics Canada (2006) Aquaculture statistics. *Catalogue no. 23-222-XIE,* , 46.

Sutherland, T.F., Martin, A. J. and Levings, C. D. (2001) Characterization of suspended particulate matter sorrounding a salmonid net-pen in the Broughton Archipielago, British Columbia. *ICES J. Mar. Sci.,* 58, 404-410.

Thornton, S.F. and McManus, J. (1994) Application of organic carbon and nitrogen stable isotope and C/N ratios as source indicators of organic matter provenance in estuarine systems: Evidence from the Tay estuary, Scotland. *Est. Coast. Shelf Sci.,* 38, 219-233.

Tominaga, O., Uno, N. and Seikai, T. (2003) Influence of diet shift from formulated feed to live mysids on the carbon and nitrogen stable isotope ratio ($\delta\ ^{13}C$ and $\delta\ ^{15}N$) in dorsal muscles of juvenile Japanese flounders, *Paralichtys olivaceus*. *Aquaculture,* 218, 265-276.

Tucker, J., Sheats, N., Giblin, A. E., Hopkinson, C. S. and Montoya, J. P. (1999) Using stable isotopes to trace sewage-derived material through Boston Harbor and Massachusetts Bay. *Mar. Env. Res.,* 48, 353-375.

Vander Zanden, M.J. and Rasmussen, J. B. (2001) Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: Implications for aquatic food web studies. *Limnol. Oceanogr.,* 46(8), 2061-2066.

Vizzini, S. and Mazzola, A. (2004) Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean. *Mar. Pol. Bul.,* 49, 61-70.

Voβ, M. and Struck, U. (1997) Stable nitrogen and carbon isotopes as indicator of eutrophication of the Oder river (Baltic sea). *Mar. Chem,* 59, 35-49.

Wada, E. and Hattori, A. (1976) Natural abundance of ^{15}N in particulate organic matter in the North Pacific Ocean. *Geochim. Cosmochim. Acta,* 40, 249-251.

Wong, W.W. and Sacket, W. M. (1978) Fractionation of stable carbon isotopes by marine phytoplankton. *Geochim. Cosmochim. Acta,* 42, 1809-1815.

Yamada, Y., Yokoyama, H., Ishihi, Y. and Azeta, M. (2003) Historical feeding analysis in fish farming based on carbon and nitrogen stable isotope ratio in sediment. *Fisheries Science,* 69, 213-215.

Ye, L.X., Ritz, D. A., Fenton, G. E. and Lewis, M. E. (1991) Tracing the influence on sediments of organic waste from a salmonid farm using stable isotope analysis. *J. Exp. Mar. Biol. Ecol.,* 145, 161-174.

Yeats, P.A. (2002) Trace metal tracers of fish farm wastes. 2411, 80-83. Environmental Studies for Sustainable Aquaculture (ESSA): 2002 workshop report. Can. Tech. Rep. Fish. Aquat. Sci.

Yoshii, K., Melnik, N. G., Timoshkin, O. A., Bondarenko, N. A., Anoshko, P. N., Yoshioka, T. and Wada, E. (1999) Stable isotope analyses of the pelagic food web in lake baikal. *Limnol. Oceanogr.,* 44(3), 502-511.

Yoshioka, T., Wada, E. and Hayashi, H. (1994) A stable isotope study on seasonal food web dynamics in a eutrophic lake. *Ecology,* 75(3), 835-846.

Appendices

Appendix A: Discriminant Analysis of the isotopic signatures of our materials and unpublished data from several locations in Georgian Bay, Lake Huron (data provided courtesy of Professor Tom Johnston, Ontario Ministry of Natural Resources).

Due to the amount of information available, the results are presented by location instead of as a whole for the entire Georgian Bay area. For convenience, the names of the locations and organisms sampled will be referred to by the codes used in Table 11. Of the seven locations tested, only the two with the fewest observations did not show any similarities in their signatures. In other locations where the number of samples and species (groups) analyzed was larger, the signatures were more similar. In every case, and dependently on the sample sizes and number of species included, the analysis was able to detect even minor variations in the signatures' ranges. Therefore, each group was reclassified, resulting in a reclassification of every material, including ours. The number of observations identified as misclassified represent percentages that reached almost 47% of the total number of observations analyzed (Table 12a). Most of the misclassifications happened among the samples of each one of Georgian Bay locations; there were very few misclassifications between those and our samples. Because the objective of the analysis was to differentiate our values from the Georgian Bay values, emphasis is placed on the observations

where the signatures of the Georgian Bay samples and those of our materials were similar (Table 12a, last column).

The sample types in each location which were most often misclassified are shown in Table 12b. In some cases, the sample size per group was only 3 or 4, which forced the similarity to reach very high percentages. Nevertheless, it was obvious that the groups shared very similar isotopic signatures. In some cases, the probability of posterior membership was as high as 80% or more, indicating a close affinity of the signatures, in some others the probability could be as low as 20%. The type of material also played an important role in the percentage of correct classifications.

Table 11. Coding of our data and of the locations and groups sampled by Professor Tom Johnston in Georgian Bay, Lake Huron in October 2002, June 2004 and October 2004. Numbers represent sample size.

	ORIGIN OF MATERIALS							
MATERIAL* (Group)	Our trial	Meldrum Bay (MB)	Hog Island (HI)	Bell Cove (BC)	Eagle Rock (ER)	Fisher Harbour (FH)	Eastern (EA)	Wabuno Channel (WB)
Muscle 1 (m1)	9							
Muscle 2 (m2)	9							
Muscle 3 (m3)	9							
Muscle commercial (mc)	3							
Feces 1 (f1)	9							
Feces 2 (f2)	6							
Feces 3 (f3)	9							
Feces commercial (fc)	3							
Feed diet 1 (d1)	6							
Feed diet 2 (d2)	6							
Feed diet 3 (d3)	6							
Feed commercial (dc)	3							
Cisco P (Cs1)								4
Cisco R (Cs2)								4
Crayfish P (Cy1)					6	4	9	10
Crayfish R (Cy2)			10	10		2	5	10
Ewos feed (EW)							3	
Hexagenia R (Hx1)				3				4
Hexagenia P (Hx2)							4	
Martin Mills feed (MT)							5	
Lake trout P (Lt1)					9	5		
Lake trout R (Lt2)					3	6		

Table 11 continued.

Rainbow smelt body P (Rs1)							2	7
Rainbow smelt body R (Rs2)		5	13					10
Rainbow smelt muscle R (Rs3)					4	4	4	
Rainbow smelt muscle P (Rs4)					4	4	4	
Rainbow trout muscle P (Rt1)					5	4	4	
Rainbow trout gut P (Rt2)						4	4	
Rainbow trout fin P (Rt3)						4	4	
Rainbow trout body P (Rt4)							5	5
Spottail shiner body P (Ss1)							5	5
Spottail shiner body R (Ss2)				5				5
Spottail shiner muscle R (Ss3)					4	4	4	
Spottail shiner muscle P (Ss4)					4	4	4	
Stickleback muscle P (Sb1)					4	4		
Stickleback muscle R (Sb2)					4	4		
Trout perch muscle P (Tp1)					3	8	4	4
Trout perch muscle R (Tp2)					4	7	4	4
Trout perch body R (Tp3)				6				
Yellow perch body P (Yp1)								10
Yellow perch body R (Yp2)								8
Yellow perch muscle R (Yp3)							4	
Yellow perch muscle P (Yp4)							4	
Walleye muscle P (WL1)					3	4		
Walleye muscle R (WL2)					3	1		
White sucker muscle P (Ws1)						4	4	
White sucker muscle R (Ws2)						3	3	
Zebra mussel viscera P (Zm1)							10	10
Zebra mussel viscera R (Zm2)			9	10	3			10
Totals	78	5	32	34	63	80	99	110

Table 12. Probability of posterior membership for all groups tested according to the discriminant analysis.

a) Percentage of misclassified observations (MO) between our samples (OD) and Georgian Bay locations (GBL)

GBL	Total observations (OD and GBL)	Total MO (OD and GBL)	%	GBL	MO GBL	%	OD vs. GBL MO	%
HI	110	15	13.64	32	1	3.13	1	0.91
BC	112	25	22.32	34	8	23.53	9	8.04
ER	141	49	34.75	63	27	42.86	17	12.06
FH	158	64	40.51	80	45	56.25	28	17.72
EA	177	83	46.89	99	62	62.63	44	24.86
WC	188	87	46.28	110	64	58.18	38	20.21

b) Groups with the largest probability of misclassification. Numbers expressed as %

Material (Group)	Bell Cove (BC)	Eagle Rock (ER)	Fisher Harbour (FH)	Eastern (EA)	Wabuno Channel (WB)
Cs1					50
Cs2					50
d1					33.3
EW				100*	
Fc	33.3*		66.6*		
m3					22.2
Mc					33.3*
MT				40	
Rt1				75	
Rt2			75	75	
Rt3			50	50	
Rt4				100	60
Rs4			50		
Ss1				100	60
Ss2	80				80
Ss3		50	50	50	
Ss4		50	25		
Tp1		66.6*	50	75	100
Tp2		50	42.85	75	100
Tp3	33.3				
Ws1				75	
Yp1					50
Yp2					50
Yp3				50	
Yp4				50	

* Less than 4 observations per group

Appendix B: Isotopic signatures of some commercial fish feeds and experimental diets with low or null percentages of marine ingredients.

The idea of using stable isotope signatures as tracers for aquaculture waste relies on the assumption that the feeds have distinct carbon and nitrogen ratios due to the marine ingredients. The experimental trials presented herein supported such an assumption. However, the trend in fish nutrition is to minimize or even eliminate fish meal in the feeds. It is possible then that the signatures of feeds elaborated with only terrestrial ingredients may loose their distinctiveness. In an attempt to determine whether such a loss of distinctiveness would occur, the signatures of a group of commercial diets were compared to experimental "vegetarian" formulas (Table 13). The results confirmed the suppositions mentioned above, where the $\delta^{15}N$ was significantly different between the samples belonging to diets with animal ingredients and those belonging to diets with vegetarian ones. It is important to mention that the formulas with animal ingredients included both marine and terrestrial sources and that the exact amount of each was not disclosed. This limited the strength of the comparisons because it was not possible to discriminate between terrestrial animal sources and marine or mixed ones. An interesting follow up could include a more exhaustive analysis of feeds with known amounts of animal sources.

Table 13. General composition and isotopic signatures of some commercial and experimental diets.

Brand	Group	Protein (%)		Lipids (%)		Fiber (%)	Signature	
		Marine, animal	Vegetable	Marine, animal	Vegetable		$\delta^{13}C$ ‰	$\delta^{15}N$ ‰
Martin Mills Mike's Farm	AN	45		22		1.5	-20.12	6.51
BioMar Ecolife 19	AN						-23.30	8.40
Unifeed	AN						-21.58	7.15
Skretting Orient LP	AN						-18.99	6.17
EWOS 7 pt	AN	43		14		2	-20.71	7.98
Corey Vigor Sinking 5 pt	AN	41		23		2.1	-21.14	7.92
Shur Gain signature 5 pt	AN	41		24		4	-19.84	3.63
Shurgain Retention*	VG	No	yes	no	yes	n/a	-20.32	4.04
Shurgain Retention*	VG	No	yes	no	yes	n/a	-19.65	2.47
Shurgain Retention*	VG	No	yes	no	yes	n/a	-22.78	4.30
Shurgain Retention*	VG	No	yes	no	yes	n/a	-20.26	4.01
Shurgain Retention*	AV	No	yes	yes	no	n/a	-20.33	4.24
Shurgain Retention*	AV	No	yes	yes	no	n/a	-19.36	2.17
Shurgain Retention*	AV	No	yes	yes	no	n/a	-21.35	4.19
Shurgain Retention*	AV	No	yes	yes	no	n/a	-22.85	4.26

* experimental diets, confidential composition. AN = animal; VG = vegetarian; AV = mixed: animal/vegetarian

Appendix C: Observations on the fecal isotopic signature shift when the fish fed the experimental diets were switched back to the commercial formulation.

Due to differential feeding behaviors, the fish on Diet 1 and Diet 3 were fed for a slightly longer period of time after the first 56 days until all the fish in each treatment consumed all the formulated feed. The fish fed each treatment diet consumed the same amount of feed during the trial. The fish were then switched back to the commercial formulation and extra samples of feces were collected at Day 73 for making empirical observations on the permanence of the experimental diet's isotopic signature after the switch.

Table 14. Isotopic signatures of feces after each treatment was switched back to commercial formulation

Diet	Initial signatures			Days*	Signatures after the switch to commercial diet		
	n	$\delta^{13}C$ ± (SD) ‰	$\delta^{15}N$ ± (SD) ‰		n	$\delta^{13}C$ ± (SD) ‰	$\delta^{15}N$ ± (SD) ‰
1	9	-21.65 (0.21)^{a}	9.27 (0.50)^{e}	13	3	-21.68 (0.42)^{k}	10.3 (0.26)^{m}
2	6	-20.98 (0.19)^{b}	8.08 (1.08)^{f}	17	3	-21.97 (0.16)^{k,l}	11.28 (0.72)^{m,o}
3	9	-20.50 (0.27)^{c}	6.65 (0.59)^{g}	6	3	-21.92 (0.04)^{k,l}	11.52 (1.21)^{m,o}
Com	3	-23.22 (0.16)^{d}	8.74 (0.50)^{e,f}		3	-23.22 (0.16)	8.74 (0.50)

Com= commercial; * number of days that the fish from each treatment fed on the commercial diet. Values in a same column or row sharing the same superscript are not significantly different (P>0.05).

Switching the diets of the fish in each treatment back to the commercial formulation at the end of the trial allowed making observations on how fast the signatures in the feces change. The feces produced by the fish after the switch

showed no statistical difference ($P<0.05$; n= 12), regardless of the treatment the fish were fed before or the time the fish were fed the commercial formulation (Table 14).

The speed at which the signatures changed is evidence that a shift in the isotopic signature of the diet shows up in the feces after only a few days, which may interfere with the principle of stability that a signature needs to have to be considered a tracer. Nonetheless, the new signatures still exhibit isotopic ratios characteristic of, and related to, the fish feeds and their ingredients.

A statistically significant change in fecal $\delta^{15}N$ was observed when the fish were switched from treatment to commercial diet, regardless of the amount of time the fish were fed the commercial diet (Table 14). Curiously, the group that exhibited the largest enrichment was the one which was fed the commercial formulation for the shortest period, which happened to be the group that exhibited the smallest $\delta^{15}N$ values when fed the treatment diet (Diet 3). In contrast, the group which fed on the feed with the highest $\delta^{15}N$ signature (Diet 1) was the one that showed the smallest enrichment in the fecal $\delta^{15}N$ after being fed the commercial diet for an intermediate period (Table 14). It appears then, that the fecal $\delta^{15}N$ after the switch were more related to the isotopic ratio of the original treatment diet than to time being fed the commercial diet. The group originally fed Diet 2, which consumed the commercial diet for the longest period (Table 14), produced feces with $\delta^{15}N$ values well within the values of the fish fed Diets 1 and 3.

The feces produced after the switch in all treatments seemed to reach a common $\delta^{15}N$ signature with no statistical differences between them regardless of the time and original treatment. It looks as though some additive effect happened since the isotopic values of the fecal matter after the diet switch in all treatment fish were statistically higher than the initial fecal values (Table 14).

In the same manner, it appeared that the $\delta^{13}C$ in the feces produced after the switch also reflected a common $\delta^{13}C$ signature with no statistical difference between treatments regardless of the time since the switch (Table 14). The $\delta^{13}C$ of the feces produced after the diet switches were more related to the original $\delta^{13}C$ of the commercial feed (means: -21.85‰ for feces of all treatments vs. -21.08‰ from the commercial feed). This could also explain the lack of statistical differences between the $\delta^{13}C$ of the feces from diet 1 and the feces after the switch, considering that the mean value of diet 1 feces was very similar (Table 14). However, although the $\delta^{13}C$ in each of the treatment diets reached that common signature, they were still far from the ratios of the feces taken from fish fed exclusively on the commercial formulation. This could also be the result of an excessive fractionation of the commercial sample since it was taken from a tank where the feces were sitting for periods longer than 24 hours.
Although the previous assumptions are mere observations with little or no scientific value due to a lack of a formal scientific design it is important to mention them as facts to consider for further studies.