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Metabolomic profiles of the endangered St. Lawrence Estuary beluga population and associations with organohalogen contaminants

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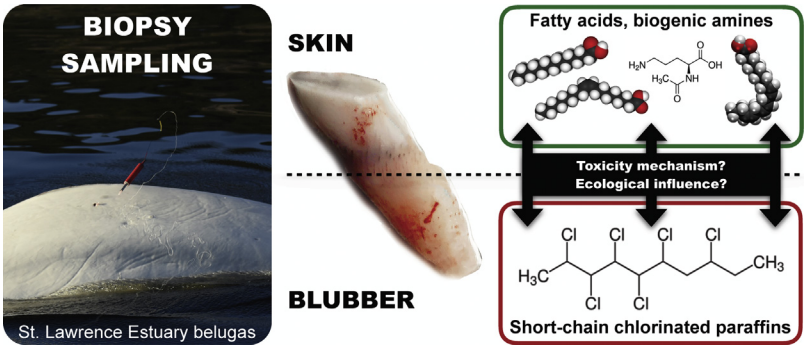
HIGHLIGHTS

- Metabolomic profiling was performed in endangered St. Lawrence Estuary belugas.
- Belugas showed different metabolite levels depending on their foraging habitat use.
- Diet and hydrographic variables may have influenced metabolomic profiles in belugas.
- SCCP concentrations correlated with those of five fatty acids and acetylornithine.

GRAPHICAL ABSTRACT



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ABSTRACT

The endangered beluga (*Delphinapterus leucas*) population residing in the St. Lawrence Estuary (SLE; Eastern Canada) is declining. The elevated tissue concentrations of a wide range of organohalogen contaminants might play a role in the non-recovery of this whale population. Organohalogens have been reported to impair the regulation of several metabolic products from cellular reactions in mammals such as amino acids and fatty acids. The objective of this study was to investigate a suite of organohalogens including polychlorinated biphenyls, organochlorine pesticides, short-chain chlorinated paraffins (SCCPs), polybrominated diphenyl ethers, and selected emerging flame retardants in blubber (biopsy) collected from 40 SLE male belugas, and their relationships to skin concentrations of targeted metabolites (i.e., 21 amino acids, 22 biogenic amines, 18 fatty acids, and 17 energy metabolites). A cluster analysis based on metabolomic profiles distinguished two main subgroups of belugas in the upper and lower sector of their summer habitat in the SLE. These results indicate that ecological factors such as local prey availability and diet composition played a role in shaping the metabolite profiles of belugas. Moreover, SCCP concentrations in SLE male belugas correlated negatively with those of four unsaturated fatty acids (C16:1ω7, C22:5ω3c1, C22:5ω3c2, and C22:6ω3), and positively with those of acetylornithine (biogenic amine). These findings suggest that biological functions such as lipid metabolism represent potential targets for organohalogens in this population, and further our understanding on potential health risks associated with elevated organohalogen exposure in cetaceans. Our results also underscore the necessity of considering ecological factors (e.g., diet and habitat use) in metabolomic studies.

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1. Introduction

The St. Lawrence Estuary (SLE; Eastern Canada) is known to be an important feeding ground for many marine mammal species, including a small resident population of belugas (*Delphinapterus leucas*) (Lesage et al., 2007; Mosnier et al., 2010). A decline of approximately 1% per year has been documented in this population from the early 2000s to 2012 at a time when it was estimated at 889 individuals (Mosnier et al., 2015). This declining trend has likely continued since this last census given the abnormally elevated number of calves found dead since 2008 (DFO, 2017). As a result, the status of this population was changed from threatened to endangered in 2014 by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2014), a status echoed under the Species at Risk Act in 2017 (DFO, 2017). Contaminant exposure, noise pollution, disturbance from recreational and whale-watching vessels, toxic algal blooms, food scarcity, and climate warming have been identified as factors potentially contributing to the non-recovery of this population (DFO, 2014). The SLE is part of the St. Lawrence Seaway and is located downstream of the Laurentian Great Lakes and St. Lawrence River in Canada and the USA where several large cities and agricultural regions are found, thus chronically exposing SLE belugas to multiple organic and inorganic contaminants that accumulate in their tissues.

Since the 1980s, elevated concentrations of polychlorinated biphenyls (PCBs) and organochlorine (OC) pesticides and industrial by-products have been reported in the blubber of SLE belugas (Lebeuf et al., 2014; Sergeant, 1980). However, since their ban in Canada more than four decades ago, PCB and OC pesticide concentrations have declined markedly in SLE beluga blubber (Lebeuf et al., 2014). In contrast, levels of a widely used halogenated flame retardant (HFR) class, the polybrominated diphenyl ethers (PBDEs), increased exponentially during this period in SLE beluga blubber, reaching a peak in the mid-1990s (Lebeuf et al., 2014; Simond et al., 2017). PBDE levels have remained stable in blubber of this population up until the last assessment that included samples from 2013 (Simond et al., 2017). The addition of PBDE commercial mixtures (i.e., Penta-, Octa-, and Deca-BDE) to the Annex A of the Stockholm Convention on Persistent Organic Pollutants (POPs) in 2009 and 2017 (Stockholm Convention, 2009, 2017) led to market usage of alternative chemicals known as emerging HFRs (Covaci et al., 2011). Several of these chemicals (e.g., chlordene plus, dechlorane-602 and -604 Component B, and hexabromobenzene) were recently quantified in the blubber of stranded SLE beluga carcasses (Simond et al., 2017).

Despite the marked temporal decline of PCB and OC pesticide concentrations in SLE beluga blubber, concentrations of these legacy organohalogens remain several-fold greater than PBDEs or any other HFRs (Simond et al., 2019). Other chemicals such as short-chain chlorinated paraffins (SCCPs) were quantified in SLE beluga blubber at concentrations 100-fold lower than PCBs in the late 1980s (Tomy et al., 2000). SCCPs are complex mixtures of alkanes of varying chain length (i.e., C_{10} to C_{13}) and chlorine content (30–70% by mass) that have been used mainly as lubricant, metal-cutting fluid, plasticizer, and flame retardant (Feo et al., 2009). SCCPs are listed as toxic substances under the Canadian Environmental Protection Act, and their manufacture, use, sale or import are prohibited in Canada since 2012 (Canada Gazette, 2013). SCCPs are also listed under Annex A of the Stockholm Convention on POPs since 2017 (Stockholm Convention, 2017). These compounds have decreased significantly in lake trout (whole homogenate) from lake Ontario in the Laurentian Great Lakes between 2001 and 2011 (Saborido Basconcillo et al., 2015). However, SCCP trends in belugas or any other species from the SLE have not been investigated.

Several of the organohalogens reported in SLE beluga blubber (i.e., PCBs, OC pesticides, PBDEs, emerging HFRs, and SCCPs) may represent a threat to their health as many of these chemicals are known to disrupt the regulation of thyroid and steroid axes in human, rodents, and marine mammals (e.g., Liu et al., 2016; Okoro et al., 2017; Reijnders et al., 2009; Zhang et al., 2016). As such, plasma concentrations of PCBs, PBDEs and OC pesticides have been correlated with several endocrine variables (i.e., plasma steroid and thyroid hormone levels and/or liver thyroid-related gene transcripts) in Fennoscandian and Norwegian Arctic ringed seals (*Phoca hipsida*) (Routti et al., 2010) and Norwegian Arctic polar bears (*Ursus maritimus*) (Ciesielski et al., 2017). Recently, concentrations of PCBs, OC pesticides and emerging HFRs in SLE male beluga blubber (biopsy) were shown to correlate with skin transcript levels of genes coding for nuclear receptors and proteins involved in the regulation of thyroid (*Dio2*) and steroid hormones (*Esrα*, *Hsd11β2*, and *Nr3c1*) as well as the metabolism of xenobiotics (*Ahr*) (Simond et al., 2019). Consistent findings have been reported in studies of transient and northern resident killer whales (*Orcinus orca*) from British Columbia (Canada) and belugas from the Canadian Arctic, which showed correlations between PCB concentrations and transcripts of genes involved in the regulation of thyroid and estrogen axes as well as xenobiotic responses (Buckman et al., 2011; Noël et al., 2014).

Several studies suggest that metabolomic profiles in mammals may be impacted by exposure to organohalogen contaminants. For instance, correlations between blubber concentrations of PCBs, OC pesticides and PBDEs, and lipid metabolism-related compounds (e.g., phosphatidylcholines and arachidonic acid) were reported in two Canadian Arctic polar bear sub-populations (Morris et al., 2019). Similarly, serum levels of amino acids involved in energy, lipid, amino acid or immune pathways were altered in human exposed to PCBs and mice dosed with BDE-209 (~97% of Deca-BDE mixture) (Eguchi et al., 2016, 2017). Additionally, in vitro studies in rodent have shown that SCCP dosage induced liver damage and fatty acid degradation, and affected several lipid metabolic pathways (Geng et al., 2015; Wang et al., 2017; Wyatt et al., 1993). Lipids play an essential role in energy storage, buoyancy and temperature regulation in cetaceans, while fatty acids are involved in the regulation of multiple biological processes including membrane structure and functions, intracellular signaling pathways, gene expression, and production of bioactive lipid mediators (Calder, 2015). Therefore, whale populations that are highly exposed to organohalogens might experience lipid metabolism disorders that may, in turn, impact energetic metabolism and ultimately the animal's health and reproductive success (Iverson and Koopman, 2018).

Investigating the mechanisms of toxicity associated with environmental contaminant exposure in free-ranging cetaceans is challenging as it requires access to fresh tissue via biopsy sampling. These samples are generally limited in size (<1 g per biopsy) and number of individuals they can be collected from. As a result, a growing number of studies investigating contaminant exposure-related effects in cetaceans rely on "omics" methods (Mancia, 2018). These approaches require small amounts of tissue and are amenable to multiple analyses, yielding a maximum of biological information to further our understanding on the mechanisms of toxicity and related metabolic pathways (Godard-Codding and Fossi, 2018). As such, metabolomic profiling allows for screening a large suite of low-molecular weight (<1000 Da) metabolites (e.g., amino acids, fatty acids, amines, and sugars) produced by cells that are specific to certain metabolic pathways (Burgess et al., 2014). Given that diet is the main uptake pathway for contaminants in marine mammals and that it can also influence concentrations of certain metabolites such as amino acids and fatty acids (Arab, 2003; Poesen et al., 2015), feeding ecology must therefore be considered when assessing metabolomic profiles. The summer habitat of SLE belugas is highly heterogeneous in terms of bathymetry, salinity, and temperature (Therriault et al., 1990). These characteristics may influence the structure of prey communities including their relative abundance and diversity. This is expected to influence metabolomic and contaminant profiles of SLE belugas depending on their prey selection, degree of dietary specialization, and preferred habitat for foraging.

The objective of this study was to examine the potential mechanisms of toxicity and metabolic disruption in the highly organohalogen-

exposed SLE beluga population. A targeted metabolomic approach was used to characterize profiles of amino acids, biogenic amines, fatty acids, sugars and energy metabolites in skin of SLE beluga males, and to examine their relationships with blubber concentrations of major organohalogens (PCBs, OC pesticides, PBDEs, emerging HFRs, and SCCPs), while accounting for ecological factors such as habitat use.

2. Materials and methods

2.1. Field sampling

Given the known effect of sex on plasma biochemistry (e.g., triglyceride, glucose, and cholesterol levels) and organohalogen concentrations in beluga tissues (Lebeuf et al., 2014; Norman et al., 2013; St. Aubin et al., 2001), only male SLE belugas were included in this study. A total of 40 males were biopsied (skin and blubber) in September 2015 and 2016 using sharpened 8 × 25 or 8 × 35 mm stainless steel tips pre-cleaned with acetone, 95% ethanol and Virkon, and fired from a MK24C Paxarms dart projector with 0.22 caliber blank charges (Domett, New Zealand). Epidermis and connective tissues were generally predominant relative to blubber in biopsies due to skin thickness and occasional oblique hit angle of the dart; "blubber" therefore refers hereafter to the hypodermis and the intermediate fibrous layer between the epidermis and blubber (i.e., dermis). Immediately after sampling, blubber was separated from the epidermis using disposable DNAse/RNAse-free scalpels and forceps pre-cleaned with acetone and 70% ethanol, wrapped in solvent-rinsed aluminum foil, flash-frozen in liquid nitrogen, and stored in a Cryovial at −80 °C in the laboratory until chemical analysis (Section 2.2). The epidermis was flash-frozen in liquid nitrogen in a Cryovial and stored at −80 °C in the laboratory until sexing according to published methods (Simond et al., 2019) as well as metabolomic analysis (Section 2.3). The GPS coordinates of the biopsy sampling location were recorded for each beluga. Age of the animals was not determined.

This study was conducted under permits granted by Parks Canada (SAGMP-2013-14734) and Fisheries and Oceans Canada (IML-2015-13 and IML-2016-021). Sampling methods were approved by the animal care committee of Fisheries and Oceans Canada, which is accredited by the Canadian Council on Animal Care (Ottawa, ON, Canada).

2.2. Chemical analysis

Blubber samples were analyzed for 41 PCBs, 23 OC pesticides and industrial by-products, 35 PBDEs, 11 emerging HFRs, and 24 C_{10} to C_{13} SCCPs (Tables S1 to S5). Sample extraction and clean-up were performed according to methods described by Simond et al. (2017) without modification. Briefly, blubber samples (30–100 mg) were homogenized with diatomaceous earth and spiked with 100 μL of a 200 ng/mL internal standard solution (BDE-30, BDE-156, ^{13}C-BDE-209, and ^{13}C-*anti*-DP). The total extractable lipid content in samples was determined gravimetrically. Identification and quantification of PBDEs and emerging HFRs were performed using a gas chromatograph (GC) coupled to a single quadrupole mass spectrometer (MS) (Agilent Technologies 5975C Series, Palo Alto, CA, USA) operating in electron capture negative ionization mode (GC/MS-ECNI).

Extracted blubber fractions generated for PBDE/HFR analysis (^{1}st aliquot) were spiked with performance standards consisting of neutral PCBs (CB-9, -136, and -204) and polycyclic aromatic hydrocarbons (naphtalene-d8, phenanthrene-d10, and chrysene-d12), and reanalyzed for PCBs and OC pesticides by AGAT Laboratories (Montreal, QC, Canada). Identification and quantification of PCBs were performed using a 6890N GC coupled to an 5975B Inert mass selective detector (GC/MSD) (Agilent Technologies) operating in selected ion monitoring mode (SIM), while for OC pesticides this was performed using a 7890A GC coupled to a 5975C MSD (Agilent Technologies) operating in SIM mode. The analytical column (30 m × 0.25 mm × 0.25 μm) was a fused silica RXI-5SIL MS capillary column (Restek Corporation, Bellefonte, PA, USA).

Blubber extracts generated for PBDE/HFR analysis (^{2}nd aliquot) were also analyzed for C_{10}-C_{13} SCCPs by the National Laboratory for Environmental Testing, Environment and Climate Change Canada (Burlington, ON, Canada). Analytical methods were adapted from Tomy et al. (1997), and quantification was performed using a Q Exactive™ GC Orbitrap™ GC–MS/MS system (Thermo Fisher Scientific, Mississauga, ON, Canada) with a TraceGOLD TG-5SilMS GC column (30 m × 0.25 mm × 0.25 μm; Thermo Fisher Scientific). The instrument operated in negative chemical ionization mode at a mass resolution of 60,000. A total of 24 SCCP mass-to-charge ratio (*m*/*z*) M-CL ions were extracted from total ion chromatograms (Table S5). Specific *m*/*z* values corresponding to the molecular formulas of $[M - Cl]^-$ ions of all major C_{10}-C_{13} formula groups were monitored concurrently. Corrections were made for the fractional abundance of specific *m*/*z* values and number of Cl atoms. Quantification was performed by comparing the response of specific *m*/*z* values in the sample to that of an authentic standard, which was a C_{10}-C_{13} technical mixture containing 55.5% Cl (DRE-X23105500CY, LGC Standards, Augsburg, Germany).

Quality control and assurance procedures included analysis of procedural method blanks, duplicate blubber samples and standard reference material (SRM; NIST 1945 Whale Blubber, Gaithersburg, MD, USA) for each batch of ten samples. Internal standards (BDE-30, BDE-156, ^{13}C-BDE-209, and ^{13}C-*anti*-DP) were used for PBDE/HFR quantification, and thus all analytes were inherently recovery-corrected. Concentrations of PCBs and OC pesticides were corrected using recovery percentages of the internal standard ^{13}C-*anti*-DP. Internal and performance standard recoveries can be found in Table S6. The percentages of variation from certified concentrations in SRM are available in Table S7. Information on methods limits of detection (MLODs) and quantification (MLOQs) for SCCP, PCB/OC and PBDE/HFR compounds can be found in Supplementary Materials. Blank contribution in samples for SCCPs required a blank correction for all congeners. All contaminant concentrations are reported in ng/g wet weight (ww). Lipid-corrected (lw) concentrations are listed in Table S8.

2.3. Metabolomic analysis

A total of 21 amino acids, 22 biogenic amines, total hexose, 18 fatty acids (FAs), and 17 energy metabolites were analyzed in skin (Table S9) by SGS AXYS (Sidney, BC, Canada) following a targeted metabolomic approach described in Benskin et al. (2014) without modification. Briefly, metabolites were extracted from skin samples (~200 mg) ground in methanol using a bead blender. Amino acids and biogenic amines were derivatized prior to analysis. All metabolites were quantified using a Agilent 1100 high performance liquid chromatography (HPLC) system coupled to an API4000 triple quadrupole MS (Applied Biosystems/Sciex, Concord, ON, Canada). Quantification was made by isotope dilution using authentic standards and identical (or homologous) isotopically-labeled internal standards (Table S9), and a quadratic metabolite-specific calibration curve. Specifically, at the start of a MS analysis, eight calibration samples containing internal and authentic standards were run. Ratios of the authentic standard peak areas and the surrogate peak areas were used to create a calibration curve specific to each metabolite. MLODs and MLOQs were based on the calibration samples containing the lowest metabolite concentrations, and were specific to both the metabolite and the sample itself.

Quality control and assurance procedures comprised triplicate analyses of procedural method blanks and SRM (*Oncorhynchus nerka* liver; SC6106). Recovery percentages of internal standard in the SRM varied between 88% and 105% for biogenic amines and amino acids (acetylornithine, alanine, glutamine, glutamic acid, glycine, histidine, methionine, serine, and taurine), between 77% and 108% for fatty acids (C20:4ω6, C20:5ω3 and C22:6ω3), and between 29% and 84% for energy metabolites (hexose-phosphate and succinic acid). Metabolites

that were detected in one or more blank samples at concentrations >30% of the mean sample concentration (i.e., FA C16:0, FA C18:2, spermine, phosphoenolpyruvate, and kynurenine) or that showed >30% variation in concentrations between triplicates in the SRM (i.e., succinic acid, hexose phosphate, tetrose-phosphate, spermidine, and cAMP) were excluded from further analyses. Analytes that could not be quantified reliably based on the calibration curve from the standards were also excluded from further analyses (i.e., hexose-phosphate and lactic acid). The final dataset therefore included 46 metabolites (21 amino acids, eight biogenic amines, total hexose, 10 fatty acids, and six energy metabolites), which were all blank-corrected (Table S10).

2.4. Statistical analysis

Because the distribution of most variables did not meet the assumption of normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test) even after log-transformation, non-parametric tests were used unless stated otherwise. Only organohalogen compounds and congeners for which at least 65% of the SLE beluga samples had concentrations above the MLOQs (PBDEs/HFRs) or MLODs (SCCPs and PCBs/OC pesticides) were included in statistical analyses (Tables S1 to S5, and S10). Concentrations of Σ_{18}SCCP, the six major PBDE and PCB congeners and all emerging HFRs and OC pesticides that were detected in ≥65% of SLE male beluga blubber samples were used for comparisons between SLE beluga groups (see below). In addition to the 46 metabolites, the ratios of omega-3 to omega-6 fatty acids (ω3:ω6) were also compared between individuals as it can be indicative of health and/or dietary changes (Käkelä and Hyvärinen, 1998; Simopoulos, 2016). The statistical effect of sampling year was not taken into account given the low annual sample size and consistency in sampling period (September) between the two consecutive sampling years.

A series of multivariate analyses using log-transformed metabolite concentrations were used to examine similarity patterns among SLE male belugas. A principal component analysis (PCA) and a hierarchical cluster analysis on principal components (HCPC) were performed using the package *FactoMineR* (Lê et al., 2008). HCPC is an unsupervised classification method that emphasizes the similarities between individuals. A hierarchical tree was generated using Euclidian distances, and the optimal number of clusters was determined based on the highest relative loss of inertia ($i_{(\text{cluster n})} - i_{(\text{cluster n+1})}$). Differences in organohalogen and metabolite concentrations between clusters were examined only for the two clusters (groups) showing the highest number of individuals using a Mann-Whitney-Wilcoxon *U* test. Volcano plots were then used to highlight variables that significantly differed (i.e., fold-change > 1.5; $p \le .05$) between these two main clusters.

The strength of the relationships between contaminant and metabolite concentrations was also examined using Spearman's rank correlation. To limit the number of simultaneous hypothesis testing and family-wise error rate, organohalogens were grouped into three chemical classes for this specific analysis: Σ_{34}HFR (sum of PBDEs, HBB, Dec-604 CB, DP, and PBEB), Σ_{18}SCCP and Σ_{41}PCB/OC (sum of PCBs, HCB, *p,p*′-DDE, and *trans*-nonachlor).

To reduce risks of Type I errors, *p*-values were adjusted using the classical one-stage false discovery rate (FDR) method following Pike (2011) and using a FDR of 0.05. Raw *p*-values were considered significant only if they remained significant after FDR adjustment. Adjusted *p*-values were referred to as *q*-values to avoid confusion with raw *p*-values. All statistical analyses were carried out using R version 3.2 (R Core Team, Vienna, Austria) and a level of significance set to $\alpha = 0.05$.

3. Results

3.1. Metabolite concentrations in SLE male belugas

Among all metabolite classes determined in SLE male beluga skin, total hexose was the most abundant (41 ± 1.9%; mean ± SEM), followed by amino acids (34 ± 0.7%), biogenic amines (13 ± 0.3%), fatty acids (10 ± 0.7%), and energy metabolites (2.5 ± 0.1%). The five individual metabolites that had the greatest concentrations, accounting for 67% of the sum of all metabolites, were in decreasing order: total hexose (3963 ± 182 μg/g ww; mean ± SEM), taurine (1078 ± 26.0 μg/g ww), alanine (533 ± 13.2 μg/g ww), valine (447 ± 11.9 μg/g ww), and oleic acid (418 ± 34.2 μg/g ww).

The HPCP analysis based on all metabolite concentrations determined in SLE male beluga skin yielded three main clusters (Fig. 1). Mapping biopsy sampling locations revealed that male belugas categorized in cluster 1 were sampled mainly downstream of the Saguenay River in the Lower Estuary (referred hereafter to "downstream belugas"; $n = 17$). Belugas in cluster 2 were sampled mainly in the sector of the SLE located just off the Saguenay River mouth ("intermediary belugas"; $n = 5$), while those in cluster 3 were sampled mainly in the Upper Estuary upstream of the Saguenay River, or in or off the Saguenay River mouth ("upstream belugas"; $n = 18$) (Fig. 2).

Each SLE male beluga cluster generated through HPCP analysis was characterized by concentrations of certain metabolites that differed significantly from the overall mean (Table S11). Specifically, male belugas sampled downstream of the Saguenay River generally exhibited greater concentrations of fatty acids (except stearic acid), whereas those sampled in the upstream sector had lower concentrations of these same fatty acids. In contrast, male belugas in the intermediary sector exhibited distinct amino acid profiles, with lower concentrations of nine amino acids. Given that the intermediary group of male belugas was constituted of only five individuals, this group was not used in subsequent statistical analyses.

Beluga males sampled in the sectors of the SLE located upstream and downstream of the Saguenay River mouth differed in concentrations of several metabolites (Fig. 3A). With the exception of stearic (C18:0) and osbond acids (C22:5ω6), concentrations of fatty acids (1.7- to 3.4-fold) and the two energy metabolites α-ketoglutaric and oxaloacetic acids (1.6-fold) were significantly greater ($252 \le U \le 306$; $p < .001$) in male belugas sampled downstream compared to upstream of the Saguenay River mouth. In contrast, concentrations of the biogenic amine acetylornithine (Ac-Orn) were 1.8-fold greater in upstream belugas ($U = 12$; $p < .001$). The ω3:ω6 fatty acid ratios did not differ between the upstream and downstream male belugas ($p = .48$).

3.2. Organohalogens in blubber of SLE male belugas

Blubber concentrations of Σ_{18}SCCP and major PCB/OC pesticide and HFR compounds are listed in Table 1. Σ_{18}SCCP represented 74 ± 15% (mean ± SEM) of the sum concentrations of all organohalogens, followed by Σ_{41}PCB/OC (24 ± 3.1%) and Σ_{34}HFR (1.7 ± 0.1%). A total of 18 SCCP congeners were detected in all but two individuals. $\Sigma_5C_{11}Cl_{5-9}$ (60 ± 1.9%) contributed largely to Σ_{18}SCCP concentrations, followed by $\Sigma_5C_{12}Cl_{5-9}$ (18 ± 1.0%), $\Sigma_5C_{10}Cl_{5-9}$ (13 ± 2.0%) and $\Sigma_3C_{13}Cl_{5-7}$ (2.8 ± 2.9%). The five most abundant SCCP congeners were in decreasing order: $C_{11}Cl_6$ (1372 ± 334 ng/g ww; mean ± SEM), $C_{11}Cl_5$ (1049 ± 165 ng/g ww), $C_{11}Cl_7$ (1022 ± 428 ng/g ww), $C_{10}Cl_6$ (824 ± 224 ng/g ww), and $C_{10}Cl_7$ (660 ± 173 ng/g ww).

Concentrations of PCBs, OC pesticides and industrial by-products, PBDEs and emerging HFRs have been reported in a companion investigation that included the majority of the present SLE male beluga samples (Simond et al., 2019), and thus will not be described in details here. Briefly, concentrations of Σ_{37}PCB accounted for the greatest proportions (64 ± 7.6%; mean ± SEM) of Σ_{41}PCB/OC, followed by *p,p*′-DDE (27 ± 4.1%), *trans*-nonachlor (5.5 ± 0.7%), and HCB (1.2 ± 0.1%). While Σ_{29}PBDE concentrations accounted for 97% of all HFRs, PBDE concentrations were 14- and 43-fold lower than Σ_{37}PCB and Σ_{18}SCCP, respectively. The most abundant emerging HFR was ΣDP (sum of *syn*- and *anti*-DP), followed in decreasing order by Dec-604 CB, HBB, and PBEB.

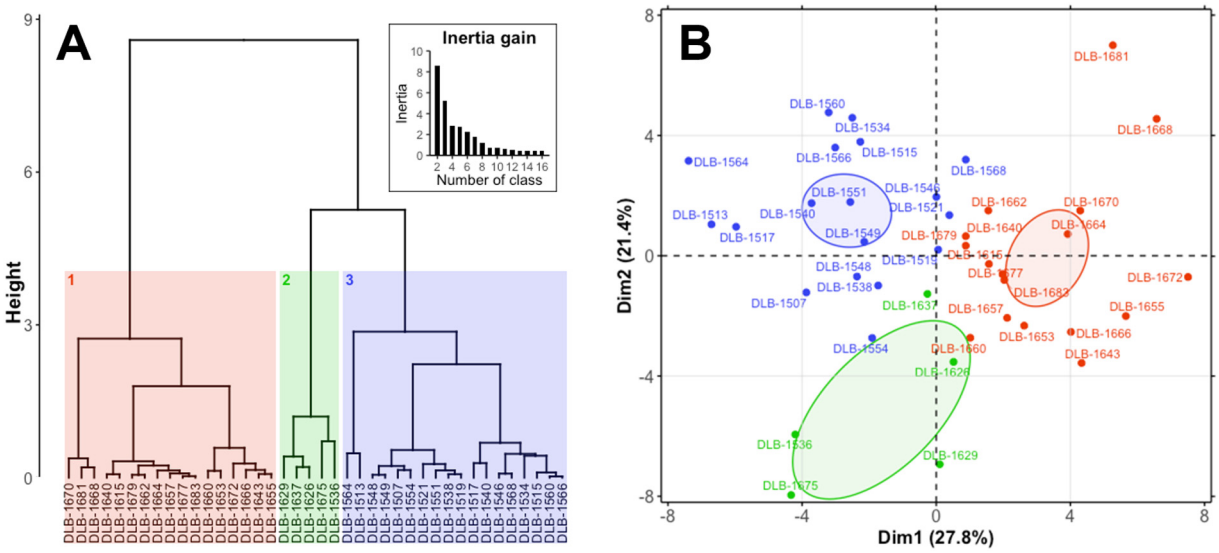

Fig. 1. Hierarchical tree (A) and score plot with 95% confidence ellipses from the HCPC and PCA analyses (B) generated based on skin metabolite concentrations of male St. Lawrence Estuary belugas. Following clusters were identified: cluster 1 (red; $n = 17$), cluster 2 (green; $n = 5$), and cluster 3 (blue; $n = 18$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

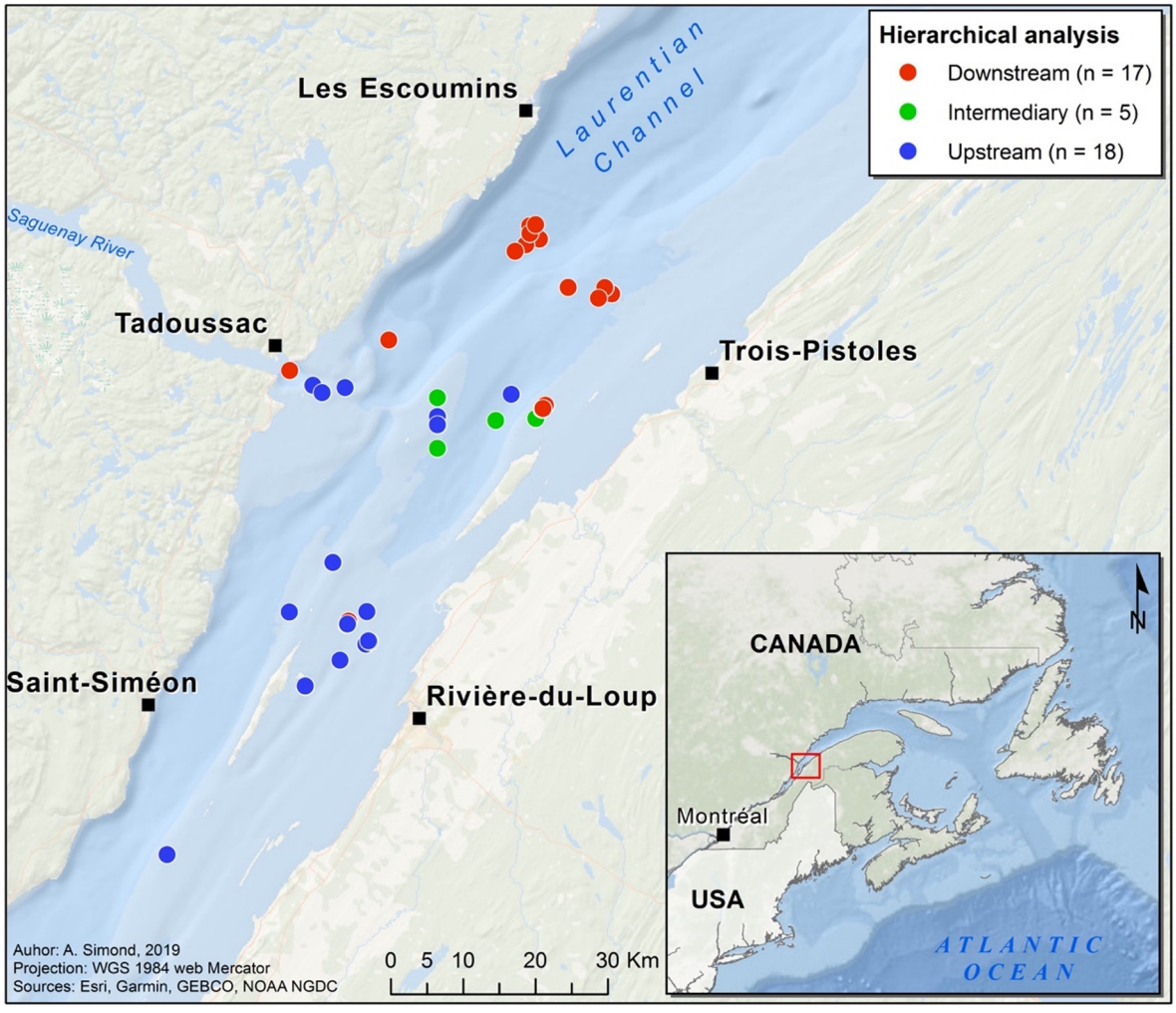

Fig. 2. Biopsy sampling sites of male belugas in the St. Lawrence Estuary (SLE; $n = 40$) categorized using hierarchical cluster analysis on principal components analysis of skin metabolite concentrations. Clusters generated from this analysis are identified using different colors and corresponded to specific sectors in the SLE: upstream (blue dots) and downstream (red dots) of the Saguenay River mouth, and in or off the Saguenay River mouth (green dots). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

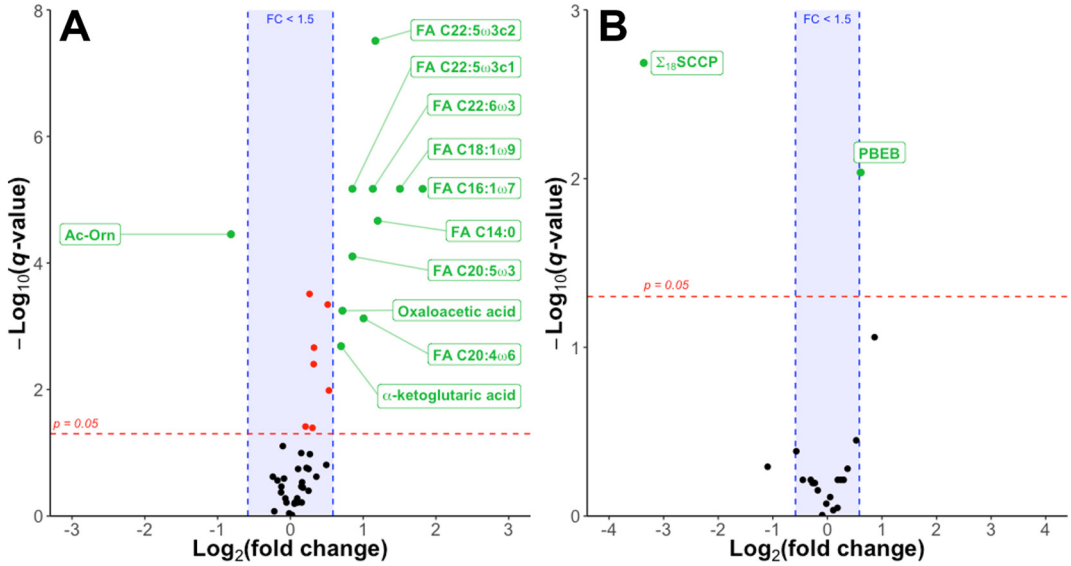

Fig. 3. Volcano plots showing concentrations of metabolites (A) and organohalogens (B) that significantly differed (fold-change > 1.5; $p \leq .05$) between upstream ($n = 18$) and downstream ($n = 17$) SLE male belugas. Fold-changes are expressed relative to individuals sampled in the upstream sector, i.e., a positive fold-change indicates higher concentrations in downstream belugas, while a negative fold-change indicates higher concentrations in upstream belugas. Concentrations that differed significantly between male belugas sampled upstream and downstream of the Saguenay River are identified in green and those that significantly differed although with a lower magnitude (fold-change <1.5) are identified in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Concentrations of two organohalogens significantly differed between male belugas sampled downstream and upstream of the Saguenay River mouth (Fig. 3B and Table 2). Specifically, Σ_{18}SCCP concentrations were four-fold greater (U = 38; $p < .001$) in upstream relative to downstream male belugas. PBEB concentrations were 2.6-fold greater in male belugas sampled downstream compared to upstream (U = 161; $p = .002$). Σ_{34}HFR and Σ_{41}PCB/OC concentrations did not differ between these two beluga groups ($p = .42$ and .55, respectively).

Table 1
Mean (±SEM) concentrations (ng/g ww) and ranges of SCCPs, PCBs and OC pesticides, and HFRs determined in the blubber of male St. Lawrence Estuary belugas ($n = 40$). Means were calculated only when concentrations exceeded the method limit of detection (MLOD) or quantification in at least 65% of the beluga samples.

	Mean ± SEM (range)
Blubber lipid content (%)	13.5 ± 1.17 (1.50–39.6)
Short-chain chlorinated paraffins	
Σ_{18}SCCP[a]	6545 ± 1325 (<MLOD - 30,745)
PCBs and OC pesticides[b]	
Σ_{37}PCB[c]	1386 ± 162 (150–4171)
HCB	27.0 ± 2.93 (<MLOD - 88.5)
p,p′-DDE	586 ± 86.3 (55.8–2456)
Mirex	(<MLOD - 249)
Trans-nonachlor	119 ± 13.9 (<MLOD - 395)
Σ_{41}PCB/OC	2154 ± 273 (254–6961)
HFRs[b]	
Σ_{29}PBDE[d]	149 ± 12.6 (28.9–386)
PBEB	0.20 ± 0.04 (<MLOD - 1.60)
HBB	0.92 ± 0.14 (<MLOD - 3.70)
Dec-604 CB	1.02 ± 0.12 (<MLOD - 3.50)
syn-DP	0.53 ± 0.18 (<MLOD - 6.50)
anti-DP	1.93 ± 0.46 (0.27–12.8)
ΣDP[e]	2.46 ± 0.59 (0.36–19.3)
Σ_{34}HFR	154 ± 13.0 (30.6–395)

[a] Sum of $C_{10}Cl_5$, $C_{10}Cl_6$, $C_{10}Cl_7$ $C_{10}Cl_8$, $C_{10}Cl_9$, $C_{11}Cl_5$, $C_{11}Cl_6$, $C_{11}Cl_7$, $C_{11}Cl_8$, $C_{11}Cl_9$, $C_{12}Cl_5$, $C_{12}Cl_6$, $C_{12}Cl7$, $C_{12}Cl_8$, $C_{12}Cl_9$, $C_{13}Cl_5$, $C_{13}Cl_6$ and $C_{13}Cl_7$. Congeners that were not detected in any samples: $C_{10}Cl_{10}$, $C_{11}Cl_{10}$, $C_{12}Cl_{10}$, $C_{13}Cl_8$, $C_{13}Cl_9$, and $C_{13}Cl_{10}$.

[b] Partial data from Simond et al. (2019).

[c] Sum of CB-17, -18, -28, -31, -33, -44, -49, -52, -70, -74, -82, -87, -95, -99, -101, -105, -110, -118, -128, -132, -138, -149, -151, -153, -156, -158, -170, -171, -177, -180, -183, -187, -191, -194, -195, -199, and -209. Congeners that were not detected in any samples: CB-169, -205, -206, and -208.

[d] Sum of BDE-7, -10, -17, -28, -47, -49, -66, -77, -85, -99, -100, -126, 138, -139, -140, -153, -154, -180, -183, -184, -191, -196, -197, -201, -203, -204, -207, -208, and -209. Congeners that were not detected in any samples: BDE-15, -71, -119, -171, -205, and -206.

[e] Sum of *syn*- and *anti*-DP.

3.3. Relationships between metabolites and contaminants

Blubber concentrations of Σ_{34}HFR, Σ_{41}PCB/OC and Σ_{18}SCCP in SLE male belugas were correlated with skin concentrations of several amino acids, hexose, biogenic amines, and fatty acids. However, only a few of these relationships remained significant after FDR adjustment (Table S12). Specifically, blubber Σ_{18}SCCP concentrations correlated positively with those of acetylornithine (biogenic amine) and negatively with those of four unsaturated fatty acids (C16:1ω7, C22:5ω3c1, C22:5ω3c2, and C22:6ω3) (Fig. 4). The ω3:ω6 fatty acid ratios did not correlate with any contaminants (Table S12).

4. Discussion

4.1. Organohalogen concentrations in male SLE belugas

Contaminant research and monitoring on the SLE beluga population over the last decades has focused primarily on PCBs, OC pesticides, and PBDEs (Lebeuf et al., 2014; Simond et al., 2017). PCB concentrations have consistently dominated contaminant profiles in SLE beluga blubber despite their marked decline over the last 30 years. However, our results show that blubber concentrations of a previously investigated class of plasticizer and flame retardant, the SCCPs, largely surpass those of PCBs in this population. In fact, a previous study on SLE belugas reported SCCP concentrations in males found dead in 1988 (493 to 915 ng/g lw;

Table 2
Mean (±SEM) concentrations (ng/g ww) of the three main organohalogen classes determined in the blubber of male belugas sampled in the St. Lawrence Estuary upstream ($n = 18$) and downstream ($n = 17$) of the Saguenay River.

	Upstream belugas	Downstream belugas
Σ_{34}HFR	146 ± 23.6	161 ± 17.7
Σ_{41}PCB/OC	2344 ± 454	1749 ± 293
Σ_{18}SCCP	10,363 ± 2234	2509 ± 812

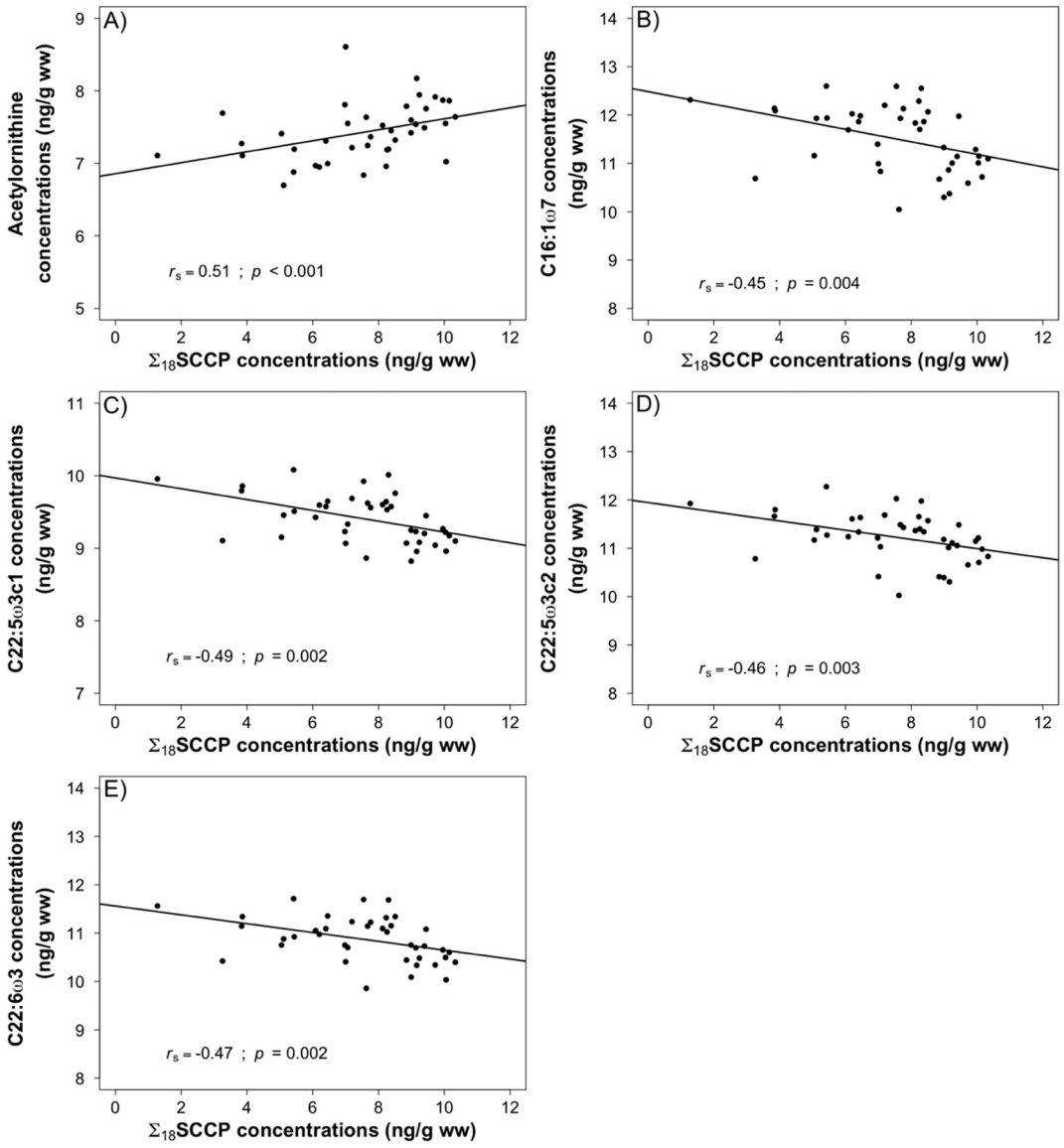

Fig. 4. Correlations between log-transformed blubber concentrations (ng/g ww) of Σ_{18}SCCP and skin concentrations (ng/g ww) of A) acetylornithine, B) C16:1ω7, C) C22:5ω3c1, D) C22:5ω3c2, and E) C22:6ω3 in male belugas sampled in the St. Lawrence Estuary. Only correlations with raw *p*-values that remained significant after applying the false discovery rate criterion are presented. Adjusted *p*-values (*q*-values) are listed in Table S12.

Tomy et al., 2000) that were >65-fold lower than those reported in the present study. Assuming that blubber concentrations determined in beluga carcasses and biopsy samples are comparable on a lipid weight basis, this suggests a strong increase in SCCP concentrations over the past three decades in this population. Also, because the maximum lifespan of belugas is around 80 years (Lesage et al., 2014; Stewart et al., 2006), SCCP exposure in certain older individuals may go back as far as the 1950s. Interestingly, Σ_{18}SCCP concentrations in SLE belugas from the present study were among the greatest reported in marine mammals worldwide: 3- to 1800-fold greater than those reported in Indo-Pacific humpback dolphins (*Sousa chinensis*) and finless porpoises (*Neophocaena phocaenoides*) from the South China Sea (570 to 24,000 ng/g lw; Zeng et al., 2015), seals and harbor porpoises (*Phocoena phocoena*) from the Baltic Sea (34 to 300 ng/g lw; Yuan et al., 2019), polar bears and ringed seals from Greenland (370 to 2700 ng/g ww; Vorkamp et al., 2017), polar bears from the Canadian Arctic (75 to 207 ng/g ww; Letcher et al., 2018), or humpback whales (*Megaptera novaeangliae*) foraging in Antarctic waters (10 to 46 ng/g lw; Casà et al., 2019). Moreover, present Σ_{18}SCCP concentrations were >300-fold greater than those documented during the 2000s in Canadian Arctic and Greenland beluga populations (Vorkamp et al., 2019). Fish from lake Ontario and the St. Lawrence River also exhibit much lower SCCP concentrations (5 to 34 ng/g ww; Houde et al., 2008; Saborido Basconcillo et al., 2015) compared to SLE male belugas.

There is currently no information available on the temporal trends of SCCP concentrations in the St. Lawrence basin nor on recent production volumes and use of SCCPs in North America. Nevertheless, considering that the production and use of SCCPs have been regulated in Canada since 2012 (Canada Gazette, 2013) and in the USA since 2014 (US EPA, 2017), it can be predicted that SCCP concentrations will eventually decline in SLE beluga tissues. However, consumer goods imported to North America or in the waste-phase represent a non-negligible source of SCCPs (van Mourik et al., 2016) that may significantly delay their decline in the environment. Given the toxicity of these compounds (e.g., oxidative stress, endocrine disruption, metabolism disturbance) (Wang et al., 2019b), current elevated SCCP concentrations in male SLE beluga blubber can be regarded as preoccupying. Therefore, a continued attention should be paid on the temporal and spatial variations of these compounds in addition to the medium- and long-chain chlorinated paraffins that were not determined in the present study.

4.2. Differences in metabolites and contaminants in belugas between sectors of the SLE

Concentrations of metabolites that were significantly different between male belugas sampled upstream and downstream of the Saguenay River mouth in the SLE were mainly fatty acids, which were documented to originate primarily from dietary intake (Iverson et al., 2004). This suggests the existence of ecological and/or hydrographic factors within this system that influence local prey availability and diet composition. The sector upstream of the Saguenay River in the SLE is characterized by shallow water (depth from 20 to 50 m), salinity ranging from 10 to 25‰ and high turbidity (euphotic zone <5 m), whereas the sector downstream of the Saguenay River exhibits more contrasting features: in its northern portion, the deep (>300 m) Laurentian Channel ending abruptly at the mouth of the Saguenay river causes an upwelling of more saline (>25‰) and cold water near the surface, while the southern portion of the Lower Estuary is shallower and receives water flowing out of the Saguenay River and the Upper Estuary, and thus is warmer and less salty than water from the Laurentian Channel (Mosnier et al., 2010; Therriault et al., 1990). These characteristics likely lead to differences in community structure, although these are currently poorly described (but see de Lafontaine, 1990; Therriault et al., 1990). It was reported that the primary productivity is higher in the Lower Estuary compared to the Upper Estuary due to the intense tidally-induced mixing (upwelling) of surface freshwater and deep saltwater at the head of the Laurentian Channel, resulting in higher levels of nutrients (Simard, 2009). This productivity cascades through the trophic web, and attracts large marine predators such as whales (Lacroix-Lepage, 2018; Lavoie et al., 2000; Lesage et al., 2007; Marchand et al., 1999). The lower concentrations of acetylornithine in male belugas sampled downstream of the Saguenay River also suggest that these individuals rely on different prey, as it has been shown that levels of this biogenic amine could vary in serum of mammals depending on their diet (Ghaffari et al., 2019). While belugas are likely to move around between the different sectors of the SLE, differences in metabolite profiles between males sampled upstream and downstream suggest a certain level of fidelity to these sectors for foraging and relatively similar diet within these.

Diet and hydrographic differences between upstream and downstream sectors could also influence foraging behaviors (e.g., dive depth, feeding frequency, and amount of food ingested) of belugas, and consequently their metabolic activity. Several metabolites that had greater concentrations in belugas sampled in the downstream sector are involved in various energy metabolic pathways. For instance, oxaloacetic and α-ketoglutaric acids are involved in urea and citric cycles and the biosynthesis of amino acids, fatty acids, and proteins. Furthermore, the fatty acids C14:0, C16:1ω7, C18:1ω9, C22:5ω3c1 and C22:5ω3c2 can be synthesized both from biosynthesis of amino acids as well as diet (Iverson et al., 2004; Iverson and Koopman, 2018). Consequently, differences in concentrations of these metabolites may reflect dietary sources exhibiting different contents of these fatty acids, and/or a differential biosynthesis or metabolic activity among groups of belugas from these two sectors. It has been shown in human that diet composition or physical activity can both affect plasma lipid and amino acid levels (Suárez et al., 2017).

Differences in PBEB and Σ_{18}SCCP concentrations between upstream and downstream SLE male belugas could also be related to dietary differences. Moreover, co-ingestion of sediments while foraging on benthic organisms (e.g., polychaetes, plaice, and rattail) (Lesage et al., 2017) could represent a non-negligible source of exposure to these chemicals. Although PBEB and SCCPs have not been monitored in sediments or preys of belugas in the SLE, these contaminants are known to accumulate in sediments at occasionally elevated concentrations (Chen et al., 2011; Ganci et al., 2019) and biomagnify in aquatic food webs (Huang et al., 2019; Tao et al., 2019). Several studies have shown that sediments is a significant sink for SCCPs, and thereby exposure source for benthic and bottom-dwelling organisms (Ma et al., 2014a, 2014b; Sun et al., 2017). It can be suggested that male belugas using the Upper Estuary were exposed to greater levels of these contaminants given the higher proximity of the Upper Estuary to the Great Lakes compared to the Lower Estuary. Another hypothesis would be that benthic or demersal prey is more important in the diet of male belugas feeding primarily in the Upper Estuary. A better understanding of these differences within the SLE beluga population would require more data on the concentrations of these contaminants in sediments and prey.

Age could also influence metabolite and contaminant concentrations in SLE male belugas. Tsai et al. (2016) showed that serum biochemistry profiles changed with age in captive belugas in Taiwan. Similarly, hepatic levels of amino acids, fatty acids and energy metabolites varied depending on age class (i.e., adults vs. subadults) in American black bears (*Ursus americanus*) (Niemuth and Stoskopf, 2014). Organohalogen concentrations are strongly related to age in male belugas as their excretion may be limited compared to females that can eliminate these via placental transfer and lactation (Cadieux et al., 2015; Desforges et al., 2012; Reijnders et al., 2018). Moreover, according to Michaud (1993), the sectors identified in the present study (i.e., upstream and downstream) generally are visited by herds of belugas with different gender composition and age structure during the summer. The sector upstream of the mouth of the Saguenay River is mostly frequented by small herds of adult females, juveniles and/or calves. Groups downstream of the Saguenay River mouth vary in size and are mainly composed of large adults (likely males) in the Laurentian Channel area, while in the southern portion the two types of herds are observed. As a result, male belugas sampled in the upstream sector of the SLE were likely younger than those sampled downstream given the higher occurrence of large adults in the downstream portion of the SLE. The greater concentrations of SCCPs quantified in male belugas (potentially younger individuals) sampled in the upstream sector may indicate that these individuals likely were born from female belugas that had been more exposed to these chemicals compared to downstream belugas that may belong to younger generations. Conversely, PBEB concentrations may be greater in downstream male belugas (potentially older individuals) as there has been a decline in the production of this chemical in North America since 1986 (Hoh, 2005). Differences between belugas from the two sectors may therefore be at least partly age-related.

4.3. Correlations between contaminants and metabolites in belugas

Among all organohalogens quantified in SLE male beluga blubber, only SCCPs significantly correlated with skin metabolite concentrations. The correlations between SCCP and fatty acid concentrations in these belugas might indicate a disruption of lipid metabolism. Recently, rats orally dosed with SCCPs showed an increase in liver fatty acid oxidation leading to a decrease in their concentrations (mostly unsaturated and long-chain fatty acids), which was associated with an up-regulation of the peroxisome proliferator-activated receptor alpha (PPARα) (Gong et al., 2019). PPARα is a ligand-dependent transcription factor known to be involved in the β-oxidation of lipids (Kersten, 2014) that could be activated by SCCPs due to their efficient binding affinity to PPARα (Gong et al., 2019). Similarly, an up-regulation of fatty acid degradation also occurred in a human cell line (HepG2) and in liver of rats and mice dosed with SCCPs (Geng et al., 2015; Wyatt et al., 1993). A change in blubber fatty acid composition might alter their thermoregulatory capacity and ultimately survival. In addition of being a source of energy, fatty acids are also involved in other physiological processes such as the inflammatory response, insulation and buoyancy (Fritsche, 2015; Iverson and Koopman, 2018). For example, an increase in saturated fatty acid content may impact the health of belugas as these fatty acids are able to trigger hepatic lipotoxicity by inducing hepatocyte lipoapoptosis (Chen et al., 2018). In adult mice dosed with SCCPs via gavage, an up-regulation in fatty acid β-oxidation induced immunotoxic effects including an increase of white blood cell, neutrophil, lymphocyte, monocyte, and blood serum

pro-inflammatory cytokine levels (Wang et al., 2019a). Moreover, as C22:6ω3 is known to have health benefits in relation to inflammatory conditions in mammals (Muhlhausler, 2018), a decrease of its bioavailability might affect the inflammatory response. Although mechanisms of toxicity of SCCPs in male belugas cannot be identified using the present correlative approach, results from the present study suggest that SCCP exposure could alter their fatty acid profiles. As described previously, all fatty acids (C16:1ω7, C22:5ω3c1, C22:5ω3c2, and C22:6ω3) that correlated with SCCP concentrations in male SLE beluga blubber can be acquired through diet (Iverson et al., 2004; Iverson and Koopman, 2018). Due to a high degree of similarity in molecular structures between fatty acids and SCCPs, the correlations observed between these compounds may also indicate that SCCPs somewhat behaved similarly to fatty acids in belugas and/or their prey (bioaccumulation and biomagnification) and/or during sample extraction (i.e., pressurized liquid extraction; see Section 2.2). In the event where our hypothesis of a difference in diet depending on sectors is verified, the correlations observed between fatty acids and SCCPs could be related to these ecological differences rather than to deleterious effects of SCCPs on fatty acids.

Acetylornithine, for which the concentrations correlated positively with those of Σ_{18}SCCP in SLE male belugas, is an intermediate compound in the biosynthesis of arginine from glutamate, and is involved in arginine and proline metabolism (Morizono et al., 2006). Significant disorders in arginine and proline metabolism were documented following exposure of the human liver cells HepG2 to SCCPs (Geng et al., 2015), which was suggested by these authors to be a possible adaptive response to oxidative stress. SCCPs might change the fatty acid profiles of SLE belugas as well as induce oxidative stress that could, in turn, impact amino acid metabolism.

5. Conclusions

This study showed that metabolomic profiles of beluga skin varied depending on sampling sectors in the SLE. Moreover, elevated concentrations of organohalogen contaminants, largely dominated by SCCPs, were quantified in blubber samples of male SLE belugas. Significant correlations between SCCP concentrations and those of four fatty acids and acetylornithine suggested potential impacts of SCCP exposure on lipid or amino acid metabolic pathways in SLE belugas. In addition, concentrations of several organohalogens and metabolites significantly differed between belugas sampled upstream and downstream of the Saguenay River mouth. These geographical differences in metabolite and contaminant profiles could be related to the unique environmental characteristic of these sectors, including prey availability and diet composition. However, these organohalogens should be monitored in the SLE beluga food web and environment (e.g., sediments and water) to verify this assumption, and will be the subject of an upcoming study. Also, it cannot be completely disregarded that these results could have been influenced by age, which is a known confounding factor to contaminant concentrations and/or metabolite profiles in belugas (Lebeuf et al., 2014; Norman et al., 2013; Stern et al., 2005; Tsai et al., 2016).

The markedly elevated SCCP concentrations in male SLE belugas, which are among the greatest reported in marine mammals worldwide, raise concerns about the potential toxicity of these chemicals in this endangered population. Future research is warranted to investigate environmental concentrations and mechanisms of toxicity of SCCPs in apex predators from the marine food web. Further investigations should also deepen our knowledge on the potential effects of organohalogens on the fatty acid metabolism of marine mammals. Finally, a more holistic approach is critically needed to better understand the health implications of highly-exposed cetacean populations to organohalogens. As such, there is a strong need to develop new tools capable of providing simultaneously a maximum of biological and chemical information (e.g., age, "omics" profiles, contaminant concentrations, nutritional status, etc.) from cetacean biopsy samples.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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