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Partial parameterization of a Physiology-based Pharmacokinetic (PBPK) model for Atlantic halibut (*Hippoglossus hippoglossus*)

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Sammendrag

Reduksjonen av havis i den Arktiske regionen fører med seg en økt kommersiell interesse for områder som tidligere ikke var fysisk tilgjengelige. En utnyttelse av disse nye områdene øker presset på denne mer urørte delen av verden. I tillegg fører klimaendringer og økende globale utslipp med seg tilstedeværelse av nye stoffer arktiske havområder. Det høye antallet av ulike stoffer gjør at arbeidet med å teste alle stoffene på alle ulike arter ikke er gjennomførbart. Derfor er det et behov for verktøy innen miljørisikoanalyse som kan predikere bioakkumulering og toksiske effekter av disse stoffene. Forurensningen i Arktiske hav utgjør en trussel både mot fiskebestander og for overføringen av farlige stoffer til fiskekonsumenter. Fysiologibaserte farmakokinetiske (PBPK) modeller kan estimere indre vevskonsentrasjoner over tid, basert på ytre eksponeringskonsentrasjoner i omgivelsene. På denne måten kan altså vevskonsentrasjoner i spesifikke målorgan i predikeres. Modeller som dette kan gi en kvantitativ beskrivelse av prosesser for absorpsjon, distribusjon, metabolisme og eliminering (ADME) av miljøgifter i biota. Formålet med denne oppgaven var å utvide nytten av PBPK-modeller til den arktiske regionen. Det ble dermed gjennomført en karakterisering av fysiologiske parametere, for å initiere utviklingen av en PBPK-modell for den marine og arktiske arten, atlanterhavskveite (*Hippoglossus hippoglossus*).

I løpet av denne studien ble det etablert en foreslått modellstruktur for atlanterhavskveite, basert på tidligere publiserte PBPK-modeller for fisk. Kriterier satt for inkludering av spesifikke vev i modellstrukturen var enten deltakelse i ADME-prosesser eller annen toksikokinetisk relevans, som målorgan. Modellstrukturen bestod av ni vevsstrukturer: arterielt blod, venøst blod, hjerne, gjeller, gastrointestinal trakt (GIT), nyrer, lever og inndeling av resterende vev i de lavt perforert av blodårer (PPT (poorly perfudes tissues); muskel, skinn, bein) og sterkt perforerte (RPT(richly perfused tissues); gonader, hjerte, milt). Hvert vev ble definert av volum, lipid- og vanninnhold og blodstrøm til organene. Selve karakteriseringen av fysiologiske parametere omhandlet måling av vevsvolum, lipidinnhold og vanninnhold. I tillegg ble verdier som ikke var mulig å måle direkte i løpet av dette studiet samlet fra tilgjengelig litteratur. Kvantifisering av parametere involverte analyse av datafordeling, korrelasjonsanalyser og regresjonsanalyser for presis beskrivelse av data. Karakteriseringen av fysiologiske parametere er et innledende trinn i utvikling av en PBPK-modell for Atlanterhavskveite, som legger grunnlaget for neste steg i utviklingen av en modell til bruk i arktiske omgivelser.

Abstract

The reduction of sea ice in the Arctic region is leading to increased commercial interest in areas that were previously inaccessible. Exploration of these new areas increases the pressures and anthropogenic impacts on this more pristine part of the world. Climate change and rising global emissions are introducing new substances into the Arctic waters. The large number of different substances makes it unfeasible to test all substances on all biota. Therefore, there is a need for tools in environmental risk assessment that can predict the bioaccumulation and toxic effects of these substances. This pollution of the Arctic ocean poses a threat to both fish populations and the transfer of hazardous substances to fish consumers. Physiologically based pharmacokinetic (PBPK) models serve as tools in environmental risk assessment to estimate internal tissue concentrations over time based on external concentrations. Models like these can provide a quantitative description of absorption, distribution, metabolism and excretion (ADME) processes of substances in biota and predict doses at specific target sites in the organism. In the attempt to expand the utility of PBPK modelling into the Arctic region, the aim of this thesis was to characterize physiological parameters necessary for the development of a PBPK model parameterized for a fish species inhabiting the Arctic region, specifically the Atlantic halibut *(Hippoglossus hippoglossus).*

During this study, a conceptual model structure for Atlantic halibut was established based on previously published PBPK fish models. The structure consisted of nine tissue/organ compartments; arterial blood, venous blood, brain, gills, gastrointestinal tract (GIT), kidney, liver, poorly perfused tissues (PPT) and richly perfused tissues (RPT). Each compartment is defined by its volume (as a fraction of total body weight), total lipid and water contents (as fraction of tissue wet weights), and blood flow (% of cardiac output) to the compartment. The criteria set for tissue inclusion revolved around participation in ADME processes and toxicokinetic relevance. The characterization of physiological parameters involved measurements of compartment volumes, relative lipid contents and water contents. Additionally, values that were not feasible to measure directly during this study were collected from available literature. Quantification of parameters involved correlation and regression analyses for the most precise description of the data structure, followed by calculations of blood flow to organs. The characterization of physiological parameters is an initial step in the development of a PBPK model for Atlantic halibut, laying the foundation for the next stage in the development of a model for use in Arctic environments.

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Abbreviations

ADME	Absorption, distribution, metabolism and elimination
AOP	Adverse outcome pathway
ECHA	The European Chemicals Agency
EFSA	The European Food Safety Authority
EXPECT	In silico and experimental screening platform for characterizing environmental impact of industry development in the Arctic
GIT	Gastrointestinal tract
IMR	Norwegian Institute of Marine Research
IVIVE	In Vitro In Vivo Extrapolations
LC50	Lethal concentration 50
LogKow	Logarithmic octanol-water partition coefficient
NIVA	The Norwegian Institute of Water Research
NMBU	Norwegian University of Life Sciences
NOEC	No observable effect concentration
PAHs	Polycyclic hydrocarbons
PBPK	Physiology-based pharmacokinetic model
PC	Partition coefficient
PC1 and PC2	Principal component 1 and 2
PCBs	Polychlorinated biphenyls
PFAS	Polyfluoroalkyl substances
POPs	Persistent organic pollutants
PPT	Poorly perfused tissues (low blood perfusion)
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RPT	Richly perfused tissues (high blood perfusion)
STOP	Source To Outcome Pathways
TK	Toxicokinetics

1.Introduction

The Arctic region plays a critical role in regulating global climate and supporting diverse ecosystems. Its unique environment harbours a rich array of wildlife, including valuable species like Atlantic halibut; however, this region is subjected to an increasing range of human pressures (Box et al., 2019; Overland et al., 2019). Lately, the utilization of Arctic ecosystems has grown more intensive, due to improved accessibility, especially in areas that used to be periodically covered by ice (Anisimov, 2007). The Arctic is experiencing a rapid climate change and is forecasted to undergo the most significant warming of any region globally (Ford et al., 2021). Increased riverine nutrient influx due to permafrost thaw and erosion (Tank et al., 2012), sewage, waste, long-range atmospheric and oceanic transportation, tourism, shipping, commercial fisheries, resource utilization of oil and gas, are all part of the long list of factors leading to aquatic pollution in the Arctic oceans (Dietz et al., 2019). Many of these processes and activities are intensified by climate change to a greater extent than in temperate regions. A rapid decline in area covered by sea ice and rise in sea-temperatures lead to possibilities for expansion of areas exploited by aquaculture and industrial activities and introduction of new shipping routes, enlarging the risk of oil spills. (J. C. Comiso, 2023; NSIDC, 2024) The expectation is that Arctic wildlife and fish populations will encounter an expanded array of pollutants at higher concentrations compared to present levels. Contaminants such as heavy metals and persistent organic pollutants (POPs), which are toxic chemicals that persist in the environment, bioaccumulate in living organisms (Campbell, 2022) pose significant risks to Arctic wildlife and indigenous communities (Adams et al., 2019; Letcher et al., 2018), highlighting the urgent need for efforts to protect this fragile ecosystem. In response to this, The Norwegian Institute of Water Research has launched the EXPECT project to assess environmental risks from upcoming Arctic industrial developments. EXPECT aims to identify key pollutants, toxicity factors and vulnerable organisms and enhance the development of robust methods and approaches for tracking pathways of pollutants from emission to environment.

Anthropogenic activities, including mining, industry, agriculture and combustion are recognized origins of persistent organic POPs (Simukoko et al., 2023). Chemicals of concern that pose significant risk to Arctic ecosystems include POPs, which includes compounds such as polychlorinated biphenyls (PCBs) and polycyclic hydrocarbons (PAHs). Due to their semi-volatile characteristics and extended environmental half-lives, these substances undergo long-range transport and achieve global distribution (Wania & Mackay, 1993). In biological

systems, POPs tend to partition into lipids in organs and adipose fat, due to their high lipid solubility. This lipophilic and hydrophobic nature yield the potential to accumulate in fatty tissues of aquatic organisms, and from there lead to biomagnification (Deribe et al., 2011; Letcher et al., 2010; Sharma et al., 2009). Additionally, heavy metals like mercury, copper and lead are also of particular concern due to their persistence and bioaccumulation potential. Emerging contaminants, such as pharmaceuticals and per- and polyfluoroalkyl substances (PFAS), present novel challenges to Arctic ecosystems (Dietz et al., 2019).

The direct usage of these chemicals of concern in the Arctic region has been limited, but the POPs, their precursors, metabolites and degradation products are transported into the Arctic from lower latitudes (Kallenborn et al., 2012). Assessing the effects of these chemicals on Arctic wildlife is crucial for understanding their impacts. Conducting effect assessments in the Arctic poses several challenges. Field studies are logistically and economically difficult, due to the remote and harsh environment. Additionally, complex interactions between contaminants, climate change and other stressors complicate the assessment of chemical effects on Arctic wildlife (Dietz et al., 2019). Traditional research methods for sampling and experimentation raise ethical concerns regarding disturbance and potential harm inflicted on the already vulnerable wildlife populations (Leary, 2010).

In addition to serving as one of the major protein sources for humans globally, fish populations generate a long list of ecosystem services, such as regulation of food web dynamics and nutrients cycling (Holmlund & Hammer, 1999). Therefore, it is crucial to prioritize the preservation of fish populations and develop robust risk assessment tools for fish, to ensure the sustainability of aquatic ecosystems. Fish are in a strong ecological interdependence with their habitat. The surrounding water is essential to several life processes, such as respiration and osmoregulation, and serves as a medium for excretion and reproduction. The physiological adaptations enabling fish to flourish in an aquatic environment strongly impact their interactions with xenobiotic chemicals. While mammalian lungs are exposed to volatile or particulate chemicals, gills are exposed to water-soluble compounds during gas-exchange. Branchial gill uptake of necessary gases requires diffusion across a hydrophobic membrane. A hydrophobic environment is excellent for non-polar, hydrophobic chemicals which may diffuse into the blood stream. Hydrophobic chemicals have a higher affinity for transitioning from the aqueous phase to a more lipophilic environment, such as the lipids present in biota. Uptake of substances can occur from

different sources alone or combined as fish are exposed to both chemical branchial uptake and through ingestion (Matthee et al., 2023; Nichols, 2024). The rising exposure to anthropogenic xenobiotics makes it unrealistic to determine their environmental impact from individual testing of each compound. It is therefore necessary to develop tools to help make predictions of these environmental impacts (Nichols et al., 1990).

An important support in developing alternative test methods is introducing toxicokinetics (TK) to provide a framework for understanding how pollutants behave within organisms. Toxicokinetics examine the absorption, distribution, metabolism and elimination of toxic substances in biological systems. Through the application of TK, we can achieve deeper insights into the internal exposure levels of pollutants and their potential effects on Arctic wildlife. By utilizing TK-models, as detailed as physiology based pharmacokinetic (PBPK) models, the movement of pollutants within the organism can be simulated, without the need for excessive animal testing (Nichols, 2024). A range of PBPK models have been developed for fish, each with unique focus and application. However, the potential for PBPK models for marine and Arctic environments remains largely unexplored, limiting the capacity to effectively assess the impacts of pollutants on Arctic species.

2. Background information

2 . 1 From entry to action: Absorption, distribution, metabolism and elimination

The toxicity of a chemical to fish is essentially determined by the absorption, distribution, metabolism and elimination of the chemical, abbreviated as ADME processes. These processes are changing the effective chemical concentration at a target site over time, affecting the whole-organism dose-response relationships. Pharmacokinetics is the description of the quantitative study of ADME processes of drugs in relation to time, while TK include all chemicals that cause adverse effects in exposed organisms (Nichols, 2024). In this manner, TK characterizes the time-course of chemical concentrations at different sites in an organism.

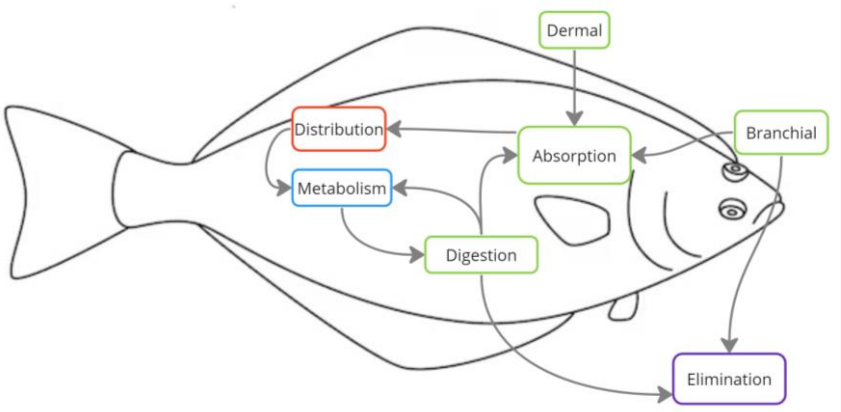

Figure 1: Outline of ADME processes in fish. Absorption of chemicals occur through branchial and dermal uptake, in addition to digestion and seawater drinking. The chemicals are distributed to metabolizing organs and eliminated through branchial, faecal and through renal excretion. Schematics constructed in Miro (2024).

As illustrated in Figure 1, absorption is the process by which chemicals enter an organism, either through ventilation, ingestion or skin contact. Distribution describes the movement of a substance throughout an organism, often via bloodstream to various organs and tissues. Metabolism involves the breakdown of compounds that have been absorbed into the tissue, while excretion refers to the elimination of the original compound or its metabolites form the organism through urine, faeces or exhalation (Campbell et al., 2022).

2. 2 Toxicokinetic (TK) modelling

Well established in aquatic toxicology is the use of mathematic models to better understand and describe the adverse effects caused by exposure (Johanson, 2010). The objective of these models is to relate an external concentration to observed whole-organism adverse effects, by calculating the parallel internal concentrations. In TK-models, time must be included as a factor when describing the uptake of chemicals in organisms, and steady-state cannot be

assumed, since environmental exposure will vary over time, and constant concentrations will rarely be established internally in organisms (Ockleford et al., 2018). Modelling non-equilibrium processes is more effectively handled using dynamic models that track changes in chemical concentrations throughout the lifespan of an organism. Dynamic models offer the advantage of integrating essential aspects of life history, such as growth and reproduction, as well as the seasonal factors of temperature and food availability, resulting in gain or loss of lipid mass (Hodson, 2022; Kooijman, 2000).

2.2.1 Compartmental toxicokinetic modelling

In terms of compartmental TK-models, there are the simple one-compartment models, which assumes uniform chemical concentrations across all tissues and view the organism as one single compartment, and multicompartment models, which account for variations in chemical concentrations among different tissues. Assuming that an organism consists of only one single compartment with a homogenous distribution of the chemical throughout the organism might be sufficient for simple small organisms, but unrealistic for more complex multicellular organisms, including fish (Hermens, 2019). The one-compartment model is useful for predicting whole-body concentrations of a chemical, while multicompartment physiology-based pharmacokinetic (PBPK) models are more relevant in estimating tissue-specific concentrations (Stadnicka et al., 2012). During a comparison study of two one-compartment TK-models and a multicompartment PBPK model by Stadnicka et al. (2012), the PBPK model accurately simulated chemical concentrations across various tissues, and additionally outperformed the one-compartment models in simulating whole-body concentrations. This difference arises from a more detailed description of gill exchange in the PBPK models, where factors like partition coefficients (PCs) between water and blood and oxygen consumption rates influences the final whole-body concentration.

2.3 Physiology based pharmacokinetic (PBPK) models

In the field of environmental risk assessment, the exposure is compared to a quantification of the potential harm of the chemical, a hazard metric, for example the lethal concentration (LC50) or no observable effect concentration (NOEC). The most common method for quantifying exposure is to use the external dose measured in the species surroundings. Physiology-based pharmacokinetic (PBPK) models offer predictions of the chemical concentration over time by integrating information related to ADME processes along with the physiological and anatomical characteristics of the fish (Grech, 2018; Wang et al., 2022). PBPK models are developed to improve the quantification of dose-response by integrating

pharmacokinetics and estimating tissue-specific internal doses from external, thereby improving the accuracy of measurements. Understanding organ-specific accumulation is important for understanding toxicity pathways that affect specific target sites within certain organs. Additionally, food-web biomagnification of edible parts (i.e. muscle tissue, liver tissue), particularly if predators have a preference for consuming specific organs (as orcas (*Orcinus orca*) targeting lipid-rich livers (Engelbrecht et al., 2019; Towner et al., 2024)) and fish oil production from fat reserves for various purposes, such as human consumption, fish and animal feed (Rustad et al., 2011) and biodiesel (Tayib et al., 2024).

As PBPK models are based on the physiology of the species, relevant organs and tissues are represented as individual compartments, or in grouped compartments. Each compartment is defined by its volume (as a fraction of total body weight), total lipid and water contents (as fraction of tissue wet weights), and blood flow (% of cardiac output) to the compartment. The total body weight of the organism is included for allometric scaling to account for physiological inter-individual variations of for example total blood flows (ml/min) and metabolic rates, which significantly influences the rate of metabolism and elimination of chemicals, allowing for more precise predictions of chemical clearance (Brown et al., 2000; Clarke & Johnston, 1999).

The uptake and distribution of chemicals in each compartment are described by a set of differential equations. Consequently, PBPK models can forecast the concentrations of substances in the entire organism and in various tissues at any given time during exposure. The level of complexity of the different models are dependent on available experimental data for parameterization and calibration. Some of the models are generic, developed to predict the average behaviour of a class of compounds or substances with similar physicochemical properties, while other models are developed to be rather chemical specific (Brinkmann et al., 2017; Krishnan, 2009). PBPK models demand a vast amount of information for parameterisation, and this type of data is often lacking or limited in literature (Grech, 2018). If the variation of toxicity between different species is assumed to be negligible, then similar levels of exposure in tissues should lead to similar effects, independent of species and exposure route. The model can therefore be used to make extrapolations for species, based on data for related species, and by this, further reducing the needed animal experiments (Clewell & Clewell, 2008).

2.3.1 Physiology-based pharmacokinetic models parameterized for fish

The origin of fish PBPK models dates back to the Nichols et al. (1990) adaption of an inhalation model originally developed for rats by Ramsey and Andersen (1984), to model the uptake of waterborne chemicals in rainbow trout *(Oncorhynchus mykiss).* Features unique to fish were added to tailor the model to fish physiology. This included portal blood flows to liver and kidney and a model for gill description developed by Erickson and Mckim (1990).

The chemical flux at fish gills is modelled as a counter current process controlled by gill ventilation, blood perfusion and partitioning of the chemical between blood and water. The fish gill description connects flux to an exchange coefficient and the difference in chemical activities in venous blood and inspired water. Blood and water flows are separated by a diffusion barrier formed by the gill epithelium and stagnant boundary layers in adjacent blood and water channels. In the context of counter current exchange, this implies that arterial blood achieves equilibrium with inspired water while venous blood reaches equilibrium with expired water (Erickson & Mckim, 1990; Nichols et al., 1993). The predicted branchial uptake is therefore influenced by the oxygen consumption rate, determining the effective respiratory volume (Schmieder & Weber, 1992; Stadnicka et al., 2012).

The effective respiratory volume defines the volume of inspired water that comes into equilibrium with blood in gill lamellae per unit of time (McKim et al., 1999), and has an inverse relationship with dissolved oxygen concentration (Erickson & Mckim, 1990). Decreased oxygen levels in inspired water requires fish to increase their effective respiratory volume to ensure adequate oxygen uptake. The greater volume increases the quantity of contaminants in equilibrium with blood in the gill lamella, thereby accelerating branchial chemical uptake (Salmina et al., 2016).

After the initial development of the fish PBPK model framework, Nichols later examined the possibilities of interspecies extrapolation from the rainbow trout model for waterborne organic chemicals to channel catfish *(Ictalurus punctatus)* (Nichols et al., 1993) and later advanced the model to include dermal absorption of organic chemicals (Nichols et al., 1996). The model has been modified to describe maternal transfer of TCDD in brook trout *(Salvelinus fontinalis)* (Nichols et al., 1998), dietary uptake of hydrophobic compounds (Nichols et al., 2004) and incorporated effects of growth and temperature (Grech et al., 2019). The basic framework of the original model remains fairly consistent for different fish species

and both organic compounds and metals have been specifically modelled. The catalogue of developed models is expanding and improving, as summarised in Appendix 2.

The existing fish PBPK models are predominantly focused on modelling freshwater and temperate species. Efforts have even been directed towards the development of a multispecies PBPK model for a surrogate species made up by the combination of values from a variety of fish species, tailored for freshwater fish (Brinkmann et al., 2016; Mangold-Doring et al., 2021). However, absent from the list of species are those occurring in the Arctic region, adapted to colder, marine environments. Additionally, none of the parameterized species are flatfish species.

2.4 Compartment selection in a multicompartment model

The compartments selected for the parameterization of a PBPK model are determined by their involvement in ADME processes or their relevance to toxicokinetics, as illustrated in Figure 1 and 2. In order to explain the absorption, relevant uptake routes must be incorporated, which in the case of fish are dermal (skin), branchial (gills) and/or by digestion (gastrointestinal tract). Tissues involved in distribution are primarily the circulatory system (blood), facilitating transport of the substance through the organism. Metabolizing organs, such as the liver, play a crucial role in potentially biotransforming chemicals to subspecies with distinct physiochemical properties from the original compound (Krishnan, 2009; Matthee et al., 2023; Nichols, 2024).

Furthermore, the inclusion of storage organs, such as muscle or lipid-rich tissues (e.g., liver), along with potential target organs or tissues that commonly take part in key events in mode of action frameworks or are typical target organs for pollutants are also incorporated. For example, the brain should be included in a compartment in a study of neurotoxicants, while the kidneys are important for studies of nephrotoxic substances, and the liver for hepatotoxic substances (Aggarwal et al., 2022; Hussain et al., 2019; Jaeschke et al., 2002). Organs involved in the elimination of chemicals are essential for explaining excretion by the PBPK model, which may occur through various routes, including faecal, urinary and branchial excretion via gills (Krishnan, 2009; Matthee et al., 2023; Nichols, 2024).

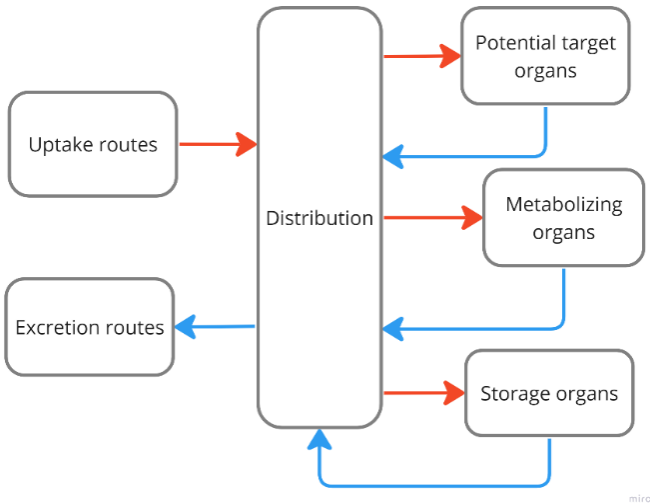

Figure 2: Framework of necessary organs that are advantageously included in a PBPK model. The red arrows represent uptake of chemicals, and in essence arterial blood flow, while the blue arrows show routes for elimination of chemicals from different compartments and the organism in total, which usually are venous blood flow. In a finished model, target organs, metabolizing organs, storage organs, uptake routes and excretion routes can be represented by one or more compartments. The schematics are constructed in Miro (2024).

Tissues not selected as individual compartments are divided according to the degree of blood perfusion: one compartment for richly perfused tissues (RPT; heart, spleen etc.) and one for the poorly perfused tissues (PPT; muscle, bones, adipose fat, etc.), often referred to as the "rest of body"- compartment (Krishnan, 2009). The calculated concentration of a chemical in a grouped compartment is then said to be valid for each of the tissues included in the pooled compartment separately (Grech et al., 2019).

2.5 Physiological parameters required in a fish PBPK model

2.5.1 Estimation of chemical partitioning

As blood is assumed to be the primary medium of transport for the chemical of interest, blood is the link for exchange between different compartments; therefore, an important part of the PBPK models are the blood-to-water and blood-to-tissue partition coefficients (PCs). These coefficients describe the transfer from water to blood in gills and subsequently from blood to each tissue within a fish. PCs are equilibrium ratios of a chemical's concentration in two phases, thereby characterizing the distribution of a chemical in environments and organisms (Tremblay et al., 2012). The octanol-water PC ($\log K_{ow}$) is used to express lipid solubility as the ratio between chemical concentrations in n-octanol to water at equilibrium. N-octanol serves as a proxy for organic material, as it mimics the lipophilic properties of organic compounds. LogKow values typically range from - 3 (hydrophilic) to +10 (hydrophobic) (Cumming & Rücker, 2017).

PCs between blood and tissues serve as indicators of the distribution of a chemical within the organism (Hodson, 2022). Given the limited availability of experimental blood to tissue partitioning data for aquatic vertebrates, estimation is often necessary (Bertelsen et al., 1998). A common method for estimation involves establishing a connection between the partitioning behaviour of a chemical, to the total lipid content and the LogKow (Mackay, 1982; Oliver & Niimi, 1983; Schmitt, 2008; Veith et al., 1979). Hydrophobic contaminants generally distribute within tissue lipids (Schmitt, 2008). To account for the polar constituent in tissues, each compartment may be treated as two-phase systems consisting of lipids and water (Bertelsen et al., 1998).

2.5.1.1 Determination of total lipid and water content in biological materials

Lipids serve as the major compartment for the partitioning of neutral organic chemicals within biological material (Honeycutt et al., 1995). As previously described, the lipid content of each of the tissues included as compartments in a PBPK model is necessary for estimating the partitioning of chemicals between blood and tissue (Peyret & Krishnan, 2011). A straightforward method for lipid assessment involves extracting and concentrating lipids to dryness for quantification. The extraction process involves disintegrating tissues to access all lipids, followed by the use of a solvent mixture of nonpolar and polar solvents. Traditional solvents include chloroform and methanol, while more safe and environmentally friendly solvents include cyclohexane and isopropanol (Saini et al., 2021). Quantification the total water content of a sample can be efficiently achieved through dry weight determination, followed by calculating the water content as the difference between the wet weight and dry weight of homogenized samples (Lantry & O'Gorman, 2007).

2.5.4 Summary of physiological parameters required for parameterization of a fish PBPK model

A complete PBPK model comprises both physiological and chemical-specific parameters. Physiological parameters required as input in a PBPK model include compartment specific factors such as relative volumes, lipid contents, water contents and blood flows, alongside general parameters, such as cardiac output, oxygen consumption rate, effective respiratory rate and total weight, as listed in Table 1.

Table 1: Summary of the physiological input parameters necessary for parameterization of a fish PBPK model.

Compartment-specific		**General**	
Parameter	**Unit**	**Parameter**	**Unit**
Relative volume	fraction of total weight	Cardiac output	ml/min/kg
Relative lipid content	fraction of tissue wet weight	Oxygen consumption rate	mlO2/min/kg
Relative water content	fraction of tissue wet weight	Effective respiratory volume	ml/min
Blood flow	% of cardiac ouput	Total weight	g

2.6 Use of toxicokinetic models in science and regulation

In order to protect the environmental and consequently human health from the adverse impacts of exposure to hazardous substances, legislation has been established globally. The European REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation (Regulation, 1999) is an example of this. In accordance with the REACH regulations, producers and importers are mandated to register chemicals within a centralized database, with details on their physicochemical properties and their potential human and environmental risk (Brinkmann et al., 2017).

2.6.1 Reducing animal experimentation

A critical aspect of aquatic risk assessment are toxicity data for representative species across all trophic levels. The REACH regulations identified 30,000 chemicals of concern out of 100,000 already in use in Europe (Wolf & Delgado, 2003). Generating toxicity data for decomposers, producers, invertebrate and vertebrate consumers means a substantial testing requirement, and a significant number of animal experiments. The European Chemicals Agency (ECHA) is committed to minimizing the reliance on such experiments. This commitment entails the substitution of animal experiments with suitable alternatives whenever feasible. Along with this comes a need for more accurate non-experimental methodologies, such as PBPK models, and the use of existing knowledge about a chemical to predict toxicological effects based on its physicochemical properties or by assuming similar effects of similar structures (ECHA, 2011; Gilbert, 2011; Spielmann et al., 2011).

2.6.2 *In vitro in vivo* extrapolations (IVIVE)

Alternative methods, primarily *in vitr*o bioassays, utilize cells or other biological materials, outside their biological context to investigate the impacts of chemicals on biological

processes, thereby avoiding the need for live animal experimentation. The outcomes derived from bioassays typically cannot be directly extrapolated to the responses of entire organisms *in vivo* (Lilienblum et al., 2008; Yoon et al., 2012). This limitation contributes to their current limited acceptance in regulatory ecotoxicology, despite their ethical appeal. Addressing this requires reliable methods for quantitative *in vitro-in vivo* extrapolations (IVIVE). Preliminary to this, there is need for pharmacokinetic models, such as PBPK models, to delineate the "baseline disposition" of a chemical to facilitate the extrapolation of *in vitro* studied processes to the *in vivo* level (Yoon et al., 2012).

2.7 Atlantic halibut (*Hippoglossus hippoglossus*)

The Atlantic halibut (*Hippoglossus hippoglossus*) largest among all flatfish species. This is a demersal species present in both the North Atlantic Ocean and in regions of the Arctic Ocean that primarily feeds on crustaceans (crabs and shrimps) during its juvenile stages and transitions towards a piscivore diet as it matures (Atlantic cod (*Gadus morhua*), herring (*Clupea harrengus*), mackerel (*Scomber scombrus*), among others) (Cargnelli et al., 1999; Link & Almeida, 2000). Their slow growth rate and late maturation make them particularly vulnerable to overfishing (Sigourney et al., 2006). Being a long-lived and predatory species of considerable size (up to 3.6m and 350kg (Berg, 2020)), positioned at a high trophic level contributes to bioaccumulation and biomagnification of contaminants (Burreau et al., 2006; Polak-Juszczak, 2012). As demersal species, they reside on the seabed potentially exposing them to contaminants that have settled and concentrated in sediments (Baumard et al., 1998; Bellas et al., 2016; Grosvik et al., 2018; Parolini et al., 2020). It is therefore not recommended to consume the largest wild caught specimens (Maage, 2017). Atlantic halibut occupy a high position in the marine food web, however they are preyed upon by marine mammals and sharks, such as Greenland sharks (*Somniusus microcephalus*), seals and spiny dogfish (*Squalus acanthus*), in addition to being targeted by human fisheries (Cargnelli et al., 1999).

3. Objectives

To assess the potential environmental risks from the anticipated industrial developments in the Arctic, The Norwegian Institute of Water Research (NIVA) and partners from United States (Clemson University), United Kingdom (Newcastle University) and Norway (Akvaplan-niva, SINTEF and NMBU) has launched a project named "In silico and experimental screening platform for characterizing environmental impact of industry development in the Arctic (EXPECT)". The EXPECT project aims to develop more robust methods and approaches to trace the pathways of the polluting substances from emission to environment and characterise environmental impact through implementation of wholistic assessments built upon "Source To Outcome Pathway" (STOP) considerations. This project will utilize various methods to identify the main pollutants, toxicity causing factors, and vulnerable organisms associated with industrial development in the Arctic. As part of the EXPECT project, an intermediate objective is to develop a PBPK model for relevant Arctic fish species, as a toxicokinetic tool for predicting internal tissue concentrations of different environmental pollutants from external exposure concentrations and supporting development of alternative test methods that limit use of experimental animals.

The objectives of this thesis was to acquire physiological parameters necessary for the parametrization of a PBPK model for the marine fish Atlantic halibut *(Hippoglossus hippoglossus),* a predatory, large sized species inhabiting the Arctic region. An assessment of the data availability for species of commercial significance in the Arctic region, including the Atlantic halibut, will be conducted along with suggestion of a model structure capturing ADME-processes of Atlantic halibut. Additional experimental data (lipid contents, water contents and relative volumes) of the included compartments will be collected for quantification of input parameters in a PBPK model. Finally, limitations and uncertainties associated with the obtained data will be identified and recommendations for future research and parameter refinements will be proposed.

4. Materials and methods

In this thesis, an approach to characterizing physiological PBPK model parameters for an Arctic fish species, specifically Atlantic halibut, is presented. The methodology in Figure 3, was initiated by a literature review of previously published PBPK models to identify relevant parameters and model structures. A literature search was conducted to assess data availability of existing parameter values for a list of commercially relevant fish species present in the Arctic region (Appendix 1), to evaluate the possibilities for expansion to an Arctic multispecies model, as the one by Mangold-Doring et al. (2021). A criteria for the PBPK model was the inclusion of Atlantic halibut and, due to the detected limited availability of parameters for this species, (as outlined in Table 3) it became necessary to conduct experimental measurements, including tissue volumes, lipid contents and water contents, through laboratory analyses and dissections, for the required physiological parameters. This approach addresses the absence of PBPK modelling efforts for marine species in the Arctic region, in addition to flatfish species. The inclusion of Atlantic halibut was influenced by the alignment of other research objectives within the EXPECT project.

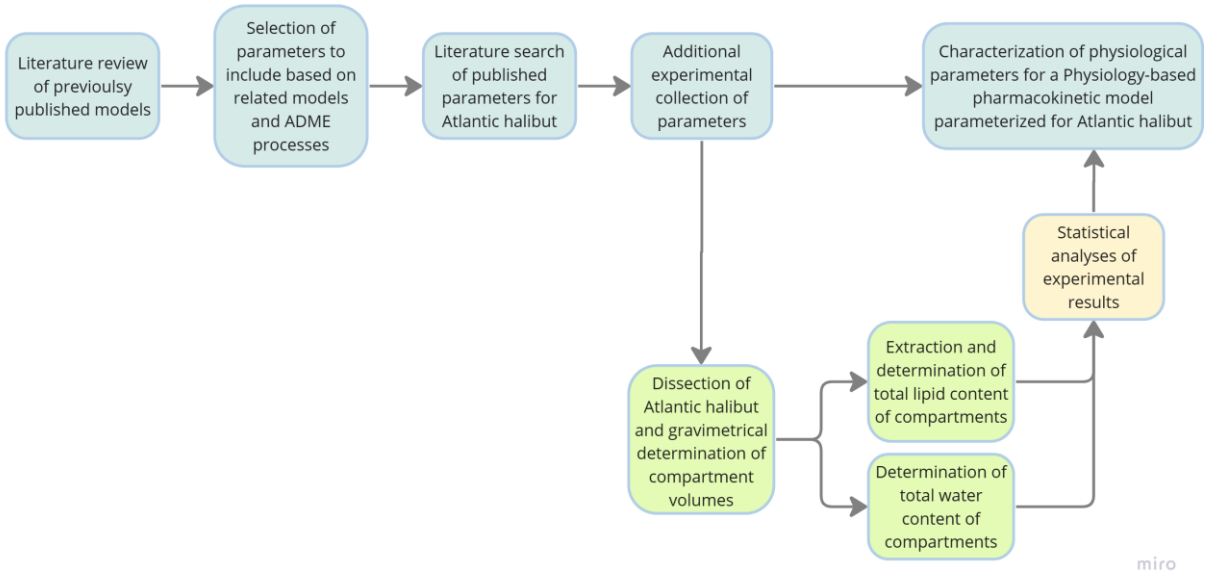

Figure 3: Flowchart illustrating the procedures conducted in the Materials and Methods section. The workflow outlines the sequential steps followed in the study for characterization of physiological parameters for a PBPK model parameterized for Atlantic halibut (Hippoglossus hippoglossus), including literature searches, sample collection, experimental work, statistical analyses and final parameter characterization. Schematic construction in Miro (2024).

4.1 Literature search

To assess the data availability of physiological parameters in existing literature for commercially relevant fish species in the Arctic region, a literature search was conducted using the Web of Science database (Web of Science, 2022). The literature search for data

availability for physiological parameters in Canadian freshwater species, conducted by Mangold-Doring et al. (2021), employing literature search strategies from a European Food Safety Authority guidance document (EFSA, 2011), was used as a template. The physiological parameters included in the initial literature search, were therefore the same as in the literature search by Mangold-Doring et al. (2021). These were relative lipid contents and volumes of tissues, blood flows to tissues and cardiac output. Additionally, oxygen consumption rates were retrieved from the FishBase database R package rfishbase (C. Boettiger, 2012) using RStudio (R Core Team, 2022). The search terms for physiological parameters included common synonyms (lipid content, fat content or total lipid). This was combined with the selection of Arctic and North Sea marine or anadromous fish (Appendix 1), including Atlantic halibut.

4.2 Conceptual model structure

The literature review of published models constructing the table of existing fish PBPK models in Appendix 2, led to the establishment of a model structure, with organs selected for inclusion. The model developed by Grech et al. (2019) based on the Nichols et al. (1990) rainbow trout model, was emphasized. The nine compartments include arterial blood, venous blood, brain, gills, liver and kidney, and three grouped compartments. The gastrointestinal tract (GIT), consisting of the stomach, intestines and pyloric caeca. Poorly perfused tissues (PPT) consist of muscle with skin, fins, bones and eventual adipose fat, while richly perfused tissues (RPT) comprise heart, gonads and spleen.

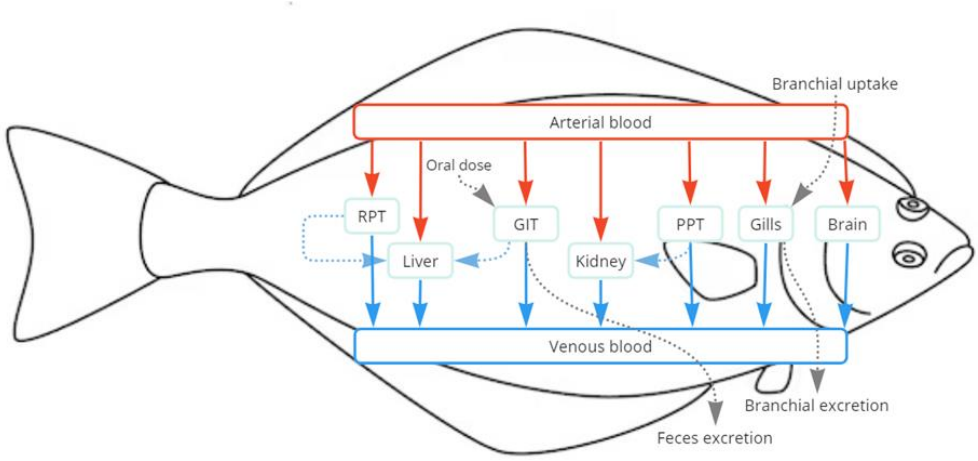

Figure 4: Conceptual model for the framework for the PBPK model parameterized for Atlantic halibut (Hippoglossus hippoglossus). Uptake and excretion routes are represented by stabled grey arrows. Arterial blood flow are red arrows, while venous blood flow is represented by blue arrows. The RPT-compartment (richly perfused tissues) consist of heart, spleen and gonads. PPT-compartment (poorly perfused tissues) consist of muscle, skin, adipose fat, fins and bones and represents the «rest of body». The illustration is constructed in Miro (2024) based on the illustration of model structure by Wang et al. (2022).

The model displayed in Figure 4, accounts for respiratory exposure with gills added as a separate compartment. Uptake is also modelled through dietary exposure and seawater drinking through GIT. The chemical is transferred to blood and distributed to the rest of the compartments by the blood flow. After exchange and metabolism, the remaining metabolites and/or chemicals are transported into venous blood and excreted through gills. Elimination also occurs through kidney and GIT, and accumulation is assumed to occur in muscle and liver (Stadnicka et al., 2012; Zhang et al., 2019). All compartments are considered well-mixed and limited by blood flow. Volume of arterial blood in fish is assumed to be 1/3 of the total blood volume (Péry et al., 2014). Arterial blood is pumped from gills towards the rest of the compartments. Venous blood from RPT and GIT is directed into the liver and a fraction of venous blood from PPT is directed into the kidneys (Nichols et al., 1990), as illustrated with dotted blue arrows in Figure 4. Therefore, the liver and kidneys receive both an arterial blood flow, and in addition a portal blood flow. In addition to its role as a metabolizing organ, Atlantic halibut deposit substantial amounts of lipids in the liver (Tocher, 2003), which makes the liver an even more interesting organ to include as a compartment, in terms of lipophilic substances.

4.3 Parameterization of physiological input parameters

The measurements obtained during the experimental collection of key physiological parameters is summarized in Table 2 and included total body weight, length, relative tissue volume, lipid content and water content. These measurements are required for estimation of PCs and scaling of extrapolated arterial blood flow. The gill compartment was described by oxygen consumption rate and effective respiratory volume and therefore not included the sampling and subsequent laboratory analysis. The relative blood volume (% of total weight) of Atlantic cod was used as proxy value (Skov & Steffensen, 2003).

Table 2: Summary of measurements obtained during sampling procedures to generate physiological input parameters for a PBPK model, accompanied by their respective units, and what organs were included in grouped compartments.

Parameter	Unit	Compartments measured	Variable name in dataset	Grouped compartments	
Total body weight	g		"total_w"	GIT:	Gastrointestinal tract; pyloric caeca, stomach and intestines
Length	cm		"length"		
Relative tissue volume	Fraction of total body volume	All compartments, except for blood	"volume_compartment"	PPT:	Poorly perfused tissued; muscle, skin, bones, fins, adipose fat
Relative lipid content	Fraction of tissue volume	Blood, brain, GIT, kidney, liver, PPT and RPT	"lipid_compartment"		
Relative water content	Fraction of tissue volume	Blood, brain, GIT, kidney, liver, PPT and RPT	"water_compartment"	RPT:	Richly perfused tissues; gonads, heart, spleen

4.3.1 Fish and culturing conditions

The Atlantic halibut was selected as a focal point for the EXPECT project, due to its presence in the Arctic, economic significance, and lengthy lifespan. To assess organ volumes and lipid content, seven halibut specimens were sacrificed at NIVA's research station in Drøbak, Solbergstrand (Norway), alongside an additional 30 halibut sourced from Sterling White Halibut's aquaculture facility at Helland, Rogaland (Norway).

The fish at Solbergstrand were collected from one single tank with a total of 9 specimen. Meanwhile, those obtained from Sterling White Halibut were derived from three different tanks with a higher density of fish, all maintained under about the same culturing conditions. All individuals from both locations were aged between two and three years. Tank temperatures were maintained at 8.5°C and water oxygen concentration ranged between 83% and 86% (9.71 to 10.06 mgO_2/l).

4.3.1.1 Sample sizes

Given the limited availability of comparable data for Atlantic halibut, the sample size was set as the default of 30 specimen (Indrayan & Mishra, 2021). Seven compartments were collected from the seven halibut specimen raised at Solbergstrand. However, due to compartment alterations after this dissection, the heart was not included in the RPT compartment, and brain sampling did not occur. Thus, there are only 30 observations for relative volume of these two

compartments. The 30 remaining individuals from Sterling Halibut were dissected into all eight tissues of the conceptual model structure (Figure 4), with the heart included in the RPT compartment. Lipid and water content determinations faced constraints on time and total volume for analysis, as both methodologies required approximately 2g of sample. Tissues with higher initial variance, such as the liver, were prioritized for measurements. The final sample sizes for each variable are listed in Appendix 4.

All individuals collected at Sterling Halibut were female, while five of the individuals from Solbergstrand were male. None of the male gonads have been measured for lipid and water content, and their relative volumes were excluded. This makes the physiological parameters specific for female specimen.

4.3.2 Relative compartment volumes – Fish dissection

In order to obtain physiological parameters required to populate the halibut PBPK model, 37 halibut specimens were dissected. Through the dissection of a sufficient number of fish, the objective was to gather accurate, species-specific and representative data on critical physiological parameters, including relative tissue volumes, lipid content and water content, as described in Table 1. This was done to facilitate more precise predictions regarding the uptake, distribution and elimination processes in Atlantic halibut.

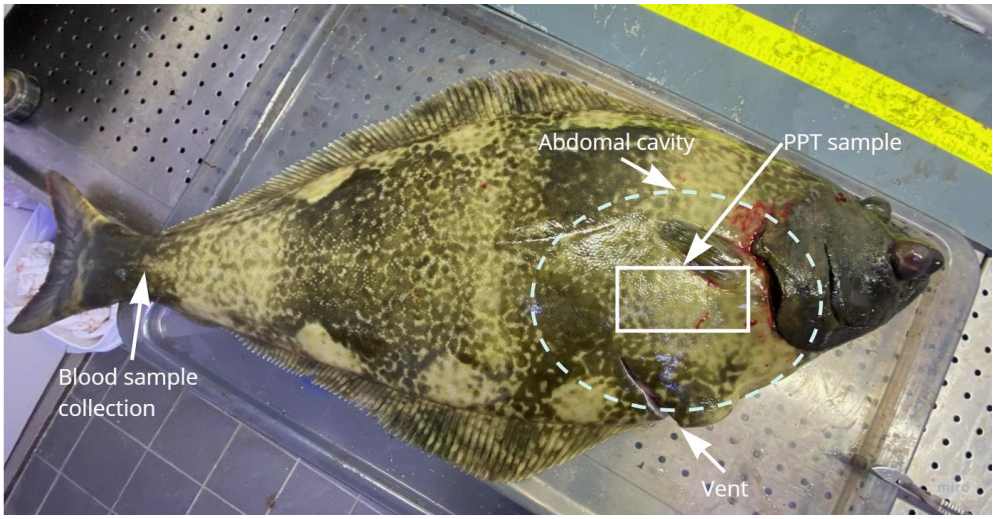

Figure 5: Locations referred to in dissection description. The blood samples were collected below the lateral line at the tail, the fish was cut from the vent along the abdominal cavity, and the PPT-sample was collected from the white marked square.

At Solbergstrand, fish were euthanized by a swift blow to the head using an aluminium tube, whereas at Helland, fish were given a lethal dose of the aquaculture tranquilizer tricaine mesylate (Pharmaq AS, Overhalla, Norway, Lotnr.619788). Initially, total weight and fork

length were recorded, followed by the extraction of 2 ml of blood from the caudal vein using a 25 Ga hypodermic needle (B. Braun, Avantor, VWR catalognr. 613-4952), positioned behind the anal fin, as illustrated in Figure 5. Then, scissors were employed to make an incision from the vent, extending along the abdominal cavity, marked in Figure 5, to expose the inner organs. Careful dissection with scissors and scalpel was performed by cutting mesenteries, the oesophagus and isolate the different tissues from one another.

The organs were grouped and weighed according to the predefined compartments for inclusion in the PBPK model (Figure 4). Larger organs were homogenized on-site with an isotonic solution (0.9% NaCl) using a grinder (OBH Nordica, Twister, China), while organs with a size insufficient for grinding were wrapped in aluminium foil, placed in zip-lock bags and stored on dried ice. Gender determination was based on visual inspection of the gonads. The head was detached with a knife to facilitate dissection of heart, brain and kidney (including head-kidneys).

The PPT- sample was extracted from the “B-section”, which represents the average composition of the rest of the edible parts of the halibut as per the sampling protocol of Institute of Marine Research (IMR), and National Institute of Nutrition and Seafood Research (NIFES) (Maage, 2017; Nortvedt & Tuene, 1998). Each of the compartments were expressed as fractions of total body weight. The volume of the PPT compartment was calculated as the difference between total body weight and the summed volumes of the other compartments (Stadnicka et al., 2012). All tissues were assumed to have a specific gravity of 1.0, hence the volume of the compartment is then equal to the measured wet weight (g) (Nichols et al., 1990). The samples were transported to NMBU (Ås, Norway) in an expanded polystyrene box filled with dried ice, where they were stored in a dark freezer (-20°C) until further analysis.

4.3.3 Random selection of samples for laboratory analysis

The samples were randomly selected for analysis by the use of the sample() function in RStudio (R Core Team, 2022). A continuous assessment of the sample prioritization was conducted, where tissues exhibiting a higher initial variance in lipid and water content were given priority, resulting in a larger sample size compared to tissues with relatively consistent lipid and water contents.

4.3.4 Sample homogenization and preparation for lipid and water content determinations

In order to access all lipids, obtain representative data for the entire compartment and optimal contact between the extraction solvent and the sample, tissue homogenization was performed prior to the lipid extraction and water content determination. The homogenized samples, initially prepared in the field with a grinder as outlined in the dissection section, were gently thawed to facilitate weighing in smaller portions. Tissues wrapped in aluminium foil in the field were partially thawed and sectioned into smaller pieces using a scalpel or knife. Liquid nitrogen (N2) was applied to enhance porosity and facilitated pulverization in a mortar. Brain and blood samples were homogenized in an ultrasonic bath (ELMA Ultrasonic T460, Germany). Blood samples were treated for 60 seconds, and brain samples were initially cut with a scalpel, then treated for 60 seconds.

The procedures for lipid extraction and water content determination originally demanded 2.0 g of sample each. For tissues that were sufficient in material (> 4.0 g), about 2.0g was weighed in 50 ml centrifuge tubes (Cellstar, Greiner bio-one, Austria), while the remaining portion was stored in zip-lock bags in a dark freezer until water content determination. For tissues with a total weight less than 4g, the initial tissue samples were reduced to yield material for both analyses. Despite this reduction, the volume of the extraction solvent was not decreased proportionally with the decrease in sample weight. This decision aimed to simplify the analysis, ensuring that the solvent remained in excess through the process.

4.3.5 Relative lipid content of compartments

A simple method for assessing the total lipid content involves an extraction and concentration to dryness for gravimetric quantification. The extraction of lipids is in essence a mass transfer from tissue to solvent. A suitable solvent to extract lipids from biological tissues should be a mix of a nonpolar organic solvent to solubilize neutral lipids, and a polar solvent, to extract the polar lipids (Saini et al., 2021). Isopropanol interferes with electrostatic forces or hydrogen bonds among proteins and lipids, and its primary function is to make lipids bound up in lipoproteins etc. accessible for extraction by cyclohexane (Pati et al., 2016). Cyclohexane facilitates the diffusion and mass transfer of lipids from cells. Additionally, the hydrophobic nature of cyclohexane contributes to the creation of a biphasic system (dos Santos et al., 2015). After the mass transfer, the cyclohexane-extract is concentrated to dryness, and lipid content can be determined gravimetrically (Allan et al., 2013).

4.3.5.1 Preparation of solvents

An extraction solvent comprising 1:1 cyclohexane (Cyclohexane, EMSURE, Millipore, Oslo), and isopropanol (2-propanol, Sigma Aldrich, United Kingdom) was mixed by combining equal volumes in a glass container inside a fume hood. Additionally, 0.5% and 0.9 % NaCl solutions were prepared by dissolving the appropriate amount of NaCl (99.5-100 %, AnalaR NORMAPUR, VWR Chemicals, Belgia) in water. To simplify logistics in field homogenization, regular tap water was used for the isotonic 0.9% NaCl solution for sample homogenization.

4.3.5.2 Extraction of total lipids from biological tissues

The methodology employed in this study was adapted from (Allan et al., 2013). An overall workflow of the procedure is illustrated in Figure 6, and numbers "(x)" in methodology refer to this figure.

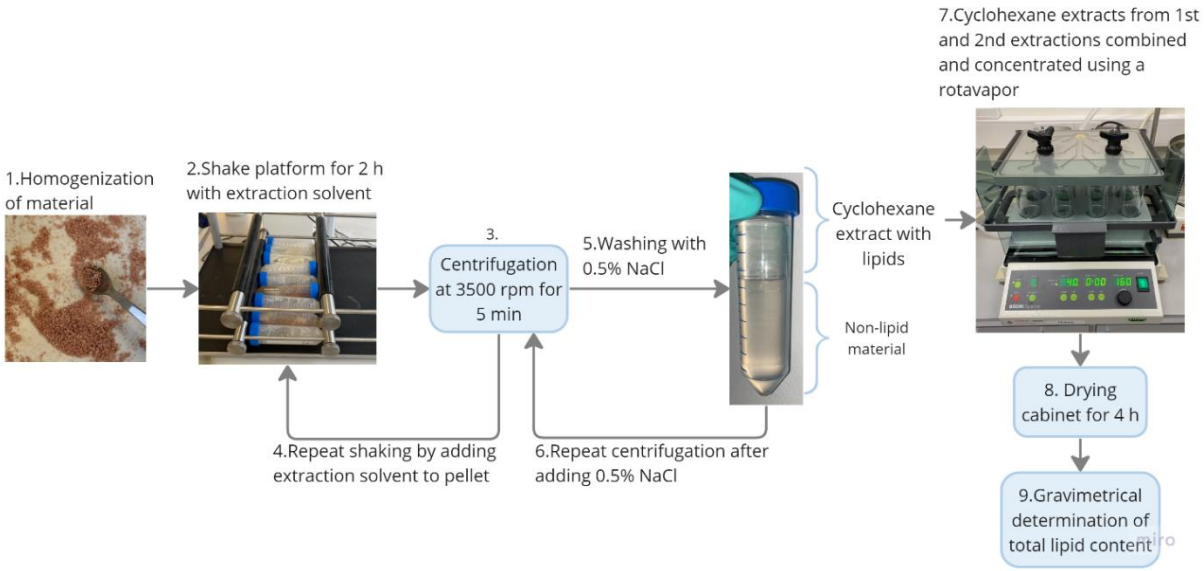

Figure 6: An overview of laboratory procedure for extraction of total lipid content using cyclohexane and 2-propanol and gravimetric determination of total lipid content in biological material. The shaking and centrifugation is repeated by adding extraction solvent to the pellet form the initial extraction. Subsequently, the cyclohexane extracts from first and second extractions are combined and concentrated using a rotavapor, before dried in a drying cabinet and gravimetrically determined. Schematic construction in Miro (2024).

To initiate the extraction process, 40 ml of the extraction solvent was added to each of the tubes. The centrifuge tubes were then positioned horizontally on a PSU-20i orbital shake platform (Grant Instruments, Grant bio) at 200 rpm for 2 hours in room temperature (2). Following this, the samples underwent centrifugation using an Eppendorf Centrifuge (Eppendorf™ Model 5430R Microcentrifuge) at 3500 rpm and 20°C for 5 minutes (3), after which the supernatant was decanted into fresh 50 ml centrifuge tubes. The extraction was then repeated by adding an additional 30 ml of the extraction solvent to the pellet, followed by a

further 2 hours of shaking and 5 min centrifugation (4, 3). The supernatants from each of the extractions were added 10 ml of the 0,5% NaCl solution (5), followed by another round of centrifugation at 3500 rpm for 5 minutes (6).

The uppermost layer of the cyclohexane-extract from the washing with 0.5% NaCl solvent of the first and second extraction was then combined and transferred with a 0.5-5ml automatic pipette (Thermo Scientific™ Finnpipette™ F1 Variable Volume, US) to a glass tube (250ml, Büchi Syncore™, Switzerland) for evaporation in a rotavapor (Büchi Syncore™ Polyvap/Analyst 250ml Parallel Evaporation Concentrator, Switzerland) with a vacuum controller (Büchi Syncore™, V-855, Switzerland). The rotavapor was set to a cyclohexane program of 90 ppm and 40°C, and with a rotation of 160 rpm for about 1.5 hours (7).

Upon reaching near dryness, the remaining liquid was transferred to pre-weighed disposable weighing boats (VWR, temperature resistant > 70°C) using a 1000µl automatic pipette (Thermo Scientific™ Finnpipette™ F2 Fixed Volume, US). Both the pipette tip and Büchi tube were rinsed with a few drops of extraction solvent, ensuring transfer of any residual material to the weighing boat. The weighing boats were placed in a drying cabinet (Binder FDL 115, Germany) set at 60°C for 4 hours, or overnight (8). The samples were weighed then placed 1 hour in the same cabinet environment, before they were re-weighed. This was repeated until the weight was stable within a tolerance of ±0.005g, when the total lipid fraction of the tissue wet weight was calculated (9).

4.3.6 Relative water contents of compartments

In biological systems, water serves not only as a passive solvent, but also actively interacts with chemicals, thereby potentially influencing their stability and flexibility. About 80% of biological material is water (Privalov & Crane-Robinson, 2017). Incorporating the relative water content of tissues into the estimation of PCs accounts for the polar aquatic tissue constituent, alongside lipids which represents the non-polar fraction (Salmina et al., 2016). A commonly employed method for quantifying water content is through measurement of dry tissue weight (Lantry & O'Gorman, 2007).

About 2 g was placed in pre-weighed weighing boats and set in a drying cabinet at 60°C for 96h as described as the optimal temperature for drying of fish tissues by Lantry and O'Gorman (2007). The tissues were weighed, then placed back into the cabinet for 1 hour and re-weighed until the weight was stable (±0.005g). The total water content was estimated as the difference between wet and dry weights of tissue.

The added percentage of physiological saltwater (0.9% NaCl) to facilitate homogenization was then calculated and subtracted according to equation 1 and 2.

$$\text{Added percentage (\%)} = \frac{\text{Added solution (g)}}{\text{Wet weight of tissue (g)}} \text{ x } 100 \quad \text{(Eq1)}$$

$$\text{Water content (\%)} = \left(\frac{\text{Weight of sample(g)} - \text{dry weight(g)}}{\text{Total weight ofsample(g)}} \text{x} 100\right) - \text{added percentage (\%)} \quad \text{(Eq2)}$$

4.3.7 Extrapolated values and proxies

Physiological parameters that were not feasible to directly obtain, or access from existing literature were extrapolated from other species or proxies were used for halibut data. The literature search for physiological parameters conducted in Materials and Methods section 4.1, resulted in no values for cardiac output, oxygen consumption or blood flows to compartments measured in Atlantic halibut. Therefore values from similar species, preferably marine flatfish species were used. Compartment fractions of arterial blood flow were measured in rainbow trout and estimated for Atlantic halibut by assuming proportionality between blood flow and organ volume (Grech, 2018). A detailed description of blood flow estimation is presented in Appendix 7. The blood flows (% of cardiac output) were calculated as fractions of cardiac output quantified in winter flounder *(Pleuronectes americanus)* at 10 ℃ of 15.5 ml/min/kg by Joaquim et al. (2004). The fractions of arterial blood flow measured in rainbow trout was sourced from the study by Barron et al. (1987), as done in several existing PBPK models (Grech, 2018; Nichols et al., 1990; Stadnicka et al., 2012). For the relative volume of blood, a value recorded in Atlantic cod *(Gadus morhua)* of 5.1 % was used (Skov & Steffensen, 2003).

Oxygen consumption rates were collected from the FishBase database, from the available marine flatfish species in the R-package rfishbase (C. Boettiger, 2012). Data was filtered to measurements obtained with no applied stress and standard metabolic rate. This resulted in a mean value drawn from European plaice (*Pleuronectes platessa*), lemon sole (*Microstomus kitt)*, common dab (*Limanda limanda*) and American plaice (*Hippoglossoides platessoides*), as elaborated in Appendix 3. The effective respiratory volume is estimated as a function of body weight, water temperature and concentration of dissolved oxygen in the inspired water, as described in Stadnicka et al. (2012) and in equation 3 (Eq3)

$$\text{Effective respiratory volume: } \frac{\dot{V}O_2}{C_{OX} - 0.2 * C_{OX}} * \text{Total weight(kg)}^{0.75} \text{ , L/h} \quad \text{(Eq3)}$$

where $\dot{V}O_2$ is the oxygen consumption rate (mlO_2/h) and C_{OX} is the dissolved oxygen concentration in inspired water (mgO_2/ml).

4.4 Statistical analysis

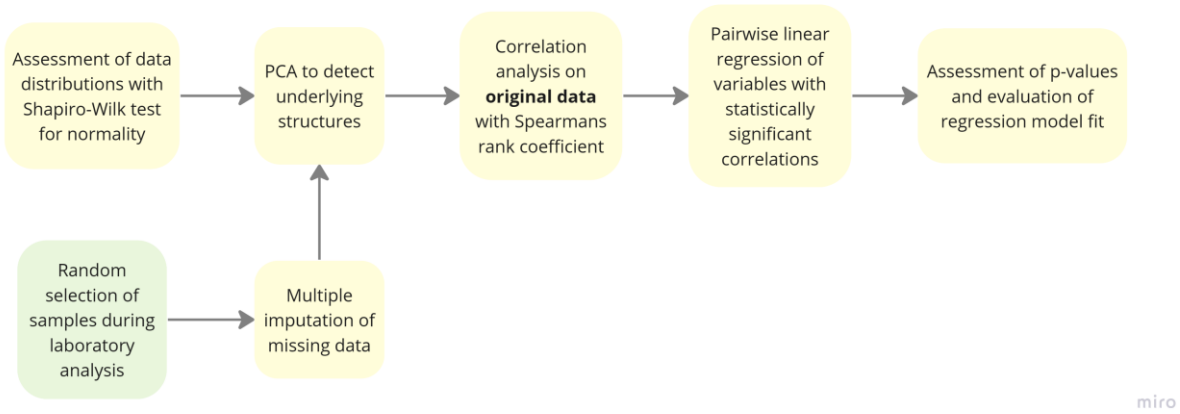

*Figure 7: Schematic workflow constructed in Miro (2024) of statistical analysis conducted to determine the most appropriate representation of measured tissue volumes and lipid and water content as input parameters in a physiology-based pharmacokinetic (PBPK) model developed for Atlantic halibut (*Hippoglossus hippoglossus*).*

The purpose of the statistical analysis was to determine the most appropriate characterization of the measured physiological parameters as input in a PBPK model. This involved investigating whether the mean value captured the tendency in the data, or if a more detailed description was necessary. The analysis aimed to explore potential correlations between physiological parameters (e.g., total weight, length, etc.) and how they affected organ volumes, lipid and water contents. By examining these relationships, the role of individual factors, interdependencies and the need for adjustment and predictions to provide input values to the PBPK model was assessed.

4.4.1 Software

In this study, R programming language (R Core Team, 2022) was employed for all data manipulation and statistical analysis. The graphical illustrations were constructed by using the ggplot2 package (Wickham, 2016), and put together with commands from the gridExtra package (Auguie, 2017). The dplyr package was employed for data manipulation (Wickham, 2023).

4.4.2. Assessment of data distributions

As part of the explorative data analysis, assessment of distributions of all variables was conducted to ascertain whether the normal distribution of either the original data or the log-transformed data adequately characterized the variable. This analysis helped gain insight of the suitability of the mean and standard deviation of each measured variable as an input parameter in a PBPK model.

4.4.2.1 Shapiro-Wilk test

A Shapiro-Wilk test provides a formal method for assessing whether sample data is likely to have been drawn from a normal distribution. This test is particularly suitable for small sample sizes. Essentially, it serves as a goodness-of-fit test to evaluate how closely the sample data is to a normal distribution (Monter-Pozos & González-Estrada, 2024). This was performed using the shapiro.test() function in RStudio. This test examines the degree of similarity between the sample data and a normal distribution. The null-hypothesis is that the sample is drawn from a normal distribution, while the alternative hypothesis suggests otherwise. If the p-value from the test is less than the chosen significance level (p-value < 0.05), then the null hypothesis can be rejected. This indicates that the sample data deviates significantly from a normal distribution and should not be considered normally distributed.

4.4.3 Principal component analysis (PCA)

Given that Principal Component Analysis (PCA) is sensitive to missing values (Jolliffe & Cadima, 2016), imputation techniques were employed to estimate missing values based on the available information within the dataset. With the missMDA package (Josse, 2016), missing values are imputed based on the observed data patterns. Due to this, the underlying structure of the dataset is preserved, but the robustness of the statistical analysis is enhanced by removing the uncertainty of missing data.

To ensure uniform contributions of variables to the PCA, the imputed data was scaled. Subsequently, correlation analysis was conducted on the scaled data to compute a correlation matrix. PCA was then preformed on the correlation matrix to identify patterns and structures in data. The original values were transformed into linear combinations of the original variables to capture the maximum variance in the data and identifying the most significant features driving variation (Jolliffe & Cadima, 2016). Multiple imputation was only employed to preform PCA, while the following statistical analyses were conducted using the original, measured data.

4.4.4 Correlation analysis

The initial dataset underwent preprocessing, specifically, the column listing the relative volume of blood (“volume_blood”) was removed, due to constant values, which could potentially interfere with subsequent analyses. To quantify the relationships between all 23 variables, correlation analysis was performed on the original dataset, using the rcorr() function from the Hmisc package (Harrell, 2023). Spearman’s rank correlation coefficient was

employed, which is based on the relative rank of observations, making it suitable for data with outliers (Sedgwick, 2014). The focus was then aimed on identifying significant correlations by filtering the p-values from the correlation analysis to retain only those below the threshold of 0.05, which indicates statistical significance. The p-value for each of the correlation coefficients was utilized to determine which relationships were necessary to quantify with pairwise linear regression models.

4.4.5 Pairwise linear regression

For the inspection of parameter-relationships, pairwise linear regression analysis was implemented for each of the combinations of variables with significantly relevant correlation coefficients. The linear regressions were fitted using the lm() function in RStudio (R Core Team, 2022), with one variable of the correlation as the response variable and the other as the predictor variable. The p-values were extracted to evaluate the statistical significance and R-squared (R^2) was calculated to assess the proportion of variance in the response variable explained by the predictor variable. Furthermore, to ensure robustness of the analysis, number of combined observations with both variables was counted as sample size. Regression models that yielded a low p-value (<0.05) and a high R^2 were inspected with a scatter plot visualization. The observed and predicted values were plotted against each other using ggplot2, with regression lines displayed to compare observed and predicted values

Evaluation of the regression models included an examination of residual distribution through QQ-plots and histograms (exemplified in Appendix 6.1), alongside metrics such as R^2, correlation coefficients, p-values and sample sizes. Practical considerations were crucial when selecting predictor variables for regression models to aid in characterizing physiological parameters for a PBPK model, in line with the objective of minimizing animal experimentation in environmental and human risk assessment.

5. Results

This study was focused on characterizing essential physiological parameters required as input for a PBPK model tailored for Atlantic Halibut. Due to limitations in available data in open literature and databases, the key parameters lipid content, water content and tissue volumes were obtained from farmed fish. Subsequently, the data obtained from these procedures was then processed and examined to assess their suitability to parametrize an Atlantic halibut PBPK model.

5.1 Data availability of physiological parameters from commercially relevant fish species in the Arctic region

The literature search for physiological parameters for the list of relevant Arctic fish (see Appendix 1) primarily yielded data om lipid content in muscle and liver, as well as the volume of gonads and liver. These values exhibited a high degree of variability, as displayed by mean values and standard deviations in Appendix 1.2, likely due to the mix of species, diverse environmental and culturing conditions, including variations in diet, and the mix of wild and farmed specimens. Much of the research identified was related to aquaculture studies investigating the impact of different diets. Collected data from the literature search is available at Zenodo (https://doi.org/10.5281/zenodo.11184933). Additionally, the availability of published values for Atlantic halibut, summarised in Table 3, mostly contained data on lipid content in muscle, from reports by the Norwegian Institute for Marine Research (IMR).

Table 3: A summary of the values available for Atlantic halibut *(Hippoglossus hippoglossus)* collected during a literature search conducted in the Web of Science database, along with searches in local databases at Norwegian Institute of Marine Research and Norwegian Institute for Water Research.

Available published data for Atlantic halibut (Hippoglossus hippoglossus)

Tissue	**Relative lipid content**			**Relative volume**	**Blood flow** (% of cardiac output)	**Cardiac output** (ml/min/kg)	**Oxygen consumption rate** (mlO2/min/kg)
	Mean	**sd**	**n**				
GIT	0.0014	NA	1	NA	NA	NA	NA
Liver	0.24	NA	1				
Muscle	0.045*	0.0037	200				

*Data retrieved from the Norwegiean Institute of Marine Research. Collected from wild fish as part of environmental surveillance programs.

5.2 Data distributions of sampled physiological data

In order to evaluate the distributions of essential variables, an initial data exploration was conducted. This laid a foundation for subsequent analyses, providing insights into the underlying characteristics and structures of the dataset available at Zenodo (https://doi.org/10.5281/zenodo.11186574 , *"Measured_data_Atlantic_halibut"*)

5.2.1 Distribution of data and normality test

An assessment of data distribution and a Shapiro-Wilk test for normality was conducted. This was performed for the relative volumes, lipid contents and water content for seven PBPK model compartments (Figure 4, Materials and Methods section 4.2), except for the relative volume of blood, which was a constant value.

5.2.1.1 Relative volume of physiological compartments

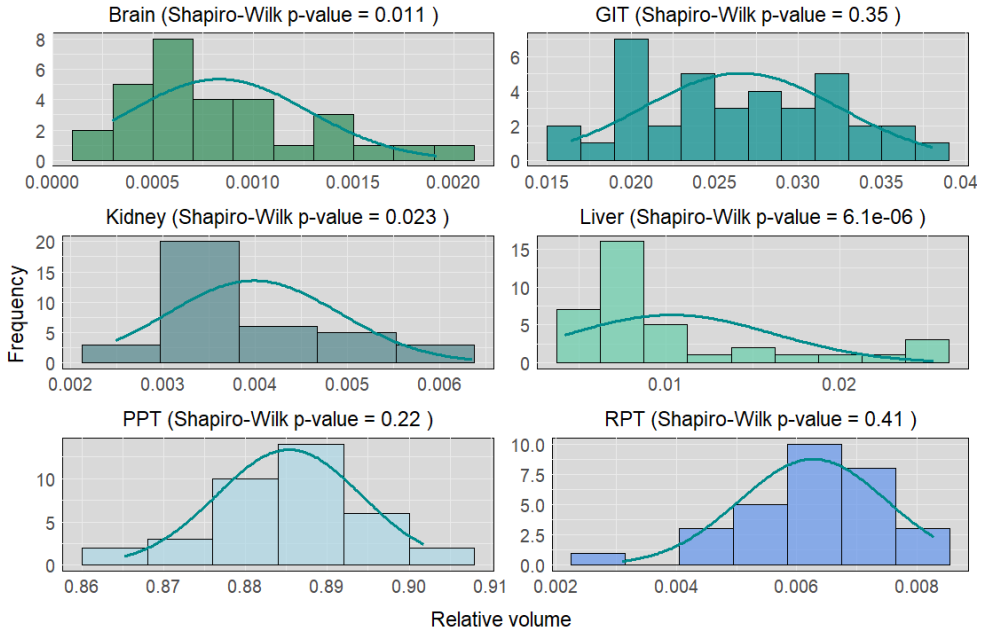

Figure 8: The distributions of relative volumes for six physiological compartments with an added density curve displaying the data fit to normality. In addition, the p-value from a Shapiro-Wilks test assessing normality is provided, where values above 0.05 indicate that data exhibit a normal distribution along the mean (average).

The relative volumes of physiological compartments, initially calculated from tissue wet weights exhibit a heterogenous pattern across compartments. Specifically, the distributions of brain, kidney and liver appear to be left-skewed, while RPT shows a weak right-skewness. In contrast, the distributions of PPT and GIT display a relatively balanced distribution. Normality testing using Shapiro-Wilk test confirmed that three of the six compartments (GIT, PPT and RPT) followed a normal distribution.

5.2.1.1 Relative lipid content

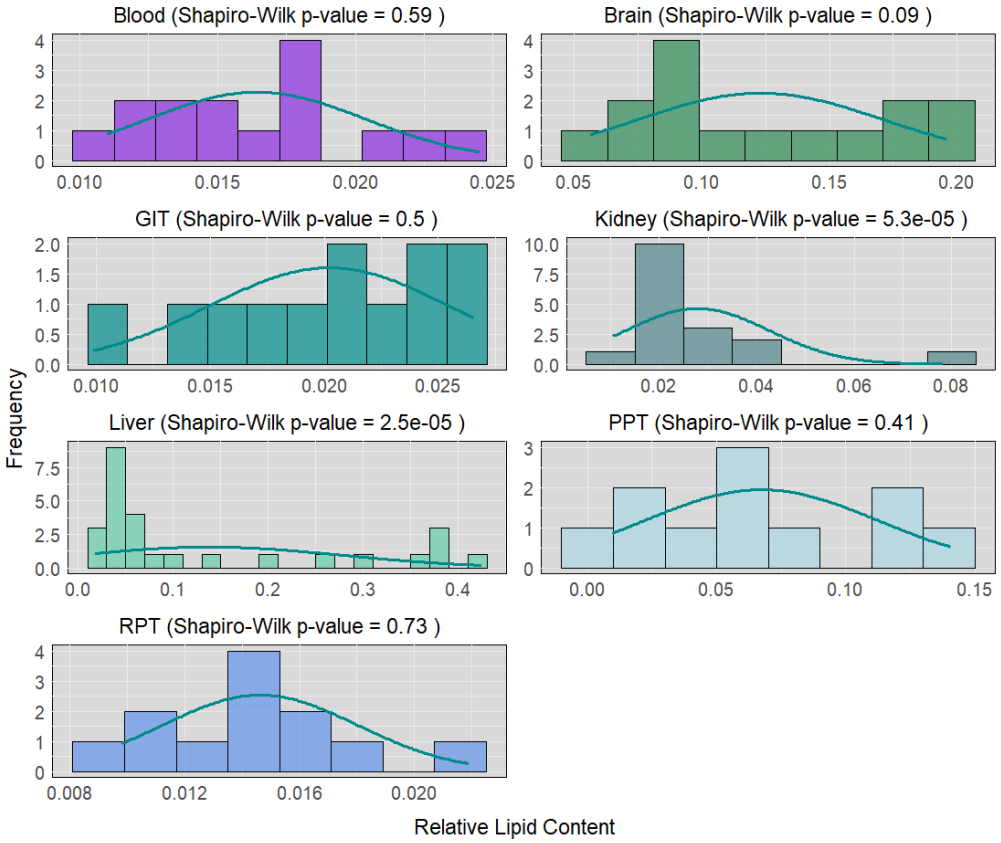

Figure 9: The distributions of relative lipid contents for seven physiological compartments with an added density curve displaying the data fit to normality. Shapiro-Wilks test provides a p-value, indicating whether the distribution is likely drawn from a normal distribution, with a value exceeding the significance level of 0.05 suggesting normality.

The distributions of relative lipid content, calculated as fractions of tissue wet weights, reveal varying patterns across compartments. Three compartments (RPT, PPT and blood) exhibit balanced distributions, while brain, kidney and liver distributions appear left-skewed. Additionally, the GIT data distribution shows a slight right-skewness. Normality testing of these distributions confirmed normality for five out of seven compartments (blood, brain, GIT, PPT and RPT), while liver and kidney distributions deviated from normality.

5.2.1.2 Relative water content

Repeating the process for relative water, calculated as fractions of tissue wet weights, revealed another set of distributions.

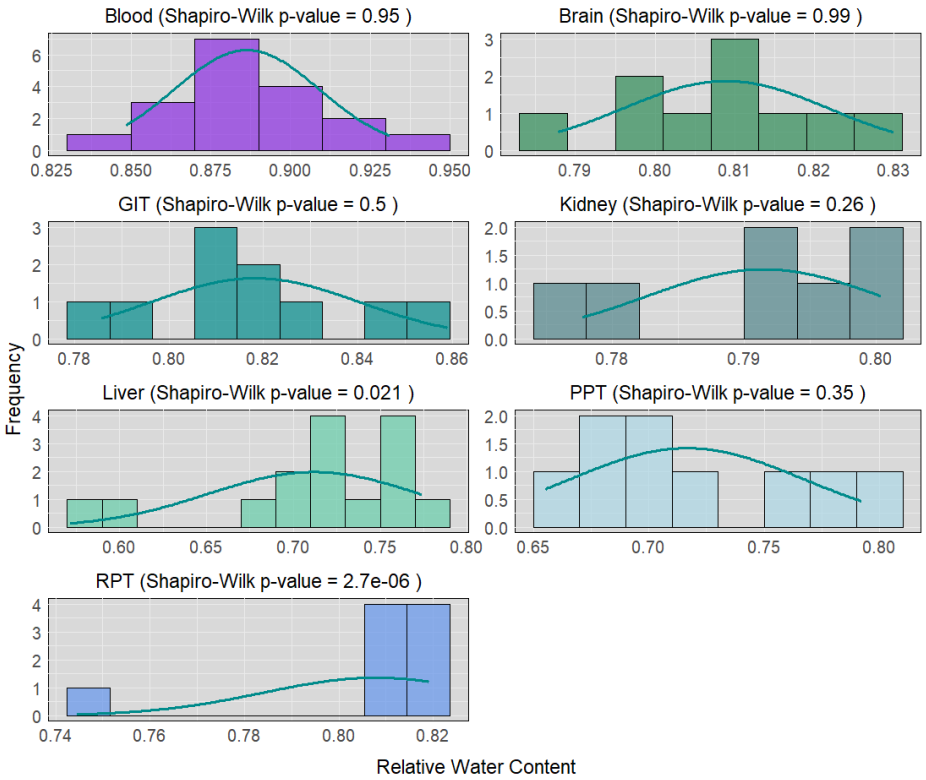

Figure 10: The distributions of relative water content in the analysed tissues. The line is added as a fitted normality curve and the p-value from a Shapiro-Wilks test assessing normality provided in the title, where values above 0.05 indicate that data exhibit a normal distribution along the mean (average).

Upon visual inspection, the distributions for blood, brain and GIT exhibit a relatively bell-shaped curve. Meanwhile, kidney and liver distributions skew to the right and PPT to the left. RPT appears to be right-skewed with an apparent outlier to the left. Shapiro-Wilk tests confirmed normal distributions for five out of seven compartments (blood, brain, GIT, kidney and PPT), whereas liver and RPT did not adhere to a normal distribution.

5.3 Principal component analysis (PCA)

To gain insight to the underlying patterns and structures within the dataset, a Principal Component Analysis (PCA) was conducted from imputed data and illustrated by a PCA variable plot (Figure 11). To mitigate potential errors caused by missing data, imputed data was employed to gain insight in potential patterns present in the original dataset.

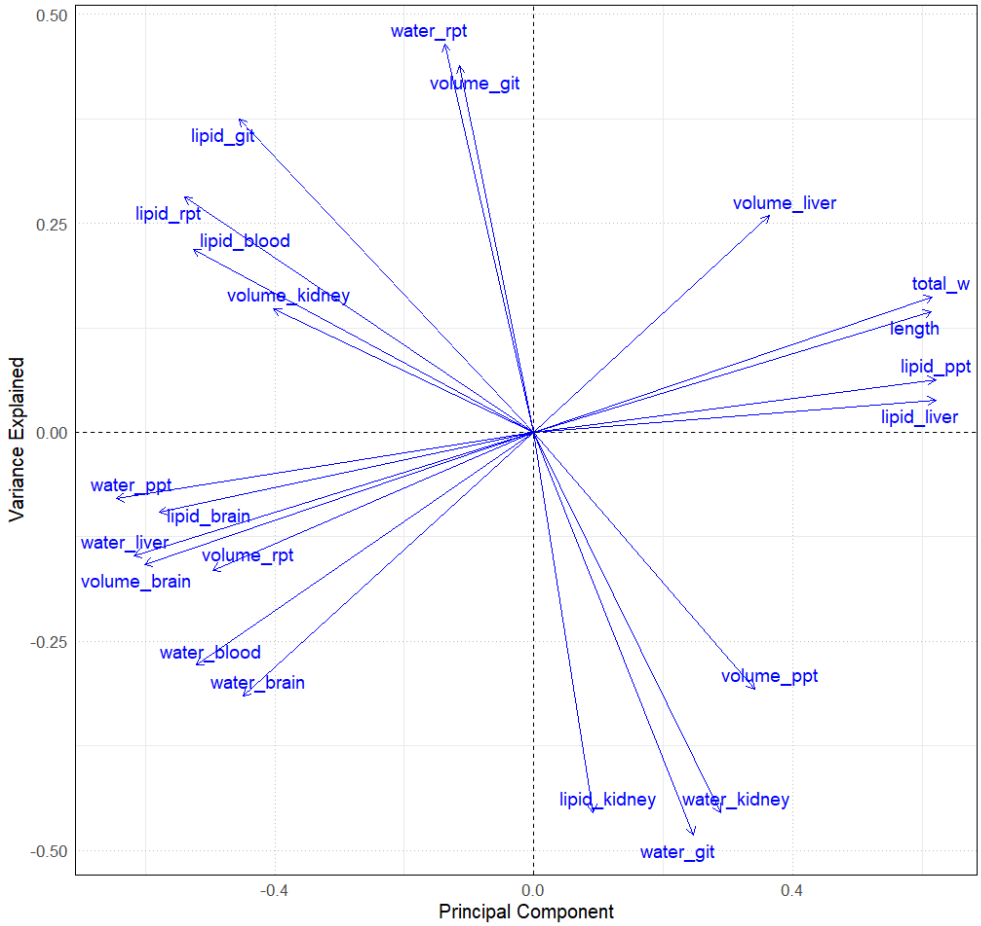

Figure 11: A PCA variable plot to provide visual representation of the relationships between the original variables and the principal components generated by the PCA. The arrows represent different variables and point along the direction of maximum variance in the dataset. Variables pointed in the same direction are considered positively correlated, while variables pointing in opposite directions are considered negatively correlated. Explanation abbreviations: lipid_compartment i (e.g. lipid_brain, where i = brain, is the relative lipid content of brain), water_compartment_i (e.g. water_brain, where i = brain is the relative water content of brain), volume_compartment i (e.g. volume_brain, where i = brain, is the relative volume of brain). The relative volume of brain was removed prior to the PCA analysis, due to constant values. Imputed data available at https://doi.org/10.5281/zenodo.11186574, sheet: "Imputed_data_for_PCA".

In the PCA variable plot (Figure 11), there appears to be four clusters. Total weight and length display a clear positive correlation, co-clustered with the lipid content of liver and PPT. The cluster housing total weight encompasses variables likely positively correlated with fish size, whereas those clustering in the opposite direction represent variables negatively correlated with total weight. The remaining two clusters, positioning right-angled to these, appear independent to total weight. Notably, the water content of the kidney shows a positive correlation with the lipid content of kidney, which are slightly negatively correlated with the volume of kidney. Four of the compartments (liver, GIT, RPT and PPT) exhibit a consistent pattern, with variables describing volume and lipid content aligning in one direction, while water content of the same compartment aligning oppositely. In the case of brain, volume, lipid content and water content values converge within the same cluster.

The majority of variables are clustered around 0 to 0.25 and -0.25 of variance explained, which indicates moderate contributions to the principal components. However, some arrows extend all the way to 0.5 of variance explained. These correlations are well illustrated by a heatmap of the PCA correlation matrix in Appendix 5, and a visual inspection of this heatmap confirms the indication of correlations in the PCA variable plot in Figure 10. To better understand the relative importance of each principal component in capturing variance withing the dataset, a scree plot was constructed in Figure 12.

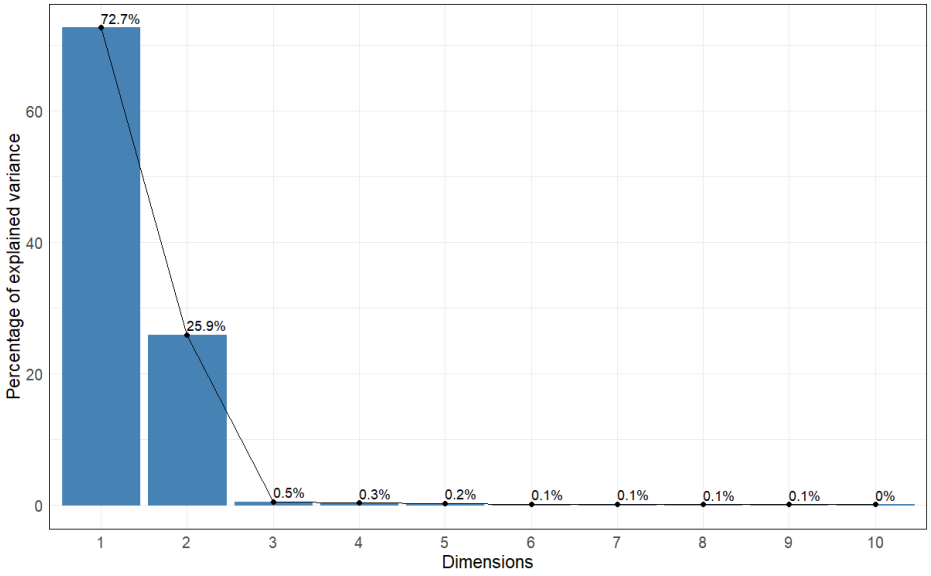

Figure 12: Scree plot illustrating the percentage of explained variance by each principal component in the PCA analysis. Eigenvalues are plotted against the number of principal components. The plot shows the portion of variance explained as we move from the first to the last principal component. According to this, about 99% of the variability in the data is captured by the first two principal components.

The scree plot of the PCA in Figure 12 reveals that principal component 1 (PC1) accounts for a substantial proportion of the total variance, explaining 73% of the variability in the dataset, thus capturing the most significant patterns or correlations between variables. Additionally, principal component 2 (PC2) contributes to 26% of the total variance, enhancing the explanation of underlying data structures. Together, the first two dimensions collectively explain 99% of variance, indicating that these two components effectively summarise the relationships between variables. The minimal contribution of subsequent principal components suggests that including them in the analysis may not yield substantial insights. This suggest that despite the original data having high dimensionality, a large portion of its variability can be explained by a smaller set of underlying factors.

5.4 Correlation analysis

A correlation analysis was performed on the original dataset, and revealed relationships among various variables within the dataset.

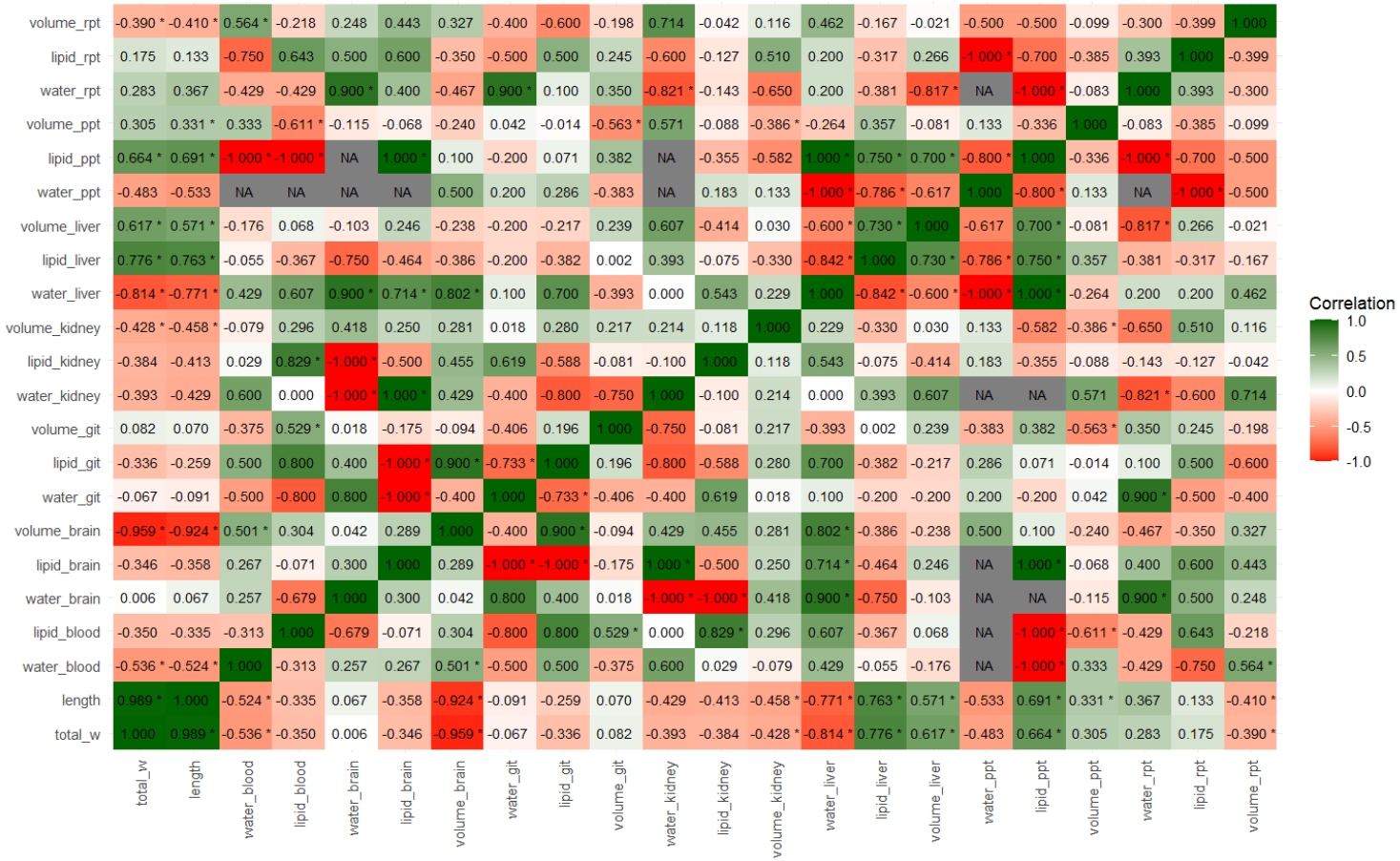

Figure 13: Heatmap of correlation coefficients between pairs of variables. Each cell in the heatmap represents the correlation coefficient between two of the variables on the x- and y-axis. The colour intensity indicates the strength of correlation. The colour scale ranges from red (negative), to green (positive). White cells indicate no correlation. The statistically significant correlations (p-value <0.05) are marked with a «» notation. Raw data of correlation coefficients and all p-values are available at (https://doi.org/10.5281/zenodo.11186574, sheet: "Corr_coeff_original_data" and "p_values_correlation_org_data")*

Symmetry along the diagonal is maintained, ensuring consistency in variable relationships. Notably, variables demonstrate equivalent correlations with both total weight and length, which again are strongly correlated. Weak correlations (correlation coefficient of -0.3 to 0.3) lack statistical significance and are not marked with "*". Furthermore, stronger correlations (-0.8 > rho and rho > 0.8) that are statistically significant include the relationship between total weight and brain volume, as well as liver water content and between length and brain volume. Within individual compartments, RPT, PPT, liver, GIT and brain exhibit recurring patterns of two similar and one opposing correlation, between their physiological parameters and both total weight and length (for instance volume and lipid content of PPT are positive, while water content of PPT is negative).

5.5 Pairwise linear regressions

Pairwise linear regression was conducted for variables exhibiting statistically significant correlations. Additionally, the regression models relevant for the characterization of physiological parameters were tested by predicting the values of the response variable, using the predictor variable in Figure 14. A linear regression of the relationship between length and total weight was also added, as the relative volume of the kidney was best explained by length. All statistically significant linear regression models for all correlating variables are listed in Appendix 6.

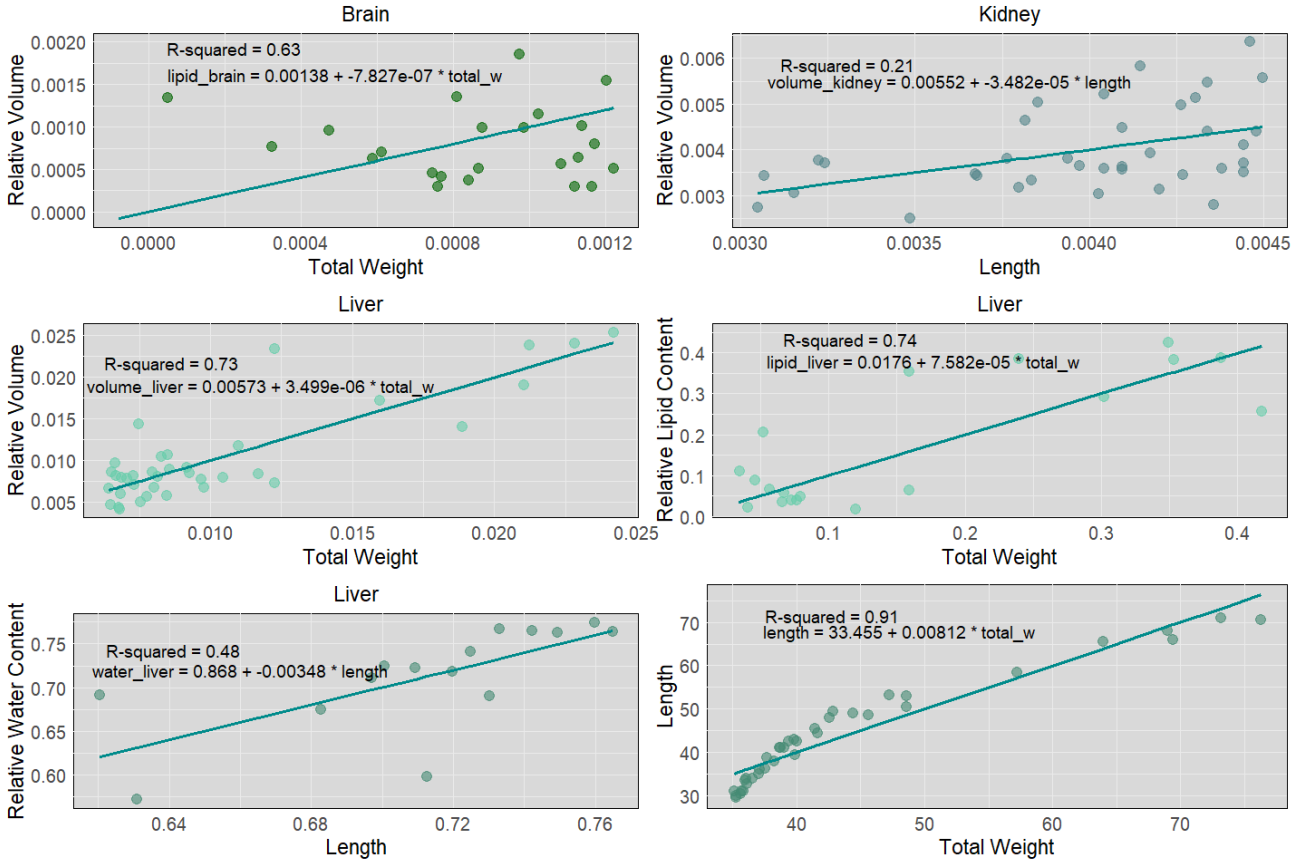

Figure 7: Values predicted from linear regressions displayed as lines and observed values represented by points. Each of the plots are visualizations of a linear regression. The tissue of interest is noted in the title and variables along the x- and y-axis of the individual plots.

Upon examination of the regression lines displayed in Figure 13, it becomes evident that the regression equations for the relative volumes of brain and kidney yield predictions of both higher and lower values than the actual observations. The regression line predicting the relative volumes of the liver emerges as one of the more accurate predictors, with occasional deviations. The regression equations for the relative lipid content of the liver consistently underestimates the observed values. A similar trend is observed in the case of the relative water content of the liver, with the majority of observed values surpassing the predicted values.

The lipid content in kidney and water content in RPT exhibit non-normal distributions (Figure 8 and 9) and lack statistically significant correlations with any of the measured variables (Figure 13). Due to the limited sample size, the means of these variables were utilized as input parameters, despite of the inability to explain their underlying structures and trends.

5.6 Summarising the physiological parameters of an Atlantic halibut PBPK model

The main objective of this study was to characterize the physiological parameters needed for the development of a PBPK model for arctic fish species. Throughout the methodology (Figure 3), data on said physiological parameters have been collected from published literature or measured. For parameters in need of more invasive methodologies, as cardiac output and oxygen consumption rate, data from related species was used as proxies. The correlation and regression analyses resulted in a set of linear regression models that can be employed to calculate physiological parameters. As total weight and length are correlated, the length may also be measured to calculate the remaining input parameters. In Table 4 and 5 there is an example of the use of the equations with the values calculated for a fish of 500g. The regression equations employed in the characterization are marked in bold in Appendix 6.

Table 4: General physiological input parameters estimated for Atlantic halibut. Effective respiratory volume is calculated with dissolved oxygen concentrations of 9.89 mg/L, as a mean value of the observed concentrations at the Sterling Halibut aquaculture facility at 8.5℃.

General physiological parameters				
Parameter	**Input value**	**Unit**	**Calculation or proxy species**	**Source**
Cardiac output	15.5	ml/min/kg	Winter flounder	(Joaquim et al., 2004)
Oxygen consumption rate	44.74	mgO2/h/kg	Average standard value of flatfish species available from FishBase (Appendix 3)	R-package rfishbase (C. Boettiger, 2012)
Effective respiratory volume	3.36	l/h	Calculated from oxygen consumption rate and water oxygen concentration by Equation 3.	(Stadnicka et al., 2012)

Table 5: Compartment-specific input parameters for the parameterization of a halibut PBPK model for a female fish of 500g.

Compartment-specific physiological parameters						
Parameter	**Unit**	**Compartment**	**Input value**	**sd**	**Calculation**	**Source**
Relative volume	Fraction of total wet weight	Venous blood	0.00171		1/3 of total blood volume (5.10 %)	Values for Atlantic cod (Nichols et al., 1990; Skov & Steffensen, 2003)
		Arterial blood	0.00340		2/3 of total blood volume (5.10 %)	
		Brain	0.000989		Regression equation	Dissection (Section 4.3.2.1)
		Kidney	0.00421			
		Liver	0.00747			
		RPT	0.00628	0.00123	Average	
		GIT	0.0266	0.00587		
		PPT	0.885	0.00885	Total weight – (collective volume of the rest of the measured compartments)	
Relative lipid content	Fraction of tissue wet weight	Blood	0.0164	0.00395	Average	Lipid extraction (Section 4.3.5)
		Brain	0.123	0.0481		
		GIT	0.0202	0.00523		
		Kidney	0.0279	0.0147		
		PPT	0.0673	0.0451		
		RPT	0.0146	0.00340		
		Liver	0.0555		Regression equation	
Relative water content	Fraction of tissue wet weight	Blood	0.886	0.0228	Average	Water content determination (Section 4.3.6)
		Brain	0.809	0.0128		
		GIT	0.818	0.0221		
		Kidney	0.791	0.00897		
		PPT	0.808	0.0238		
		RPT	0.717	0.0507		
		Liver	0.739		Regression equation	
Blood flow	ml/min	Brain	0.0282		Assumed proportionality with tissue volume and calculated from % of cardiac output outlined in Appendix 7	Values for fractions of cardiac output measured in Rainbow trout (Barron et al., 1987; Grech, 2018)
		GIT	0.366			
		Kidney	0.351			
		Liver	0.0627			
		RPT	6.910			
		PPT	0.0316			

6. Discussion

This thesis outlines an approach to characterizing physiological parameters for the development of a physiologically based pharmacokinetic model for an Arctic fish species, the Atlantic halibut. The workflow (Figure 3) began with a literature review of existing PBPK models to identify essential parameters and compartments, which resulted in a suggested conceptual model structure for a halibut model in (Figure 4) and the physiological parameters in Table 3. The characterization of physiological parameters necessitated experimental collection of tissue volumes, relative lipid contents and water contents through a combination of sampling of fish and tissues at an aquaculture facility (fish farm) and dedicated laboratory analyses. These steps enabled the integration of both literature-derived and experimentally obtained values to provide parameters for a halibut PBPK model. In the following subsections, individual approaches undertaken in the workflow, the model limitations, alternative approaches, advantages of the model and future directions are discussed.

6.1 Data availability of physiological parameters in literature

The available data on physiological parameters collected during the literature and database searches, was mostly related to food quality or safety, therefore focusing predominantly on tissues intended for consumption, particularly muscle and liver. Species commonly utilized by aquaculture and fisheries, such as Atlantic salmon *(Salmo salar)* and Atlantic cod (*Gadus morhua*) were extensively represented in the available literature, likely due to their significance in food quality and human safety considerations, as well as a widespread availability (see Appendix 1.2). Their prevalence in aquaculture facilitates easier access for scientific studies.

6.1.1 Physiological parameters requiring invasive methodologies

The availability of physiological parameters in existing literature and databases varied across parameters. For instance, a study of blood flow or cardiac output involves more invasive methods (Axelsson & Fritsche, 1991; Iversen et al., 2010) and requires detailed animal use protocol applications. Consequently, there are fewer published articles on these physiological parameters, compared to lipid contents and tissue volumes, where there is no need for surgical procedures. No values for cardiac output and blood flows were published for Atlantic halibut, leading to the use of related species, preferably marine flatfish as proxies for data.

6.1.2 Differences of individual compartments

The coverage of data for lipid content in tissues relevant for consumption surpasses that of tissues of more ecotoxicological interest, such as the kidneys and brain. As listed with average values for tissues in Appendix 1.2, only two observations were uncovered for the relative lipid content of brain measured in Arctic char *(Salvelinus alpinus),* and Atlantic herring (*Clupea harengus*) and further two observations for the relative volume of brain across relevant Arctic species measured in Atlantic salmon (*Salmo salar*). Considering the brain's importance for neurotoxicological studies, its inclusion as a compartment in a PBPK model holds particular interest for assessing chemicals affecting the nervous system (Aggarwal et al., 2022). Similarly, there was only one recorded observation for the relative volume of the kidneys (for Atlantic salmon) and three for the relative lipid content (Arctic char). Given the critical role of kidneys in both detoxification and renal excretion (Matthee et al., 2023; Pritchard & Bend, 1991), their inclusion is essential for physiologically based models such as PBPK. In terms of Atlantic halibut, the only tissues with observed values were GIT, liver and PPT, illustrating the data gap this thesis aimed to fill.

Enhancing the coverage of these parameters across relevant species inhabiting the Arctic region, constitutes an initial step in expanding upon the potential for developing an Arctic multispecies model, as done for Canadian freshwater fish (Mangold-Doring et al., 2021). The values collected for relevant Arctic and North Sea fish species in this study are also valuable for the later assessment of other fish species, and even for the potential of an Arctic multispecies model.

6.2 Data obtained through laboratory analysis

The summarised physiological parameters collected either from existing literature or through experimental methodologies, detailed in Table 4 and 5, provide the opportunity to expand the applicability of fish PBPK models to Arctic species. The assessments of distributions (Figures 7, 8 and 9) for physiological parameters experimentally measured in sampled fish are particularly reliable for variables with larger sample sizes, clear data distributions and where key parameters do not vary throughout life (e.g. age, weight, volume, length etc.) or with changes in environmental factors (e.g. food availability, habitat occupation, temperature etc.). For variables with smaller sample sizes, and large variations, the interpretation becomes more biased and challenging, exemplified by water content in kidney (Figure 10) where the accuracy of visual assessment was limited.

Throughout the experimental methodology, data on 23 distinct physiological parameters across individual fish were collected. To unravel the potential relationships among these variables, both PCA and correlation analysis was performed. These analytical approaches were employed to discern the extent and nature of associations between the various physiological parameters. In the scree plot from the PCA analysis (Figure 12), about 99% of the variance is accounted for by PC1 and PC2. The substantial variance captured by a small number of components suggests either significant correlation among the original variables or a robust underlying structure within the data. Essentially, this could indicate that the data naturally segregates into groups or categories. Further support for this interpretation is provided by the PCA variable plot (Figure 11), which reveals the presence of two large and two smaller clusters. Notably, the two clusters that encompass the majority of variables exhibit relationships with total weight and length, indicating either negative or positive correlations. This pattern suggests that certain physiological characteristics may be associated with the overall size of the specimens. This is confirmed by the correlation analysis, with total weight and length displaying the most apparent correlative pattern in the heatmap in Figure 13.

While correlation analysis offered valuable insight to the general associations among variables, pairwise regression analysis was performed to explore the specific relationships between individual pairs of physiological parameters. This allowed for quantification of the influence of one parameter on another. When selecting predictor variables for regression equations to aid characterization of physiological parameters, practical considerations were key. Correlations leading to regression equations using predictor variables needing experimental measurements were deemed less relevant for a PBPK model (see Appendix 6). Therefore, in line with the objective of minimizing animal experiments by developing a PBPK model, regression equations with predictor variables that do not require sacrifice of fish such as total weight and length, were chosen to show their potential for subsequent reuse.

As described in results section 5.5, the regression models utilized in the summary of input parameters (Table 5), have all demonstrated statistical significance in describing the relationship between the response and predictor variables. Although the regression model for the R^2value for relative volume of kidney is 0.21, it is statistically significant (p-value 0.0045). While this is not particularly high, it still suggests some degree of association between the volume of kidney and length. The predicted values from all regression equations

show both overestimations and underestimations compared to the observed values. The equations demonstrate consistent predictive accuracy across a broad spectrum of fish sizes, encompassing both smaller and larger specimens. The majority of fish utilized in the experiments (30 out of 37) weighed less than 2000g, yet the regression equations appear equally effective in predicting values across the entire size range of 200g – 5000g.

6.3 Factors potentially interfering with obtained data

In the experimental phase, a total of 37 fish were dissected. Among these specimens, seven originated from NIVA's research station at Solbergstrand, Drøbak, while the remaining 30 were bred at a Sterling Halibut aquaculture facility. This underscores a substantial diversity in upbringing and variations in diet. Despite their comparable ages, ranging between 2 to 3 years, the fish exhibited significant differences in size, spanning from about 200g to 5kg. The inclusion of total weight as a physiological parameter in a PBPK model is essential for accurate predictions of disposition of chemicals within a specimen, as total weight influences metabolic rates, blood flows and the rate of clearance and elimination of chemicals (Clarke & Johnston, 1999; White et al., 2006)

Another factor influencing chemical concentrations are growth rates. High growth rates affects growth dilution, where chemicals are dispersed over a larger body mass, potentially leading to lower concentrations (Simoneau et al., 2005). Growth rates are highly controlled by diet, hierarchy dynamics and intraspecies competition (Ward et al., 2006), where dominance is strongly correlated with relative body size (Hamilton & Benincasa, 2022; Hughes, 1992). The aquaculture fish were raised in more populous conditions compared to their research station counterparts, and follow a max-profit diet geared toward optimizing growth. This diet makes the physiological parameter values and regression equations applicable primarily to aquaculture-raised halibut rather than wild fish.

At the aquaculture facility, fish undergo annual size-based sorting, with batches exceeding an average weight of 1500g transferred to sea pens (Tungland, 2024). The fish selected for dissection originate from sorted land tanks, indicating slower growing individuals. Ideally, to achieve the most accurate predictions of internal chemical concentrations, the growth rate should be incorporated. This area is still under development, and will be discussed later. Female Atlantic halibut typically achieve greater sizes at younger ages (higher growth rates)

(Sigourney et al., 2006). Fish cultured for commercial interest, are preferrably selected to be female specimens, which explains why all fish sampled from aquaculture were female.

6.3.1 Proxy species

For blood flow values (% of cardiac output), proportionality with tissue volumes was assumed, and rainbow trout values measures by Barron et al. (1987), were scaled based on tissue volumes. Due to limited data availability on blood flow values for flatfish, the accuracy of allometric scaling of trout values to halibut cannot be adequately evaluated, although it has been proven effective in several other species, as zebrafish, fathead minnow and stickleback (Grech, 2018). However, quantifying blood flow values for flatfish remains a potential area of future research.

The cardiac output value used in the model was obtained from winter flounder, as this was the sole flatfish species with directly measured values available. Mendonça et al. (2007) conducted a comparison of cardiovascular function in winter flounder, Atlantic cod and Atlantic salmon (*Salmo salar*), revelaing that despite the winter flounder having a significantly smaller heart size (40% lower relative ventricular mass), it exhibited the largest maximum stroke volume (ml/g ventricle) observed. Remarkably, it therefore maintained a similar maximum cardiac output (ml/min/kg) to the other two species. This observation suggests that the selection of proxy speices for extrapolating cardiac output values may not be crucial. However, the considerations of oxygen consumption makes the selection a bit more intricate.

Unlike most teleost species such as rainbow trout, where oxygen absorbed by the skin is primarily utilized by the skin itself, flatfish possess distinct physiological traits. About 33% of their total oxygen consumption occurs through skin absorption, with the blind side demonstating particular efficiency in oxygen uptake, as evidenced in sole (*Solea solea*) (Joaquim et al., 2004; Nonnotte & Kirsch, 1978). Reusing measurements from rainbow trout or relying on allometric scaling will possibly result in a less precise PBPK model. Given the correlation between oxygen consumption rate and cardiac output in several species (Brodeur et al., 2001; Webber et al., 1998), the dermal uptake of oxygen potentially complicates extrapolation of cardiac output and oxygen consumption rates from non-flatfish species. This efficient skin absorption also raises questions of whether dermal uptake needs to be more thoroughly integrated into a flatfish PBPK model.

6.3.2 Lipid extraction methodologies

The accuracy of PBPK models in simulating the fate and distribution of chemicals within an organism often relies heavily on the quality of the physiological parameters (Khazaee & Ng, 2018). While metrics like the total lipid content of a tissue may seem straightforward, the methods used to derive these values can vary significantly, potentially impacting measured outcomes. Additionally, many of the measurements are conducted using "modified" techniques, without validation and description of the modifications (Karl & Ruoff, 2008; Neregard et al., 2008; Papiol et al., 2017; Valente et al., 2007). Combining measurements collected with different methods obtained from the literature may decrease the reliability of the PBPK model (Khazaee & Ng, 2018).

When it comes to lipid measurements, the most frequently used methods for extraction of lipids from marine fish tissues are Folch (Folch et al., 1957), or Bligh and Dyer (Bligh & Dyer, 1959). The extraction of lipids from tissues utilizes the properties of diverse organic solvents. The principal is that the solvent blend has to be at a level of polarity to dissociate lipids from their connection with cell membranes and other tissue components, but not polar enough to hinder the dissolution of triglycerides and other nonpolar lipids. A chloroform/methanol/water phase system was first developed by Folch et al. (1957), and later a reduced solvent/sample ratio was developed by Bligh and Dyer (1959). The Bligh and Dyer method has become the preferred method for extraction of lipid from tissues in marine fish, because of economic benefits and time efficiency (Iverson et al., 2001).

The lipid extraction method utilized in this study is essentially a modified version of the Bligh and Dyer method, with less toxic and cheaper solvents. Notably, the solvent used facilitates rapid separation during the washing step, and its low density enables centrifugation as an efficient alternative to the filtration process employed in the traditional Bligh and Dyer or Folch methods (Hara, 1978). In a comparative study conducted by Iversen et al. (2010), it was demonstrated that samples with less than 2% lipid content showed no notable difference between the results obtained from the two methods. However, in samples with lipid content exceeding 2%, the Bligh and Dyer methods consistently produces significantly lower estimates. As the lipid content increased, so did the observed discrepancy. For the samples with highest lipid content (26.6%), the Bligh and Dyer method underestimated the lipid content by up to 50%. This raises questions about the comparability of values obtained from

different extraction methods, as well as those utilized in this thesis, which were measures using a modified Bligh and Dyer technique, as done by Allan et al. (2013).

6.4 Exploring opportunities for data reutilization

6.4.1 Comparative analysis of species-specific data

Samples of PPT were procured from the same section of the fish as done in protocols followed by the Norwegian Institute of Marine Research (IMR). IMR has previously conducted analysis of the nutritional composition of halibut fillets sourced from Sterling Halibut. Specifically, IMR reported a lipid content value of 7.8% for a "small" halibut specimen (Erstad, 2024), whereas this study yielded lipid content measurements of 6.7% for PPT samples for fish in between 200g to 5000g. Furthermore, the search for Atlantic halibut values in literature and databases maintained by IMR and NIVA resulted in the average lipid content in muscle tissue to be 4.5%, however with all values derived from wild-caught fish specimens (Table 3).

Regarding liver lipid content, the sole published value identified during the literature search was 23.8% in a study by Nortvedt and Tuene (1998), while experimentally measured values produced an average liver lipid content of 14.6%, which is a deviation of 40% compared to the published value. Nonetheless, it's crucial to highlight that the correlation analysis and assessment of normal distributions, as detailed in the results sections 5.2 and 5.4, underscores the inadequacy of relying solely on mean values to characterize liver lipid content in Atlantic halibut. The reported liver lipid content was measured in fish weighing around 2.4 kg (average 2353g from n = 7) (Nortvedt & Tuene, 1998). Employing this total weight in the regression equation for liver lipid content derived from this study (Figure 14 and Appendix 6) yielded a liver lipid content of 19.6 %, reducing the apparent deviation to 17.6 %.

6.4.2 Cross-species re-use of measured data

Examining the average physiological measurements (Appendix 1) sourced from published literature on species inhabiting the Arctic and North Sea regions, distinct variations emerge in the relative tissue volumes compared to the species list's average metrics (Appendix 1.2). The relative volumes of brain, GIT and liver in Atlantic halibut apparently diverge from the Arctic species averages with factors of more than 100% (brain: 120%, GIT: 166%, liver: 120%), whereas the relative volumes of kidneys exhibit closer proximity (3.7% difference). The relative lipid content of the kidneys shows similarity (9.7%), while the remainder of the parameters (i.e. brain, GIT, liver, PPT) were fairly dissimilar (47.8 to 71.5 %).

Flatfish, such as Atlantic halibut, possesses distinctive physiological traits. A circular curl of the GIT and the body cavity (Guntherz, 2021), an absent swim bladder (Desoutter-Meniger & Chanet, 2009) and small-sized hearts (Mendonça et al., 2007). Therefore, cross-species extrapolation and re-use of data from this study might be most suitable for other flatfish species. When selecting species to be included in the species list for data availability assessment (Appendix 1) priority was given to species of significant commercial interest. Considering the physiological traits of flatfish, the focus of the data availability assessment should perhaps have been directed more towards flatfish species rather than encompassing Arctic species as a whole.

There were two flatfish species included in the literature search of published parameters, Greenland halibut (*Reinhardtius hipppoglossoides*), and European plaice *(Pleuronectes platessa).* Lipid content values from wild Greenland halibut were found, ranging from 7.5% to 14% for fish weighing 350-750g (Karl et al., 2018). While these values were higher than the 4.5% lipid content observed in wild Atlantic halibut, they are fairly closer to the 6.7% measured in Atlantic halibut from aquaculture.

The applicability to other non-flatfish, may be limited due to the mentioned inherent physiological differences. As lipid content is the main driver for partitioning of organic contaminants into tissues (Mackay & Fraser, 2000), re-use of measured data should be directed towards fish with similar lipid storing strategies, thus other lean, white fish. The average lipid content of the lean fish included in the data search, including Atlantic cod, pollack (*Pollachius virens*), and haddock (*Melanogrammus aeglefinus)* (Skåre et al., 2014) was 44.6% for liver, which is a 67% deviation and 2,7% for PPT, marking a deviation of 148% from the measured PPT lipid content in halibut of 6.7%. Given that most of the values in the dataset were derived from Atlantic cod, it raises the possibility of a data bias, making them tell more about the ability of reuse for parameterizing Atlantic cod rather than representing the broader category of lean fish.

6.5 Parameter specifications: Female aquaculture Atlantic halibut

6.5.1 Female Atlantic halibut (*Hippoglossus hippoglossus)*

The physiological parameters experimentally obtained in this thesis originate from female specimens. The most notable disparity between male and female halibut, in terms of the conceptual halibut PBPK model structure in Figure 4, is the RPT-compartment containing

gonads. Female halibut tend to attain larger sizes at younger ages (Sigourney et al., 2006), likely due to lower metabolic rates, as observed for species such as lake whitefish (*Coregonus clupeaformis*), European hake (*Mercillus mercillus*), lake trout (*Salvelinus namaycush)* (Bodiguel et al., 2009; Madenjian et al., 2010; Madenjian et al., 2015). This results in a higher rate of food consumption in male fish and higher concentrations of pollutants (Madenjian et al., 2016). Additionally, distribution and elimination of chemicals has been related to spawning and release of eggs (Guiney et al., 1979; Nichols et al., 1998), which hinders the possibility of one accurate model for both genders.

6.5.2 Modelling wild fish with values derived from aquaculture specimens

Similar to other flatfish, Atlantic halibut predominantly store lipids in their liver (Tocher, 2003). Dissection observations revealed no presence of adipose fat among the internal organs of specimens collected either at NIVA (Solbergstrand) or Sterling Halibut. Aquaculture fish that accumulate lipids in liver and are fed high-energy diets for maximum growth, tend to exhibit enlarged livers and higher lipid levels than their wild counterparts (Grant et al., 1999; Kennedy et al., 2007). Consequently, combining data from both wild and farmed fish may lead to a model that lacks precision for either group. On the base of this, a model populated with values measured as part of this thesis is likely to produce the most reliable results for aquaculture fish.

6.5.2.1 Parameterizing wild fish

Lipids serve as a crucial energy reservoir, with their presence and concentration in an individual being influenced by factors such as food availability and hierarchical position within the ecosystem (Hamilton & Benincasa, 2022). This dynamic is particularly significant for wild fish specimens, which actively forage for food, in contrast to aquaculture fish that are typically fed to maximize growth (Arnason et al., 2009). A challenge in developing accurate models for wild fish lies in accounting for seasonal and geographical variations in lipid content. For instance, studies on wild Atlantic halibut have revealed migratory patterns (Le Bris et al., 2018), and lipid content of migrating fish is observed to decrease during autumn and winter as a result of wintering and spawning migration periods (Hamre et al., 2003). These variations in lipid contents might complicate the modelling of chemical partitioning in wild fish, which relies heavily on lipid contents.

6.6 Alternative methods and approaches

6.6.1 Random selection of individuals and multiple regression

The predetermined sample size for fish dissection was 30 individuals. Due to unforeseen delays and time-demanding methods for lipid extraction and water content determination, the sample size had to be reduced prior to laboratory work. Samples were selected randomly based on sample number rather than fish ID, resuling a dataset with randomly missing values. Due to incomplete data for all fish, simple linear regression was conducted instead of multiple regression. Simple regression involves one dependent variable and one independent variable, whereas multiple regression may include several independent variables. While a simple linear regression model requires less data and assumptions, it may overlook complexities and interactions in real-world phenomenons and exclude other factors affecting the dependant variable (Marill, 2004a; Marill, 2004b).

An alternative apporach could involve employing random selection of individuals and analysing all compartments from the selected fish. This method would offer the opportunity to uncover deeper structures in data with multiple regression analysis. However, given that the objective of developing a PBPK model is to reduce animal experiments, developing complex regression equations for later re-use, especially those relying on animal experiments for predictor variables, is not a major advancement in this context. Therefore, the most valuable predictor variabes for reuse of regression models are the total weight and length, which can be readily obtained without sacrificing fish. Regression equations requiring a limited number of laboratory measurements nontheless hold significance in terms of reducing time-intensive meaurements or utilizing published data to compute variables not explicitly provided. For example, if a significant correlation exists between kidney and liver variables, the measurement of only one of these compartments suffices to estimate the other, thereby effectivising parameterization efforts.

6.6.2 Specimens raised in separate facilities and culturing conditions

The primary objective during laboratory work was to obtain measurements across a broad weight range to identify potential correlations with total weight that required consideration. Consequently, fish sampled both from NIVA (Solberstrand, Norway), ranging from 1800g to 5300g, and from Sterling Halibut (Helland, Norway), spanning 200g to 1800g, were included in the same dataset. These two cohorts of fish were reared under distinct conditions, including differences in population size, feeding regimen, diet and culturing conditions. Such variations

may impact the collected data in manners not explored within this study. An alternative approach would have involved excluding the fish from Solbergstrand, resulting in a narrower weight range. While this could have minimized instances of missing data by allowing for greater focus on fewer fish, it may also have limited the documentation of potential correlation between physiological parameters and fish size.

6.7 Additional physiological measurements enhancing a PBPK model

6.7.1 Additional parameters for estimating partition coefficients

PCs between blood and tissue are crucial chemical specific parameters estimated from physiological parameters, affecting the precision and reliability of PBPK model outcomes. (Salmina et al., 2016). The simplest of models for estimation rely solely on logKow and lipid contents (Veith et al., 1979), while more advanced versions incorporate different biological sorptive phases, including polar and neutral lipids, as well as water, albumin and total protein content of tissues (Endo et al., 2013). Throughout this methodology, measurements of water and lipid content have been conducted, enabling the treatment of each compartment as a two-phase system consisting of polar and non-polar phases when estimating PCs, an approach developed by Bertelsen et al. (1998), accounting for both polar and non-polar constituents of aquatic tissues. This is particularly relevant for more hydrophilic compounds (Salmina et al., 2016).

While the more traditional methods are effective for classical non-polar pollutants, they fall short in explaining the behaviour of more complex compounds. A possible improvement of physiological parameters obtained for PC estimations (i.e. lipid and water content of compartments) would involve analysing polar and neutral lipids, protein and albumin contents of the compartments, as done by Endo et al. (2013) to include additional sorptive capacities. However, a study by Salmina et al. (2016) investigating variation in predicted internal concentrations from PBPK models utilizing these three different modelling approaches for estimating chemical partitioning concluded that the equations developed by Bertelsen et al. (1998) preformed comparably well for 28 organic chemicals with log Kow ranging from 1.9 to 6.6. Moreover, they exhibit higher feasibility in providing reliable input data compared to incorporating all sorptive phases, as done by Endo et al. (2013).

6.7.2 Growth rates

A proposed extension of the fish PBPK model framework by Grech et al. (2019) incorporates environmental factors to account for the influence of growth and temperature on the model. Specifically, a sub-model for fish growth was implemented using Dynamic Energy Budget (DEB) theory (Kooijman, 2000). This integration accounts for dynamic processes that regulate bioaccumulation, like growth dilution (Grech et al., 2019; van der Meer, 2006). The sensitivity analysis conducted in the Grech et al. (2019) study, revealed significant impacts of growth and temperature on the TK of both lipophilic and polar compounds. Integration of growth and temperature is particularly interesting, given the demonstrated relationship between the growth rate of Atlantic halibut and temperature (Hallaraker et al., 1995; Shackell et al., 2022; Sigourney et al., 2006). Metabolic processes in fish exhibit strong correlations with water temperature (Volkoff & Ronnestad, 2020; Zhang et al., 2024). Temperature elevantion typically enhances metabolic rates, impacting the estimations of metabolism and elimination in a PBPK model (Anacleto et al., 2018; Horodysky et al., 2015)

6.8 Practical application in science and regulation

Fish PBPK models are applied to predict uptake, distribution and elimination of substances within fish tissues and simulate bioaccumulation processes. In terms of environmental risk assessment, these models serve as tools for evaluating the impact of hazardous substances, and offer insights that inform decisions regarding the approval or prohibition of chemicals and the regulation of emissions. The development of PBPK models parameterized for fish species inhabiting the Arctic, extends the geographical application of this modelling technique. This extension aligns with the observed accumulation of heavy metals, POPs and other anthropogenic contaminants in the Arctic region (Lagunov & Abdurakhimov, 2021; Varotsos & Krapivin, 2018). Moreover, rising sea temperatures (Carvalho & Wang, 2020; IPCC, 2014) possibly leading to accelerated growth rates (Shackell et al., 2022) and the migration of species into Arctic waters (Bailleul et al., 2012; Doney et al., 2012; Frainer et al., 2017), coupled with the imperative to minimize animal experimentation (ECHA, 2011), demands advancement of robust and dependable modelling techniques, such as PBPK models (Brinkmann et al., 2017).

6.8.1 Environmental, marine and human health risk assessment

A finalized Arctic PBPK model proves beneficial in discussions concerning the prospective of expansion of aquaculture, oil industry, shipping routes and other antropogenic activities in the Arctic region. By offering insights into the concentration levels in different fish tissues over

time, PBPK models take part in assessing the potential health impacts on halibut populations. Moreover, these models may also contribute to human health risk assessments, as halibut consumers. Predictions of transfer of hazardous chemicals to edible tissues of halibut is therefore of relevance for human health. Additionally, as prey for other marine mammals, who often face significant health risks due to accumulation of contaminants in their large lipid deposits, insight in concentrations of contaminants in Atlantic halibut tissues contribute to assessments of marine mammal health (Schaap et al., 2023).

6.8.2 *In vitro in vivo* extrapolation (IVIVE)

The physiological framework of a PBPK model offers a robust platform for conducting quantitative *in vitro-in vivo* extrapolation. The chemical-specific parameters derived from *in silico* and *in vitro* studies can be integrated into the PBPK model to predict *in vivo* ADME processes. Combining *in vitro*, and *in silico* parameter estimation with PBPK modelling enables prediction of the *in vivo* exposure conditions that would result in the same chemical concentrations within the target tissue as the concentrations where effects were observed with *in vitro* assays of tissue toxicity (Stadnicka-Michalak et al., 2014; Yoon et al., 2012).

Brinkmann et al. (2014) demonstrated the link between PBPK and IVIVE, by investigating whether results from *in vitro* bioassays using primary fish hepatocytes could accurately forecast the effects observed *in vivo* in rainbow trout. *In vivo* EC50 values from literature and modelled values generated by a PBPK model both correlated with *in vitro* EC50 values using fish hepatocytes. Using modelling methods like these would help translate cellular changes into relevant organ effect data for toxicological risk assessment (Schaap et al., 2023). The PBPK models may also serve as valuable tools in bridging the TK data with the mechanistic insights of Adverse Outcome Pathways (AOPs). AOPs offer a faster and more cost-effective way to generate mechanistic data needed for IVIVE. These frameworks establish connections between molecular disruptions and adverse outcomes, allowing for toxicity evaluation across chemical groups and species without individual testing (Ankley et al., 2010; Kramer et al., 2011).

6.9 Future directions and prospects

While initial physiological parameterization has been completed, opportunities for enhancement exist by expanding sample sizes, particularly for variables like relative lipid content of kidneys and relative water content of RPT, as these variables exhibited no significant correlations and non-normal distributions (section 5.1 and 5.4). Additionally, to achieve precise estimations of ADME processes in Atlantic halibut, a possible improvement

would involve expansion of the dataset with experimental species-specific measurements of currently extrapolated or estimated parameters, such as cardiac output, blood flow to compartments, effective respiratory volume and total blood flow. Such parametrization would be expected to enhance the reliability of a species-specific model for halibut, but also offer a valuable data set to develop a more Arctic-relevant generic PBPK model, or flatfish PBPK model. However, the experimental efforts needed should be evaluated after sensitivity analysis of a PBPK model based on extrapolated values.

A discussed expansion of the physiological parameters is integrating growth rates to incorporate growth dilution as an elimination route (Grech et al., 2019). Parameters for estimation of chemical partitioning can be refined, either through expanding to the 5-phase system of polar and non-polar lipids, albumin, protein content and water content (Salmina et al., 2016), or through in vitro assessment of chemical partitioning in halibut (Hoffman et al., 1992). Further characterization of metal rate constants would extend the model to encompass metals in addition to current organic pollutants.

Model development consists of four parts; physiological parameters, chemical-specific parameters, model construction and sensitivity analysis (Krishnan, 2009). The initial part of the parameterization has been attempted, whereas latter three parts (i.e. chemical-specific parameters, model construction and sensitivity analysis) remain for the finalisation of an Atlantic halibut PBPK model.

7. Conclusion

In conclusion, this study presents a comprehensive approach for characterizing physiological parameters for the development of a physiologically based pharmacokinetic (PBPK) model tailored to the marine and Arctic fish species, Atlantic halibut (*Hippoglossus hippoglossus*). The characterization process involved a combination of literature review, experimental data collection, and the establishment of a conceptual model structure, entailing the determination of compartments advisable to include. A combination field and lab measurements, reuse of published values and predictions was used to parameterize variables ready for implementation (Table 4 and 5) Employing PCA, correlation analysis and pairwise regression analysis facilitated a thorough exploration of the measured data, revealing underlying patterns and structures in halibut physiology. While certain physiological parameters were relatively stable across measurements, and were easily quantified and reuseable, quantifying parameters associated with the liver compartment demanded greater efforts, due to strong correlations with total weight of the fish. The collected values were specific to female aquaculture halibut, but are proposed suitable for reuse in other flatfish species, as well as species with similar lipid storing strategies. This study initiates the advancement of PBPK modelling of marine organisms and an expansion of the geographical applicability of PBPK models into the marine, Arctic region. Development of such models is envisioned to aid quantifying internal concentrations in Atlantic halibut, but it also facilitates predictions regarding transfer of hazardous chemicals from edible parts of this fish species to both humans and marine mammals. Future directions involve refinement of physiological parameters and species-specific direct measurements of the extrapolated values. Moving forward, deriving chemical-specific parameters, selecting a (PBPK) model framework and performing sensitivity analysis are next in line to advance the work towards an Atlantic halibut PBPK model.

8. Literature

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Appendix

Appendix 1: Species selection of commercially relevant fish species present in the Arctic region: A list of commercially relevant fish species present in the North sea and Arctic Oceans used for assessment of data availability. The selected species should possess commercial significance, to enhance the model's relevance. Criteria for inclusion is documented presence within the Arctic region and classification as either marine or anadromous. The presence in the Arctic is evaluated based on standardized distribution maps fetched from AquaMaps, which is a model-based tool for large-scale predictions of natural occurrences of marine species (Kaschner, 2019). The species list was inspired by fishery-statistics from Northern-Europe and Northen-America, using the software FishStatJ by (FAO, 2011) and a list of species used in previous research of commercial marine fish species in Norway (Azad et al., 2019). Details of the data is available at Zenodo: (https://doi.org/10.5281/zenodo.11184933). The literature search was conducted in Web of Science (Web of Science, 2022), with a search template developed by Mangold-Doring et al. (2021). Additionally, a data search for all species in the NIVA database, and data shared by IMR from their database for Atlantic cod (*Gadus morhua*), and Atlantic halibut (*Hippoglossus hippoglossus*), which explains the high number of observations of these species compared to the rest.

			Number of observations of available data				
Species	Latin name	Habitat	Lipid content	Tissue volumes	Cardiac output	Blood flows	Oxygen consumption rate
Arctic char	*Salvelinus alpinus*	anadromous	31	1	-	-	3
Atlantic cod	*Gadus morhua*	marine	2574	672	11	2	88
Atlantic halibut	*Hippoglossus hippoglossus*	marine	212	-	-	-	-
Atlantic herring	*Clupea harengus*	marine	40	-	-	-	-
Atlantic mackerel	*Scomber scombrus*	marine	102	-	-	-	-
Atlantic salmon	*Salmo salar*	anadromous	126	77	6	-	18
Atlantic wolffish	*Anarhichas lupus*	marine	10	-	-	-	-
Blue whiting	*Micromesistius poutassou*	marine	1	-	-	-	-
European hake	*Merluccius merluccius*	marine	4	-	-	-	-
European plaice	*Pleuronectes platessa*	marine	3	-	-	-	43
Greenland halibut	*Reinhardtius hippoglossiodes*	marine	15	-	-	-	-
Haddock	*Melanogrammus aeglefinus*	marine	26	-	-	-	6
Pollock/Pollack	*Pollachius pollachius*	marine	1	-	-	-	2
Saithe	*Pollachius virens*	marine	27	2	-	-	-
Sea trout	*Salmo trutta*	anadromous	2	-	1	-	55
Polar cod	*Boredagus saida*	marine	22	4	-	-	-
Arctic cisco	*Coregonus autumnalis*	anadromous	1	-	-	-	3
European sea bass	*Dicentrarchus labrax*	marine	11	18	5	6	-

Appendix 1.2 Average values of physiological parameters for the selected species

The physiological parameters included in the literature search for relative Arctic fish species are published on Zenodo (https://doi.org/10.5281/zenodo.11184933), while average values is listed in Appendix 2.1. In addition to the WOS literature search, IMR shared data from their local database on Atlantic cod and Atlantic halibut, and the number of observations for both lipid content and tissue volumes from these two species is affected by this additional information. Data on Atlantic cod was also available from NIVAs local database, but none of the other species.

Appendix 1.2: A summary of the available data collected from published literature including physiological parameters for fish species of commercial relevance present in the marine, Arctic environment. The parameters collected are the relative lipid content, volume, blood flow to tissue and cardiac output. The oxygen consumption rate is collected from the FishBase r-package rfishbase (C. Boettiger, 2012). The column "farmed/wild" indicate whether the collected value is measured in farmed or wild specimen, with the first number counting the number of farmed observations and the last counting the wild. NA in this matter means that it was not reported in the article or database.

Compartment-specific parameters												
	Relative lipid content				**Relative volume**				**Blood flow**			
Tissue	Mean	sd	n	Farmed /wild	Mean	sd	n	Farmed /wild	Mean	sd	n	Farmed/ wild
Brain	0.23	0.20	2	NA	0.00045	0.000071	2	2/0				
Gonads	0.056	0.032	23	1/16	0.0019	0.0033	86	0/86				
GIT	0.071	0.10	32	4/24	0.010	0.0068	30	28/2	16.3%	16.1%	7	NA
Kidney	0.031	0.0057	3	NA	0.0051	NA	1	1/0				
Liver	0.43	0.22	2341*	21/2041	0.0034	0.0036	103	46/52				
Muscle	0.039	0.049	633*	63/55								

*: 2221 of observations for liver lipid content are measured in Atlantic cod from environmental surveillance programs by the Norwegian Institute for Water Research along the Norwegian coast.
* 475 of observations for lipid content in muscle measured in Atlantic cod and Atlantic halibut from environmental surveillance programs by the Norwegian Institute for Water Research along the Norwegian coast

Generally, there is high availability of "PPT" and "total" lipid content across species. The lipid content of liver also shows good coverage, possibly due to their significance as a tissue consumed by humans, which likely explains its prominence in published literature. Values for GIT, gonads, kidney and brain are less abundant.

Appendix 1.3: A summary of the available data collected for general physiological parameters from relevant arctic and north sea fish species. Raw data is available at Zenodo (https://doi.org/10.5281/zenodo.11184933 "oxygen_cons_rate(rfishbase)" and "cardiac_output")

General physiological parameters

	Oxygen consumption rate (mlO2/min/kg)			**Cardiac output** (ml/min/kg)		
Species	Mean	sd	n	Mean	sd	n
Atlantic cod *(Gadus morhua)*	81.53	33.44	88	16.96	11.15	11
European sea bass (*Dicentrarchus labrax)*	-	-	-	40.41	11.15	5
Atlantic salmon (*Salmo salar)*	330.34	188.05	18	24.23	10.44	6
Sea trout (*Salmo trutta)*	132.12	77.25	55	34.5	3.0	1
Arctic cisco *(Coregonus autumnalis)*	202.66	28.22	3	-	-	-
Haddock (*Melanogrammus aeglefinus)*	52.66	13.56	6	-	-	-
European plaice *(Pleuronectes pletessa)*	176.32	272.03	42	-	-	-
Pollock (*Pollachius pollachius)*	261.5	30.41	2	-	-	-
Arctic char (*Salvelinus alpinus)*	50.63	26.47	3			

Appendix 2: Summary of existing fish PBPK models, the species, chemical/group of chemicals parameterized and eventual specifications for the model developed.

Fish species	Chemical	Specifications	Reference
Dogfish shark (*Squalus acanthias*)	Phenol red and glucuronide	Distribution, urinary and biliary excretion, but gills and therefore branchial uptake and excretion not included.	(Bungay et al., 1976)
Sting rays *(Dayatidae sabina and sayi*)	Methotrexate	Mouse model	(Zaharko et al., 1972)
Rainbow trout (*Oncorhynchus mykiss)*	Waterborne organic chemicals (PCE, TCE, HCE)	Adaptation from inhalation model	(Nichols et al., 1990) improved by (Stadnicka et al., 2012)
		Added dermal absorption	(Nichols et al., 1996)
	Hydrophobic organic compounds (PCB- 52)	Added dietary uptake	(Nichols et al., 2004)
	Non-neutral organic compounds (PFOS)		(Vidal et al., 2019)
	Manganese (Mn^{54})	Transfer of radionuclides to freshwater fish	(Garnier-Laplace et al., 2000)
	Organic compounds (bisphenol A, TCDD, oxytetracycline etc.)	Integration of environmental factors and a growth model based on DEB theory.	(Grech et al., 2019)
Channel catfish (*Ictalurus punctatus*)	Waterborne organic chemicals (TCE, PCE, HCE)	Intraspecies extrapolation	(Nichols et al., 1993)
		Dermal absorption	(Nichols et al., 1996)
Brook trout (*Salvenus fontinalis*)	TCDD	Maternal transfer	(Nichols et al., 1998)
Lake trout (*Salvelinus namaycush*)	Waterborne organic chemicals (TCE, HCE, PCE)		(Lien et al., 2001)
Zebrafish (*Danio rerio)*	Waterborne organic chemicals	Adapted to smaller fish	(Péry et al., 2014)
		Integration of environmental factors and a growth model based on DEB theory.	(Grech et al., 2019)
	Cd and Pb		(Zhang, Y. et al., 2019)
Fathead minnow (*Pimephales promelas*) and fathead medaka (*Oryzias latipes*)	TCDD	Two-species model	(Parhizgari & Li, 2014)
Talapia (Oreochromis mossambicus)	Cu		(Chen & Liao, 2014)
European eel *(Anguilla anguilla)*	Hydrophobic organic chemicals		(Brinkmann et al., 2015)
Terapon jarbua	Hg, MeHg		(Wang & Wang, 2015)
	Cu		(Wang & Wang, 2016)
Black seabream *(Spondyliosoma cantharus*)	MeHg		(Wang & Wang, 2017)
Common carp (*Cyprinus carpio*)	Pharmaceutical and personal care products (PCPP)	Includes metabolism of pollutants	(Zhang, S. Y. et al., 2019)
Grass carp (*Ctenopharyngodon idella*)	Doxycycline (antibiotic)		(Xu et al., 2020)
Marine medaka (Oryzias latipes)	As		(Zhang et al., 2020)
Marine grouper (*Epinephelus fuscoguttatus)*	AsB and As(V)		(Xiong et al., 2021)
Multispecies model for freshwater species	Organic compounds		(Mangold-Doring et al., 2021)
Generic model for fathead minnow, roach (*Rutilus rutilus*), zebrafish, rainbow trout and lake trout)	Neutral organic chemicals	Multispecies model	(Brinkmann et al., 2016)

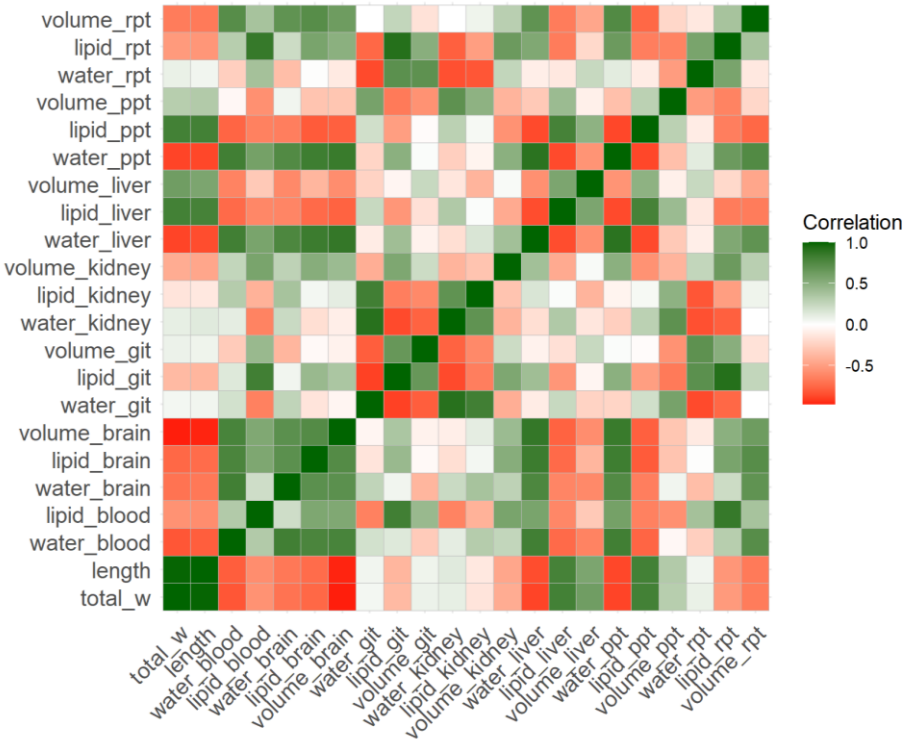
Appendix 3 Oxygen consumption rates for flatfish species: Marine flatfish species with a presence in the Arctic and North sea according to computer generated maps based on environmental conditions by Aquamaps (Kaschner, 2019). Oxygen consumption rates for flatfish species with a presence in the colder climates of the Arctic and North Sea. Retrieved from rfishbase (C. Boettiger, 2012). These flatfish species were used in a search for proxy values for oxygen consumption rates in the rfishbase database in Atlantic halibut, as there were no published values for Atlantic halibut. The results were filtered to include solely standard measurements with no applied stress. Values measured in larvae was also removed.

Flatfish species			**Oxygen consumption rate (mlO2/min/kg)**		
Common name	Latin	Habitat	Mean	sd	n
Atlantic halibut	*Hippoglossus hippoglossus*	Marine	-	-	-
Winter flounder	*Pleuronectes americanus*	Marine	-	-	-
Megrim	*Lepidorhombus whiffiagonis*	Marine	-	-	-
Norwegian topknot	*Zeugopterus norvegicus*	Marine	-	-	-
Brill	*Scophthalmus rhombus*	Marine	-	-	-
Topknot	*Zeugopterus punctatus*	Marine	-	-	-
Common dab	*Limanda limanda*	Marine	28.47	13.53	3
Lemon sole	*Microstomus kitt*	Marine	37.72	12.38	3
European plaice	*Pleuronectes platessa*	Marine	53.07	24.13	24
Common sole	*Solea solea*	Marine	-	-	-
American plaice	*Hippoglossoides platessoides*	Marine	-	-	-
Turbot	*Psetta maxima*	Marine	-	-	-
Witch flounder	*Glyptocephalus cynoglossus*	Marine	-	-	-
Average across all			**44.74**	**24.30**	**35**

Appendix 4: The sample sizes for each of the tissues were not equal across variables, these were the actual sample sizes.

	Sample size		
Compartment	**Relative volume**	**Relative lipid content**	**Relative water content**
Blood	37	15	18
Brain	30	15	10
GIT	37	12	10
Kidney	37	17	7
Liver	37	27	15
PPT	37	11	9
RPT	30	12	9

Appendix 5 Correlation heatmap from PCA correlation matrix



Appendix 6: Heatmap of correlation matrix from PCA analysis. Positive correlation between variables are colored red, while negative correlations are green, and white tiles indicate little to no correlation. The correlation matrix constructed for this heatmap is based on imputed data.

As support for the PCA variable plot in Figure 11, the PCA correlation matrix was plotted in a heatmap. A visual inspection of the heatmap shows that the variables that are clustered in the PCA variable plot have the same pattern in the heatmap (variables for blood and brain are correlating with the same variables). In general, this heatmap confirms the interpretation of the PCA variable plot as described in Results and Discussion.

Appendix 6 Statistically significant pairwise regressions: Summary of all statistically significant pairwise regressions of correlating variables. The equations of relevance to the characterization of physiological parameters and that of the same reason have been employed in the calculation of parameters in Table 5, are marked in bold. The summary includes correlation coefficients (rho) and their p-value, the regression equation and its R2 value and p-value. The sample size was also a factor of relevance for the regression equations. A heatmap of all correlation coefficients, marked with a "*" for statistical significance is in Figure 13 and all raw data, p-values and correlation coefficients are available at (https://doi.org/10.5281/zenodo.11186574)

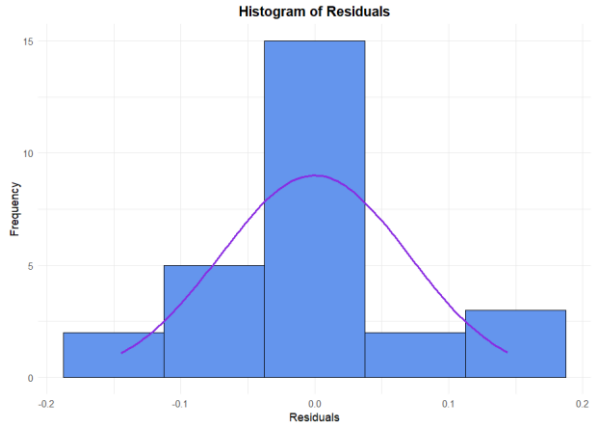
Response variable	Predictor variable	Correlation coefficient and p-value	Regression equation	R2: p-value	Sample size
Length	Total weight	Rho: 1.0 p-value: 0	length = 33.4554 + 0.008123206 * total_w	R2: 0.9125 p-value: < 2.2e-16	
Relative lipid content of blood	Relative volume of PPT	Rho: -0.6107143 p-value: 0.0155928	lipid_blood = 0.2601622 + (-0.2748794) * volume_ppt	R2: 0.4219778 p-value: 0.008767	15
	Relative volume of GIT	Rho: 0.5285714 p-value: 0.0427973	lipid_blood = 0.007448429 + 0.3431575 * volume_git	R2: 0.4156982 p-value: 0.009459	15
Relative lipid content of GIT	Relative water content of GIT	Rho: -0.7333333 p-value: 0.0158006	lipid_git = 0.1802985 + (-0.1955118) * water_git	R2: 0.5661514 p-value: 0.01204	10
Relative lipid content of liver	Length	Rho: 0.7627481 p-value: 0.0000037	lipid_liver = -0.2913549 + 0.009176289 * length	R2: 0.6934167 p-value: 7.131E-08	27
	Total weight	**Rho: 0.77637 p-value: 0.0000019**	**lipid_liver = 0.0175683 + 7.581682e-05 * total_w**	**R2: 0.73503 p-value: 1.122E-08**	**27**
	Relative water content of liver	Rho: -0.8417582 p-value: 0.0001597	lipid_liver = 1.038087 + (-1.309543) * water_liver	R2: 0.6420679 p-value: 0.0005706	14
	Relative lipid content of PPT	Rho: 0.75 p-value: 0.0199421	lipid_liver = 0.03758795 + 2.736594 * lipid_ppt	R2: 0.5492097 p-value: 0.02233	9
	Relative water content of PPT	Rho: -0.7857143 p-value: 0.0208151	lipid_liver = 2.35547 + (-2.936899) * water_ppt	R2: 0.8664226 p-value: 0.0007854	8

	Relative volume of liver	Rho: 0.7295482 p-value:0.0000158	lipid_liver = -0.07331897 + 18.60078 * volume_liver	R2: 0.7453926 p-value: 6.768E-09	27
Relative lipid content of PPT	Relative lipid content of liver	Rho: 0.75 p-value: 0.0199421	lipid_ppt = 0.02762718 + 0.200691 * lipid_liver	R2: 0.5492097 p-value: 0.02233	9
	Relative water content of PPT	Rho: -0.8 p-value: 0.0096279	lipid_ppt = 0.5461486 + (-0.6575973) * water_ppt	R2: 0.5430136 p-value: 0.02352	9
	Relative volume of liver	Rho: 0.7 p-value: 0.016471	lipid_ppt = 0.01399292 + 3.768721 * volume_liver	R2: 0.4611802 p-value: 0.02156	11
Relative water content of blood	Length	Rho: -0.523812 p-value: 0.0256698	water_blood = 0.9508291 + (-0.001631657) * length	R2: 0.2743503 p-value: 0.02568	18
	Total weight	Rho: -0.5356037 p-value: 0.0219731	water_blood = 0.9035425 + (-2.434216e-05) * total_w	R2: 0.2471228 p-value: 0.035	18
	Relative volume of brain	Rho: 0.500516 p-value: 0.0343838	water_blood = 0.8645791 + 25.12782 * volume_brain	R2: 0.3066489 p-value: 0.01711	15
	Relative weight of RPT	Rho: 0.5644995 p-value: 0.0146636	water_blood = 0.8219162 + 10.54225 * volume_rpt	R2: 0.3726051 p-value: 0.007136	18
Relative water content of GIT	Relative lipid content of GIT	Rho: -0.7333333 p-value: 0.0158006	water_git = 0.8770951 + (-2.89574) * lipid_git	R2: 0.5661514 p-value: 0.01204	10
Relative water content of liver	**Length**	**Rho: -0.7714286 p-value: 0.0007569**	**water_liver = 0.912215 + (-0.004626144) * length**	**R2: 0.4908192 p-value: 0.003626**	**15**
	Relative lipid content of liver	Rho: -0.8417582 p-value: 0.0001597	water_liver = 0.7596143 + (-0.4902993) * lipid_liver	R2: 0.6420679 p-value: 0.0005706	14
	Total weight	Rho: -0.8142857 p-value: 0.0002194	water_liver = 0.7547309 + (-3.806567e-05) * total_w	R2: 0.459529 p-value: 0.005482	15
	Relative volume of liver	Rho: -0.6 p-value: 0.0180501	water_liver = 0.7949213 + (-8.993098) * volume_liver	R2: 0.3122698 p-value: 0.03036	15

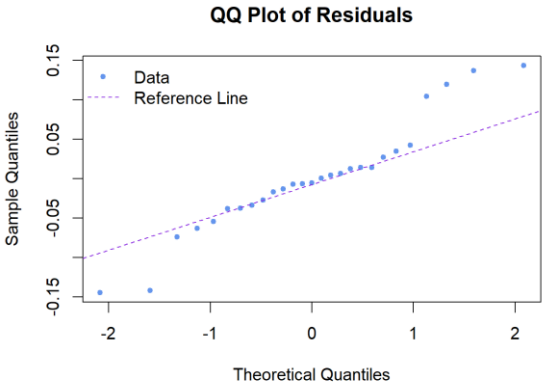
	Relative lipid content of liver	Rho: -0.7857143 p-value: 0.0208151	water_ppt = 0.7894445 + (-0.2950127) * lipid_liver	R2: 0.8664226 p-value: 0.0007854	8
	Relative lipid content of PPT	Rho: -0.3363636 p-value: 0.0096279	water_ppt = 0.7784185 + (-0.825754) * lipid_ppt	R2: 0.5430136 p-value: 0.02352	9
Relative volume of brain	Length	Rho: -0.9244995 p-value: 0	volume_brain = 0.002913641 + (-5.28505e-05) * length	R2: 0.714744 p-value: 0.714744	30
	Total weight	**Rho: -0.9590656 p-value: 0**	**volume_brain = 0.001380761 + (-7.826633e-07 * total_w)**	**R2: 0.6336584 p-value: 1.445E-07**	**30**
	Relative water content of blood	Rho: 0.500516 p-value: 0.0344	volume_brain = -0.009949429 + 0.01220356 * water_blood	R2: 0.306689 p-value: 0.01711	18
	Relative water content of liver	Rho: 0.8021978 p-value: 0.0009688	volume_brain = -0.003343737 + 0.005650123 * water_liver	R2: 0.4051042 p-value: 0.01932	13
Relative volume of GIT	Relative lipid content of blood	Rho: 0.01932 p-value: 0.0427973	volume_git = 0.006259051 + 1.211392 * lipid_blood	R2: 0.4156982 p-value: 0.009459	15
	Relative volume of PPT	Rho: -0.562826 p-value: 0.0002876	volume_git = 0.3620514 + (-0.3789167) * volume_ppt	R2: 0.325897 p-value: 0.0002245	37
Relative volume of kidney	**Length**	**Rho: -0.4579529 p-value: 0.0043698**	**volume_kidney = 0.005522234 + (-3.481617e-05) * length**	**R2: 0.2088019 p-value: 0.004488**	**37**
	Total weight	Rho: -0.4277162 p-value: 0.0082751	volume_kidney = 0.004336094 + (-2.665704e-07) * total_w	R2: 0.1692648 p-value: 0.01141	37
	Relative volume of PPT	Rho: -0.3859649 p-value: 0.0183047	volume_kidney = 0.04466208 + (-0.04594458) * volume_ppt	R2: 0.1926282 p-value: 0.006578	37
Relative volume of liver	Length	Rho: 0.5714626 p-value: 0.0002205	volume_liver = -0.006093749 + 0.0003720784 * length	R2: 0.5979081 p-value: 2.026E-08	37
	Total weight	**Rho: 0.6173908 p-value: 0.0000469**	**volume_liver = 0.005728217 + 3.498909e-06 * total_w**	**R2: 0.7311409 p-value: 1.612E-11**	**37**
	Relative lipid content of PPT	Rho: 0.7 p-value: 0.016471	volume_liver = 0.005910835 + 0.1223705 * lipid_ppt	R2: 0.4611802 p-value: 0.02156	11

	Relative lipid content of liver	Rho: 0.7295482 p-value: 0.0000158	volume_liver = 0.005830568 + 0.04007318 * lipid_liver	R2: 0.7453926 p-value: 6.768E-09	27
	Relative water content of liver	Rho: -0.6 p-value: 0.0180501	volume_liver = 0.03446509 + (-0.03472327) * water_liver	R2: 0.3122698 p-value: 0.03036	15
Relative volume of PPT	Relative lipid content of blood	Rho: -0.6107143 p-value: 0.0155928	volume_ppt = 0.9119236 + (-1.535138) * lipid_blood	R2: 0.42198778 p-value: 0.008767	15
	Relative volume of GIT	Rho: -0.562826 p-value: 0.0002876	volume_ppt = 0.9081974 + (-0.8600757) * volume_git	R2: 0.325897 p-value: 0.0002245	37
	Relative volume of kidney	Rho: -0.3859649 p-value: 0.0183047	volume_ppt = 0.9020443 + (-4.19262) * volume_kidney	R2: 0.1926282 p-value: 0.006578	37
Relative volume of RPT	Length	Rho: -0.4100225 p-value: 0.0244272	volume_rpt = 0.009228505 + (-7.481384e-05) * length	R2: 0.1890885 p-value: 0.01633	30
	Total weight	Rho: -0.3899889 p-value: 0.0331329	volume_rpt = 0.007191619 + (-1.296923e-06) * total_w	R2: 0.2297114 p-value: 0.007367	30
	Relative water content of blood	Rho: 0.5644995 p-value: 0.0146636	volume_rpt = -0.02521347 + 0.03534398 * water_blood	R2: 0.3726051 p-value: 0.007136	18

Appendix 6.1: Residual Assessment of Linear Regression Model for Lipid Content in Liver, with the Relative Volume of Liver as Predictor Variable.



Histogram of residuals from linear regression model of lipid content in liver with relative volume of liver as predictor variable. Constructed to assess their normality. Visual inspection of the histogram reveals bell-shaped distribution, suggesting a normal distribution.



Quantile-Quantile (QQ) plot generated to evaluate normality of residuals. The plot demonstrates a close alignment of the data points with the reference line, with outliers at high and low values.

Appendix 7: Extrapolations of blood flow volumes from assumed proportionality with tissue volumes Blood flow to compartments (ml/min) were extrapolated from rainbow trout values by assuming proportionality with tissue volumes. The values measured in rainbow trout by Barron et al. (1987) were divided by tissue volume to get a scaling constant to multiply with the volumes of Atlantic halibut tissues to calculate fractions of arterial blood flow. Fractions were later multiplied with cardiac output (Winter flounder values (15.5 ml/min/kg) as proxies (Joaquim et al., 2004)) to estimate ml/min out of total blood flow to each tissue for an Atlantic halibut of 500g. The fractions of arterial blood flow that are measured in tissues, which in this thesis is part of a grouped compartment (i.e. skin, gonads)), the fractions are added to the compartment they have been included in (fraction of blood flow to gonads is added to the fractional blood flow to RPT)

Tissue	Relative volumes in Rainbow trout from Grech (2018)	Blood flows (fraction of cardiac output) in Rainbow trout from Barron et al. (1987)	Scaling constant	Relative volumes of tissues in Atlantic halibut	Scaled blood flows (fractions of cardiac output) in Atlantic halibut	Estimated blood flow volumes (ml/min)
Brain	0.018	0.018	3.67	0.000989	0.0036	0.028
GIT	0.099	0.176	1.78	0.0266	0.047	0.37
Kidney	0.0076	0.0817	10.75	0.00421	0.045	0.35
Liver	0.0146	0.0158	1.08	0.00747	0.016	0.067
PPT	0.5978	0.5367	0.78	0.885	0.69	6.91
RPT	0.0257	0.0952	0.65	0.00628	0.0041	0.032
Total						= 7.76

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