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Abstract

The potential presence of PAH metabolites, expressed as PFE, in whale bile demonstrate the possibility of evaluating PAH contamination in this species using a real peak reading at the traditional excitation wavelengths.

In future investigations, the use of 3D graphs is thought to be particularly useful in determining the presence of other fluorescent compounds in whale bile.

Finally, the use of spiked samples is highly recommended when using an established method in a new species.

More thorough analytical methods are needed to confirm the results obtained in this thesis. GC-MS or LC-MS analysis of those samples are suggested as a follow up. Furthermore, it would be interesting to compare more bile samples from whales captured in other locations all around the world.

The obtained results are a promising sign to the use of this species for PAH contamination in our seas.

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1 Table of abbreviations

PAH	*Polycyclic aromatic hydrocarbon*
FF	*Fixed wavelength fluorescence*
SOP	*Standard operating procedure*
ERA	*Environmental risk assessment*
PFE	*Pyrene fluorescence equivalent*
Ex	*Excitation*
Em	*Emission*
EEWP	*Excitation/Emission wavelength pairs*

2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ring-structured organic molecules that have been identified as persistent and widespread pollutants As PAHs are often linked to mutagenic, toxic and carcinogenic effects in biota, their metabolic products have been widely studied in environmental monitoring (Harvey, 1991).

With PAHs natural occurrents in petroleum, the offshore gas and oil sector have developed the term "priority compounds. Depending on their origin, they are mainly categorized as petrogenic or pyrogenic (Pampanin & Sydnes, 2013: Pampanin, 2017: Pampanin & Schlenk, 2019). Petrogenic sources of pollution from incomplete combustion processes are the primary source of PAHs in the marine environment.

In biota, through the process of biotransformation, PAHs are broken down into their respective metabolites. These metabolites are then known to concentrate in fluids and tissue. For the purpose environmental analysis, bile is often sampled due to its major role in the metabolic process and accumulation of PAHs. (Dissanayake & Galloway, 2004)

A number of biomarker investigations of PAH exposure in vertebrate species have been conducted, primarily of the biliary metabolites in fish. These studies have shown to be useful in assessment of environmental contamination relating to bioavailability and exposure. The expeditious and inexpensive screening method fixed wavelength fluorescence, or FF for short, utilizes the fluorescent properties of PAHs for analysis. While most biliary compounds give off non or little emission, suspended PAH metabolites fluoresce upon excitation. This way it is possible to semi-quantitively detect potential contaminants.

The purpose of this study was to show how FF spectrometry can be used to analyse PAH contamination in long-finned pilot whale (*Globicephala melas*) bile. Referencing previous studies on FF, this method of PAH analysis has not been tried with this whale bile. Due to the erratic migration pattern of these large aquatic vertebrates, they are an interesting choice for environmental analysis. Showing promising results, they could be employed in future monitoring studies as possible bioindicators of overall PAH contamination in our waters.

During the spring of 2018 and 2015 7 long-finned pilot whales were killed during Faroese traditional whaling (figure 4). The bile from these whales were subsequently sampled by The Environmental Agency of the Faroe Islands and shipped to Core laboratories at UIS. The samples were kept at -80 degrees Celsius until they were analysed. Following the protocols of Aas et al. (1998), the Hitachi F-7000 spectrophotometer was used to perform fixed fluorescence analysis with 4 preparatory procedures: pure bile, centrifugated bile, spiked bile, and spiked and centrifuged bile. Bile samples were routinely diluted 1:1600 with 50% methanol prior to analysis. With the excitation/emission slit width set to 2.5nm, naphthalene-, pyrene-, and benzo[a]pyrene-type metabolites where detected with the designated Ex/Em wavelength pairs of FF290:335, FF341:383, and FF380:430, respectively. Pyrene fluorescence equivalents were used to quantify PAH metabolites.

3 Theory

3.1 Polycyclic aromatic hydrocarbons

PAHs are ring structured molecules and some of the most widespread organic pollutants. Organic pollutants are primarily made up of hydrogen and carbon bonds and recognised as persistent anthropogenic contaminants. Anthropogenic PAHs are pollutants originating in human activities, and mainly categorized by their emission source as petrogenic or pyrogenic (Pampanin & Sydnes, 2013: Pampanin, 2017: Pampanin & Schlenk, 2019). Origin from thermal decomposition of materials is what we call pyrogenic sources, while petroleum-related sources are defined as petrogenic.

PAHs are often linked to mutagenic (mutation inducing), toxic, and carcinogenic (cancer inducing) effects in biota, and are readily absorbed via consumption. With their natural occurrents in petroleum, and ubiquitous character, they receive substantial attention in environmental monitoring (Harvey, 1991).

3.1.1 Properties of polycyclic aromatic hydrocarbons

The simplest form of PAHs consists of two and three ring structures such as naphthalene and anthracene. However, they may also contain up to six and seven-membered rings, the most predominant containing five or six (Feng, 2009). These molecules consist of fused cyclic conjugates with carbon-carbon single and double bounds, forming a distinguishable aromatic ring structure.

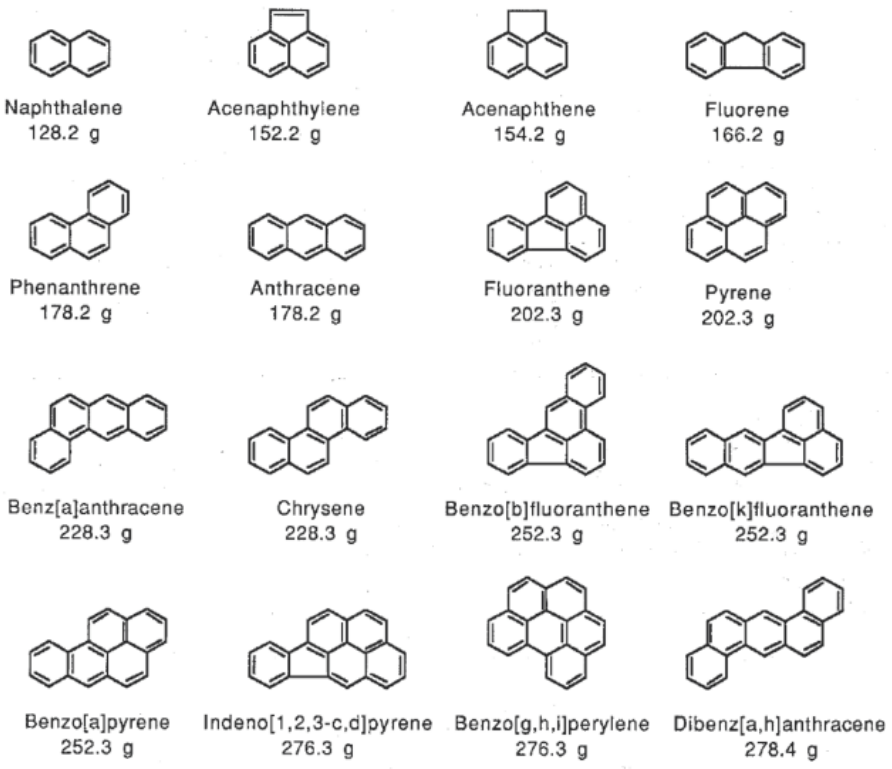

Figure 1 Structures and nomenclatures of the 16 PAHs reported as priority pollutant list in the USA Environmental Protection Agency (EPA). From (Yan et al, 2004)

Depending on the hybridization, PAHs may appear both planar and non-planar. For instance, naphthalene and anthracene appear in an achiral symmetric plane due to the σ-bonds that result from sp^2-hybridization. Other PAHs may appear bowl-shaped as a result of stiffness in the carbon-carbon bonds that complete the ring-structure (Marina V. Zhigalko O. V., 2004).

Indeed, PAHs share the primary conjugate cyclic structure, however as shown in *figure 1*, the ring configuration varies. The simplest form of a cyclic hydrocarbon is the organic compound *benzene.* Benzene is solely made up of carbon and hydrogen in a single carbon-carbon cyclic structure, with the chemical formula C_6H_6. In chemistry, benzene is often depicted as a hexagonal shape with alternating single and double bonds. However, it is equally common to substitute the double bonds with a simple circle (*figure 2*). This is due to delocalization of electrons within the molecule, where bonding cannot be illustrated by a single structure. These are what we call resonance structures, or structural mesomerism. Molecules with multiple resonance structures will in general be more stable than those lacking this property.

In turn, due to PAH mesomerism, electrons of the cyclic structure are easily excited. An exited electron will temporarily occupy a greater energetic state than its ground/relaxed state. When the electron returns to its lower energetic state, the energy is released in the form of a photon. Consequently, this gives fluorescent properties to PAH compounds.

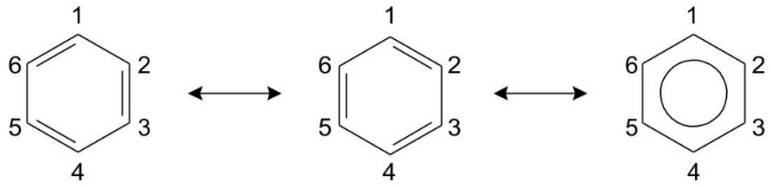

Figure 2 Resonance structure of benzene

Fluorescent characteristics depend on the position and number of aromatic rings. Moreover, with increasing ring conjugates, the fluorescent spectrum shifts with wavelength-peaks closer to the visible range (Aniela Matuszewska, 2020). This quality can be used for analytical detection (Pampanin et al, 2016). The resonance of the aromatic structure also ensures high molecular stability, which indeed corresponds to the bioaccumulation of this anthropogenic contaminant.

3.1.2 Sources of polycyclic aromatic hydrocarbons

Recognising a source of pollution, in efforts to mitigate contamination, remains one of the most important considerations in environmental monitoring.

Environmental sources of PAHs have been found to be both of natural and anthropogenic origin (Borgulat & Staszewski, 2018). Natural contributions are shown to mainly consist of seepage from coal or petroleum deposits, volcanic activities, and open burning.

(Abdel & Mansour, 2016). While natural derivations of PAHs indeed carry environmental impact, estimates indicate that anthropogenic emission greatly exceed natural sources. (Borgulat & Staszewski, 2018)

As PAHs are persistently found and associated with offshore activity, it is no surprize that oil spills play a crucial role in permeating these contaminants. Petroleum-related sources are defined as petrogenic, and both accidental oil spills (in the event of a disaster), and the deliberate release of produced water effluents, are petrogenic PAH sources. These "priority" pollutants, coined by the offshore gas and oil industry, are thus under constant consideration. Thus, significant efforts of minimizing PAHs under normal operations have been adopted.

However, anthropogenic contamination is not only limited to offshore activity. Pyrolysis, or thermal decomposition, of materials such as biomass and fossil fuels, are what we call pyrogenic sources. Transport and land-based operations are shown to introduce pyrogenic sources, appearing in activities such as catalytic cracking towers, trolling, asphalt production, and aluminium smelters (Beyer, Jonsson, Porte, Krahn, & Ariese, 2010). Furthermore, emissions are also connected to urban life, as residential heating, exhaust from motor-vehicles, and as aerosol discharge (pressured spray-cans) (Abdel & Mansour, 2016).

To distinguish petrogenic and pyrogenic origin, environmentalists exploit the fact that PAH-mixtures have ratios of alkylation. Petrogenic contamination sources predominantly consist of extensive alkylation, while less alkylation points to an abundant pyrogenic source. Moreover, distributions of both quantity of PAHS, and isometric ratios, are dictated by the type of combustion (Beyer et al, 2010).

In many cases it is difficult to back-track to one specific contamination source, as PAHs can travel long distances due to their volatile nature. Particularly, with their interaction with ultrafine particles, they can aerially "hitch-hike" extended lengths and deposit on plant surfaces, sediment or ultimately end up in aquatic circulation. (Borgulat & Staszewski, 2018). Hydrophobic hydrocarbons in a marine environment will generally become more resistant to bacterial degradation, due to their ability to adsorb strongly to particles. Aquatic concentration, in both sediment and biota, is therefore a valued source for information regarding local PAH accumulation. (Beyer et al, 2010)

3.1.3 Toxicity of polycyclic aromatic hydrocarbons

As stated, PAHs are often linked to mutagenic and carcinogenic, as well as toxic effects. Studies indicate that aquatic vertebras are no exception. Interestingly, it is shown that pyrogenic varieties are more toxic than petrogenic mixtures. This is partly due to higher levels of non-alkylated and larger PAHs with four or more aromatic rings. As such, the toxicity of PAH compound is highly contingent of structural properties. Depending on steric positioning of the aromatic benzene rings, even within isomers of the same molecule, toxicity can vary greatly from extremely toxic to non-toxic (Beyer et al. 2010).

For marine vertebras, and living organisms in general, biochemical functions are indeed

crucial. Biotransformation processes are most important for transformation and modulation of chemical compounds. However, in toxicokinetics, metabolic transformations can have a negative impact. Organisms with poor biotransformation capabilities, such as mussels, are often less effected by PAHs. Organisms with higher biotransformation capabilities such as vertebras, can metabolize PAHs and form metabolites which can be highly reactive. PAH metabolites can thus covalently bond to macromolecules such as proteins, DNA and RNA. This in turn is what gives these metabolites their carcinogenetic, mutagenetic and teratogenic/toxic tendencies. Thus, it indeed seems that toxicity is closely dependant on enzymatic biotransformation. (Beyer et al, 2010)

3.1.4 Metabolism of polycyclic aromatic hydrocarbons

The aquatic vertebrates' metabolism is often separated into two phases of biochemical reaction: phase I and phase II, with the liver being the major biotransformation site. During phase I metabolism, PAHs are efficiently converted to epoxides (three-carbon cyclic ether) and hydroxylated derivatives by well-developed enzyme systems. Furthermore, to aid excretion, phase II metabolism involves the convergence of these products into highly water-soluble conjugates. Subsequently, the metabolic products are retained in the bile of the gallbladder. Upon ingestion, the breakdown of lipides and other compounds in the gut are assisted by the release of bile into the alimentary tract. In a phenomenon known as enterohepatic circulation, conjugated PAH metabolites may become hydrophobic again due to intestinal pH conditions. This hydrolytic reaction causes reabsorption over the gut wall, and through transportation of the portal vain, the products are ultimately released back into the liver. In normal conditions, this phenomenon is crucial for the recirculation of bile acids, as they can be reused in the liver during digestion. However, as this enterohepatic circulation prolongs the organism’s exposure of PAHs metabolites, the metabolites may have more time to inflict toxic actions on the organism. (Beyer et al, 2010)

3.2 Environmental risk assessment and biomarkers

An environmental risk assessment (ERA) is a method of determining the likelihood that one or more environmental stressors, such as pollutants, invasive species, and climate change, could inflict impact on the environment. It has been established that assessing environmental risk entirely based on pollutant levels in the environment is often unreliable. Rather than the presence of a few specific substances, one must consider the impacts of the overall mixture of contaminants present (Oost, 2002). Therefore, biomonitoring systems have advantages over chemical monitoring. These methods evaluate the combined effects of the chemicals present and their bioavailability. Such methods, involving the use of biomarkers, are beneficial in a variety of situations. In general, a biomarker is referred to as any response that can be observed within an individual organism, with regards to the toxic effect or presence of a pollutant chemical. Thus, a biomarker is any biological response to a substance at the individual or sub-

individual level that indicates a change in normal status (WHO, 1993). Though the definition is often speculated in the field of ecotoxicology, fundamentally, biomarkers are used to indicate and measure pollutant stress upon an individual organism.

3.3 *Globicephala melas* – The long-finned pilot whale

Globicephala melas, commonly known as the long-finned pilot whale, is the second largest member of the dolphin family. The sexes are dimorphic, males growing significantly larger than the female counterparts. Since the Norse establishment of the Faroe Islands, this species has been subjected to the controversial «grindadráp» traditional whaling. In this tradition, long-finned pilot whales are beached and slaughtered in a sort of dolphin drive hunting. Though the Faroe Islands host the world's only remaining large-scale long-finned pilot whaling, historically, this species has been hunted globally.

3.3.1 Abundance and distribution

Both the northern and southern hemispheres are home to long-finned pilot whales. Observations in the North Atlantic range from Iceland to the eastern coasts of Canada and the United States, as well as off the northern coasts of Africa. In the southern hemisphere they are frequently encountered in the Antarctic Convergence Zone, where the frigid Antarctic waters meet the warmer subantarctic waters. It is apparent that the pilot whale distribution varies both seasonally and yearly. Observations show no seasonal migration patterns, however, the distribution shifts to deeper waters in the winter season. This may also be seen in the Faroes, where the frequency of pilot whales peak in July and August. Though long-finned pilot whales appear to be plentiful, there are no current credible estimates of their global number. However, estimates suggest a population ranging from 500,000 to 1 million (K. J. Fullard, 2000).

Individual from this species marked with satellite tags off the Faroe Islands showed rapid long distances travel, averaging a speed of 65 – 110 km per day. Movement was shown to increase between apparent feeding and breeding locations, where they would linger for days or even months. While the images displayed whales heading south to the Norwegian Sea, others headed east to Iceland's east coast or south to the Reykjanes Ridge (Bloch, Petter, Jørgensen , Stefansson, & Mikkelsen, 2003). This social species of dolphin travel in varied pod sizes with several comprising around 50 – 100 individuals, and others holding over 1000 (K. J. Fullard, 2000). The distribution in the North East Atlantic appears to be driven by the abundance and migrations of preferred prey (Desportes G. a., 1993). This has been demonstrated the coast of the Faroe Islands, Newfoundland, United States, and Iberian Peninsula. (Smith, 1990).

3.3.2 Feeding ecology

On the outer continental shelf, long-finned pilot whales are thought to be important predators. Direct observation of feeding is challenging, as it is with other pelagic cetaceans, however stomach contents can indicate relative pray importance. A study conducted by Gannon et al. (1997) found primarily squid in the stomach contents of 30 animals incidentally captured during the mackerel trolling. Most of the diet was found to be made up long-finned and short-finned squid, with the occasional appearance of other squid species. Additionally, there were several fish species present, though not as predominantly occurring as squid. The most common species were Atlantic mackerel and herring, with Silver hake less frequently turning up. (Gannon et al, 1997)

3.4 Fixed wavelength fluorescence

Fixed wavelength fluorescence is a low-cost, quick, and accessible method that provides useful data on PAH exposure. Despite that it is not completely quantitative, its simplicity has made it a popular method for determining the level of PAH pollution in aquatic vertebras. (Beyer et al, 2010)

Biliary samples are typically chosen for analysis since PAHs are known to accumulate in the bile of the gallbladder. The FF method utilizes PAHs fluorescent properties, as most biliary compounds show little or no fluorescence. For the general purpose of FF analysis, a set of Excitation/Emission wavelength pairs are recommended for the detection of the various PAHs. Small naphthalene-type 2 and 3-ring PAH metabolites are traditionally detected by FF290:335nm wavelength pair reading. Larger 5 and 6-ring PAHs, however, require less excitation energy. These benzo[a]pyrene-type metabolites are commonly detected by FF380:430nm. As for 4-ring pyrene-derived metabolites, traditionally, the recommended wavelength pair is FF341:383. Thus, the fluorescent detection at these specific EEWP could indicate the presence of the numerous metabolites in the analysed sample. (Pampanin et al, 2016; Beyer et al, 2010)

For field surveys and monitoring studies, the rather unspecific signals acquired from FF analysis are usually adequate to identify regions as contaminated, or less contaminated/ non-contaminated.

3.4.1 Inner filter effect

Inner filter effect is a common fluorescence spectroscopy problem that occurs when incoming light absorbs so strongly that fluorescence is difficult to detect. The effect occurs as it is not possible to detect the whole strength of the fluorescence in high concentrations, as shown in figure 3. Before reaching the detecting region, the incoming excitation light will be absorbed. This effect gives the illusion of decreased fluorescence with increasing sample concentration. As such, a necessary step for fluorescence spectroscopy involves dilution of the samples.

Beyer et al, 2010 study of FF analysis recommend a 1000- or 2000- fold dilution with 50% ethanol. (D. A. Harris & C. L. Bashford, 1987)

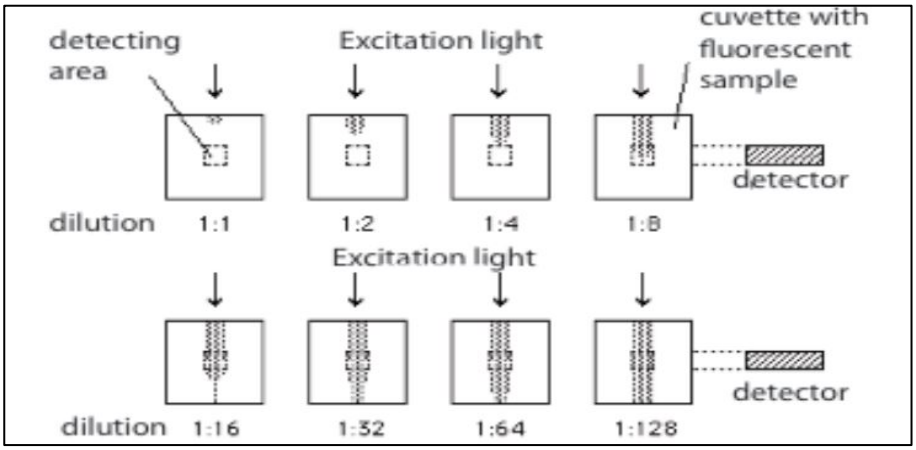

Figure 3 Demonstration of the" Inner Filter effect" with increasing dilution of the sapmles. Fluorescent detection is shown to increases with dilution. (Harris & Bashford, 1987)

4 Materials and methods

4.1 Sampling and storing

The 29th of June 2015 a subadult (Borrell, Bloch, & Desportes, 1995) female long-finned pilot whale was captured and killed in Hvannasund bay during Faroese traditional whaling. This whale, among six others (both male and female) who were subject to the same tradition in 2018, were sampled by The Environmental Agency of the Faroe Islands (Figure 4).

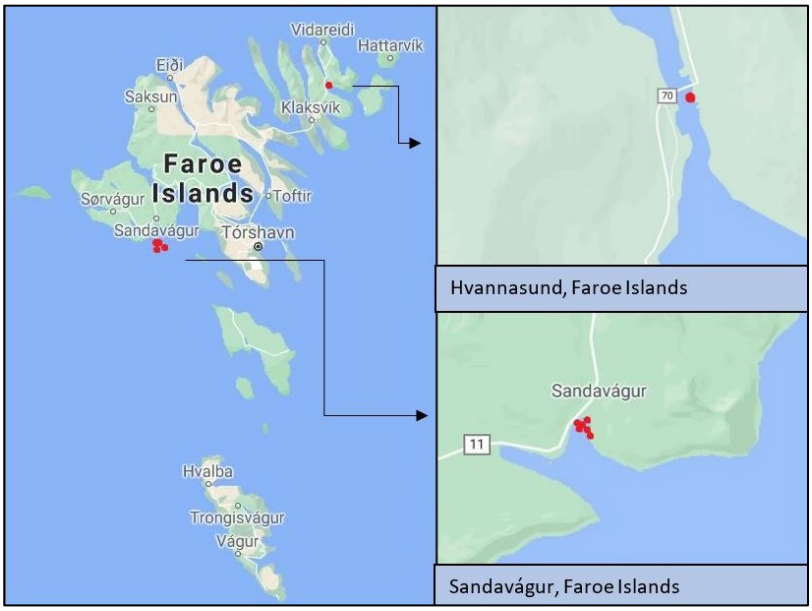

Figure 4: Capture location of whales, Faroe Islands

Bile samples were extracted, then transported in dry ice to the University of Stavanger (UiS) laboratory. Upon arrival, samples were categorized and stored in a -80º C freezer. The bile samples arrived labelled 3,4,5,6,7,10, and 13. For convenience, we chose to keep these labels. Specimen records such as capture location, ID markings, body length, and estimated maturity, were provided by the agency (table 1).

As reported in Bloch et al. (2003) and Desportes et al. (1993), until the individual reaches a length of 430cm, the age is proportionate to the standard body length. Females are thought to reach maturity after they have grown to a length of 375 cm or more. Growing substantially larger than their female counterpart, acording to estimations, males achieve maturity with a length of 494 cm. which is These calculations allow us to determine an individual's estimated maturity, which is described as subadult or adult in this case.

Bile samples	**3**	**4**	**5**	**6**	**7**	**10**	**13**
Capture location	Sandagerði (= whale bay in Torshavn)	Sandagerði (= whale bay in Torshavn)	Sandagerði (= whale bay in Torshavn)	Hvannasund	Sandagerði (= whale bay in Torshavn)	Sandagerði (= whale bay in Torshavn)	Sandagerði (= whale bay in Torshavn)
Date	30.07.2018	30.07.2018	30.07.2018	29.06.2015	30.07.2018	30.07.2018	30.07.2018
(Cause of death)	Killed in traditional whaling by cutting spine.	Killed in traditional whaling by cutting spine.	Killed in traditional whaling by cutting spine.	Killed in traditional whaling by cutting spine and/or blood supply to brain.	Killed in traditional whaling by cutting spine.	Killed in traditional whaling by cutting spine.	Killed in traditional whaling by cutting spine.
Standard body length	410	555	388	345	253	311	456
Estimated age of individual*	adult	adult	adult	subadult	subadult	subadult	subadult
Sex	female	male	female	female	female	female	male
ID by Faroese Environment Agency	300718-3	300718-4	300718-5	290615-6	300718-7	300718-10	300718-13

*Table 1 Sample Information long-finned pilot whale samples 3, 4, 5, 6, 7, 10 and 13 provided by The Environmental Agency of the Faroe Islands. Capture location, date of capture, cause of death, standard body length, age estimate, sex, and ID markings are described. * Females are classified as mature with body lengths equal or greater than 375 cm. Furthermore, male classified maturity is defined with a body length greater or equal to 494 cm. The body length is proportional to age until physical maturity is reached at approximately 430 cm (Bloch et al., 1993; Desportes, 1993)*

4.2 Sample preparation evaluation

Since this is the first time the fluorescent analysis of PAH metabolites has been done in bile from this species, additional preparation steps were considered.

Because of the biliary viscosity, centrifugation of the samples was of particular interest. This approach could reveal the presence of fluorescent emission interference. Furthermore, by spiking a portion of the samples, the biliary PAHs could be compared to spiked concentrations and determine the fluorometric recovery.

In total 4 sample preparation were compared:

1. Pure bile
2. Centrifuged bile
3. Spike bile
4. Centrifuged and spiked bile

1. For the pure bile samples, 1µl of bile was added to the bottom of an eppendorf tube and diluted with 50% methanol. Then, the tube was twirled and transferred to the quartz cuvette for analysis.

2. For the centrifuged samples, 100 µl of bile was added to a tube and centrifuged at 5000 xg at 4^{o} C for 5 mins. Thereafter, the sample followed the same steps as for pure bile.

3. The spiked samples consisted of 1µl of pure bile to a tube, and a 1.5 µl of spiking solution. The spiking solution was made by mixing a ratio of 1:1 51 mg/L OH-naphtalene solution and 51 mg/L OH-pyrene solution. Subsequently, 5ml of solution was diluted with 5 ml 70% MeOH, (1:1600 dilution).

4. The last sample preparation, named centrifuged and spiked bile, was made by combining the centrifugation step with the addition of the spiking solution.

4.3 Fixed wavelength fluorescence

The fixed wavelength fluorescence analysis performed in this thesis is based a commonly used method for the evaluation of PAH-metabolites (Pampanin et al, 2016; Aas et al. 1998).

A standard operating procedure (SOP) was made in order to report all the steps and be able to reproduce the present study data (Appendix A).

Roughly one hour leading up to the analysis, bile samples were retrieved from the -80° C freezer, and the Hitachi F-7000 spectrophotometer was turned on. This allowed sufficient time for the samples to thaw on ice, and for the xenon lamp of the fluorometer to warm up. The spectrophotometer was set to emission scan mode, with a slit width of 2.5 nm for both emission and excitation. Furthermore, the voltage was fixed at 7000,

with a scan speed of 1200nm/min, and an excitation scan-range of 310nm-550nm. The seven bile samples each underwent four procedures. To ensure homogeneous samples, 1ul of bile was pipetted into an Eppendorf tube and diluted 1:1600 with 50% methanol. The balnk consisted of 1ul distilled water, diluted 1:1600 with methanol. Thereafter, diluted samples were added to a quartz cuvette, swirled, and carefully placed in the fluorometer. The scan-cycle consisted four scans of FF290:335, FF341:383, FF380:430, and a full 3D scan. The FF290:335 wavelength has been mainly used to indicate naphthalene-type metabolites (2- and 3-ring PAHs). Pyrene-derived metabolites have been mainly detected by FF341:383 pair reading, and benzo[a]pyrene-type metabolites (5- and 6-ring PAHs) are best detected by the FF380:430 pair reading. Acetone was thus used to clean the cuvettes by gently wiping the outer surfaces and swirled several times. (Pampanin et al, 2016)

A recent paper has shown that the indicated excitation and emission wavelengths for the FF analysis do not contain the maximum emission wavelengths for these metabolites, resulting in an incorrect estimation of PAH exposure (Pampanin et al. 2016). Due to these findings, it was of interest to record both the absorbance values using the traditional method, and also record the global emission scan peaks.

In environmental monitoring studies, PAH metabolites are usually expressed as pyrene fluorescence equivalents (PFE). PFE is calculated by creating a pyrene standard curve. The arbitrary fluorescent unit of the fluorometer can thus be converted and provide environmental relevance.

4.4 Full fluorescent analysis (3D graphs)

In addition to the previously described FF method, full-scale 3D scans were recorded to map the excitation/emission full spectra. The fluorometer was tuned to the desired excitation/emission scope (310-550 ex/em), which produced complete spectrum images. 3D scans provide the advantage of illustrating even more detail to the assessment.

4.5 Data treatment

In the pursuit of credibility, all data were analysed in 5 replicates. Allowing for statistical variation, the recorded absorbance values were used to calculate standard deviation and standard error in Excel. Pure bile data were thus compared to the other 3 preparation methods. In addition, this paves way for comparison between the biliary contents of each pilot whale.

The data was treated using the programs Excel, Strawberry Perl, and Gnuplot. The output retrieved from the Hitachi F7000 spectrophotometer was saved as .txt files and compiled with a simple script in Strawberry Perl. Furthermore, scatterplots were created in Gnuplot, using the mentioned compiled files from Strawberry Perl. Tables and column graphs with their calculated the standard derivation, were created by inputting the raw data into Excel.

Excel was also used to complete a simple t-test. Values were compared using a probability of 0.05 ($p < 0,05$).

In part of this thesis work, obtained results were expressed and compared as absorbance

values (expressed as the fluorescent unit). However, for the environmental assessment, data were expressed as PFE. This was done by calculating the slope of the pyrene standard curve, thus applying it to the fluorometric values of the replicates.

5 Results and discussion

5.1 Sample preparation evaluation

The FF analysis will be discussed in this section, utilizing both real peak values and the traditional reading method.

5.1.1 Sample preparation evaluation

In the fixed fluorescence analysis with FF290 excitation, the pure bile samples with and without the centrifugation seem to provide comparable values, in most cases (figure 5). However, samples from whales 3, 5, and 10 show higher levels of PAH metabolites (in term of absolute absorbance), while sample from whale 6 has lower values recorded after the centrifugation step. The remaining whale samples have no particular change in absorbance when comparing pure bile and centrifuged pure bile.

To better assess the presence of PAH contamination in whale bile, samples were spiked to show a peak related to naphthalene and pyrene type of metabolites in our emission spectra. The use of spiked samples is particularly important in the case of new matrix when using a method established for other species.

Only some spiked samples show a consistent behaviour according to the increased presence oh naphthalene metabolite, i.e. whale samples 3, 5 and 10.

This could be explained by the difficulty experienced while dissolving the PAH metabolites in methanol. As the powder tend to remain as undissolved precipitate, it was necessary to sonicate the spiking solution. However, the process might have created some inconsistency.

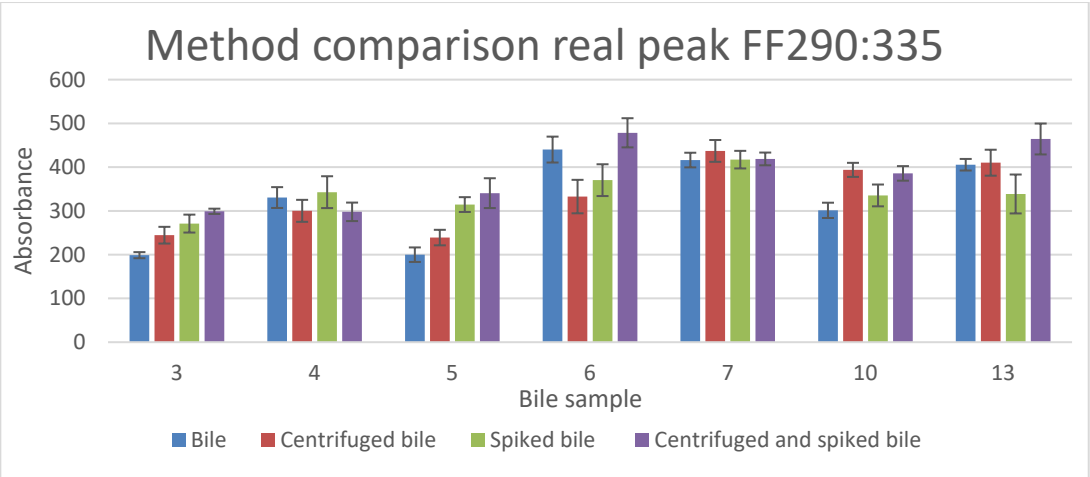

Figure 5 Biliary samples from seven whales (3, 4, 5, 6, 7, 10 and 13) and their peak absorbance at the FF290:335 pair. The graph reports mean values ± standard error (n = 5).

Since most pure bile samples were not substantially different from centrifuged ones, the centrifugation step appears to be unnecessary when performing this procedure for the fixed fluorescence analysis at the pair FF290:335 (associated to 2-ring PAH metabolites).

Figure 6 shows the results related to the FF341:383 pair reading, lower values were recorded for the pure bile with or without centrifugation in comparison with the spiked samples. This suggests that the centrifugation step might not be necessary for a good sample reading. The low value for the whale bile samples also indicates low presence of pyrene-type metabolites.

It is interesting to note that the spiking worked very well for this pair reading, highlighting the presence of 4-ring PAH metabolites (as samples where spiked with OH-pyrene).

The data recorded for the last pair FF380:430 (figure 7) have similar trend as the one just described (figure 6). Also in this case, the centrifugation step does not seems to change the value,

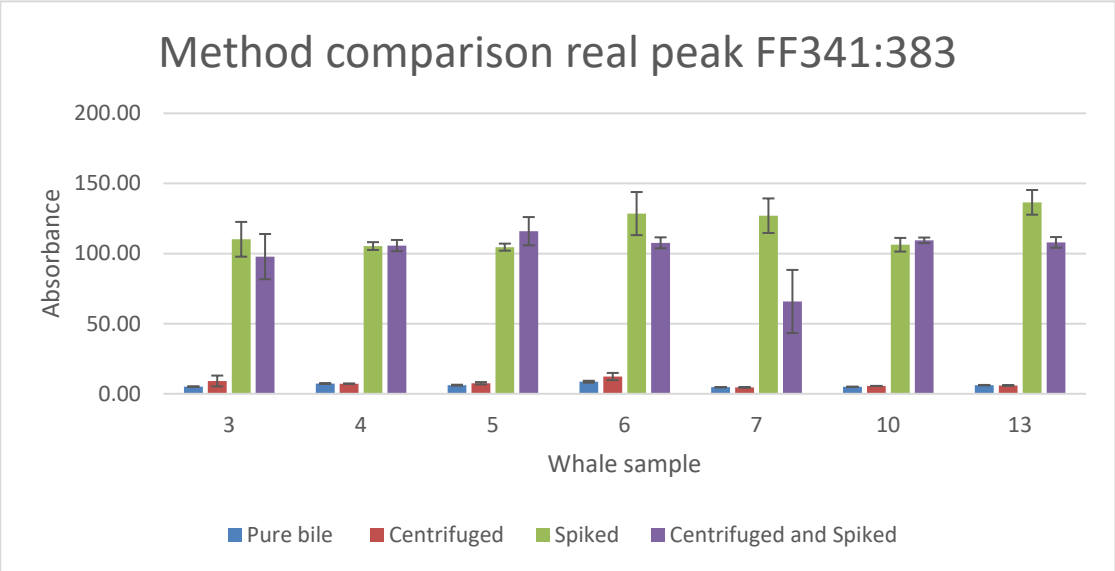

Figure 6 Biliary samples from seven whales (3, 4, 5, 6, 7, 10 and 13) and their peak absorbance at the FF341:383 pair. The graph reports mean values ± standard error (n = 5).

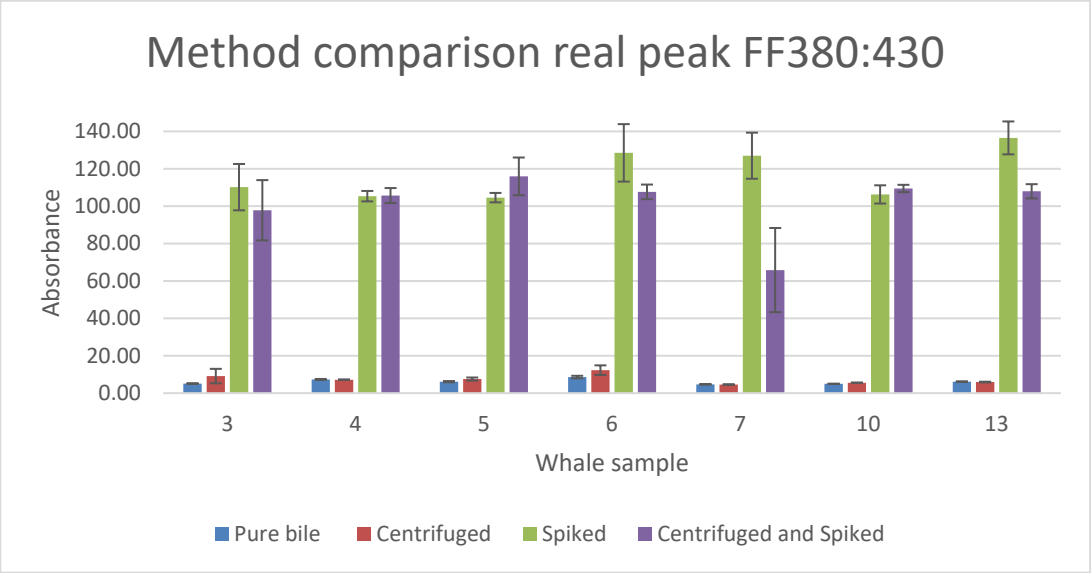

Figure 7 Biliary samples from seven whales (3, 4, 5, 6, 7, 10 and 13) and their peak absorbance at the FF380:430 pair. The graph reports mean values ± standard error (n = 5).

5.1.2 Traditional reading *vs* real peak reading

As previously mentioned, according to the research of Pampanin et al. (2016), excitation and emission wavelength pairs used for traditional FF analysis might exclude the maximum emission peaks of the metabolites. Upon examining the traditional and real peak FF data, evidently, this occurs here as well.

As shown in the scatterplots presented in Appendix B, the peaks do not precisely align with the suggested wavelength pairs in the traditional method. Since the emission is measured at the shoulder of the peak, rather than the maximum, the results will be slightly underestimated.

For instance, as shown in figure 8, lower levels of absorbance occur with the FF341:383 pair. Here the recommended ex/em pairs should reflect absorbance from metabolites of 4-ring PAHs. However, using the traditional pair reading, that would exclude valuable data. In turn this from of incorrect estimation could reflect lower PAH exposure thus tainting the perception. This slight variation of the actual peak is evident in all three wavelength pairs as depicted in Appendix C.

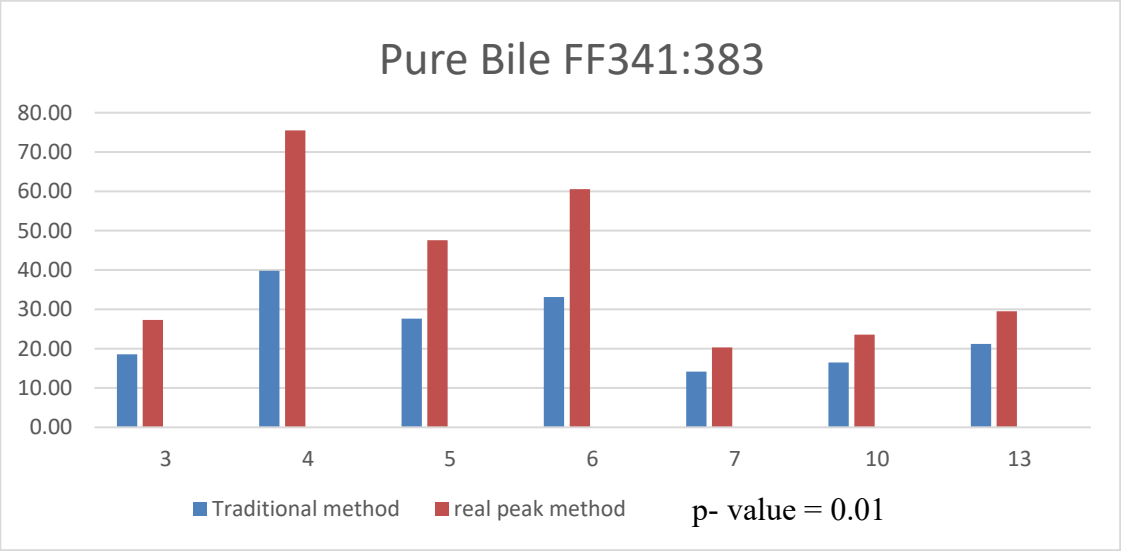

Figure 8 Biliary samples from seven whales (3, 4, 5, 6, 7, 10 and 13) and their peak absorbance at the FF341:383 pair. The graph reports mean values ± standard error (n = 5).

Furthermore, this outcome is described through statistical analysis. Student's t-distribution, described in Appendix D, indicates significant data loss for all three wavelength pairs. With p-values lower than the 0.05 alfa-value, there appears to be significant a significant difference between the two methods.

5.2 Environmental levels of PAH metabolites in whale bile

Pilot whales could be interesting bioindicators, revealing widespread contamination challenges in our seas. They in fact travel great distances and to inhabit both open and coastal areas. Though fixed wavelength fluorescence analysis is a commonly used method in environmental monitoring studies. However, at present it seems like there are no similar investigations carried out in this species to evaluate the presence of PAH metabolites tin bile samples. FF analysis is primarily a quick and affordable tool for semi-quantitative examination, therefore, the results in this study are treated as such. As an overall they can provide a good screening tool, indicating the necessity of further evaluation with more advance approaches.

Figures 9, 10 and 11 illustrate the presence of PAH metabolites in the biliary samples of 7 pilot whales. The real peak readings are plotted and expressed as PFE for the FF290:335, FF341:383, and FF380:430 pairs. The numbers represent the individual whale according to the info reported in Table 1.

As presented in figure 9, whales 6, 7 and 13 expressed the highest values for naphthalene-type metabolites. It is interesting to note that of the three matured pilot whales (3,4 and 5), two of them expressed the lowest levels. Since the presence of PAH metabolites is reflecting a short-term contamination, this is not so surprising. A correlation between age/size and presence of PAH metabolites could be further evaluated. With a larger sample size, it would be possible to compare body length and PAH accumulation in a statistical manner.

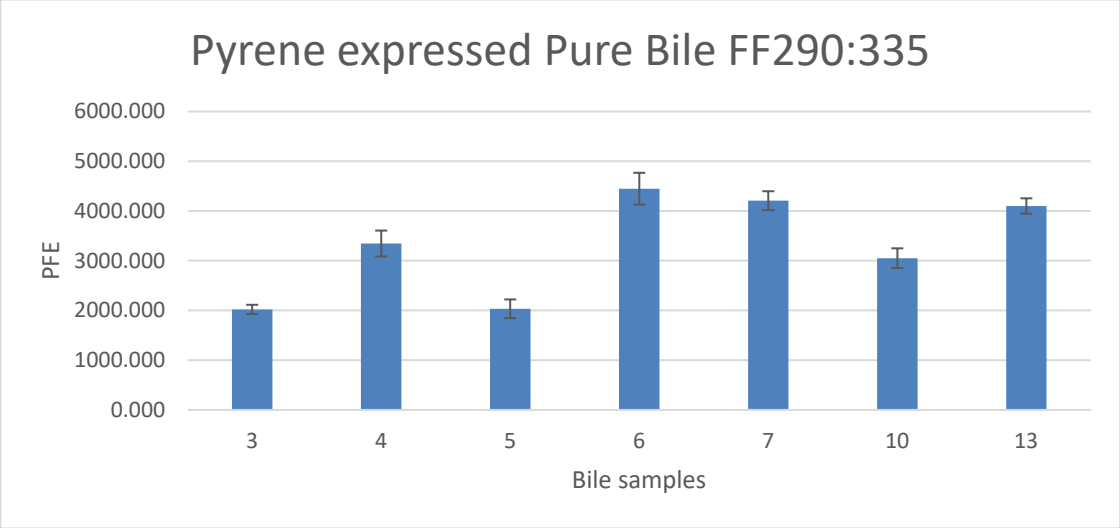

Figure 9 Biliary samples from seven whales (3, 4, 5, 6, 7, 10 and 13) and their peak absorbance at the FF290:335 pair. The graph reports mean values ± standard error (n = 5).

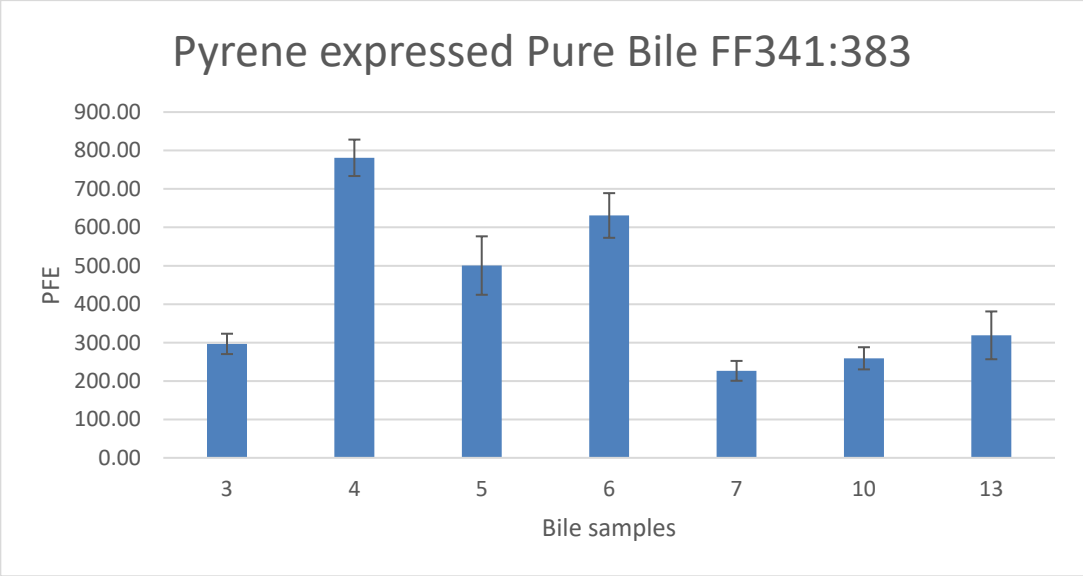

Figure 10 Biliary samples from seven whales (3, 4, 5, 6, 7, 10 and 13) and their peak absorbance at the FF341:383 pair. The graph reports mean values ± standard error (n = 5).

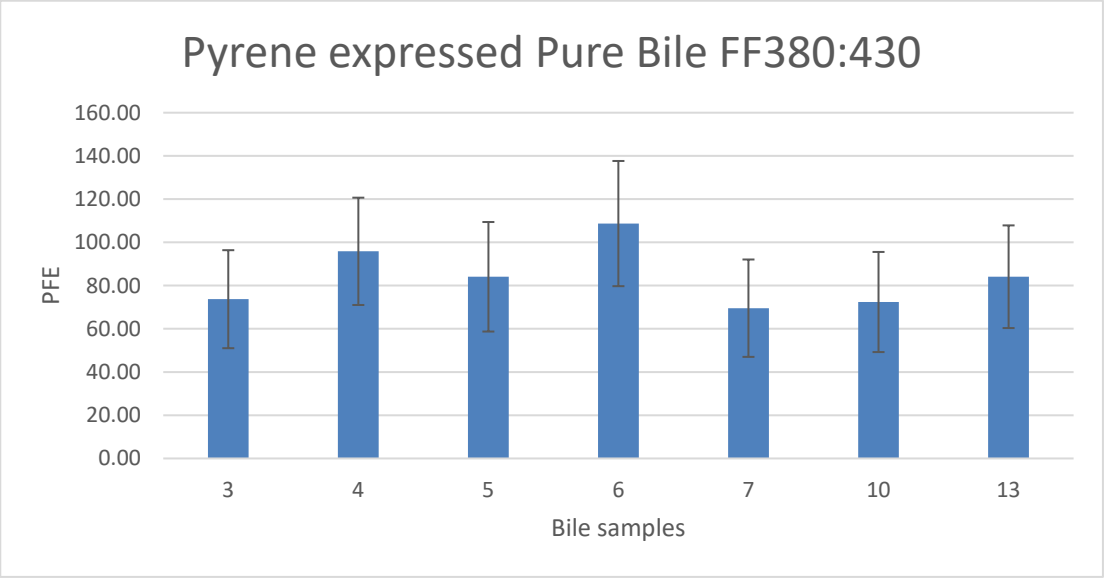

Figure 11 Biliary samples from seven whales (3, 4, 5, 6, 7, 10 and 13) and their peak absorbance at the FF380:430 pair. The graph reports mean values ± standard error (n = 5).

5.3 Full fluorescent analysis (3D graphs)

The purpose of creating 3D graphs is to investigate absorption throughout the spectrum, to see the presence of peaks which are not normally included in the FF method. According to both peak values and 3D graphs the following observation are reported. While whales 3, 10, and 13 looks like it can contain 2- and 4-ring PAH metabolites, whale 7 indicates concentration of 2- and 3-ring ones. Whales 4, 5, and 6 has a peak in the region where one would expect 5-ring compounds. However, more evaluation is needed to confirm those findings.

After the evaluation of both 2-d and 3D spectra, to fully quantify the presence of PAH metabolites potentially present according to the FF results, gas chromatography–mass spectrometry (GC-MS) would be needed. This method utilizes the combined features of mass spectrometry and gas chromatography and is known for its selectivity in separation of complex mixtures.

Figure 12 reports example of the 3D scan of whale bile. The use of these graphs will help further analysis to evaluate the presence of other fluorescent compounds in the whale bile.'

In figure 13, it is possible to notice how the spiked samples present a quite different 3D graphs, showing that the evaluated peaks are due to PAH metabolites.

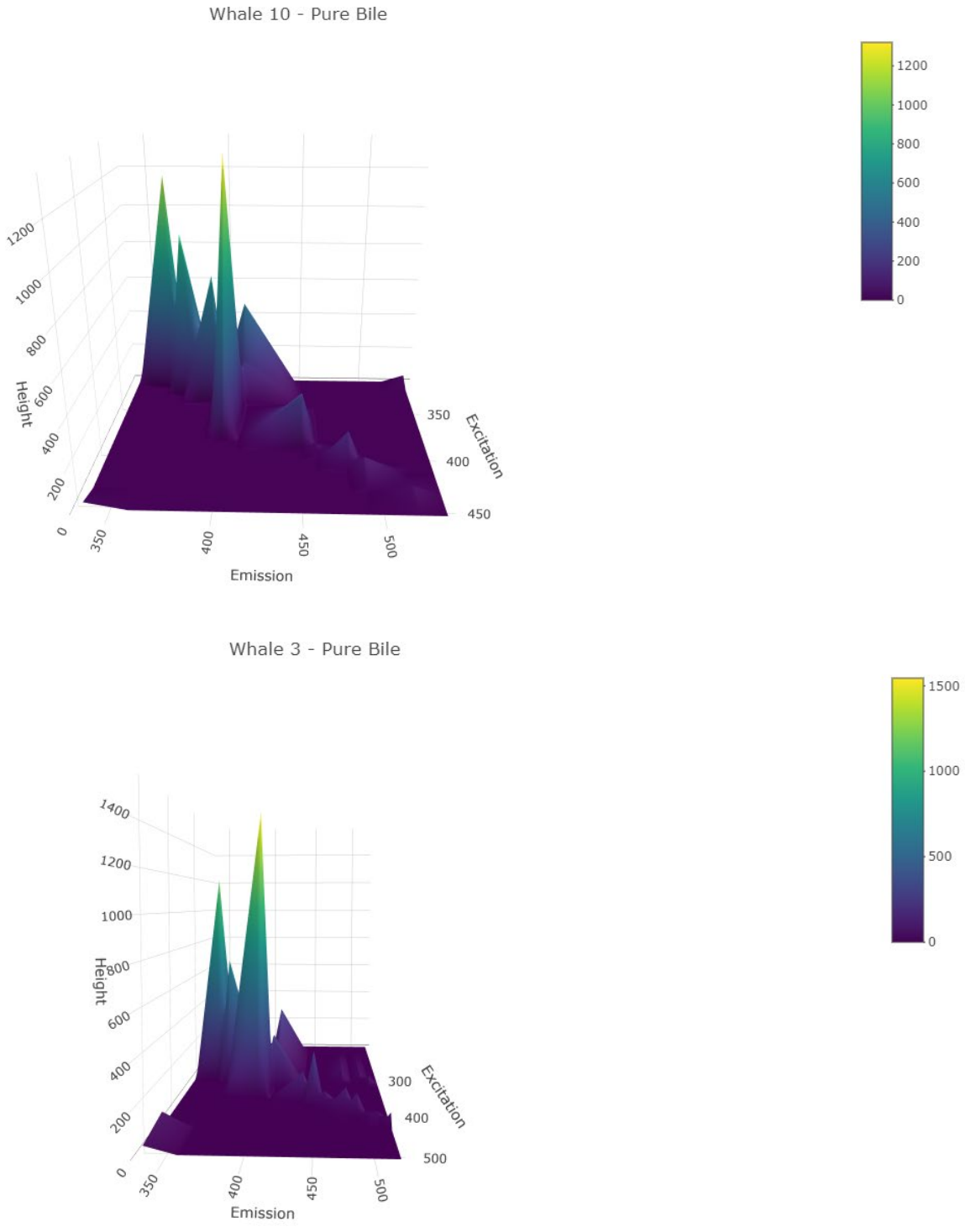

Figure 12 Example of 3D full scan graphs from whale 3 and 10.

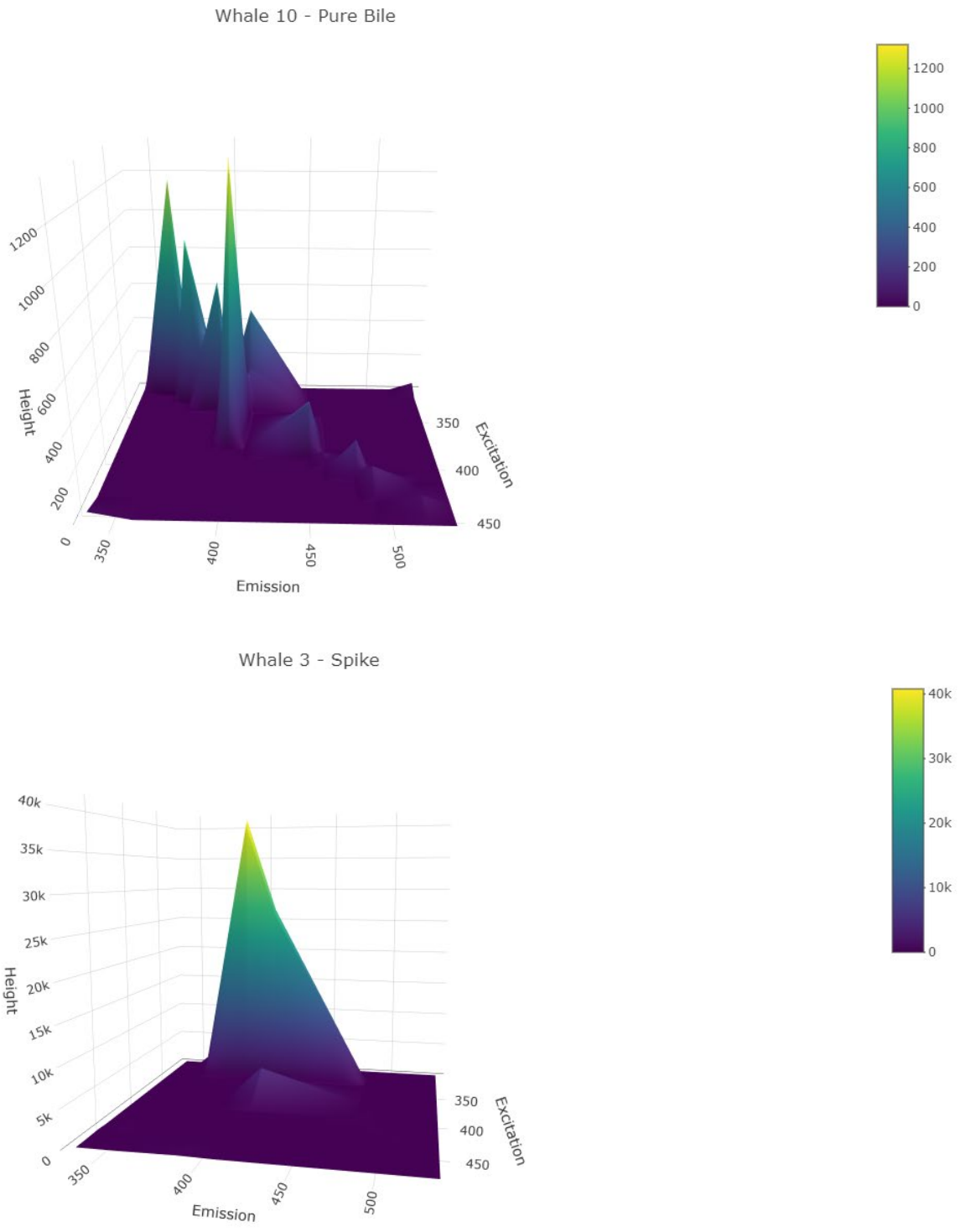

Figure 133 Comparison of 3D full scan graphs of pure bile samples non-spiked and spiked.

6 Conclusion

Fixed wavelength fluorescence is a frequently used method for analysing fish bile. In this study, FF was used for reviewing the exposure levels of petrogenic and pyrogenic derived polycyclic aromatic hydrocarbons in whale bile, with promising results.

In this thesis work, four preparatory processes were utilized. These included pure bile, centrifuged bile, spiked bile, and centrifuged and spiked bile. The centrifugation step appears to be the least beneficial and somewhat unnecessary. Data shows no significant difference between centrifuged samples and pure bile, and whale bile samples could be analysed in their natural state. However, spiking the samples appears to be a useful step. Creating a spiking solution with predetermined PAH concentration is an effective way of estimating the concentration of metabolites present in the pure bile samples.

The em/ex pairs utilized in this study, consisting of FF290:335, FF341:383, and FF380:430, indicate naphthalene-type, pyrene-derived, and benzo[a]pyrene-type metabolites, respectively.

Even though there seems to be metabolites present in the samples, it is difficult to conclude. This method of PAH analysis is primarily a semi-quantitative assessment, merely portraying an exposure overview. Also, so far there are no similar studies of this species on PAH metabolites to compare with.

Nevertheless, as pilot whales are known to travel long-distance, covering thousands nautical miles of both coastal shores and deep ocean, there is the possibilities that they have been exposed to PAH contamination.

7 Further perspective

More thorough analytical methods are needed to precisely estimate the quantities of various PAH metabolites in whale bile samples. GC-MS or LC-MS analysis of those samples is suggested as a follow up of this thesis work.

Furthermore, it would be interesting to compare more bile samples from whales captured in other locations all around the world.

The obtained results are a promising sign to the use of this species for PAH contamination in our seas and the analysis of a larger number of samples would be the next obvious step.

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9 APPENDIX A

Standard Operating Procedure
Fixed wavelength fluorescence analysis of whale bile

Based on: Aas et al, 1998

Author: William Arnli _____ 3.25.2021

Introduction

Using fixed wavelength fluorescence as a screening tool for polycyclic aromatic hydrocarbons (PAH), samples from 7 long finned pilot whales are evaluated. The samples are scanned with the respective FF290:335 nm, FF341:383 nm, and FF380:4390 nm excitation/emission pairs to discriminate between 2-, 4-, and 5-ring structures of the PAH metabolites. A sample dilution of 1:1600 serves the advantage of bypassing the common problem of inner filter effect in fluorescent spectroscopy. Furthermore, a pyrene standard curve was created.

Equipment

Equipment	***Remarks***	***Location***
Hitachi F-7000 spectrophotometer	Mode - emission scan Slit width - 2,5nm emission/ excitation Voltage - 7000 Scan speed - 1200 nm/min	Måltidets hus D-231
Centrifuge 5430 R	5000 xg at 4C for 5 mins	Måltidets hus G-206
Ultrasonic Bath		Måltidets hus D-132

Chemicals

Chemical	*CAS number*	*Location*	*Product specifications*
1-Hydroxypyrene	5315-79-7	D-109, fridge	Acros Organics BVBA
Methanol	67-56-1	D-132, Poison/fire Cabinet	VWR International
2-Naphthol	135-19-3	D-109, fridge	Thermo Fisher (Kandel) GmbH
Aceton	67-64-1	D-132, Poison/fire Cabinet	Merck Life Science AS

Solutions

Solutions	*Preparation and storage*
50% MeOH	1:1 ratio of pure grade methanol and distilled water using serological pipets Stored in sealed bottle under fume hood
70% MeOH	7:3 ratio of pure grade methanol and distilled water using serological pipets Stored in sealed bottle under fume hood
51 mg/L OH-Pyrene in 50% MeOH	-Stock solution: 5.1mg OH-Pyrene in 100 ml 70% MeOH, sonicated for 5 minutes Stored in sealed bottle in fridge, wrapped in aluminium foil
25.5 mg/L PAH (OH-Pyrene/ OH-Naphtalene) in 70% MeOH	-51 mg/L OH-Naphtalene solution: 5.1mg OH-Naphtalene in 100 ml 70% MeOH, sonicated for 5 minutes -Mixed solution: 1:1 ratio of 51 mg/L OH-Naphtalene solution and 51 mg/L OH-Pyrene solution using serological pipets -Final solution diluted by mixing 5 ml mix solution with 5 ml 70% MeOH Stored in sealed bottle in fridge, wrapped in aluminium foil

Safety

The procedure was carried out with regards to the following safety precautions:

- Use of appropriate personal protective equipment such as coats, gloves, and eye protection.
- Protective equipment changed and disposed of when contaminated.
- When working with potentially hazardous materials hands must be washed before leaving the laboratory.
- Eating and drinking prohibited in the laboratory.
- General safety precautions while handling of sharp objects (i.e., broken glassware, scalpels, pipettes).
- Clean laboratory equipment and work surfaces routinely
- Proper disposal of infectious or hazardous materials.

Procedure

Pyrene standard curve

A stock solution of 1-Hydroxypyrene (51 mg/l) was diluted with 70% methanol, creating four standards. The stock solution and the four standards were then further diluted to a concentration of 1:1600 with 50% methanol.

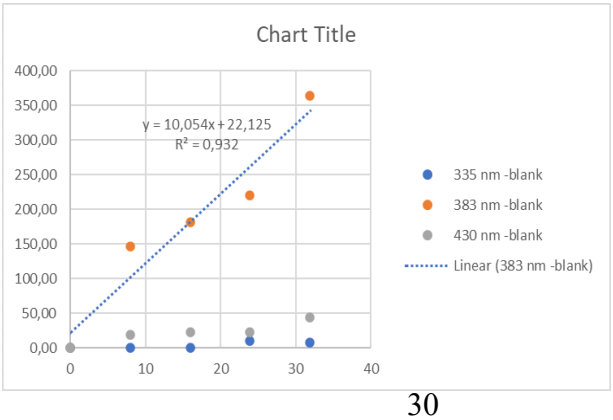
Pipetting scheme for pyrene standard curve

Pipetting scheme		**Theoretic pyrene conc** before dilution (mg/L)	**Pyrene conc after dilution (1:1600) (mg/L)**
stock	0,0051 g pyrene in 100 ml 70% MeOH	51	0,032
std1	7,5 ml stock + 2,5 ml 70 % MeOH	38,25	0,024
std2	5 ml stock + 5 ml 70 % MeOH	25,5	0,016
std3	2,5 ml stock + 7,5 ml 70 % MeOH	12,75	0,008
std4	only MeOH	0	0,000

Each sample was centrifuged and homogenized in an Eppendorf tube, before transferred to a quartz cuvette and run through the fluorometer. The emission was recorded and plotted to finalize the standard curve.

Plotted fluorescent values for standard curve

Raw data				Fluorescent values			
	335 nm	383 nm	430 nm	pyrene conc. (mg/L)	mean 335 nm	mean 383 nm	mean 430 nm
stock	16,96	431	55,62	0,032	15,68	371,57	47,24
	16,33	355,1	44,66	0,024	17,50	227,90	25,81
	13,74	328,6	41,43	0,016	8,00	189,37	25,67
std1	13,81	225,3	17,22	0,008	8,12	154,23	21,24
	22,62	236,1	30,62	0,000	7,73	7,82	2,68
	16,07	222,3	29,58				
std2	8,746	183,5	25,07	Calibration curve values			
	7,29	171,2	23,14	pyrene conc (µg/L)	335 nm - blank	383 nm - blank	430 nm -blank
	7,955	213,4	28,79	31,875	7,95	363,75	44,56
std3	10,56	105,9	14,58	23,90625	9,77	220,08	23,13
	7,001	217	29,35	15,9375	0,27	181,55	22,99
	6,787	139,8	19,79	7,96875	0,38	146,41	18,56
std4	7,702	7,667	2,782	0	0,00	0,00	0,00
	8,215	7,77	2,541				
	7,278	8,026	2,711				



Fixed fluorescent

Bile samples were removed from the -80° C freezer and defrosted on ice. A 25.5 mg/l solution spiking was created with a 1:1 ratio of 1-hydroxypyrene and hydroxynaphthalene in 70% methanol. The solution was sonicated to ensure maximum solvation. The sample preparation involved adding 100ml of bile to two separate Eppendorf tubes. The first tube was centrifuged at 5000xg at 4° C for five minutes, while the other was set aside. 10ul of bile from each tube was pipetted into four new tubes, two spiked with the PAH solution, completing the four preparations. The samples were diluted 1:1600 with 50% methanol before transferred to a quartz cuvette. The following scheme was than applied to all seven whales. The scheme represents the four preparations with respect to the five replicates for each of the seven whales.

10 ul of bile (pure bile)	**10 ul of bile and centrifuge + spiked 1600 ng/ml**	**10 ul of bile and centrifuge**	**10 ul of bile + spiked 1600 ng/ml**
1a	2a	3a	4a
1b	2b	3b	4b
1c	2c	3c	4c
1d	2d	3d	4d
1e	2e	3e	4e

Sample preparation scheme

The Hitachi F-7000 spectrophotometer was set to emission scan and a scan speed of 1200 nm/min. Moreover, the voltage was set at 7000, with a slit width of 2,5nm for both emission and excitation.

Fixed fluorescent (FF) was measured at the excitation/emission pairs FF290:335, FF341:383, and FF380:430, respectively. The FF290:335 wavelength mainly indicate naphthalene-type 2/3-ringed metabolites usually associated with petroleum products. Benzo[a]pyrene-type 5/6-ringed metabolites are best detected by FF380:430, while pyrene-derived metabolites are mainly detected by FF341:383. In addition, full-scale 3D scans were also recorded to map the full excitation/emission range.

References

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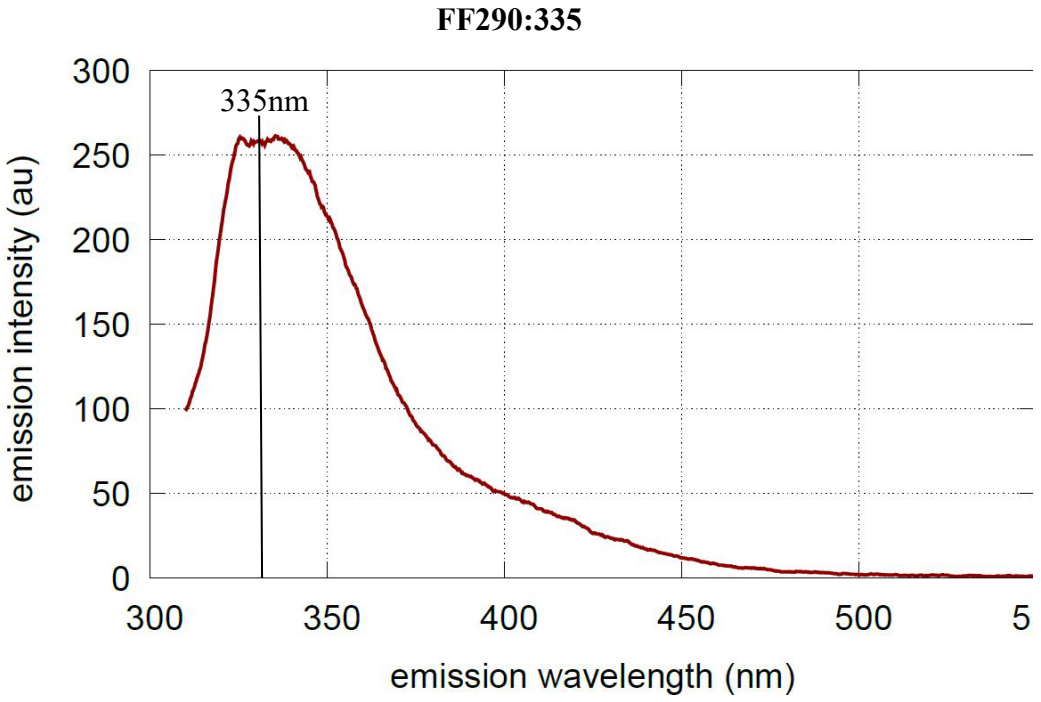
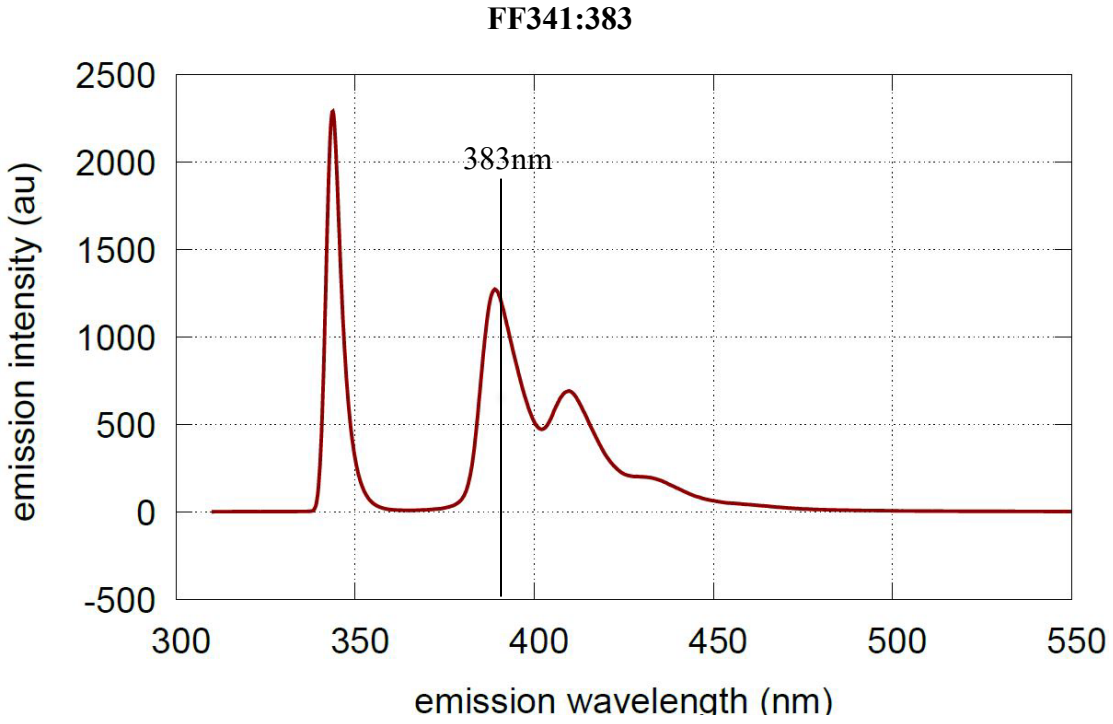
10 Appendix B

Exsamples of fluorometric scans

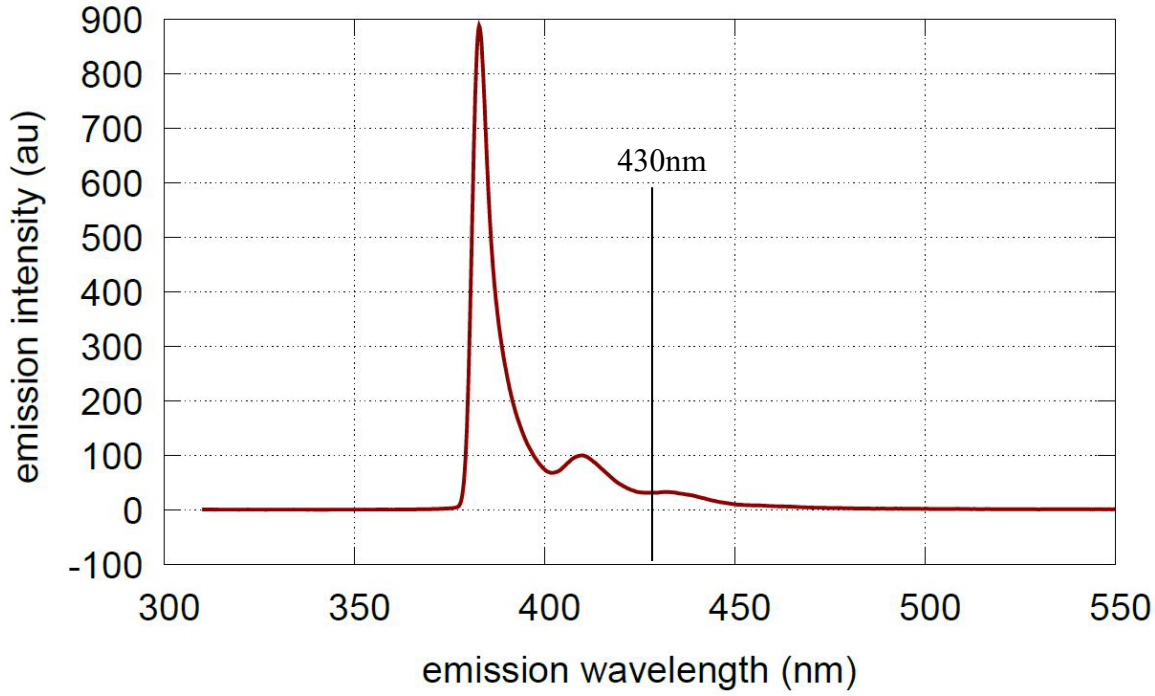


Exsample graph showing a scan of sample 3 pure bile at FF290:335 derived from the Hitatchi F7000 photospectrometer. The graph was ploted with gnuplot and strawberry perl.



Exsample graph showing a scan of sample 3 pure bile at FF341:383 derived from the Hitatchi F7000 photospectrometer. The graph was ploted with gnuplot and strawberry perl.

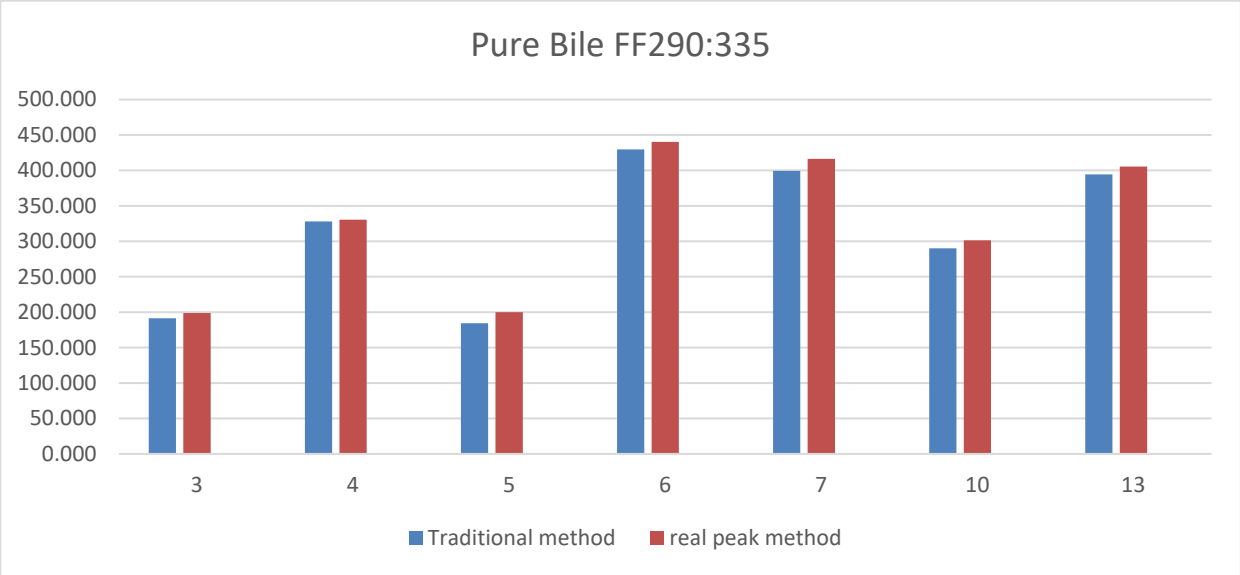
FF380:430



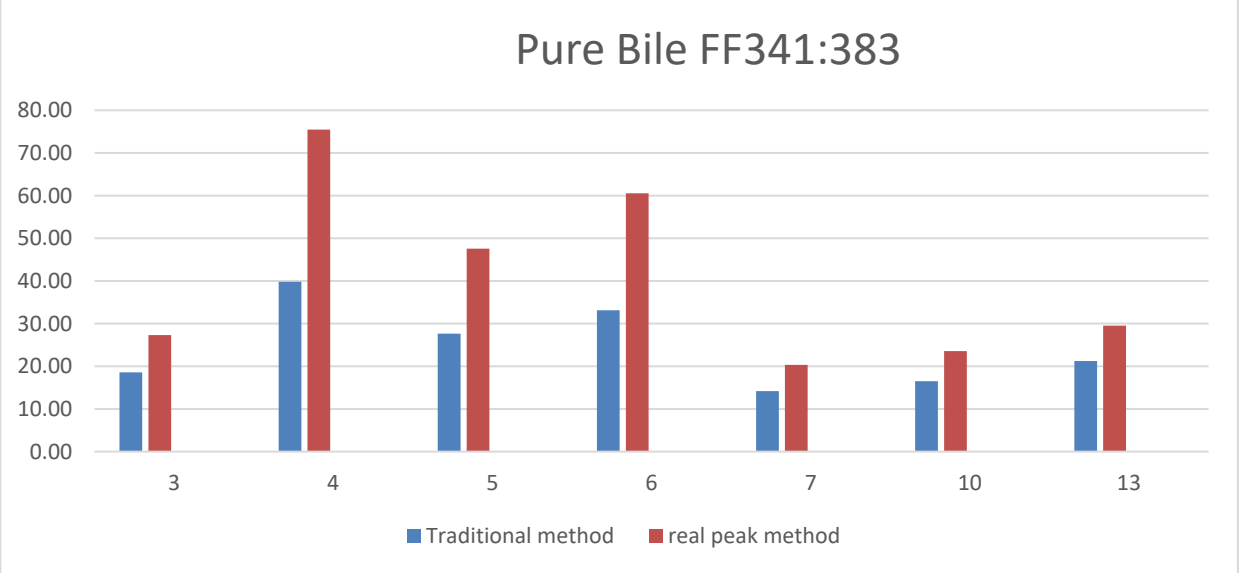
Exsample graph showing a scan of sample 3 pure bile at FF380:430 derived from the Hitatchi F7000 photospectrometer. The graph was ploted with gnuplot and strawberry perl.

11 Appendix C

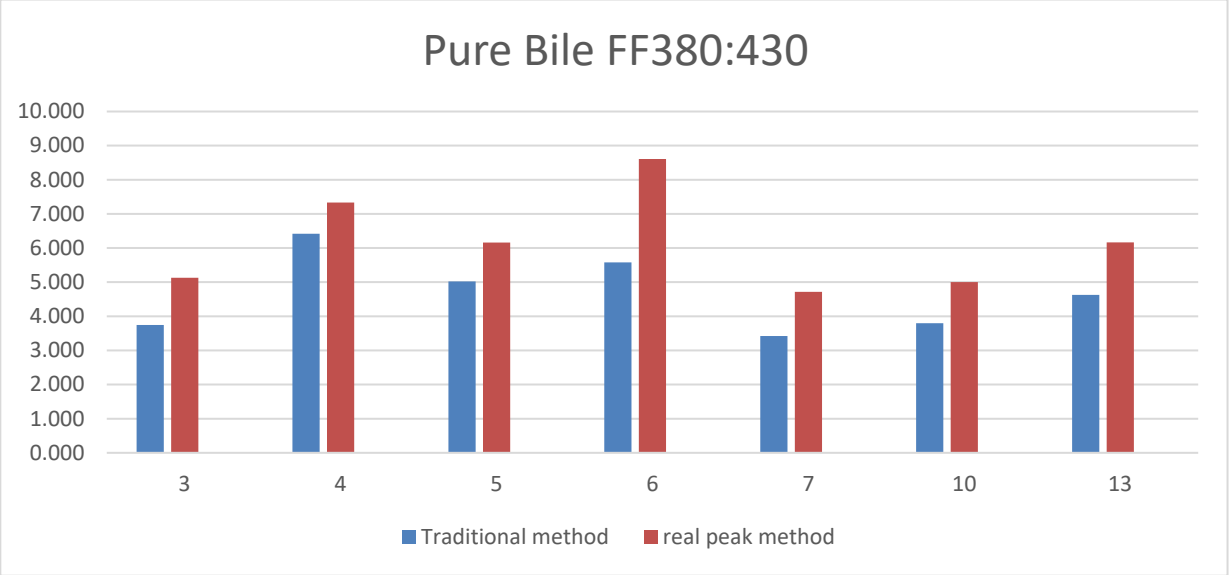
Traditional method vs real peak



Biliary samples frrom 7 whales, their peak absorbance, and traditional vlue at the fixed wavelength of FF290:335. Numbers represent the individual whale according to the info reported in Table 1.



Biliary samples frrom 7 whales, their peak absorbance, and traditional vlue at the fixed wavelength of FF341:383. Numbers represent the individual whale according to the info reported in Table 1.



Biliary samples frrom 7 whales, their peak absorbance, and traditional vlue at the fixed wavelength of FF380:430. Numbers represent the individual whale according to the info reported in Table 1.

12 Appendix D

Traditional method vs real peak -Statistical analysis

Paired Sample t_test using Traditional method and real peak method
Null Hypothesis: H0A = H0B
Alternative Hypothesis: H0A ≠ H0B

t-Test: Paired Two Sample for Means **Purebile FF290:335**

	Traditional method	*real peak method*
Mean	316,75	327,52
Variance	9943,07	10026,99
Observations	7,00	7,00
Pearson Correlation	1,00	
Hypothesized Mean Difference	0,00	
df	6,00	
t Stat	-5,93	
P(T<=t) one-tail	0,00	
t Critical one-tail	1,94	
P(T<=t) two-tail	0,00	
t Critical two-tail	2,45	

t-Test: Paired Two Sample for Means **Purebile FF341:383**

	Traditional method	*real peak method*
Mean	24,43	40,62
Variance	89,15	443,99
Observations	7,00	7,00
Pearson Correlation	1,00	
Hypothesized Mean Difference	0,00	
df	6,00	
t Stat	-3,66	
P(T<=t) one-tail	0,01	
t Critical one-tail	1,94	
P(T<=t) two-tail	0,01	
t Critical two-tail	2,45	

t-Test: Paired Two Sample for Means	**Purebile FF380:430**	
	Traditional method	*real peak method*
Mean	4,66	6,16
Variance	1,20	1,98
Observations	7,00	7,00
Pearson Correlation	0,87	
Hypothesized Mean Difference	0,00	
df	6,00	
t Stat	-5,66	
P(T<=t) one-tail	0,00	
t Critical one-tail	1,94	
P(T<=t) two-tail	0,00	
t Critical two-tail	2,45	