Method development for serial-extraction of contaminants in fish followed by species comparison of PCB 153 levels in Tench and Roach from Källby waste water treatment pond

Julia Grönholdt Palm

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Supervisor: PhD student Marja Boström, Unit of Aquatic Ecology, Lund University, Sweden
Co-Supervisor: Senior Lecturer Olof Berglund, Unit of Aquatic Ecology, Dept. of Biology, Lund University, Sweden

Environmental Science

Lund University

Sweden

Abstract

Two studies were performed in this paper. The first study was a method development examining the possibilities to do a serial extraction of lipids, PCB 153, NSAIDs (non-steroidal anti-inflammatory drugs) and selective serotonin reuptake inhibitors (SSRIs) from a single sample. Five different treatments were tested with three replicates from each individual fish. All samples were first freeze-dried and then the following treatments were executed: First sample series were only cut and PCB-extracted, second sample series were cut, lipid-extracted and PCB-extracted, third sample series were homogenized, filtered, lipid-extracted and PCB-extracted, forth sample series were homogenized, freeze-dried, lipid-extracted and PCB-extracted and the fifth sample series were homogenized, membrane-extracted, freeze-dried, lipid-extracted and PCB-extracted. The treatments were assessed using an analysis of variance (ANOVA) with Tukey-Kramer post hoc tests. Results showed that there was a significant effect of type of treatment ($p=0.008$) to the amount of extracted lipids. Filtering treatment had a high standard deviation and low mean lipid yield implying that the method is inappropriate for analytical studies. Freeze-drying was the best treatment after cutting with almost as high lipid yields as the cutting treatment. The full membrane extraction treatment however had a low lipid yield. A possible explanation is that the lipids were hydrolyzed from the acid HP-LPME-treatment and therefore partly dissolved in the water phase. However this demands further research and modeling.

The second study was a species comparison of PCB 153 concentrations in roach and tench from Källby sewage treatment pond. The study's main aim was to assess two species with different feed and foraging behaviour's tendency to accumulate PCBs. Lipids and PCB were extracted using the cutting treatment (as above) and the results were then analysed for difference using a Mann-Whitney test. The stable isotopes $\partial^{13}C$ and $\partial^{15}N$ were also measured and analysed using two sample, unpaired, t-tests. Result showed that both $\partial^{13}C$ and $\partial^{15}N$ were higher in the tench. The species comparison showed no significant results although after lipid normalization the roach indicated higher concentrations of PCB 153 with a p-value of 0.072. The higher concentration of PCB in roach could not be explained although one study in Poland assessing mercury had found similar results (Misztal-Szkudlinska, 2008). Therefore the results are believed to be an effect of either inter- and intra-species variations or possibly experimental flaws such as failure in assessing PCB yield from tench or insufficient amount of replicates.

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1. Introduction

1.1. Problem and aim

In toxicological studies there is often a desire to study more than one contaminant but the amount of samples available can be a limiting factor. This study will examine the possibility to do serial extractions from a single sample. The contaminants that will be assessed were selected based on a current PhD study by Marja Boström; "Uptake and bioaccumulation of ionisable pharmaceuticals in aquatic organisms". Boström is among other things studying bioaccumulation of non-steroidal anti-inflammatory drugs (NSAIDs) as well as selective serotonin reuptake inhibitors (SSRIs). These compounds will be extracted from samples of fish and other aquatic organisms from a pond receiving water from Källby waste water treatment plant (WWTP) through a membrane extraction method (Boström, 2012). After the compounds have been extracted there is also a need to extract lipids and analyse PCB-levels. This study is therefore aiming to examine if it is possible to do lipid and PCB extractions in series with the membrane extraction method on a single sample.

The study also aims to examine PCB-levels in roach (Rutilus rutilus) and tench (Tinca tinca); two species with different feed and foraging behaviours from Källby WWTP. The hypothesis is that tench will have a higher PCB-level due to its preference for benthic feed. The results from the PCB-measurements can also be used as reference substance for quantitative studies of biomagnification in Boström's project.

1.2. Method development for serial extraction

Besides the pH depending toxicity Boström is also assessing possible bioaccumulation and biomagnification of NSAIDs and SSRIs where an extraction method for ion-trapping with hollow fibre liquid-phase microextraction (HP-LPME) is used (Boström, 2012). In Boström's study the BMF (biomagnification factor) for NSAIDs and SSRIs will be calculated using concentration measurements from different organisms from Källby WWTP. The BMF will then be compared with the legacy contaminant PCB 153 as a reference substance for quantitative studies of biomagnification. In this study PCB levels will be measured in roach and tench. Since the samples are scarce there is a desire to be able to do several extractions from the same samples which will be assessed in this study. To make the HP-LPM method possible the sample needs to be homogenized with water (Sagrista et al., 2012). Samples must then be dried to make lipid-extraction possible, using the bench mark method of Bligh and Dyer (Bligh and Dyer, 1959). Finally PCBs will be extracted by removing all organic matter with sulfuric acid (Jansson et al., 1991). Today there is no method for doing a serial extraction of pharmaceuticals (using HP-LPME) and PCBs/lipids. The development of this method could dramatically lessen the amount of samples needed and the time spent in similar projects where serial extraction is required.

Below is a figure showing the extractions that would ideally be carried out using only one sample.

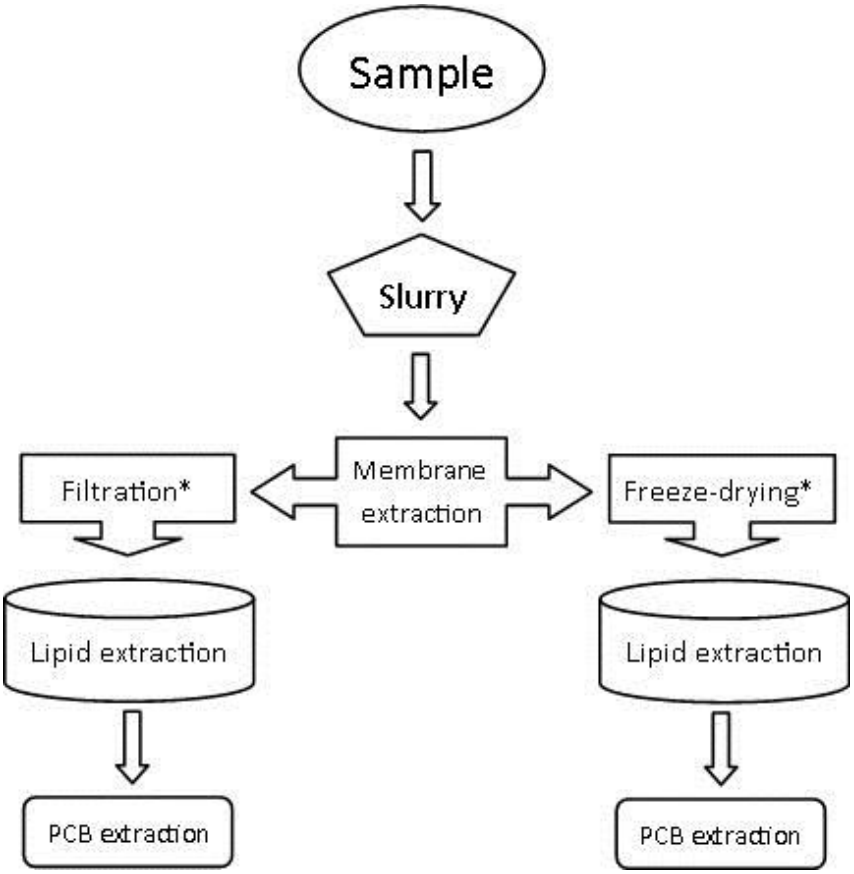

Figure 1. The model shows the serial extraction that will be assessed in this study. *Treatments will be assessed and the method that is proven best will be used.

Each separate treatment could possibly affect the end results. To be able to detect critical parts of the serial-extraction five separate treatments were conducted, adding one step in each, starting with only PCB-analysis to the full serial extraction (Figure 2.). The samples that are only PCB-extracted are mainly conducted as reference samples for the more processed samples.

1.3. Species comparison of PCB-levels in Roach and Tench

1.3.1. *PCB*

PCBs are one of the twelve chemical groups classified as persistent organic pollutants (POPs) under the Stockholm convention (Muir & Sverco, 2006). They have been proven to have a wide range of toxic effects in organisms e.g. carcinogenic-, immune-, reproductive-, neurological- and endocrine effects (EPA, 2013a). PCBs are manufactured organic chemicals that started being used in 1929. They have been phased out since the 1970s in industrialized countries but are still being used in many developing countries. They have several desirable properties; chemical stability, non-flammability, high boiling point etc. that led to their heavy use in e.g. electrical and heat-transfer-equipment, carbonless copy paper, plastics and paint (as plasticizer) etc. (EPA 2013b). As the name implies PCBs are persistent and therefore many countries are still experiencing environmental issues as a result of previous heavy use and long-range transport (Muir & Sverco, 2006).

PCBs are non-volatile organic compounds with a vapour pressure of 10^{-12}-10^{-4} in 25°C (atm). They have a high K_{ow} ranging between 4,5 - 8 and low water solubility resulting in their tendency to absorb to unpolar material like fatty tissues and sediments (UNEP, 2007). The concentration of PCBs in sediment has been shown to be positively correlated with oil-concentration (Bedard, 1996). The degree of chlorination and molecular position of chlorines in general determines the physiochemical properties of the PCB. Henry's constant is higher for less chlorinated PCBs (~0.8 atm L/mol) which means they will mainly be found in gas phase. More chlorinated PCBs adsorbs to particulate matter and can be removed from the atmosphere with wet or dry deposition and typically ends up in water ways (UNEP, 2007). Biodegradation of PCB by microbes occurs through aerobic respiration, anaerobic respiration or anaerobic fermentation and the rate and level of biodegradation is reduced with rising degree of chlorination (Liu et al., 2006 and UNEP, 2007). In organisms PCBs are first hydroxylated to make them polar and possible to excrete. The rate of transformation depends on chlorine content, replacement pattern and amount of certain enzymes (UNEP, 2007). Half-life of different congeners ranges between eight days to more than one year (R. Leschber, 2006). PCB 153 is a common type of PCB frequently found in for instance adipose tissues, breast milk, human serum and fish and has a half-life of approximately 6,8 years.(Sinkkonen and Paasivirta 2000, Venkatesha 2010). Studies of diverse fish species in Sweden has shown that concentrations of PCB 153 correlates well with levels of the regulation-relevant CB-congeners (28, 52, 101, 118, 138 and 180) with correlation coefficients varying between 0.9752 – 0.9998 (Atuma et al. 1996). This makes PCB 153 a suitable CB-indicator and it was thus chosen to represent overall PCB exposure in this study.

1.3.2. *PCB-levels in Källby sewage treatment ponds*

To study the accumulation of PCB a chronically exposed location was chosen. Källby sewage treatment plant, operated by VA SYD, handles sewage water coming from Lund, Vallkärra, Stångby and Stora Råby and totally 83 200 people are connected to the plant (VA SYD, 2010). The WWTP treats about 30 000m^3 daily (Saleh et al. 2011). The sewage is treated by screen raking, sand catch, main sedimentation, biological treatment, anaerobic and aerobic denitrification and tertiary treatment. Part of the sludge from secondary treatment is lead to the inlet and treated again and some is mixed with tertiary and main sludge for additional treatment (Saleh et al. 2011).
The levels of PCB in sludge from Källby sewage treatment plant has been documented by VA SYD since 2008 and mean values of 0,03-0,05mg/kg TS (total solids) and total amounts of 0,07-0,1kg/year has been recorded (VASYD, 2013). The PCB-level in Källby is fairly low compared to the mean concentrations in Swedish treatment plants 2005 measuring 0,1mg/kg (Thuresson and Haapaniemi, 2005). However, few external studies concerning PCBs in Källby's sludge were found and no studies were found regarding PCB levels in organisms living in the treatment ponds which made it a suitable site to assess.

1.3.3. PCB accumulation in fish

Organic chemicals accumulate in fish through at least three different pathways; direct uptake through the gills, absorption through the skin or intake via diet (Swartz and Lee, 1980). The level of bioaccumulation is determined by lipid and protein content, age, size, purification rates, metabolic processes, temperature, feeding tactics etc. (Mackay, D. 2000, Sharpe, S. 2000, van der Oost, R. 2003). PCBs and other POPs (persistent organic pollutants) have commonly been detected in concentrations higher than what would be predicted from only bioconcentration factors (Fisk et al., 2001). Since PCBs are hydrophobic, once it enters an organism, it dissolves in the fatty tissues. Therefore the octanol-water partition coefficient (K_{OW}) and lipid-water partition coefficient (K_{LW}) of

the substance has been a main indicator used to predict level of bioconcentration of POPs (Mackay, 2001). Apart from bioconcentration, food-chain studies have shown that POPs can accumulate through the diet and biomagnify along food chains i.e. trophic magnification. PCB has a food chain magnification factor (FCMF) significantly >1 which implies that it will not only bioconcentrate in organisms but also biomagnify along the food chain (Gobas et al. 1999, Nfon et al. 2008).
This was explained by Gobas et al. 1999 by an altered fugacity ratio in the gastrointestinal tract in guppies. As the food is digested and lipids are removed from the tract in a higher rate than the chemical, the fugacity capacity for the chemical drops and the fugacity raises in the GIT (above that in the diet) which leads to passive diffusion into the organism where it is accumulated in fatty tissue (Gobas et al. 1999). The bioaccumulation depends on predator-prey fugacity ratio. If the ratio is larger than one biomagnification will most likely occur (Binelli and Provini, 2003).Substances with a K_{OW} higher than five and that are hard to metabolize has an elevated tendency to biomagnify in food chains (Mackay, 2001). Since POPs are lipophilic, once a substance has been taken up by an organism excretion is hindered (Nfon et al. 2008). This might also be the reason that organisms with higher lipid content has been found to have a higher toxic load than organisms with lower lipid content (Berglund et al., 2001).

1.3.4. Measuring of stable isotopes

To determine the trophic level of an organism a commonly used method is to assess the ratio of nitrogen isotopes; $^{15}N/^{14}N$, expressed as $\partial^{15}N$ (‰). For each tropic level $\partial^{15}N$ increases and the highest values are found in top predators (Broman et al., 1992). A study of bioaccumulation of OCs (organochlorines) in a freshwater food web in Canada found clear positive correlations between $\partial^{15}N$ and lipid–adjusted concentration (ng/g wet weight) of OCs (Kidd et al. 1998). In contrast, the carbon isotope $\partial^{13}C$ does not seem to change along the food chain and are therefore frequently used to determine carbon source in food webs (Nyström et al. 2003). The $\partial^{13}C$ signature differ in benthic and pelagic primary producers and can be used to assess the diet of fish species where a high $\partial^{13}C$ indicates lager share of benthic feed (e.g. ephilitic algae) and a lower $\partial^{13}C$ indicate a larger share of pelagic feed (e.g. zooplankton) (France, 1995, Kidd et al. 1998). Although both roach and tench are omnivorous fish that feed on both zooplankton and benthos the tench in generally known to live and obtain a larger part of its diet in benthic areas (Wanke, 2011). Based on this and PCBs tendency to accumulate in sediments (UNEP, 2007) the hypothesis is that there will be a higher concentration of PCB in tench than roach. Measures of $\partial^{15}N$ and $\partial^{13}C$ from sampled individuals will in this study be used to represent the feed of roach and tench in Källby treatment pond.

2. Method

2.1. Chemicals

Dichloromethane was purchased from VWR International (Leuven, Belgium). N-Hexan was purchased from Kebo lab (Spånga, Sweden). Sulfuric acid was obtained from Acros organics (Geel, Belgium). Chloroform (pro analysis) and sodium sulphate was purchased from Merck (Darmstadt, Germany) and methanol was obtained from Merck Schunchardt OHG (Hohenbrunn, Germany).

2.2. Sample preparation and analysis

Below is a model showing the different treatments that will be performed in this study;

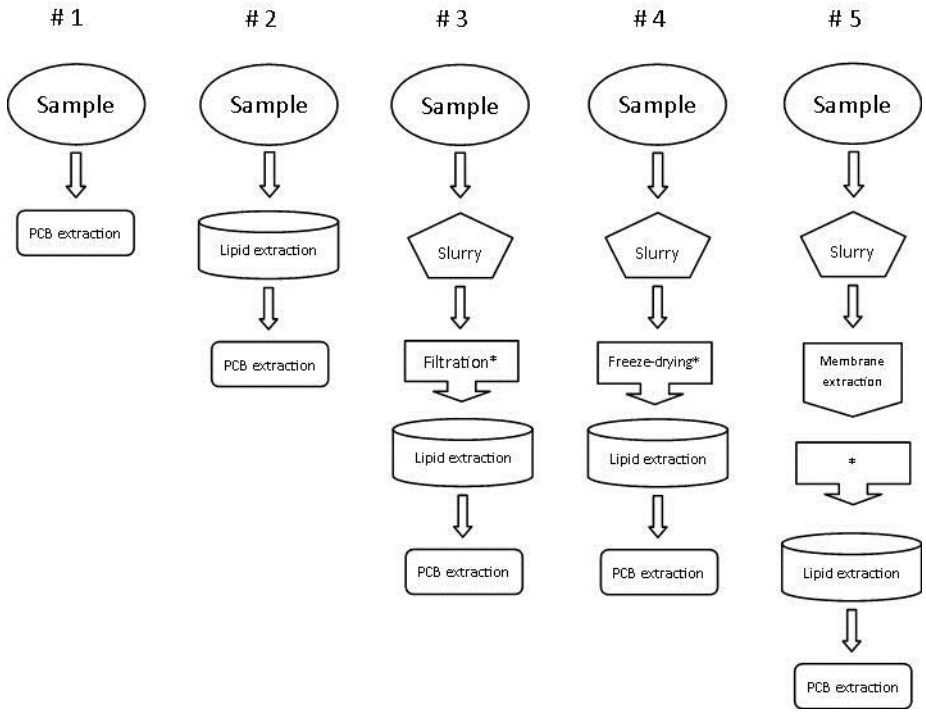

Figure 2. The model illustrates the different treatments that will be performed.
***Treatments will be assessed and the method that is proven best will be used for the membrane extraction treatment (#5).**

2.2.1. Method development for serial-extraction

15 samples from three different roach individuals were cut in sizes equivalent to approximately 0,5g wet weight. All samples and weighting boats were weighted three times and were freeze-dried overnight covered with aluminium. Samples were then weighted three times together with the weighting boats to retain dry weight. All pre used glass containers were burnt in an oven (350°C) for at least 5h covered with aluminium.

2.1.1.1. Slurry (#3, 4, 5)

Samples for the filtering, freeze-drying and membrane-extraction treatment (# 3, 4, 5) were put in separate 100ml bottles and each sample were homogenised with 30ml Milli-Q water using a IKA Dispersing device T 25 Digital (Staufen, Germany) for 10 minutes. Residues of the sample on the device were washed off with 20 ml Milli-Q water (totally 50ml/sample).

2.1.1.2. Membrane extraction (#5)

Weak acid pharmaceuticals were extracted from samples using a method for ion-trapping with hollow fiber liquid-phase microextraction (HP-LPME) (Boström, unpublished work, Sagrista et. al. 2012).

Results from lipid-extraction and PCB-analysis of filtered (#3) and freeze-dried (#4) samples were then awaited to determine which treatment is more appropriate to use for the membrane extracted samples.

2.1.1.3. Filtration (#3)

Slurry samples were filtrated using Whatman No. 3 filter paper (5,5 cm) (Whatman International Ltd., Maldstone, England). The original bottles and the funnel were washed with methanol and Milli-Q water and passed through the filtering system before the filter was removed to minimize the loss.

2.1.1.4. Freeze-drying (#4)

Slurry samples were freeze-dried.

The results from the lipid-extraction (below) showed a higher lipid yield from the samples that were freeze-dried then from the samples that were filtered. Therefore it was assumed to be a better method and used also for the membrane extracted samples.

2.1.1.5. Lipid extraction (#2, 3, 4, 5)

Lipids were extracted in accordance with the Bligh and Dyer method (Bligh and Dyer, 1959), with some modifications.

Day one:

Cutting treatment (#2):

Samples were cut into small pieces and transferred to a 15ml Kimax tube. 7,5ml of Bligh and Dyer-solution (B&D) was added to the tube.

Filtering treatment (#3):

Filters from samples were transferred to 15ml Kimax tubes and 7,5ml of B&D-solution was added to the tube.

Freeze-dried treatment (#4):

The freeze-dried slurry was transferred to a 15ml Kimax tube. 2ml of B&D-solution was added to the tube and 5,5ml was used to wash the bottle and transfer the remaining sample to the tube.

Membrane extraction treatment (#5):

Same process as for the freeze-dried treatment.

All samples were then sonicated for 10 minutes and left overnight in room temperature.

Day two:

2ml of hexane and 2ml Milli-Q water was added to each sample. The samples were vortexed for 10 sec and centrifuged for 2-4 minutes (1600rpm) until there was a clear phase separation. The organic phase (bottom layer) was then extracted and transferred to a pre-weighted 15 Kimax tube. The steps were then repeated two more times adding only 2ml of hexane each time (no Milli Q). The hexan was then evaporated from the pre weighted tubes using nitrogen gas (35°C) and the tubes were weighted again to retain lipid weights.

2.1.1.6. PCB-extraction and analysis (#1, 2, 3, 4, 5):

2ml hexane and 1-2ml of sulphuric acid was added to the tubes. Samples were shaken and left for a couple of minutes until there was a clear phase separation and the sulphuric acid had adopted a brown/yellow colour. The top layer (hexane) was then transferred to a new 15ml

Kimax tube and the steps were repeated until the sulphuric acid was clear. The hexane was then transferred to a new 15ml Kimax tube and was dried with sodium sulphate. Approximately 1,5 ml was transferred to a 2ml bottle. All samples were then analysed using a Bruker Scion TQ GC/MS/MS system (Bruker Cooperation, Fremon, CA) with CP-8400 autosampler and VF-1MS column (30m x 0;32mm i.d., 0;25µm film thickness). Chromatographic conditions were as follows: The source temperature was held at 230°C. Injector and detector retained at a temperature of 280°C and flow rate was set to 1.5ml/min. The column was set to 50°C for 2 min then increasing (40°C/min) reaching 300°C at 6.75min and finally held at 300°C until totally 15min had passed. PCB was identified based on mass and retention time and was then quantified using standards of known concentrations.

The results showed that the samples might be contaminated with water and therefore they were fully evaporated. 200µl of hexane was used to wash the bottles and were then transferred to vials with 500 µl inserts. The PCB samples were then run again as above.

2.2.2. Species Comparison

Ten samples of same type as in previous test were collected from different individuals of tench and seven from roach (since three individuals were already analysed in previous test by cutting treatment #2). Three extra samples were taken from a random individual of roach to be used for measuring standard yield of PCB from the employed method. 200µl of a 200ng/ L PCB/hexane solution was applied to these samples before they were cut and transferred to the tubes. Lipids and PCBs were then extracted from all samples using the same procedure as for samples #2 in previous test. A slight change was made for the PCB extraction where instead of adding sodium sulphate and using only 1,5ml of the sample; all of the hexane was extracted and evaporated in 1,5ml Kimax bottles in two sequences. Thereafter 200µl of hexane was used to extract the PCB from the bottle and was transferred to vials with 500 µl inserts. This was done to avoid any contamination of water. Samples were then analysed in GC/MS/MS as in section 2.1.1.6. $\partial^{13}C$ and $\partial^{15}N$ isotopes from 12 of the sampled individuals were also measured.

2.3. Statistical methods

For the method development (sect. 2.1.) an analysis of variance (ANOVA) with Tukey-Kramer post hoc tests were used to study the relationship between the lipid content (dependent variable) and the different extraction methods.

For the species comparison (sect. 2.2.) a standard curve was plotted from peak areas of known PCB concentrations and was used to derive unknown concentrations in the samples. The concentrations were recalculated to ng/g dry weight and also lipid normalised to ng/g lipid. A Mann-Whitney test was employed to test differences in PCB content between roach and tench. Both tests were completed in IBM SPSS Statistics Standard program (21.0) for Windows (IBM, New York, United States).

Two sample, unpaired, t-tests were conducted to assess the relationships of $\partial^{13}C$ and $\partial^{15}N$ isotopes and the species.

3. Results

3.1. Method development for serial extraction

Despite that the PCB samples were fully evaporated and dissolved in a smaller volume it was not possible to determine PCB-levels in the samples from the filtered, freeze-dried and membrane extracted treatments. However, based on the strong correlation between lipids and PCB-levels in organisms (Grafton 2006, Berglund 2005) the amounts of extracted lipids were used to determine the success of the various treatments.

Both the cut and the freeze-dried samples showed high mean values; 12.14 and 11.88 (g lipids/ g dry weight) compared to the filtered and membrane extracted samples with means of 5.02 and 5.93. They also had a small standard deviation (SD=1.35 and SD=1.06) (Figure 3). The membrane extracted samples had the lowest standard deviation (SD=0.60) and the filtered treatment had the highest (SD=3.77).

The ANOVA conducted on the amount of extracted lipids showed a significant effect of type of treatment to the amount of extracted lipids ($F(3, 6) = 10.738$, $p = 0.008$). The Post hoc comparisons (Tukey HSD test) implies that the cutting treatment is significantly different to both the filtered and membrane-extraction treatment (p=0.016 resp. p=0.036). The freeze-drying treatment is only significantly different to the filtering treatment (p=0.022) although the p-value is also low (p=0.054) for membrane extraction treatment (Figure 3.).

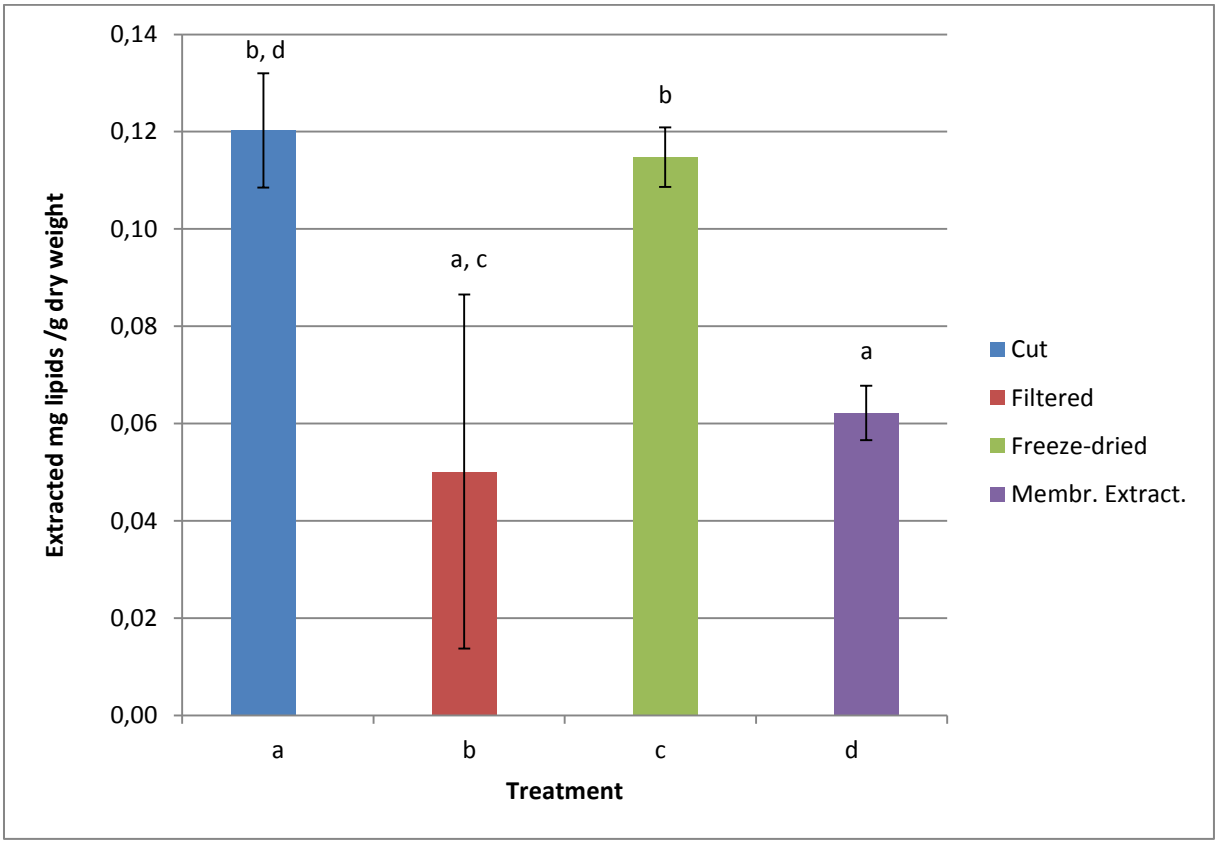

Figure 3. The graph demonstrates extracted lipids normalized to dry weight (g lipids/ g dry weight) resulting from the different treatments. The bars represent the mean value of the three roach individuals (n=3) and the whiskers represents the standard deviations (Cut: M=0.120 SD=0.012, Filtered: M=0.050 SD=0.036, Freeze-dried: M=0.115 SD=0.006, Membrane extracted: M=0.062 SD=0.006). Letters above the bars represent the treatments that are significantly different.

3.2. Species Comparison

Since the method for PCB extraction was slightly changed in the second study the three roach samples from the method development was excluded from the species comparison. One sample of tench was lost resulting in totally seven samples of roach and nine samples of tench. In two of the roach samples PCB levels were not detectable and they were therefore excluded from the statistical analysis. The PCB levels in the three samples with the known amount of applied PCBs showed a mean yield of 91,6% PCB. This was accounted for in the calculations of mean and standard deviation.

The mean concentration of PCB in ng/ g dry weight for roach was 0,019±0,019 and for tench 0,014±0,018 (Figure 4.). The Mann-Whitney test showed no significant difference between PCB content in dry mass of roach and tench (U=17.0, p=0.463, sig≤.05, 2-tailed, r=0.196).

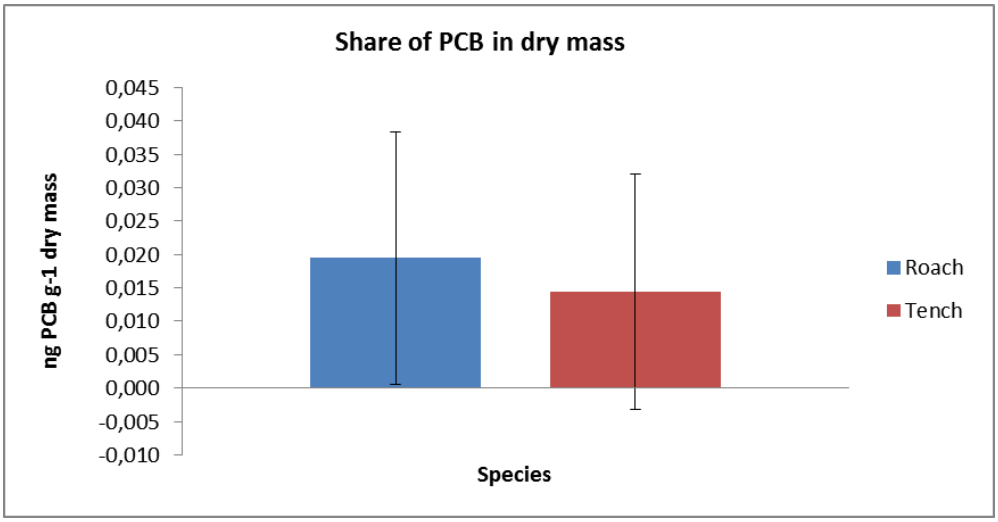

Figure 4. The graph illustrates mean concentrations and standard deviation of PCB in Roach (N=5) and Tench (N=9) (dry weight). Roach: M=0.019 SD= 0.019, Tench: M=0.014 SD=0.018.

After lipid normalization mean concentration of PCB for roach was 0.259 ±0.139 and for tench 0.129±0.138 (Figure 5.). Results from Mann-Whitney test displayed a considerably lower p-value but there were still no significant difference in PCB concentration between the two species (U=9.0, p=0.072, sig≤.05, 2-tailed, r=0.48).

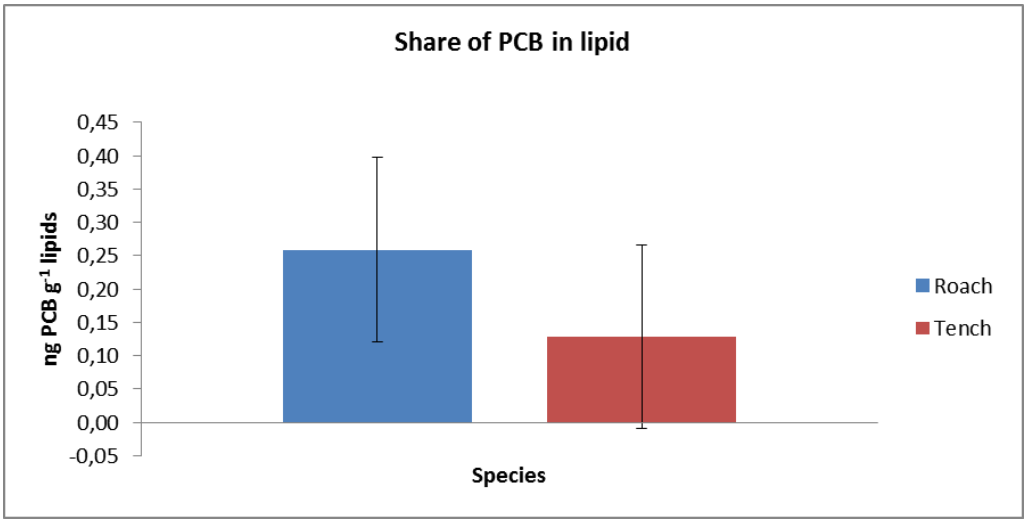

Figure 5. Lipid normalized data. The graph illustrates mean concentrations and standard deviation of PCB in Roach (N=5) and Tench (N=9) (ng/ g lipid). Roach: M=0.259 SD= 0.139, Tench: M=0.129 SD=0.138.

The t-tests demonstrated a clear significant difference between the mean $\partial^{13}C$(‰) in roach (-31.45±0.286) and in tench (-29.69±0.732); t(6)=-5.50, p=0.0015 (Figure 5.). There was also a significant difference in mean $\partial^{15}N$(‰) isotopes in roach (15.48±0.473) and tench (17.65±1,12); t(7)=-4,40, p=0.0032 (Figure 6.).

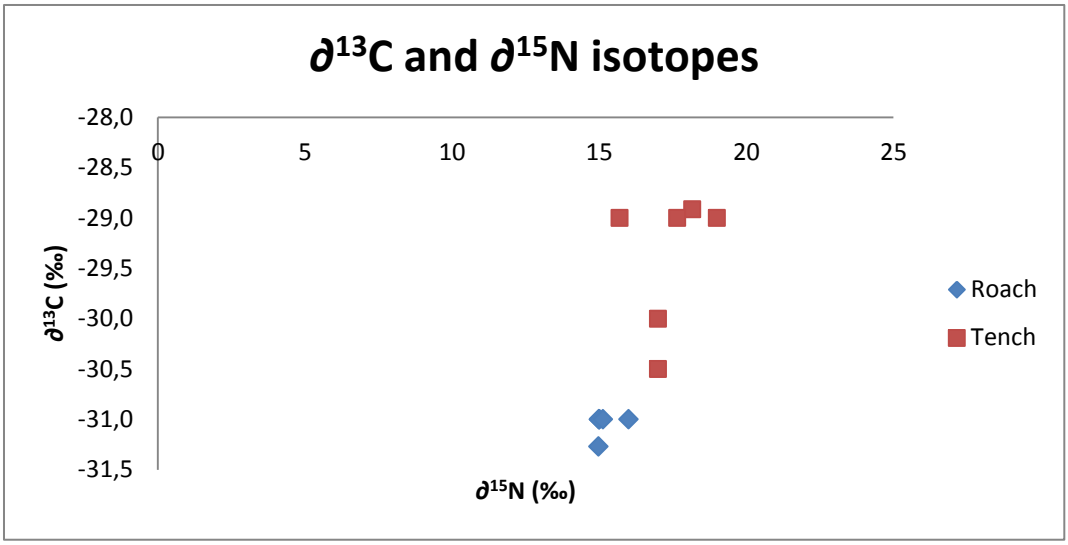

Figure 6. Carbon and nitrogen isotope ratios in roach (n=6) and tench (n=6). The species were significantly different in both $\partial^{13}C$ ratio (p=0.0015) and $\partial^{15}N$ ratio (p=0.0032).

4. Discussion

4.1. Method development for serial extraction

The ANOVA showed that there is a significant effect of type of treatment (p=0.008) to the amount of extracted lipids which confirms that there are critical steps in the serial extraction. The filtering treatment and the membrane extraction treatment both gave a near 50% less mean lipid yield compared to the cutting and freeze-drying treatments. The large standard deviation in the filtering treatment was possibly a result from lipids being trapped in the filter. Overall the high standard deviation and low mean yield implies that the method is defect and inappropriate for analytical studies. Freeze-drying was the alternative treatment to filtering to be able to extract lipids after a membrane extraction. The results from the freeze-drying treatment showed a mean yield of 0.115 (g lipids/ g dry weight) which was only 0.005g less than the mean yield from the basic cutting treatment. The standard deviation was also the lowest which suggest that it is an adequate method to use. The lipid yield from the membrane extraction was low; 0.062(g / g dry weight). In this treatment, during the lipid extraction after adding dichloromethane and Milli-Q water the second day, a white sludge appeared above the organic phase. It also had a tendency to mix with the organic phase which suggests that it contains organic substance. Hence, it is possible that a significant part of the fat was dissolved in the sludge. A possible explanation is that the lipids have been hydrolyzed from the acid HP-LPME-treatment and therefore partly dissolves in the water phase (Holliday et al. 1997). However this demands further research and modeling. The PCB in the samples from all five treatments (including the samples that were only used for PCB extractions) was hard to detect. Instead lipid yield was quantified since lipid:PCB relationship has been proven to be strongly correlated (Grafton 2006, Berglund 2005). By these means these results can serve as an indication of

the adequacy of the treatments. However for this method to be fully developed it should preferably be repeated performing the full PCB-analysis.

The desired method includes the extraction of NSAIDs, SSRIs, lipids and PCBs and thus this study does not answer the question if a serial-extraction is possible. Nonetheless, it shows that the freeze-drying treatment most likely is applicable and may be used in other studies treating water based fish slurries. Furthermore the filtration treatment has been proven inadequate unless it is modified. A possible alternative is to dry the filters before the lipids are extracted in accordance with the Weibull-Berntrop method (Schlechtriem, 2009).

4.2. Species Comparison

No significant difference ($p<0.05$) could be found in PCB concentration between roach and tench neither before nor after lipid normalization. However the p-value after normalization was 0.072 which indicate a tendency of higher concentrations of PCB in roach than in tench.

Based on the plot of $\partial^{13}C$ (‰) and $\partial^{15}N$ (‰) isotopes tench have both a significantly higher $\partial^{13}C$ ($p=0.015$) and $\partial^{15}N$ ($p=0.032$). The higher $\partial^{13}C$ supports the theory that its diet to a larger extent then the roach's consists of benthic feed (Wanke, 2011). The slightly higher $\partial^{15}N$ could imply that it is part of a higher trophic level). However, there are several possible explanations to the contradictive results. In a study by the Swedish Environmental protection agency, roach were considered to occupy a lower trophic level (2,8) than the tench (3.5) (Söderberg, 2008). Yet, it is possible that the differences in $\partial^{15}N$ (mean of roach: 15.48‰ tench:17.65‰) is not sufficient to represent a trophic level. In vitro observations by Deniro and Epstein (1981) showed increases of ~3‰ for each trophic level. This makes it questionable if the roach and tench from Källby resides different trophic levels differentiating only 2.17‰. Deniro and Epstein also showed that species despite feeding on the same diet can vary in $\partial^{15}N$. Nfon et al. found, when assessing benthic and pelagic food webs in the Baltic sea, that the benthic food chain inconsistently had a higher $\partial^{15}N$. Similar results were found in a study of fish's and invertebrate's dietary uptake from contaminated sediments in northeastern USA, where the lobster and scup had significantly higher $\partial^{15}N$ than the butterfish and squid although they were not occupying a higher trophic level (Morgan and Lohmann, 2010). According to Hoekstra (2003 a, b) this is due to a higher content of $\partial^{15}N$ in benthic feed. Using $\partial^{15}N$ for calculating trophic level in the benthic food web has even given negative fractions (DeNiro and Epstein 1991). Kidd et al. found that the $\partial^{15}N$ varied significantly in the lower parts of the food chain while the top of the food chain had more consistent levels depending on length of the food chain. Trusting these findings it is highly plausible that the roach and tench are in fact occupying the same trophy level. This is supported by the fact that the tench also has a higher $\partial^{13}C$ which implies that it to a lager extent feed from benthic sources then the roach (France, 1995, Kidd et al. 1998). Several studies have found correlation between $\partial^{15}N$ and PCB levels which contradicts the results in this study (Burreau et al., 2006, Kidd et al. 1998). However, also this relationship might be affected by dissimilarities in $\partial^{15}N$ between the benthic and pelagic food web.

The high K_{OW} and low water solubility of PCB 153 causes it to bind to unpolar material like fatty tissue or sediments and therefore the concentration in the water column is low (UNEP, 2007). It has also been shown that the main accumulation of POPs is caused by intake via diet (Nfon el. 2008). In a study of bioaccumulation of PCB congeners in western lake Erie in Canada more than 99% of the accumulated PCB 153 in fish (*Sander vitreus*) were estimated due to consumption of contaminated food (Morrison et al. 1997). Another study of dietary accumulation of PCBs from contaminated

sediments by demersal fish upheld a dietary contribution of 53 %. This experiment was conducted during 20 days and the dietary contribution seemed to enlarge with time (Rubenstien et al., 1984). Thus, possible differences in bioconcentration in roach and tench will be considered negligible when assessing the results in this study.

Studies of biomagnification of mercury in Poland showed similar results to this study where concentrations found in roach were more than twice of that found in the tench (Misztal-Szkudlinska, 2008). The cause was not assessed in the study but since mercury and PCB have similar physiochemical properties it is possible that same environmental conditions trigger the same distribution of PCB between the tench and roach in Källby STP (Powers, 2009). Conversely another study from two polish rivers connected to the Baltic Sea (Vistula River and Dead Vistula River channel) showed opposite results; 9 out of 9 metals (e.g. cadmium, mercury and lead) had higher concentrations in tench. (Wyrzykowska, 2012).

Zooplankton's diet in general consists primarily of phytoplankton and other zooplankton (Wyrzykowska, 2012). Therefore, if the results in this study are accurate, assuming that roach in Källby to a larger extent then tench feed from zooplankton then benthic invertebrates, it would imply that the phytoplankton has a higher level of PCB 153 than the benthic feed. This theory seems unlikely since PCB 153 accumulate in sediments (UNEP, 2007). Numerous of studies have shown that benthic fauna at least in lower part of the food chain has higher PCB 153 concentrations than the pelagic fauna, particularly the more chlorinated and hydrophobic PCB congeners like PCB 153 (e.g. Campfens and Mackay, 1997, Morgan and Lohmann2010, Nfon et al., 2008). Therefore the results in this study contradict most studies comparing PCB accumulation in benthic and pelagic food chains.

Studies have shown that roach feed almost exclusively of zooplankton during the ontogenetic stages (Wanke, 2011). In later stages its diet like the tench's is omnivore and both species can therefore change their diet depending on supply and other environmental factors. Both species also spend varying amounts of time in the different water zones and the results could thus reflect spatial and temporal differences (Nfon et al. 2008). Hence, it is not implausible that the result is an outcome of intra-species differences where also age and size influence outcome (Lána, 2008). Moreover it is possible that there are inter-species disparities like protein content, purification rates and metabolic processes which were not addressed in this study (Mackay, D. 2000, Sharpe, S. 2000, van der Oost, R. 2003). This would ideally be examined to be able to draw conclusions from this study

Even though the statistical analysis showed no significant result, the p-value ($p=0.072$ $p<0.05$) when normalized to lipid content indicated that there might be higher concentrations in roach. Experiment-wise it is possible that the yield of PCB from roach is higher than from tench. Therefore to improve this study, standards for PCB extraction in tench should also be assessed. Inter-laboratory surveys have shown that analytical errors are common in surveys of POP concentration in organisms (Muir & Sverco, 2006). There might be methods for lipid- and PCB-extraction that give more accurate results than the methods used in this study. Muir and Sverco (2006) suggested that examining the samples in their original state, e.g. no freeze-drying, would give more accurate result in PCB/OC analyzes.

Furthermore, to be able to draw strong conclusions a more extensive study is needed. Firstly, more replicates could reduce the effect of intra-species variations. Secondly, assessing the feed, tentatively by stomach contents analysis, could help to verify the diets (Hyslop, 2006). PCBs in the feed (e.g. zooplankton and epilithic algae) should also be measured to further corroborate the results (Wanke, 2011).

Finally, it would be interesting to compare the results from Källby STP to other places where both species reside and possibly assess larger, less contaminated areas to see how the species differentiate in a more pristine environment.

5. Conclusion

For the method development there was a significant effect of type of treatment ($p=0.008$) to the amount of extracted lipids. The filtering treatment showed both a low mean and high standard deviation which suggests it is inappropriate for analytical studies. However, the freeze-drying showed a mean of 0.120 g lipids/ g dry weight which was only 0.005g lower than the cutting treatment and indicates that it is a valid treatment. The membrane extraction treatment showed consistent but low mean lipid mass. This was probably due to lipids being hydrolysed in the HP-LPME-treatment and as a result part of the lipids dissolved in the water phase. However this demands further research.

The species comparison showed no significant results although after lipid normalization the roach indicated higher concentrations of PCB 153 with a probability of 0.072.
Both $\partial^{15}N$ and $\partial^{13}C$ were significantly higher for the tench ($p=0.032$ resp. $p=0.015$) indicating higher trophic position and higher share of benthic feed. However, the higher $\partial^{15}N$ was in other studies justified by larger share of benthic feed (similar to $\partial^{13}C$) and therefore the assumption was neglected.

The higher concentration of PCB 153 in roach could not be explained although one study assessing mercury had found similar results (Misztal-Szkudlinska, 2008). Therefore the results are believed to be an effect of either inter- and intra-species variations or possibly experimental flaws such as failure in assessing PCB yield from tench or insufficient amount of replicates.

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22362 Lund