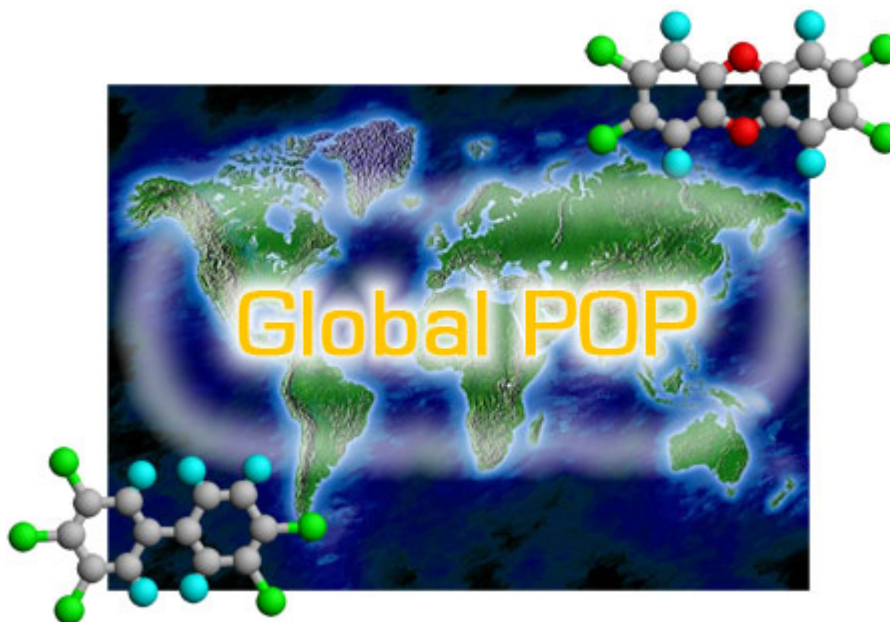


# **The IPY project Global POP**



## **International School Education**

### **Protocol: DIOXINs in local fish**

**Fall 2007**

**Phase II-IV**

**Sample preparation  
Evaluation of bioassay results  
Writing report**

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# IPY project: Global POP 2007-2008

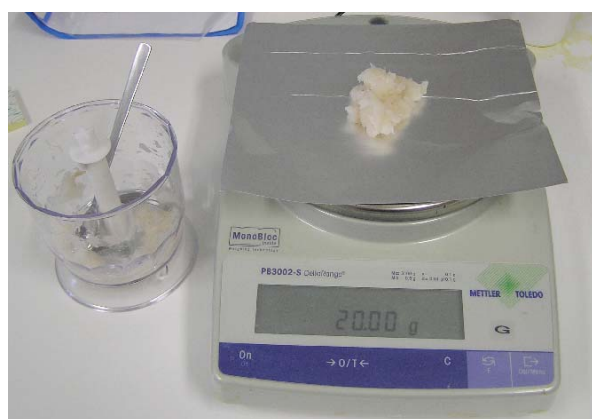
## Dioxins and dioxin-like compounds in fish

### Phase II: Example of sample preparation for POP analysis

#### 1 Homogenization

Before taking a sample for extraction, samples should be homogenized to obtain a proper (sub) sample before further use.

First the fish sample (10-25 g muscle, depending on the lipid content) is cut or minced into small pieces. Next, the small pieces of filet are homogenized in a clean blender. You may also mix the sample pieces by using a pre-cleaned spoon to create a homogenous paste into the jar.



#### 2 Extraction

The compounds of interest are extracted with an extraction solution of hexane and diethyl ether. Prior to addition of the extraction solution, water and isopropanol are added. Isopropanol is added to facilitate the penetration of extraction solution by reducing water covers of the particle surface and to open the three-dimensional (3D) structures of proteins. Water is added to suspend the solid material for better shaking efficiency.



### 3 Extraction (continues)

The glass container with the fish sample, water, isopropanol and extraction solution is then mixed thoroughly on a shaker. After shaking leave the extraction flask closed and let the layers separate. The upper hexane level is then transferred to a preweighed (0.001 gram accuracy) and cleaned collection vial. The described extraction procedure is repeated twice. The collected hexane fractions is then evaporated in the collection vial under a gentle stream of nitrogen at a temperature below 35C° C. The collection vial with the residue is then weighed in order to evaluate the samples lipid content. The amount of residue in the collection vial is defined as the samples lipid content.



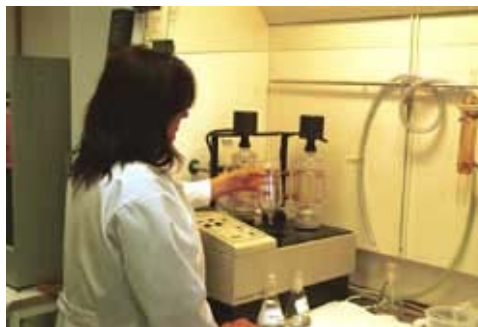
### 4 Clean-up of sample extracts

The evaporated residue is dissolved in a small amount of hexane and cleaned-up on an acid-silica column. The acid-silica column is prepared on the lab by adding sulfuric acid treated silica on a glass column. The fish extract is added the column and eluted with hexane and diethyl ether. This process will oxidize impurities in the extract and be retained on the column. The POPs will follow the solvent into the collection vial.



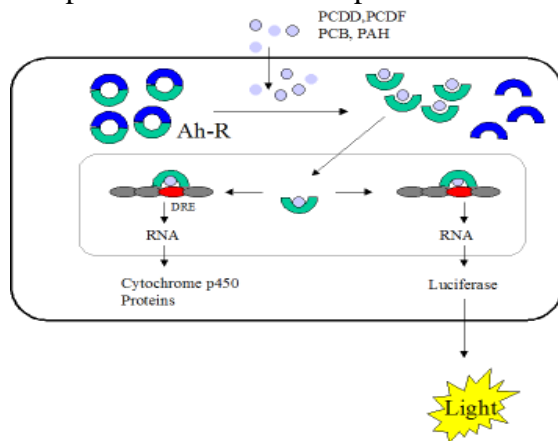
## 5 Volume reduction

After clean-up the solvent has to be removed and the sample is carefully evaporated into dryness. A system (TurboVap) that vaporizes and removes larger volumes of the organic solvents is used at NILU. One can also use a gentle stream of nitrogen as described above. The analytes will not disappear because they have a much higher boiling points and a lower vapor pressure than the organic solvents. A small amount of dimethylsulfoxide (DMSO) is added the evaporated residue and the samples are now ready for analysis.



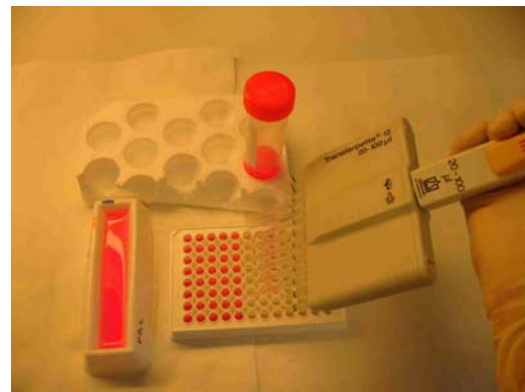
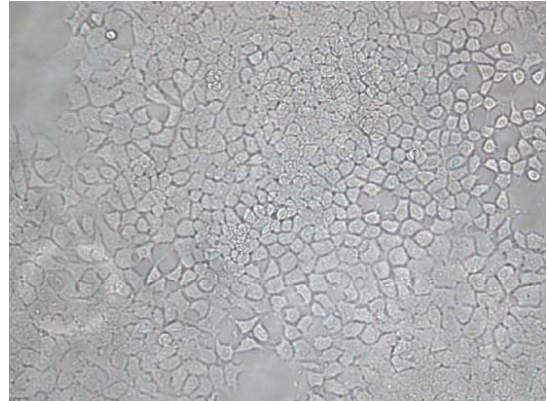
## 6 Analysis and quantification

The samples are then analyzed with the use of bioassay. Dioxin sensitive liver cells are used, which transmit light as a response of dioxin exposure. The amount of light is a measure for the amount of dioxins and dioxin-like compounds in the fish sample.



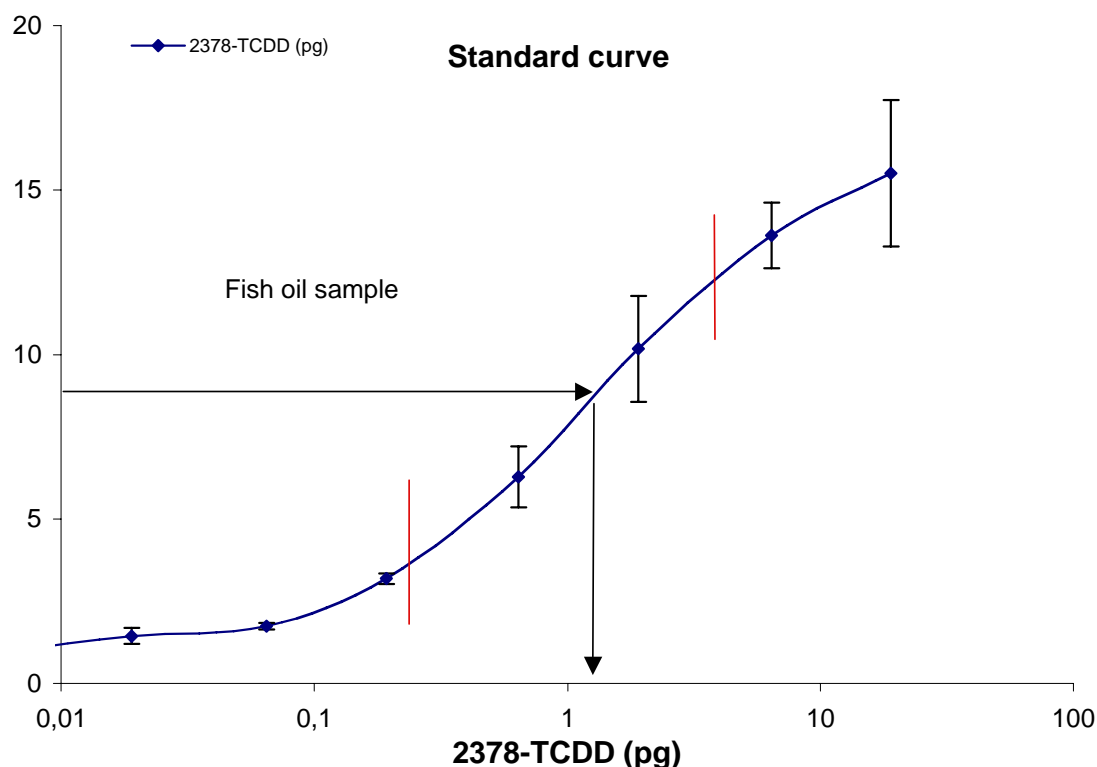
Human eyes do not see the transmitted light and to be able to measure the transmitted light one has to use a so-called luminometer, which is extremely light sensitive.

The cells are exposed to the prepared extract in small wells, which enables a high throughput of analysis. The cells are exposed in 24 hours before quantification in a luminometer. To be able to quantify the amount of dioxins in the sample, the cells are also exposed to increasing concentrations with the most toxic dioxin known (2378-TCDD). This is called a standard curve.



### Phase III: Evaluation of bioassay results

When phase II (Bioassay analysis) is done, the result for each sample can look like the figure below: The figure shows the standard curve of 2378-TCDD, which is equivalent to the WHO-Toxic Equivalents (TEQ). 2378-TCDD is the most toxic dioxin known and therefore used as a reference substance. The x-axis shows the concentration of 2378-TCDD and the y-axis shows the amount of light transmitted (RLU). When knowing the amount of light transmitted from the liver cells that have been exposed to the fish sample one can read directly from the standard curve the content of dioxins- and dioxin like chemicals in the sample.



WHO has recommended regulatory limits of dioxins and dioxin like chemicals in different food and feed as shown in the table below. If the levels of these chemicals are below the recommended limits it is not likely that any adverse effects will appear in people consuming the food. For example WHO recommends that one shall not be exposed to more than 1-4pg TEQ/kg body weight per day in food. That means that a person weighing 80kg can be exposed to 80-320pg TEQ per day through food. EU operates with a tolerable intake of 14pg

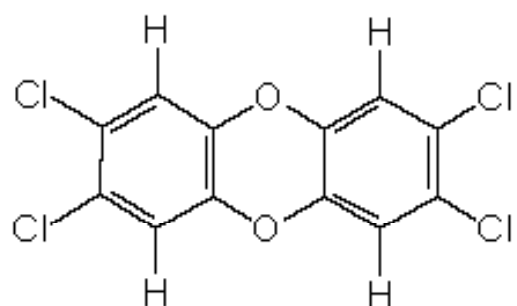
TEQ/kg body weight per week, which is a slightly more conservative estimate. If the level of dioxins and dioxin like chemicals in food is known it is easy to estimate if the food is above or below recommended levels, and how much it is recommended to eat.

Foodstuff	Maximum permitted level	Action Limits for dioxins
Meat, bovine	3pg WHO-TEQ/g fat	2pg WHO-TEQ/g fat
Poultry	2pg WHO-TEQ/g fat	1.5pg WHO-TEQ/g fat
Pig	1pg WHO-TEQ/g fat	0.6pg WHO-TEQ/g fat
Liver and derived products	6pg WHO-TEQ/g fat	4pg WHO-TEQ/g fat
Fish muscle	4pg WHO-TEQ/g whole weight	3pg WHO-TEQ/g whole weight
Milk	3pg WHO-TEQ/g fat	2pg WHO-TEQ/g fat
Eggs	3pg WHO-TEQ/g fat	2pg WHO-TEQ/g fat
Vegetable oil	0.75pg WHO-TEQ/g fat	0.5pg WHO-TEQ/g fat
Fish oil	2pg WHO-TEQ/g fat	1.5pg WHO-TEQ/g fat
Mixed animal fat	2pg WHO-TEQ/g fat	1.5pg WHO-TEQ/g fat

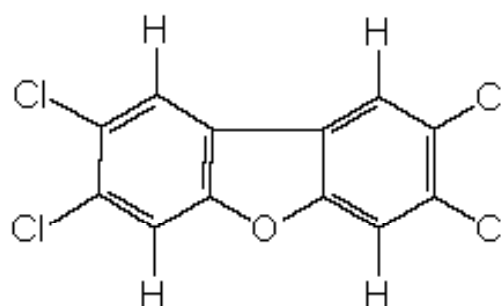
WHO	1-4pg/kg body weight/day
EU	14pg/kg body weight/week

## 1 Compounds measured with the bioassay method

The chemical compound groups, dioxins, furans and dioxin-like PCBs and PAHs are not specific types of chemical but group of chemicals with very similar chemical structures and/or effects. For example, for PCBs and dioxins, there are theoretical 209 different molecules or congeners based on their degree of chlorination and chlorination pattern. The bioassay method used in this project measures an effect and do not selectively analyze specific chemicals. Chemicals that can induce effects on the bioassay method are primarily dioxins, furans, PCBs and PAHs. The method is therefore a semi-quantitative method. However, the clean-up procedure and the exposure time of 24 hours will exclude the influence of most PAHs and the observed effect will mainly be due to dioxins, furans and dioxin-like PCBs.

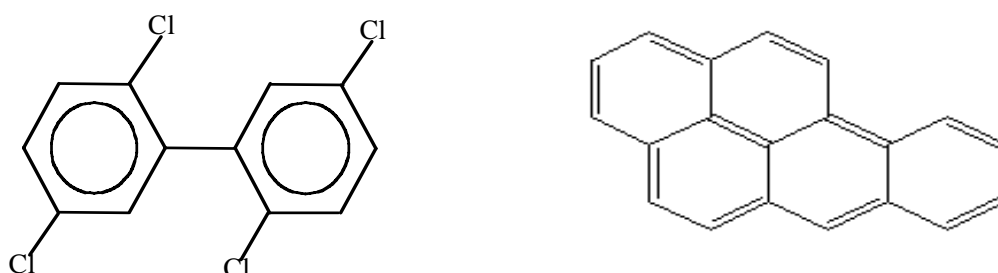


2,3,7,8-TCDD



2,3,7,8-TCDF

**Figure 1: Example of an individual dioxin and furan.**



**Figure 1: Example of an individual PCB and benzo(a)pyrene, which is recognized as the most toxic PAH.**

## 2 Understanding the received result sheets

As can be seen from below the results are given **in a table**. The first part is information on the sample itself, reference number, dates type of sample etc. Very important is the reference to the protocol Datasheet that contains all the additional facts on the sample. For the evaluation the students are to use both the Analysis result sheets and the Datasheet. Each school will receive the raw data file to be able to estimate themselves the level of dioxin-like compound in their fish samples.

### 2.1 Reference facts:

<b>School:</b>	Name of school
<b>Country:</b>	Name of country where school is located
<b>Sampling date:</b>	Date of the samples was taken by the school
<b>Type of sample:</b>	salt or freshwater, fish species + organ
<b>Sample received at NILU:</b>	Date when the sample was received
<b>NILU sample number:</b>	reference ID
<b>Sample amount:</b>	Amount of sample used by NILU
<b>Concentration units:</b>	Measurement unit used (see explanations to table)
<b>Lipid weight</b>	%
<b>Date of analysis:</b>	Date when chemical analysis was completed
<b>Response</b>	pg /g fish wet weight
<b>Response</b>	pg/g fish lipid weight

### 3 Evaluation of the results

NILU scientists will evaluate the results after the samples are analyzed. Based on this, NILU will post the result sheet on the web site for the respective school and give each school an evaluation task. These evaluations are to be written in the report in phase IV.

The details of the task for each school cannot be given before the results are available but it will follow a standard format where all schools should do some general evaluations.

Example of specific tasks schools can be given:

1. Based on recommendation from WHO as shown in table above, make an evaluation of the levels of dioxins- and dioxin like compounds in your fish samples in order to do a risk assessment for potential consumers.
  - a. If one meal is approximately 200g of fish fillet, how often can you eat the fish without exceeding the recommendations from WHO
  - b. Are your fish samples below or above the recommended action limits from WHO?
  - c. If your fish samples are above recommended action limits from WHO, try to give an explanation.
  - d. Do your country have recommendations on dioxins in fish that are different from the recommendations given by WHO?
2. Look at dioxin data from fish samples for all the Arctic schools. The levels are different. Please discuss the possible reasons for this based on geographical distribution and different biology of the analyzed fish.
3. Look at the levels in your fish samples and compare this to scientific articles on dioxin levels in fish in your area if existing, and in Arctic in general. Also look for reported time trends and see where your result fit in.



## Phase IV: Writing report

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The background for writing the reports are the work done in phase I and III. The aim of this phase is to learn how to report properly as well as collecting the evaluations and lessons learned in order to improve the protocols as well as ensuring the scientific use of the evaluations.

**The report generator will be available through the project web site on [sustain.no](http://sustain.no)**

### 1 Template example for reporting

1. Preface
2. Content list
3. Summary (half page)
4. Report of sampling (Phase I)
  - a. Description of how it was done (pictures can be included)
  - b. Documentation of scientific correct sampling through photos

- c. Photos of gonads laying next to the body cavity
  - d. What was good and what can be improved
- 5. Report on the results from bioassay (Phase III)
  - a. Description of tasks given
  - b. How the task was solved
  - c. Discussion
  - d. Conclusion
  - e. Reference list
- 6. OPTIONAL: Report on other teaching activities resulting from this protocol
- 7. Resources used (institutes, resources persons, web sites, agencies)