University of Stavanger

Faculty of Science and Technology

MASTER'S THESIS

Study program/ Specialization: MSc Environmental Monitoring and Nature Management in the Northern Oil and Gas Producing Regions	Spring semester, 2016 Open / Restricted access
Writer: IO Ada Karmelle Delgado	(Writer's signature)

Faculty supervisor: Magne O. Sydnes

External supervisor(s): Daniela M. Pampanin

Thesis title:

Biomarker responses in Atlantic cod (*Gadus morhua*) exposed to PAHs: data treatment, data interpretation and communication of results

Credits (ECTS): 30

Key words: Biomarkers Atlantic cod Polycyclic aromatic hydrocarbons (PAHs) PAH metabolites Crude oil Multivariate analysis Data treatment	Pages: 73 + enclosure: 21 pages (Appendix) + 1 USB flash drive Stavanger, 15.06.2016 Date/year

Abstract

The evaluation of multivariate statistical analysis to provide a simplified output based on biomarker responses is presented in this study. Biomarker responses obtained from two separate exposures on the effects of PAH on Atlantic cod were integrated using Principal Component Analysis (PCA), Integrated Biomarker Responses (IBR) and Bioeffect Assessment Index (BAI). The calculation of the indices was used to distinguish the different exposure groups within and between exposure setups. First exposure was designed to differentiate the effects of two PAHs and their first metabolic products in *in vivo* exposed Atlantic cod, and the second exposure was to reveal the effects of crude oil depending on the length of exposure. The sensitivity of the biomarker and the effectivity of using different suites of biomarkers in determining the variation between groups were also demonstrated. According to PCA results, the groups in the first exposure setup were not clearly discriminated but were differentiated in the second exposure, where the control group is clearly separated from the exposure groups after being exposed for 7 and 30 days. IBR was able to indicate the different groups for the second exposure setup, but only gave emphasis on the naphthalene exposed (high dose) group for the first setup. PAH metabolite biomarkers were consistently dominant in exposing the different groups in both exposures as shown by IBR and IBR/n. BAI reflected the dose-response relationship between the groups in the second exposure but was not evident on the first exposure. BAI is inclined to indicate the different health condition of the individuals in the groups in each exposure. PCA and IBR were able to acquire information about the biomarkers involved in the differentiation of groups. Based on the results, the biomarker responses were reflective of the effect of PAH on the health condition of individual samples and groups using the methods in the second exposure. The indices were able to extract consequent information out of the biomarker data and provide a simplified output despite their varying sensitivity, resolution and graphical representations.

Table of contents

Abstract I

Table of contents II

Acknowledgements IV

List of figures V

List of tables VII

Abbreviations VIII

1. INTRODUCTION 1
- 1.1 The species: Atlantic cod (*Gadus morhua*) 1
- 1.2 PAH exposure 1
- 1.3 Biomarkers 2
- 1.4 Multivariate statistical analysis 6
  - 1.4.1 Principal Component Analysis (PCA) 7
  - 1.4.2 Integrated Biomarker Response (IBR) 7
  - 1.4.3 Bioeffect Assessment Index (BAI) 7
- 1.5 Purpose of study 8

2. MATERIALS AND METHODS 9
- 2.1 Sources of data 9
  - 2.1.1 First exposure 10
  - 2.1.2 Second exposure 11
- 2.2 Biomarkers 12
  - 2.2.1 Biotransformation enzymes (EROD, GST) 12
  - 2.2.2 Oxidative stress parameters (CAT) 13
  - 2.2.3 Biotransformation products (PAH metabolite) 13
  - 2.2.4 Genotoxic parameters (DNA adducts) 14
  - 2.2.5 Physiologic and morphologic parameters (CI, LSI, GSI, histopathology) 14
- 2.3 Multivariate statistical analysis 15
  - 2.3.1 Principal Component Analysis (PCA) 15
  - 2.3.2 Integrated biomarker response (IBR) 16
  - 2.3.3 Biomarker assessment index (BAI) 17

3. RESULTS AND DISCUSSION 18
- 3.1 Data treatment 19
  - 3.1.1 Principal Component Analysis (PCA) 19
  - 3.1.2 Integrated Biomarker Response (IBR) 20
  - 3.1.3 Biomarker Assessment Index (BAI) 20
- 3.2 Data interpretation 23

3.2.1 First exposure 24
3.2.2 Second exposure (7 days) 35
3.2.3 Intraperitoneal and CFS exposures (7 days) 44
3.2.4 Second exposure (30 days) 53
3.3 Data communication 62

4. CONCLUSIONS 67

5. FURTHER RECOMMENDATION 68

REFERENCES 69

APPENDIX

A Dataset A: biomarker data from first exposure

B Dataset B: biomarker data from second exposure (sampling after 7 days)

C Dataset C: biomarker data from second exposure (sampling after 30 days)

D Stages of toxically-induced alterations of the biomarkers and corresponding BAI values

E Standardized values used for PCA calculations (datasets A-C)

F Score calculations in IBR (datasets A-D)

G Individual scores for all biomarkers in each group (datasets A-D)

Acknowledgements

The completion of this work owes a lot of thanks to my supervisors Dr. Magne O. Sydnes and Dr. Daniela M. Pampanin, for the encouragement and the time since the beginning, and for broadening my view on organic chemistry and ecotoxicology. Gratitude is also extended to my family and friends for the support and for understanding the isolation. Last but not the least, thanks to the Research Council of Norway for funding the project "Indicators for Environmental Impact of Petroleum Activities: the Next Generation of Molecular Markers" (iNEXT) where the biomarker data used in this project is based from.

List of figures

Figure 1. Schematic representation of the sequential order of responses to pollutant stress within a biological system

Figure 2. Illustration of the fate of xenobiotic compounds in the liver cell

Figure 3. Plot of loadings for dataset A

Figure 4. Dataset A: score plots of individual samples using different combinations of biomarkers

Figure 5. Dataset A: scores and loadings plot

Figure 6. IBR star plots for the different exposure groups (different sets of biomarkers)

Figure 7. IBR/n for dataset A comparing different sets of biomarkers

Figure 8. Star plots representing each biomarker in each set for dataset A

Figure 9. BAI values of each group in dataset A

Figure 10. BAI values of each group in dataset A (different sets of biomarkers)

Figure 11. Plot of scores for dataset B

Figure 12. Dataset B: score plots of the individual samples (different sets of biomarkers)

Figure 13. Dataset B: scores and loadings plot

Figure 14. IBR star plots for the different exposure groups (different sets of biomarkers)

Figure 15. IBR/n for dataset B comparing different sets of biomarkers

Figure 16. Star plots representing each biomarker in each set for the different groups in dataset B

Figure 17. BAI values for dataset B

Figure 18. BAI values of dataset B (different sets of biomarkers)

Figure 19. Plot of scores for dataset D

Figure 20. Dataset D: score plots of individual samples (different sets of biomarkers)

Figure 21. Dataset D: scores and loadings plot

Figure 22. IBR star plot for the different exposure groups in dataset D

Figure 23. IBR/n for dataset D comparing different sets of biomarkers

Figure 24. Star plots representing the biomarkers used in the index analysis of dataset D

Figure 25. BAI values of each group for dataset D

Figure 26. BAI values of dataset D (different sets of biomarkers)

Figure 27. Plot of scores for dataset C

Figure 28. Dataset C: score plots of the individual samples using different combinations of biomarkers

Figure 29. Dataset C: scores and loadings plot

Figure 30. IBR star plots for the different exposure groups (different sets of biomarkers)

Figure 31. IBR/n for dataset C comparing different sets of biomarkers

Figure 32. Star plots representing each biomarker in each set for the different groups in dataset C

Figure 33. BAI values of each group for dataset C

Figure 34. BAI values of each group in dataset C (different sets of biomarkers)

Figure 35. Presentation of the study title and analyzed biomarkers in the sentinel organism.

Figure 36. Presentation of results for the first exposure

Figure 37. Presentation of results of the biomarker data obtained 7 days after the second exposure

Figure 38. Presentation of results using biomarker data from the first and second exposures

Figure 39. Presentation of results of the biomarker data obtained 30 days after the second exposure

List of tables

Table 1. Abbreviations for all exposure groups used in all datasets

Table 2. Characterization of the single BAI stages

Table 3. Results of selected biomarkers (PAH metabolite using GC-MS method) for all groups in datasets A-C

Table 4. Average BAI values for dataset A using all biomarkers

Table 5. Average BAI values for dataset B using all biomarkers

Table 6. Average BAI values for dataset C using all biomarkers

Table 7. Average BAI values for dataset D using all biomarkers

Table 8. IBR values for each group in dataset A using different sets of biomarkers

Table 9. IBR values for each group in dataset B using different sets of biomarkers

Table 10. IBR values for each group in dataset D using different sets of biomarkers

Table 11. IBR values for each group in Dataset C in different sets of biomarkers

Abbreviations

AChE	Acetylcholinesterase
Ade	Adenoma
An	Aneurysms
Bas	Basophilic
BC	Blood congestion
CAT	Catalase activity
CI	Condition index
CFS	Continuous Flow System
EL	Epithelial lifting
EMS	Excess mucus secretion
EROD	Ethoxyresorufin-*O*-deethylase
Fib	Fibrosis
FD	Fatty degeneration
GC-MS	Gas chromatography-mass spectrometry
Gra	Granomula
GSI	Gonadosomatic index
GST	Glutathione-S-transferase
HPLC/F	High-performance liquid chromatography with fluorescence detection
Inf	Inflammation
LF	Lamellar fusion
LSI	Liver somatic index
MMA	Melano-macrophage aggregates
Nec	Necrosis
OiW	Oil in water
Par	Parasite
PEC	Proliferation of epithelial cells

1. INTRODUCTION

The marine environment is under constant pressure due to the fact that it is the ultimate sink for various types of pollutants. One of the pollutants that has been given worldwide attention is polycyclic aromatic hydrocarbons (PAHs). Sources of such contaminants can range from natural to anthropogenic and enter the environment from for example offshore oil and gas production. Its presence in aquatic organisms has been a growing concern since it can lead not only to deterioration of aquatic populations but also humans thru seafood ingestion.

One of the ways to be able to counter and formulate methods to prevent PAH's harmful effects is to be able to understand the degree of damage it can cause. The use of biological markers in organisms that are directly affected by this contaminant is one of the ways in order to increase this understanding. But the complexity of the biological processes in an organism and the unstable state of the ecosystem makes it difficult to interpret the results obtained using the markers directly. Thus the use of mathematical methods to simplify the data obtained from the markers can serve as a useful tool in providing a comprehensive assessment of the effects of pollutants in marine organisms.

1.1 The species: Atlantic cod (*Gadus morhua*)

Choosing the appropriate organism that is most suitable for the purpose of evaluating the effects of a certain pollutant in a chosen environmental compartment, often termed a sentinel, is an important preliminary step in a biomarker-based monitoring. The sentinel must demonstrate the presence of the pollutant and the extent of its exposure. It should be a model showing negative impacts in the long run, for the individual organism and the population, and to a higher extent, the ecosystem involved [1].

Fish has played an important role in monitoring programs due to its position in the trophic chain, in addition to demonstrating the aforementioned criteria [2]. Atlantic cod was selected as an eligible sentinel species because it has a high commercial value especially for countries that relies on its fishery, its abundance and is widely distributed [3,4].

1.2 PAH exposure

PAHs are aromatic, hydrophobic and organic compounds found in crude oil [5]. These compounds have been chemically converted over time from natural products like steroids [6]. Some

of the compounds can be substituted by alkyl, nitro and amino groups in their conjugated ring systems causing it to be more reactive [7].

The increasing exposure of the marine environment to crude oil has led to the growing concern of PAH contamination that can cause carcinogenic and mutagenic changes in marine organisms [8,9]. It has also been listed as a priority hazardous substance for surface waters in the Water Framework Directive [10]. Marine contamination by PAHs can either be of petrogenic or pyrogenic origin. PAHs coming from forest fires and incomplete combustion of organic compounds such as fossil fuels are grouped under pyrogenic PAHs. Direct contamination of the marine environment by industrial effluents, accidental spills and discharge from offshore oil installations are sources of petrogenic PAHs [7].

1.3 Biomarkers

A biological marker (biomarker) is identified as a biological response of an organism which deviates from the norm as an effect of exposure to contaminants [11]. Specific biomarkers can be used for contaminant analysis in aquatic organisms. They can be capable of detecting early signals of exposure or long term effects [12]. The biomarker approach in monitoring has also been suggested as a suitable method for identifying sublethal effects of pollutants due to its capability of providing early indication of possible adverse effects in the organisms [13].

Biomarkers can range from subcellular effects to whole tissue damage which can reflect the state of contamination in different levels of organization in the organism and the ecosystem (Figure 1) [2,14-16]. Therefore, the selection of a suite of available biomarkers and the integration of their responses in the form of a multivariate dataset is a relevant technique in providing a more valid basis for interpretation. These markers are important tools to evaluate the effects of contamination in an organism because the degree of contamination is not normally reflected in fish tissue accumulation alone. By using the relationships of biomarker responses for exposure and effect in different levels of organization, it is possible to analyze the overall impact of a specific pollutant (i.e. PAH) on the selected organism [17]. Due to the varying results that each biomarker can provide, combining them can provide a more encompassing evaluation of the condition of the exposed organism [15].

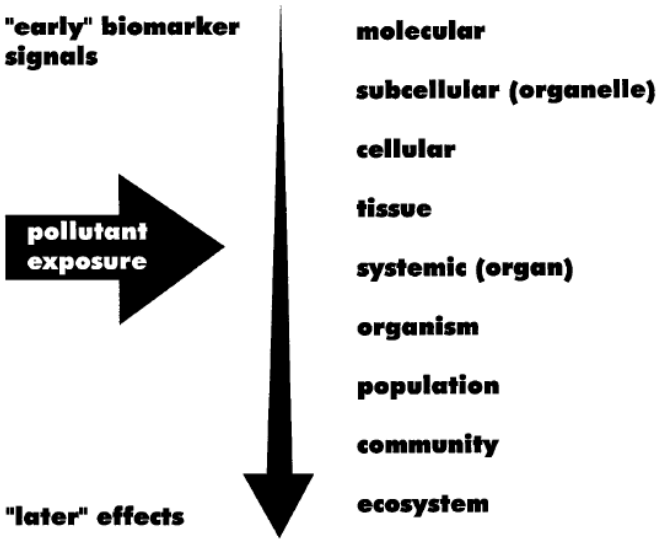

Figure 1. Illustration of the sequential order of responses of an organism to pollutant stress within a biological system. Modified from Bayne *et al.* [12,18].

Monitoring programs are increasingly including the integration of molecular level to tissue level types of biomarkers due to their rapid response and highly specific effects to environmental contamination [19,20]. These types of biomarkers are preferably administered in combination rather than singly for the thorough assessment of the biological effect of the pollutant [21-24]. Based on the study done by Van der Oost et al. [12], biomarkers can be grouped according to the different parameters involved in their response to the contaminant: biotransformation enzymes (EROD and GST), oxidative stress parameters (CAT), biotransformation products (PAH metabolites), genotoxic parameters (DNA adducts), and physiologic and morphologic parameters (CI, LSI, GSI and histopathology).

The alterations in levels and activity for biotransformation enzymes are considered to be in the list of sensitive markers. These markers are involved in the possible mechanisms of enzymatic induction following route I (Figure 2) after exposure [12]. This route breaks down into three phases wherein phase I (EROD) and phase II (GST) activities takes place in fish [12,25]. EROD activity is an early response biomarker at the molecular level that has been proven to be the most effective biomarker for detecting the effects of aromatic xenobiotic compounds in fish [2]. EROD is therefore suitable to be used as a biomarker for evaluating the immediate exposure of the organism [15]. EROD has been found to increase with increasing pollutant concentrations [2]. GST is another enzyme involved in biotransformation as the organism's defense against oxidative damage and peroxidative products of DNA and lipids. Increasing hepatic GST activity has been observed in several studies where the organism has been exposed to PAHs and other pollutants [12].

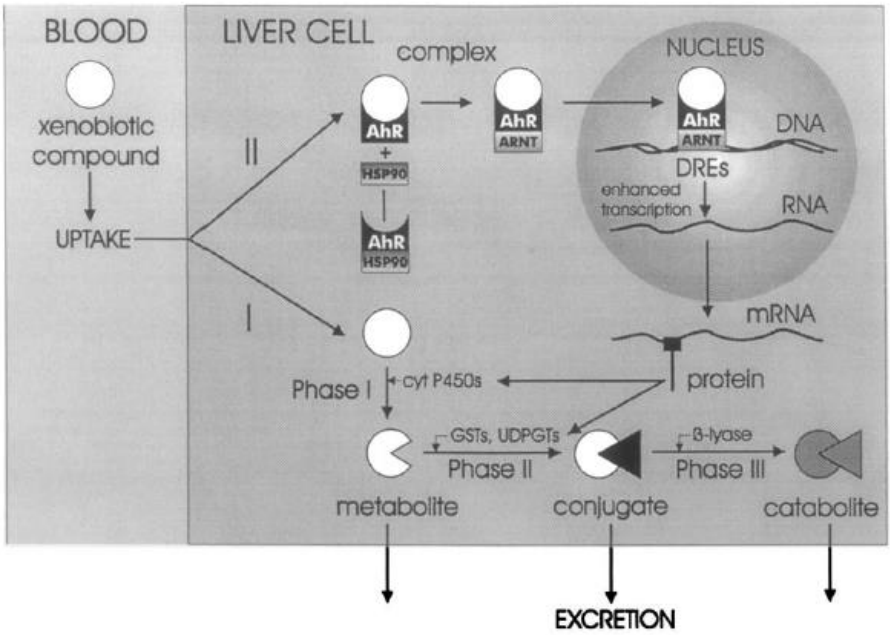

Figure 2. Illustration of the fate of xenobiotic compounds in liver cells [12].

The presence or absence of biotransformation enzymes in fish may not always be caused by contamination, therefore incorporating on a battery of tests for biological responses to contaminants will likely yield more concrete information [26]. It is also important to take into consideration the balance between bioactivation and detoxification processes caused by the enzymes in order to assess the potential hazard caused by the toxic substance [12].

Under oxidative stress parameters, evaluation of the CAT activity is among the biomarkers considered. It is the defense mechanism resulting in the release of hepatic antioxidant enzyme in fish as a response to organic pollutants [12].

The products of biotransformation due to exposure of the organism to significant amounts of the contaminant is another group of markers used in several biological assessments. After exposure, PAHs are chemically modified into their corresponding metabolites in the liver of many aquatic organisms, especially in fish [12]. After biotransformation, most of the hydrophilic metabolites are then excreted via bile [9]. Due to the rapid biotransformation of PAH compounds, PAH metabolite determination in bile are used to indicate short-term exposure to PAH contamination of up to 14 days [27]. The determination of PAH metabolites in bile rather than the parent compound is due to the possibility of underestimation of the *in vivo* exposure level of PAHs in fish [28], and to quantify the PAH flux caused by the rapid metabolism of the contaminant in the same organism [8,29]. The methods that have already been used in previous studies that are suitable for screening of PAH

metabolites are fixed wavelength fluorescence (FF) and GC-MS, or HPLC/F for a more sensitive measure and quantification [28,30].

DNA adduct quantification is a biomarker that provides an assessment of chronic effects after a long period of exposure rather than a few days, unlike the previously mentioned biomarkers. It is a biological marker of exposure to genotoxic compounds, using the liver as the most commonly studied organ when fish are used as a sentinel organism [31]. The measurement of the quantity of DNA adducts in marine organisms has been previously studied and has shown to be a promising biomarker for environmental assessment of carcinogenic and mutagenic exposures. It is also found to be a sensitive biomarker for PAH exposure due to its corresponding response to varying levels of PAH contamination [12].

Also an important parameter to be considered when assessing the effects of PAH exposure to marine organisms involves physiological and morphological factors. Analysis for these paramters can indicate damage to higher-level organizations of the organism as a result of severe cellular and chemical alterations. The damage that can be detected related to these parameters can be irreversible and is an effect of long term exposures [13]. Histopathological biomarkers are used to reflect morphological transformations due to exposures to a wide range of contaminants including PAHs, and are considered an indicator of the sentinel organism's general health [12]. These markers utilize methods such as detection of lesions, and categorizing them into groups, namely: non-specific, neoplastic and non-neoplastic toxicopathic lesions. It is a widely used biomarker of exposure, both in field studies and laboratory exposures. The advantage of using this biomarker is that the organs involved are specific for vital functions (gill, kidney and liver) and these affected organs are easily identifiable, serving as an indication of the animal's abnormal activity [32]. Detection of abnormalities in these organs can lead to abnormal growth, reproductive capabilities, and health of the individual, which eventually will result in a negative effect to the population [6].

In addition to histopathological markers, measuring gross indices are also indicative of the most sensitive members in the fish population and may provide information about the stress that the ecosystem is exposed to. The condition of the whole body as the condition factor (CF) index of growth rate and the liver as liver somatic index (LSI) are often included in measuring physiologic and morphologic parameters [33]. These condition indices are quite general and non-specific but they can be used as valuable measurements in environmental monitoring [12]. In addition to CF and LSI, the measurement of gonad size as gonadosomatic index (GSI) can also be used to assess the apparent risk for a reduced reproductive potential of the organism. Combining these three measurements is a capable supporting variable to biomarkers conducted at the whole individual,

tissue, cellular, and subcellular levels [33]. However, careful interpretation of these gross indices must be observed because they can be influenced by specific factors such as location, water temperature, and gender [34].

The interpretation of the results from a combination of the biomarkers has more advantage than interpreting single biomarker results due to the variation in mechanisms involved in the organism when they are exposed to the pollutant [35]. Three of the biomarkers mentioned previously are commonly applied and recommended biomarkers specifically for the classification of PAH contamination in fish. These biomarkers are fluorescence analysis of PAH metabolites in bile, EROD activity and formation of DNA adducts in liver [9].

1.4 Multivariate statistical analysis

Using biomarker-based monitoring for specific environmental purposes is already in itself a powerful tool that has demonstrated its significance in a number of studies [2,11,12]. But to be able to communicate the results obtained effectively, one cannot easily interpret the biomarker data directly due to the complex relationship between markers. This has led to the use of mathematical methods to incorporate the diffferent biomarker responses to single values and obtain a simplified and understandable outcome [36]. The information that is acquired when using the multivariate statistical index or models can help in understanding and interpreting the relationships between exposure and the resulting adverse effect [36]. These indices are developed in order to minimize the complications between biomarker responses that may not always be anticipated [5]. Several indices have already been tried and ranked for the mentioned objectives in the field as well as in laboratory setting [15]. Indices like PCA, IBR and BAI have been used to interpret data from studies on fish and results from different biomarkers previously [36-38]. The effectivity of these different indices have also been tested with different sentinel organisms, assessment of different types of pollutants and in different periods of exposure [17]. Furthermore, the use of different indices in a single assessment is done to avoid the oversimplification of the results obtained in one index [16]. Different indices also have the advantage of conveying various information related to the results and using different graphic representations to express them.

1.4.1 Principal Component Analysis (PCA)

PCA is a multivariate technique that was developed to simplify observations obtained from several datasets. This method extracts important information from the given quantitative variables, combines them into fewer variables, called principal components, and displays the pattern of similarity between the samples using the principal components as a basis. This pattern that is obtained from the extraction of principal components is represented as points in the map, where the distance between the points corresponds to the correlation of these points. The farther they are from another point, the bigger the difference they have [39].

1.4.2 Integrated Biomarker Response (IBR)

IBR index is another mathematical tool that has been used previously to assess the health condition of different sentinel organisms [16,36,37,38]. It was first successfully applied using 4 biochemical markers (GST, AChE, CAT, and DNA adduct) on flounder by Beliaeff and Burgeot [16]. In addition to the 4 biochemical markers, Broeg and Lehtonen [36] were able to incorporate histochemical biomarkers to calculate the index for eelpout and blue mussels.

In this index, the relative difference between each biomarker in the dataset is considered in the calculation [37]. The resulting information taken from the IBR index is represented as a star plot indicating the relative biomarker responses in the different groups being studied. The degree of damage as reflected by the biomarkers is then represented by the star plot radius of that exposed group or site. To assess the condition of the target species, the index takes advantage of using the response of a set of biomarkers. But relevant interpretation of the results obtained from IBR will only be achieved by carefully choosing the biomarkers used depending on the objective [16].

1.4.3 Bioeffect Assessment Index (BAI)

BAI is another multivariate analysis that was first created as a modification to a previous multivariate technique (HAI), originally used to assess biological health conditions also. It was designed by Broeg et al. [15] to assess the contamination condition in coastal areas by integrating several pathological endpoints measured in the liver of fish (European flounder) during a long term study of pollution exposure in the German Bight [15]. The index is based on using biological

markers on different levels of organization (subcellular, molecular, tissue level, individual) to measure the effects of pollution to the condition of the organism [15]. The idea is to evaluate the negative progression of the health of the organism from early onset to late effects. The index has later been used to assess the health status of different sentinel organisms such as mussel [15,16,36,37,38] and to monitor the long term effects of contamination by using a battery of biomarkers in eelpouts [15,36,37].

This index has been a useful quantitative tool in providing information for environmental assessments and management purposes due to its capability to statistically compare large data sets obtained from samples that are known to be exposed to pollution in varying degrees [15]. The index gives a higher BAI value to the most affected sample (individual or group) compared to the less exposed ones. It is aimed to use specific biomarkers of toxic effects in order to evaluate the alterations caused by the pollutant [15]. To be able to get a clearer interpretation of the index, existing baseline values and thresholds are taken into consideration when the values for categorizing the stages (Table 2) were done [35,37].

1.5 Purpose of study

The aim of this study was to use multivariate statistical analyses (PCA, IBR and BAI) to obtain an understandable and simplified output of the biomarker responses from two previous exposure studies. The strengths and weaknesses of the multivariate statistical analyses will also be examined in comparing groups of different exposure levels, as well as the effects of PAH compounds in different modes of exposure (injected and continuous exposure) and to apply the integrated biomarker approach to differentiate the further metabolism *in vivo* of PAH metabolites (naphthalene-diol and chrysene-diol) and their corresponding parent compounds (naphthalene and chrysene).

This study is carried out as part of a research project at International Research Institute of Stavanger (IRIS), called “Indicators for Environmental Impact of Petroleum Activities: the Next Generation of Molecular Markers” (iNEXT) funded by the Research Council of Norway. The aim of the iNEXT project is to find the effects of single and mixed PAH compounds in exposed Atlantic cod (*Gadus morhua*) in different contaminant concentrations.

2. MATERIALS AND METHODS

Evaluation of the results involved data collection, pre-processing of data and further analysis using established environmental health indices with the use of IBM SPSS 21 Statistical tool and Microsoft Excel 2013.

All results used in this study is based on two large datasets on biomarker responses obtained from individual Atlantic cod exposed to single PAH compounds and their corresponding synthetic metabolites (first exposure) and crude oil (second exposure). The single PAH (naphthalene, chrysene and the corresponding diols) in the first exposure were introduced via intraperitoneal (i.p.) exposure, and through direct exposure via continuous flow system (CFS) for the second exposure. Biomarker responses were assessed 7 days after exposure for the first and second exposure, and an additional assessment of the individual samples 30 days after the second exposure. The combination of biomarkers of exposure (EROD, PAH metabolites, CAT, GST) and toxic effects (DNA adducts, LSI, CI, GSI, gill and liver histopathology) obtained for the study will be explained in detail in the following sections.

2.1 Sources of data

Results from selected biomarkers obtained in two separate studies carried out as part of the iNEXT project were used as data in order to evaluate the integrative indices. The results for all the biomarker responses taken from the first exposure setup comprise dataset A in all analyses, and datasets B and C for the corresponding biomarker results for the second exposure setup, sampling after 7 days and 30 days, consecutively. Biomarker data for each individual fish used in all datasets are presented in Appendix A-C. Laboratory experiments were carried out with different pollutant doses and after different exposure times in the two different exposure setups. The type of exposure for each dataset will be further explained in Sections 2.1.1 and 2.1.2. An additional dataset D comprises the combined results of datasets A and B, but only for selected biomarkers which are common to both exposures (FF and GC-MS methods for PAH metabolites, EROD, GST, CAT, CI and LSI). No significant difference in biomarker baseline levels between genders was observed, thus all data were presented as mixed gender [40].

Table 1. Abbreviations for all exposure groups used in all datasets A-D.

Dataset	Abbreviation	Group
A	CONEX1	Control
	CAREX1	Carrier
	Naph L	exposed to low dose of naphthalene
	Naph H	exposed to high dose of naphthalene
	Naph DL	exposed to low dose of naphthalene-diol
	Naph DH	exposed to high dose of naphthalene-diol
	Chry L	exposed to low dose of chrysene
	Chry H	exposed to high dose of chrysene
	Chry DL	exposed to low dose of chrysene-diol
	Chry DH	exposed to high dose of chrysene-diol
B	CON7	Control
	LOW7	Exposed to 0.01 ppm OiW
	MED7	Exposed to 0.05 ppm OiW
	HIGH7	Exposed to 0.1 ppm OiW
C	CON30	Control
	LOW30	Exposed to 0.01 ppm OiW
	MED30	Exposed to 0.05 ppm OiW
	HIGH30	Exposed to 0.1 ppm OiW

Groups mentioned in the following chapters will be referred to as the abbreviations designated to the exposure group in Table 1.

2.1.1 First exposure

Sixty-six mature Atlantic cod were captured in Idsefjord in the north eastern region of Stavanger, Norway (58 female and 8 male individuals). Fish were transported to the laboratory in IRIS and kept in 1000 L glass fiber tanks with a continuous seawater flow from Byfjorden with controlled water temperature, water flow, salinity and the light/darkness duration for normal conditions. Water is pumped constantly into the containers via sand filtration and water goes out with equivalent inlet flow (8 L/min). The fish were divided into 10 groups with one group in each tank. Each group contained 7 fish except for Chry DL that contained 3 fish due to the limited amount of available synthetic compound. Exposure groups were divided according to PAH concentration, namely: high dose (2.5 mg/kg) and low dose (0.5 mg/kg) groups for the single PAH compounds (naphthalene and chrysene) and high dose (0.25 mg/kg) and low dose (0.1 mg/kg) groups for their synthetic metabolites (1*R*,2*R*)-1,2-duhydronaphthalene-1,2-diol, (naphthalene-diol) and (1*R*,2*R*)-1,2-dihydrochrysene-1,2-diol (chrysene-diol). Exposure was done by intraperitoneal injection. Two control groups included one untreated and one group injected with the carrier (DMSO/cod liver oil, 1:1, 0.47 mL/kg). The fish were then sacrificed a week after injection. Biological fluids, bile, blood,

liver and fillet tissues were collected and used for the biomarker analyses. Biomarkers included in the analysis for the first exposure include PAH metabolites (using FF and GC-MS methods), EROD, GST and CAT activities, DNA adduct, and gross indices (CI, LSI and GSI).

The selection of exposure groups, between parent PAH compound and metabolite, was carried out based on the study done by Jonsson et al. [41]. This study mentioned that, specifically for Atlantic cod, the diol accounted for up to 88% of the chrysene metabolites found in bile. It is also likely that this result is similar for naphthalene-diol making up the majority of the naphthalene metabolites found in bile, as found in the same study [40,41]. Selection of high and low doses of the synthetic metabolite of naphthalene and chrysene was also done to avoid the possible underestimation of the *in vivo* exposure level of PAHs in fish if only the detection of parent PAHs will be determined. Using PAH metabolite detection can represent the quantification of the behavior of PAHs through the individual sample's body [35].

Due to the reasons mentioned, the group exposed with a low dose of synthetic chrysene-diol wasn't included in the index analysis for dataset A.

2.1.2 Second exposure

The second exposure setup included the use of 64 Atlantic cod caught outside the coast of Stavanger and transferred to IRIS for acclimatization. The individual samples were contained in 8 1000 L tanks in the laboratory. Fish weight ranged from 105 – 1455 g and most of the fish were mature, except for 3 fish that were juvenile. To obtain normal conditions in the tanks, factors such as daily light exposure, oxygen and temperature levels and feeding times were controlled. The tanks have continuous seawater flow from Byfjorden. Water is pumped constantly into the containers via sand filtration and water goes out with equivalent inlet flow (8 L/min). CFS was used for the purpose of exposing the samples to relative stable concentrations of dispersed crude oil for an extended period of time [42].

Two tanks were designated for each exposure group with 8 fish in each tank, namely: control, low, medium and high. The control tanks contained only clean seawater while the other exposure tanks were mixed with Troll oil in different concentrations after 1-2 weeks of acclimatization. Fish were continuously exposed for 4 weeks to 0.01 ppm of OiW for the low concentration group, 0.05 ppm and 0.1 ppm for the medium and high concentration groups, respectively. Sampling was done twice in a period of 4 weeks: 32 fish were used after 7 days of exposure and another 30 after 4 weeks. Individual samples were reduced to 62 fish due to fatalities in the high exposure group before

sampling. Samples for biological and chemical tests were taken after the fish were measured for their length and weight. Blood and bile were drawn, and the liver and gills from each individual fish were stored following the SOP for the method, to be used for the biomarker analyses.

Biomarkers analyzed after 7 days of exposure were PAH metabolites (FF and GC-MS), EROD, GST and CAT activities, and gross indices (CI and LSI). The same biomarkers were analyzed for the 30-day exposure, but with an additional histopathological analysis.

2.2 Biomarkers

Fillet samples from A. cod. were analyzed to confirm successful exposure of the different groups. Each biomarker analysis is done for 63 fish for dataset A, 32 for dataset B and 30 for dataset C. All biochemical analyses were performed on bile samples and liver slices from each sampled fish. Dataset D comprises the selected biomarker responses which are common for datasets A and B.

The biomarkers used in both the exposures are carefully chosen and have currently been approved as valuable tools for the assessment of the impacts of PAH in fish based upon the six criteria that has been proposed by Van der Oost et. al. [12]. Analysis of the biomarkers are categorized according to the following parameters: biotransformation enzymes (phase I and II), biotransformation products (biliary PAH metabolites), oxidative stress parameters, genotoxic parameters, physiologic and morphologic parameters. The biomarkers not included in Van der Oost et. al. [12] report were also successfully used in previous studies as effective markers for PAH contamination [27].

The correlation between biomarker responses was determined prior to the index analysis and it showed that it is unlikely that a biomarker is responsible for any adverse effect in another biomarker response.

2.2.1 Biotransformation enzymes

Two biological markers were chosen in order to test the individual sample's response to PAH exposure via biotransformation enzyme induction, namely EROD and GST activity of the fish. EROD and GST activities were analyzed for datasets A-D. Complete description of the method is described thoroughly where data is obtained, in Pampanin et al. [40]. Briefly, 1 week after exposure liver samples were collected after sedating the fish and then a blow to the head. Liver samples were then snap-frozen in liquid nitrogen and stored at -80 °C for the biomarker analysis. The liver sample

was used to determine the EROD and GST activities. Microsome fractions of the liver sample was spectrophotometrically measured based on the method described by M. Nilsen et al. [43]. EROD activity was calculated using the values obtained for increase in fluorescence per min (F_S/min), pmol resorufin added as internal standard (R), the change in fluorescence due to the resorufin standard (F_R), volume of sample (Vs) and protein concentration of liver sample (Cs), in the following formula: EROD = $F_S/\text{min} \times R/F_R \times 1/V_S \times 1/C_S$ [40]. Resulting values are expressed as pmol/min/mg of proteins. Bradford method [44] was used for the determination of protein concentration.

As detailed in the study by Habig et al. [45], GST activity was measured using the cytosolic fraction of liver. The values used in the multivariate analysis for GST activity are expressed as international enzyme units (U) per mg protein (1 U = 1 μmol/min) and normalized against the total protein content, by measuring the GST activity spectrophotometrically at 340 nm.

2.2.2 Oxidative stress parameters

For evaluation of oxidative stress due to PAH exposure, liver catalase (CAT) activity was measured for each fish. The cytosolic fraction of the liver sample was used for this purpose. Measurement of CAT activity followed the steps used by Claiborne [46]. The values used in the indices are expressed as moles of H_2O_2 consumed per min per mg of protein (mol H_2O_2/min/mg protein). Protein concentration was determined using the Bradford method [44].

2.2.3 Biotransformation products

PAH metabolites in bile were determined using two methods, FF analysis and GC-MS. The former is conducted as described by Aas et. al. [9] analyzing for 2/3-ring PAH (2/3-ring), 4-ring PAH (4-ring) and 6-ring PAH (6-ring), while the latter is by Jonsson et al. [47]. Specific excitation/emission (ex/em) wavelength pairs were utilized for the FF method in order to optimize the detection of 2- and 3-ring, 4-ring and 6-ring PAH metabolites (i.e. metabolites of naphthalene and phenanthrene, chrysene and pyrene, and coronene, respectively). The concentration of PAH metabolites was expressed as mg pyrene fluorescence equivalents (PFE)/ml bile and the unit for GC-MS is ng/g.

Note that for GC/MS method, only naphthalene metabolites were analyzed for datasets A and D due to the exposure type and standards available for this type of analysis. These metabolites are: 1-OH-Naphthalene (1-OH-Naph), 2-OH-Naphthalene (2-OH-Naph), C1-OH-Naphthalene (C1-OH-

Naph), C2-OH-Naphthalene (C2-OH-Naph) and C3-OH-Naphthalene (C3-OH-Naph). Datasets B and C included the analysis of 1-OH-Phenanthrene (1-OH-Phenan), C1-OH-Phenanthrene (C1-OH-Phenan), C2-OH-Phenanthrene (C2-OH-Phenan) and 1-OH-Pyrene, in addition to the naphthalene metabolites similar to datasets A and D.

In dataset A, levels were often below the limit of detection (LOD) for 6-ring (FF) and C1-C3-OH-Naph (GC-MS), therefore statistical analyses excluding these values were run as one of the suites of biomarkers. When PAH concentration were below LOD, only the LOD value is presented in the results.

2.2.4 Genotoxic parameters

The environmental exposure to genotoxic chemicals can affect the organisms involved and one of the consequences for this is the formation of DNA adducts [12]. The method used for DNA adduct determination in the study was ^{32}P-postlabelling method, previously reported by Reichert and French [48]. Each analysis was conducted on 5 µg of DNA with two independent adduct measurements conducted for each DNA sample. The LOD was fixed to half the smallest DNA adduct level (Relative adduct level, RAL) calculated for an observed spot in a pattern (RAL x 10^{-8}). Therefore, for analyses without detectable adducts, the concentration of adducts is defined as < 0.01 x 10^{-8} nucleotides (i.e. below the LOD).

Only dataset A included the results for DNA adduct because it was not performed in the second exposure setup.

2.2.5 Physiologic and morphologic parameters

After the fish were first sedated and then a sharp blow in the head, they were dissected carefully to obtain the organs needed. Liver and gill samples were cut out for both snap freezing and to be put in formalin. The gonads were also obtained, snap-frozen and sex determined for all fish samples in both exposure setups. Length and total weight of fish, gonads and liver weights were obtained for all individual samples.

The full detail of the method used for markers with physiologic and morphologic parameters are explained in Pampanin et al. [40]. General physiological indices were calculated as follows: condition index (CI) = [weight (g)/length (cm)^3] x 100; liver somatic index (LSI) = [liver weight (g)/fish weight (g)] x 100; gonadosomatic index (GSI) = [gonad weight (g)/fish weight (g)] x 100.

These indices were previously established in studies done on the same sentinel organism [49,50]. GSI results are not included in datasets B, C and D.

Histopathological analysis is also included in the results used for dataset C. This biomarker is used in environmental monitoring to include examination of specific target organs, as described by Hinton et al. [51]. Histopathological response of fish to contaminants are best studied using the gills and liver [32]. After the tissue samples were prepared, they were examined for health parameters related to physiological conditions, inflammatory and non-specific pathologies and those associated with pathogen and parasite infections for histopathological analysis. A scoring system developed by Bernet et al. [52] was then used to assign the degree of damage as five main categories recommended for monitoring biological effects of contaminants in cod species. These categories are steatosis, circulatory disturbance, inflammatory changes, melano-macrophage aggregates, parasites and other pathological changes. Only dataset C included the results for histopathological assessment.

2.3 Multivariate statistical analysis

The methods used for the statistical analyses were chosen according to their performance and assessment in previous applications [15,36,37]. The three indices (PCA, IBR, BAI) were used to integrate the responses of 8 biomarkers for dataset A, 6 for datasets B and D, and 7 biomarkers for dataset C. For each dataset, different suites of biomarkers were employed as suggested by Marigómez et al. [37], depending on the concerned level of biological organization.

2.3.1 Principal Component Analysis (PCA)

Standardization of all results were accomplished using the equation ‘new data’= log (1 + ‘old data’) as explained by Zitko [53]. All principal components were obtained using the standardized values of all biomarkers for individual samples corresponding to each dataset. Biomarker data was first standardized using Microsoft Excel 2013, then the factor analysis function in IBM SPSS 21 Statistics was used to run the analysis.

PCA was generated with 7 fish for LOW7 in dataset B due to the absence of GC-MS values for one sample (fish no. 17). All the other exposure groups in the same dataset are conducted with 8 fish. For dataset C, PCA was generated with one less fish for CON30 (fish no. 47) due to the absence of GC-MS results, and for LOW30 because fish no. 39 was lacking histopathology results for both gills and liver. This results to 6 fish for CON30 and 7 fish for LOW30.

2.3.2 Integrated biomarker response (IBR)

The results from all biomarker responses in datasets A-D were standardized on a parameter mean-value 0 and standard deviation 1 prior to the calculation of the triangular star plot areas for each two neighboring biomarkers in the dataset. The star plot areas are needed to obtain the IBR value for each group.

The procedure was carried out as Broeg and Lehtonen [36] described:

For each biomarker in the dataset (1) - (5):

(1) Calculation of mean (X) for each group.

(2) Calculation of general mean (m) and standard deviation (s) of the biomarker for all stations

(3) Standardization of data for each group using the equation $Y_i = (X_i – m)/s$.

(4) Obtain $Z = Y_i$ and $Z = -Y_i$, depending on the biological effect corresponding to induction or inhibition, respectively.

(5) Addition of Z-values obtained for each group to the absolute value of the minimum Z-value for each biomarker to calculate the scores. The equation used is: $B = Z + |MinZ|$ to adjust the lowest value in the set to zero.

(6) Star plots were then generated to represent the biomarker results by multiplication of the obtained value of each biomarker (B_i) with the value of the next biomarker, arranged as a set, then dividing each product by 2.

(7) Summing up all the biomarker values: $\{[(B_1 \times B_2)/2] + [(B_2 \times B_3)/2] + \ldots [(B_{n-1} \times B_n)/2]$ to obtain the IBR value for each group.

Biomarkers that are closely related according to their response (i.e. determination of metabolic phases I and II by analyzing EROD and GST activities) and PAH metabolite biomarkers (FF and GC-MS methods and EROD) were positioned adjacent to each other on the star plot as suggested by Beliaeff and Burgeot [16].

Due to the dependence of the IBR values to the number of biomarkers in the set, after obtaining the IBR values for each group in all datasets, results were also presented as IBR/n where the calculated IBR values were divided by the number of biomarkers (n) used in each IBR calculation.

2.3.3 Bioeffect Assessment Index (BAI)

Each biomarker response for each individual sample in the group was graded according to determined parameters (Table 2). Due to the lack of established baseline data for some of the biomarkers (tissue level and whole organism biomarkers), their critical values were arbitrarily determined for this purpose. Following recommendations by Broeg et al. [15], biomarkers with different levels of biological response were used as explained in Chapter 2.2.

The corresponding numerical BAI values assigned to each biomarker are shown in Appendix D. PAH metabolite (FF and GC-MS) results were used as guideline parameters for the assessment of the other biomarkers because of its good response and stability in the analyzed samples. Reference values for GST baseline was take from Hylland et al. [27]. Reference values for the different stages used for PAH metabolites, DNA adduct and EROD were taken from ICES [54]. Following the suggestions of Marigómez et al. [37], as a preliminary approach, the median of all values from all the samples in different groups obtained in this study (according to exposure setup) was arbitrarily decided, after a trial-and-error approach, as the critical value. This was done for the biomarkers lacking concrete reference values and sufficient background data used in similar exposure to PAH.

Table 2. Characterization of the single BAI stages on the basis of studies on lysosomal membrane stability and liver histopathology [15,36].

Stage 1	Destabilisation time 20 min and longer (reference)	*Healthy*
Stage 2	Destabilisation time >10 to <20 min	*Minor diseased*
Stage 3	Destabilisation time 5–10 min	*Reversible, progressively*
Stage 4	Destabilisation time <5 min	*Irreversible, degenerative*

The integration of different biomarkers is made by substituting each biomarker response measured for each individual with a numerical value according to the degree of alteration, as follows: 10 = Stage 1, 20 = Stage 2, 30 = Stage 3, 40 = Stage 4 (Table 2, Appenidx D). These values were fixed for BAI applications using fish as the sentinel organism by Broeg et al. [15]. BAI value for each group is calculated by summing up all the BAI values for each biomarker of each individual fish and dividing them by the number of individuals analysed per group. A higher BAI value indicates a poorer health condition and vice versa [15].

In this study, critical values were not discussed in the analysis as was done with the previous application of BAI [15,36] since this is not an evaluation of environmental deterioration utilizing a fixed number of biomarkers for all exposures, rather using the index to compare the health status of

the individual fish and each exposure groups with known concentrations of the contaminant and different suites of biomarkers.

3. RESULTS AND DISCUSSION

The results used for the calculations of the indices for datasets A-C are summarized in Table 3 for PAH metabolite biomarkers (GC-MS). The whole dataset of biomarker responses for all individual fish was divided into four different sets, as explained in Section 2.1: (1) dataset A corresponds to the data from the first exposure (intraperitoneal introduction of single PAH compounds, naphthalene and chrysene, and their synthetic metabolites, naphthalene-diol and chrysene-diol); (2) dataset B contained results from the second exposure after 7 days (mixture of waterborne PAH compounds); (3) dataset C has results from the second exposure after 30 days; and (4) dataset D was used for the analysis of both setups 7 days after exposure.

Table 3. Results of selected biomarkers (PAH metabolite using GC-MS method) for all groups in datasets A-C. Values in mean ± standard deviation.

Dataset	Fish #	Group	GC-MS (ng/g)				
			1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph
A	22-28	CONEX1	20.7 ± 6.8	27.56 ± 20.2	384.29 ± 174.3	264.49 ± 106.2	697.4 ± 207.9
	29-35	CAREX1	18.21 ± 7.4	23.91 ± 9	317 ± 90.5	200	631.43 ± 143.3
	50-56	Naph L	176.25 ± 123.3	637.33 ± 420.7	345.29 ± 139.1	200	592.77 ± 128.7
	43-49	Naph H	892.22 ± 807.3	1864.03 ± 1412	327.12 ± 129.8	200	588.51 ± 89.8
	71-77	Naph DL	136.73 ± 104.8	90.16 ± 56.9	359.26 ± 213.7	227.58 ± 34.2	679.9 ± 218
	78-84	Naph DH	293.99 ± 152.3	242.41 ± 216.1	351.77 ± 148	200	650.64 ± 209.8
	57-63	Chry L	19.38 ± 7	35.82 ± 19.4	326.33 ± 121.4	211.7 ± 24	666.62 ± 143.2
	64-70	Chry H	21.87 ± 8.9	35.02 ± 21.8	361.42 ± 131.6	202.92 ± 7.7	640.36 ± 203.9
	36-42	Chry DH	17.86 ± 5.7	25.16 ± 14.1	335.79 ± 138.9	200	651.48 ± 164.7
B	2-9	CON7	13.34 ± 26	5.73 ± 3.6	173.49 ± 67.8	82.63 ± 24.7	141.11 41.3
	11-17	LOW7*	7.63 ±2.3	13.4 ± 8.6	241.53 ± 83	357.03 ± 98.2	626.05 ± 218.3
	18-25	MED7	24.42 ± 12.8	53.48 ± 34.8	598.42 ± 147.4	2273.16 ± 820.5	3204.79 ± 1700
	26-32. 1	HIGH7	32.06 ± 14.2	70.83 ± 46.3	998.99 ± 567.9	3459.53 ± 1917	4244.42 ± 1653
C	33-39	CON30	5.81 ± 3.5	2.96 ± 2.2	242.96 ± 44	68.71 ± 22.4	82.57 ± 16.6
	40-47	LOW30	8.06 ± 3.7	12.11 ± 5.2	294.63 ± 51.1	491.84 ± 231	663.63 ± 451
	48-56	MED30	20.78 ± 4.4	26.41 ± 13.3	638.81 ± 150	2268.04 ± 638	3009.58 ± 1016
	57-62	HIGH30	37.62 ± 10.8	46.6 ± 12.8	1077.54 ±275	3588.07 ± 1163	4189.58 ± 1717

*one sample without values for GC-MS

The results presented in data treatment include values of the pre-processed and standardized raw data of all datasets for each index and will be shown in detail in the following sections. Results for data interpretation include the graphical representations of each index for all datasets as explained

in Section 2.1. The application of the different indices in providing information about the biological effect of PAH in Atlantic cod is simplified and summarized in Section 3.3.

3.1 Data treatment

The pre-processed and standardized data of each individual biomarker is based on the biomarker parameters carried out in 63 fish for dataset A, 32 and 30 fish for datasets B and C, respectively. Standardization was done to allow direct comparison of the different biomarker data of groups within and between exposures.

3.1.1 Principal Component Analysis (PCA)

Dataset A represents results using the 63 x 15 matrix (63 fish and 15 variables), dataset B (32 x 16, 32 fish and 16 variables), dataset C (30 x 33, 30 fish and 33 variables) and dataset D with 95 x 12 matrix (95 fish and 12 common variables from the first and second exposures).

Preprocessing of the raw data decreased the skewness and kurtosis of the biomarker data to less than 1, except for the results of C2-OH-Naph in dataset A. GST resulted in a high skewness (4.72) and kurtosis (31.03) and CAT (2.26, 7.87) and GSI (3.90, 4.70) also had skewness and kurtosis higher than 1. The preprocessed data gave an equal weight to all variables before the PCA was performed. Preprocessing of the other datasets resulted in 6-ring (2.03, 6.47) and GST (1.25, 2.14) in dataset B, and similar result for GST in dataset C (1.26, 2.26). These mentioned values are the biomarkers with skewness and kurtosis exceeding the value for normal distribution.

The whole matrix of scaled biomarker parameters for all datasets is given in Appendix E. The data matrix consisted of 63 rows and 15 columns for dataset A. The 63 rows were subdivided into 9 groups representing the 2 control groups and 7 exposed groups. Dataset B has 32 rows and 16 columns, while dataset C has 30 rows and 30 columns. Dataset D has 95 rows and 12 columns corresponding to the combined individual fish of datasets A and B, and 12 variables common to the 2 datasets.

3.1.2 Integrated Biomarker Response (IBR)

Score calculations and values used to obtain IBR values for the groups in all datasets are presented in Appendix F (calculation) and G (score values). Scores calculated from the results in dataset A yielded a minimum value of 0.00 indicating the lowest response from the biomarker among the groups, and maximum value of 2.48 for the whole dataset, coming from Naph H. Scores for dataset A indicate that CONEX1 has only 20% with the lowest score from all biomarker results, and unexpectedly has the highest score for C1-OH-Naph (0.48), C2-OH-Naph (1.57) and C3-OH-Naph (0.66). Naph H has the highest score for 1-OH-Naph (2.33) and 2-OH-Naph (2.48) biomarkers and the lowest in 33.3% of all the biomarker scores, including CI, LSI, GSI.

Scores for dataset B indicate that HIGH7 yields the highest score for FF and GC-MS (PAH metabolite), in addition to EROD activity (Appendix G), yielding 52.9% of all highest scores. CON7 yielded the lowest score for almost all the biomarkers except for 6-ring (0.24), 1-OH-Naph (0.31), EROD (0.57) and LSI (0.12), and has the highest score for GST (0.96).

Based on the calculated scores for dataset C, HIGH30 has 51.1% of the highest response from all biomarker analyses, but the group also scored the lowest in 24.2% of the responses. On the other hand, CON30 scored the lowest (0.00) in 57.6% of the biomarker responses. CON30 also has the highest score in 21.2% of the responses, the same number of high responses for MED30.

Majority of the highest scores are calculated for HIGH7 (53.8%) and 15.4% by Naph H. CONEX1 has the most number of 0.00 among all groups in dataset D.

3.1.3 Biomarker Assessment Index (BAI)

Table 4 presents the calculated average BAI values for each sample in each group using all biomarkers in the dataset, using the designated BAI values for each biomarker data presented in Appendix D. The highest mean BAI value calculated for all the exposure groups was 30.00 and the lowest was 20.00. The highest mean BAI value was calculated for one of the samples in Chry L, and the lowest from Naph DH and Chry DH.

Table 4. Average BAI values for the 7 individual samples of each group in dataset A using all biomarker data.

Group	CONEX1	CAREX1	Naph L	Naph H	Naph DL	Naph DH	Chry L	Chry H	Chry DH
Sample 1	22.86	24.29	24.29	27.14	24.29	25.71	24.29	24.29	22.86
Sample 2	24.29	21.43	22.86	27.14	24.29	25.71	24.29	21.43	25.71
Sample 3	22.86	22.86	25.71	22.86	22.86	22.86	25.71	22.86	28.57
Sample 4	25.71	22.86	25.71	24.29	22.86	25.71	21.43	22.86	27.14
Sample 5	21.43	24.29	25.71	24.29	21.43	24.29	28.57	22.86	24.29
Sample 6	24.29	25.71	25.71	24.29	24.29	20.00	27.14	21.43	27.14
Sample 7	24.29	24.29	28.57	27.14	25.71	22.86	30.00	27.14	20.00
Mean	23.67	23.67	25.51	25.31	23.67	23.88	25.92	23.27	25.10
SD	1.29	1.29	1.61	1.66	1.29	1.98	2.69	1.83	2.74

As seen in Table 5, the average BAI value calculated for each group increases from CON7 (27.81) to HIGH7 (34.22). The same trend can be observed from BAI values calculated for each individual, where the lowest sample with the lowest BAI value of 26.25 is from CON7 and the highest is 36.25 from HIGH7.

Table 5. Average BAI values for the 8 individual samples from each group in dataset B using all biomarkers.

Group	CON7	LOW7	MED7	HIGH7
Sample 1	26.25	30.00	33.75	36.25
Sample 2	27.50	27.50	33.75	30.00
Sample 3	27.50	30.00	32.50	35.00
Sample 4	28.75	30.00	31.25	33.75
Sample 5	27.50	28.75	35.00	32.50
Sample 6	28.75	28.75	35.00	37.50
Sample 7	27.50	30.00	31.25	33.75
Sample 8	28.75	27.50	30.00	35.00
Mean	27.81	29.06	32.81	34.22
SD	0.83	1.04	1.74	2.16

Table 6 shows a similar pattern with the score calculations for dataset B where the average BAI value calculated for each group increases from CON30 (63.88) to HIGH30 (92.78). But the calculated BAI value for each individual does not follow the trend from the same exposure group because the lowest BAI value (51.25) is from LOW30, but the highest (100) is still from HIGH30. The scores are calculated using all the biomarkers that were used in the same exposure setup as dataset B, in addition to histopathological analysis.

Table 6. Average BAI values for the individual samples in each group in dataset C using all biomarker data.

Group	CON30	LOW30	MED30	HIGH30
Sample 1	65.71	70.00	62.50	96.67
Sample 2	55.71	51.25	66.25	81.67
Sample 3	64.29	61.25	66.25	100.00
Sample 4	74.29	67.50	71.25	91.67
Sample 5	60.00	75.00	66.25	101.67
Sample 6	70.00	67.50	70.00	85.00
Sample 7	57.14	61.25	78.75	-
Sample 8	-	65.00	68.75	-
Sample 9	-	-	78.75	-
Mean	63.88	64.84	69.86	92.78
SD	6.28	6.66	5.32	7.43

In Table 7, the comparison of scores using the biomarker responses from the two different exposure setups after 7 days of exposure is shown. The scores were obtained using the responses from the biomarkers that were common for both exposures as explained in Section 2.1. The calculated BAI values reveal that there is no distinct difference between the control groups and exposure groups for the i.p. exposed setup, but the pattern is increasing from the lowest (16.56) of CON7 to the highest (21.35) of HIGH7 in the second exposure setup. Although the highest mean BAI value (25.48) for the first exposure setup is from Naph L and H. For the individual samples, the lowest BAI value is 15.00 from CON7, and 60% of the exposed groups (Naph L and H, Chry L and H, Chry DH and HIGH7) had the highest individual BAI value of 28.33.

Based on the score calculations using the BAI stages and scoring, the responses from the first exposure setup didn’t give a clear distinction between the groups. Although the opposite can be observed from the second exposure setup where there is a clear deterioration of health condition in fish in the exposed groups compared to the control group.

Table 7. BAI values for individual samples in each group in dataset D using all biomarkers.

Group	CONEX1	CAREX1	Naph L	Naph H	Naph DL	Naph DH	Chry L	Chry H	Chry DH	CON7	LOW7	MED7	HIGH7
Sample 1	23.33	21.67	23.33	28.33	25.00	26.67	23.33	23.33	21.67	15.00	18.75	18.75	21.25
Sample 2	25.00	21.67	21.67	26.67	26.67	25.00	25.00	23.33	26.67	16.25	16.25	20.00	17.50
Sample 3	23.33	23.33	26.67	23.33	23.33	23.33	26.67	23.33	28.33	16.25	21.25	18.75	20.00
Sample 4	26.67	23.33	26.67	23.33	23.33	25.00	20.00	23.33	26.67	17.50	18.75	17.50	20.00
Sample 5	21.67	23.33	25.00	25.00	21.67	23.33	26.67	21.67	25.00	16.25	17.50	20.00	18.75
Sample 6	25.00	26.67	28.33	25.00	25.00	20.00	26.67	21.67	26.67	17.50	17.50	20.00	23.75
Sample 7	25.00	23.33	26.67	26.67	26.67	23.33	28.33	28.33	18.33	16.25	20.00	18.75	21.25
Sample 8	-	-	-	-	-	-	-	-	-	17.50	17.50	18.75	28.33
Mean	24.29	23.33	25.48	25.48	24.52	23.81	25.24	23.57	24.76	16.56	18.44	19.06	21.35
SD	1.63	1.67	2.30	1.85	1.85	2.09	2.79	2.24	3.53	0.88	1.60	0.88	3.38

4.1 Data interpretation

Each index generated their unique graphical representation on how they discriminate different exposure groups from each other. One advantage of such a method is for simplification of results because, mostly, the structure and interpretation of what the numerical results wants to convey can't be immediately obtained from the tabulated data. Each graph for each index for every dataset are presented and explained in the following sections.

The PCA results include the plots of loadings indicating the relationship among the given variables specific to each exposure and where the individual sample is in relation to the coordinates of the plots of scores [53]. In IBR, the line of each group in each of the axes depends on the response of the biomarker to exposure and corresponds to the relative response of the biomarker within that exposure group [16,36,37]. Boxplots generated to represent the calculated average BAI values of each groups show the interquartile range, minimum and maximum values and median values of each individual in the group for BAI analysis. Symbols for individual samples with extreme BAI values in the group (extremes) are represented by *, and ○ represents the individual samples with outlying BAI values in the group (outliers).

4.1.1 First exposure

The plots represented in this section are obtained using the individual samples and biomarker analyses outlined in Section 2.1.1. The first component of the PCA explains 94.12% of the total variance (Figure 3). Figure 3 shows the correlation of both the positive and negative control groups with chrysene and chrysene-diol exposed groups (for both low and high doses), and negatively correlated to these groups are the naphthalene and naphthalene-diol exposed groups (low and high doses). This negative and positive correlation amongst the groups is shown by the separation of the groups to the upper and lower part of the plot (Figure 3). This high percentage of the total variance explained by the first component means that the combined biomarker responses of each individual resulted into the division of groups according to the level of naphthalene or naphthalene-diol detected in the individual.

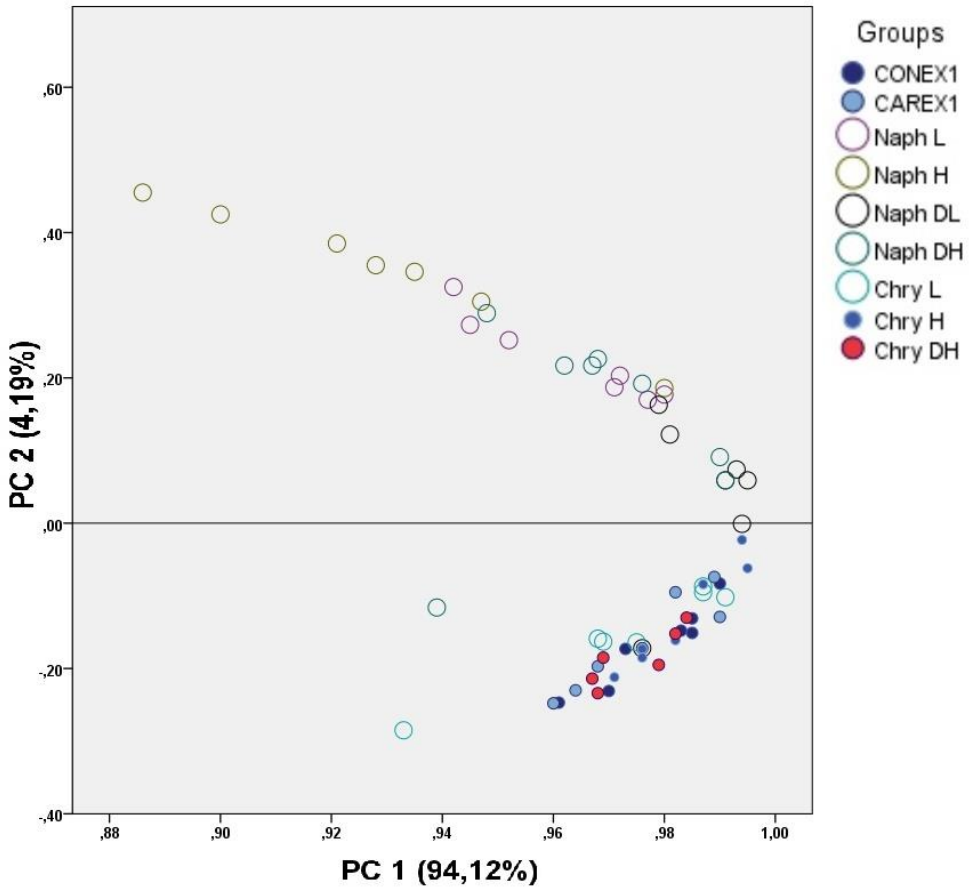

Figure 3. Plot of scores with principal components 1 (94.12%) and 2 (4.19%) of dataset A (scale: 645 x 746.25, size in points).

The first two principal components of all the score plots using the different sets of biomarkers comprise more than 85% of the total variance (Figure 4, A-D). The PCA was also able to show discrimination between the control and naphthalene exposed groups. The percentage of the total variance explained by the first two components of the set with the PAH metabolite biomarker is higher compared to the combination without it. As can be seen in Figure 4A, using only the data from the PAH metabolite biomarkers provide a PCA where the total variance of 98.6% for the two components was explained (PC 1: 90.75%, PC 2: 7.85%). Without the PAH metabolite biomarkers (Figure 4B), the two components explain 63.82% and 20.64% of the total variance for PC 1 and 2, respectively. Including the results of the PAH metabolites but removing the data below detection limit (6-ring in FF and C1-C3-OH-Naph in GC-MS) changes the percentage of the total variance to 95.63% (PC 1: 92.08%, PC 2: 3.55%) as shown in Figure 4C. This indicates that the PAH metabolite data influences the distribution of the components dominantly. The clustering of individual samples in their respective groups in Figure 4D remains for Naph H, CONEX1, and CAREX1 as the percent of the total variance explained by the first two components decrease to 88.98% (PC 1: 70.16%, PC 2: 18.82%).

As for the different combinations of biomarkers, the same trend is observed for the combination with PAH metabolites where there is clear separation between the naphthalene group and their metabolites and the other groups. Without the dominating PAH metabolite biomarkers, the distinction between groups are not obvious. Figure 4B and 4D show less correlation between the individuals in the same exposure group compared to the correlation in Figure 4A and 4C where PAH metabolite biomarkers are included. This accounts to the sensitivity of the biomarker to respond to different degrees of exposure. Figure 4 (A-D) also shows that the type of biomarker does not affect the correlation between the two control groups and Chry DH. The grouping of the individual samples within each group is more distinguished in the PCA with the PAH metabolite biomarkers, except for the outlier from the Chry DH in Figure 4C. There is no clear grouping between the high and low dose for chrysene and chrysene-diol groups, but there is a slight separation between Naph H as the individuals are separated from Naph L and both doses for the naphthalene-diol groups (Figure 4A). The scores plot using only the PAH metabolite biomarkers (Figure 4A) is also the plot that is most similar to the score plot using all the biomarker data (Figure 3), indicating the consistency of the influence of the biomarker in determining the principal components.

The PCA was able to distinguish the importance of the different sets of biomarkers (Figure 4). The health status may not have been emphasized by the PCA because there is no clear distinction between high and low doses, but the sensitivity of the biomarkers, especially that of PAH

metabolites, was pointed out. The clustering of the individual samples in the same group indicates that the index was able to discriminate between the groups using the responses of the individuals to the contaminant.

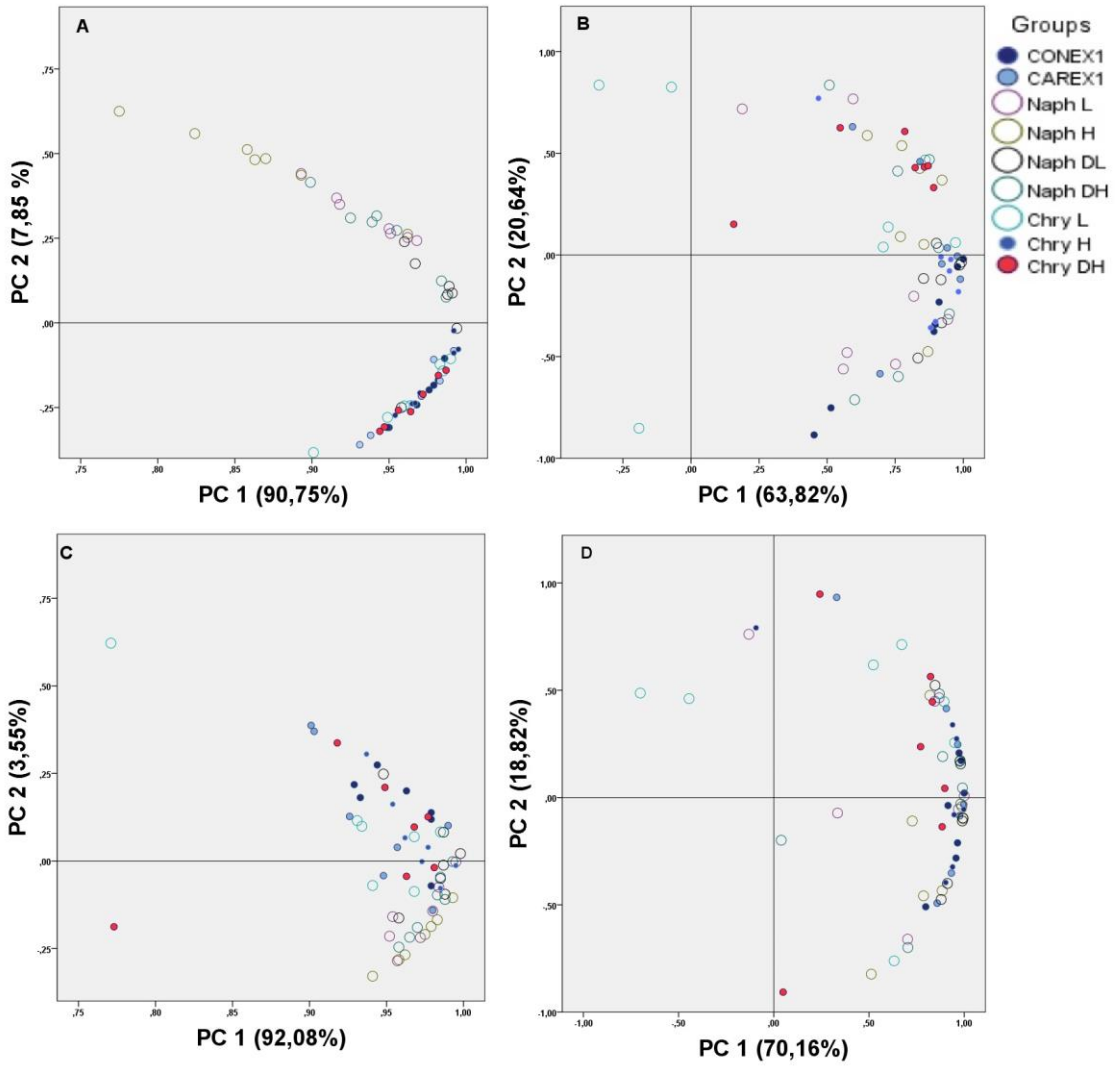

Figure 4. Dataset A: score plots using different sets of variables (biomarkers), according to the affected level of organization caused by PAH exposure: A – PAH metabolites (FF and GC-MS), B – EROD, GST, CAT, DNA adduct and general health condition indices (CI, LSI and GSI), C – without 6-ring (FF) and C1-C3-OH-Naph (GC-MS) from the PAH metabolites, D – biochemical (GST, CAT and DNA adduct) and general health condition indices (CI, LSI and GSI). Scale: 645 x 746.25, size in points.

Figure 5 shows the loading and score plots with the first two principal components for the different groups and all variables. The first two principal components in the plot for dataset A accounts for 98.6% of the total variance. As shown in Figure 5, the control groups and chrysene (both doses) and chrysene-diol (both doses) are separated from the naphthalene (both doses) and their metabolites (both doses). This was also shown in more detail in the plot of scores in Figure 3.

The positions of the individual fish in the coordinates of PC 1 and 2 show that the relationship between the exposure groups and which method for PAH metabolite determination played a role in groups' clustering. The GC-MS method determined the response of the samples for naphthalene and its metabolites only, causing the exposed groups to separate from the other groups (Figure 5).

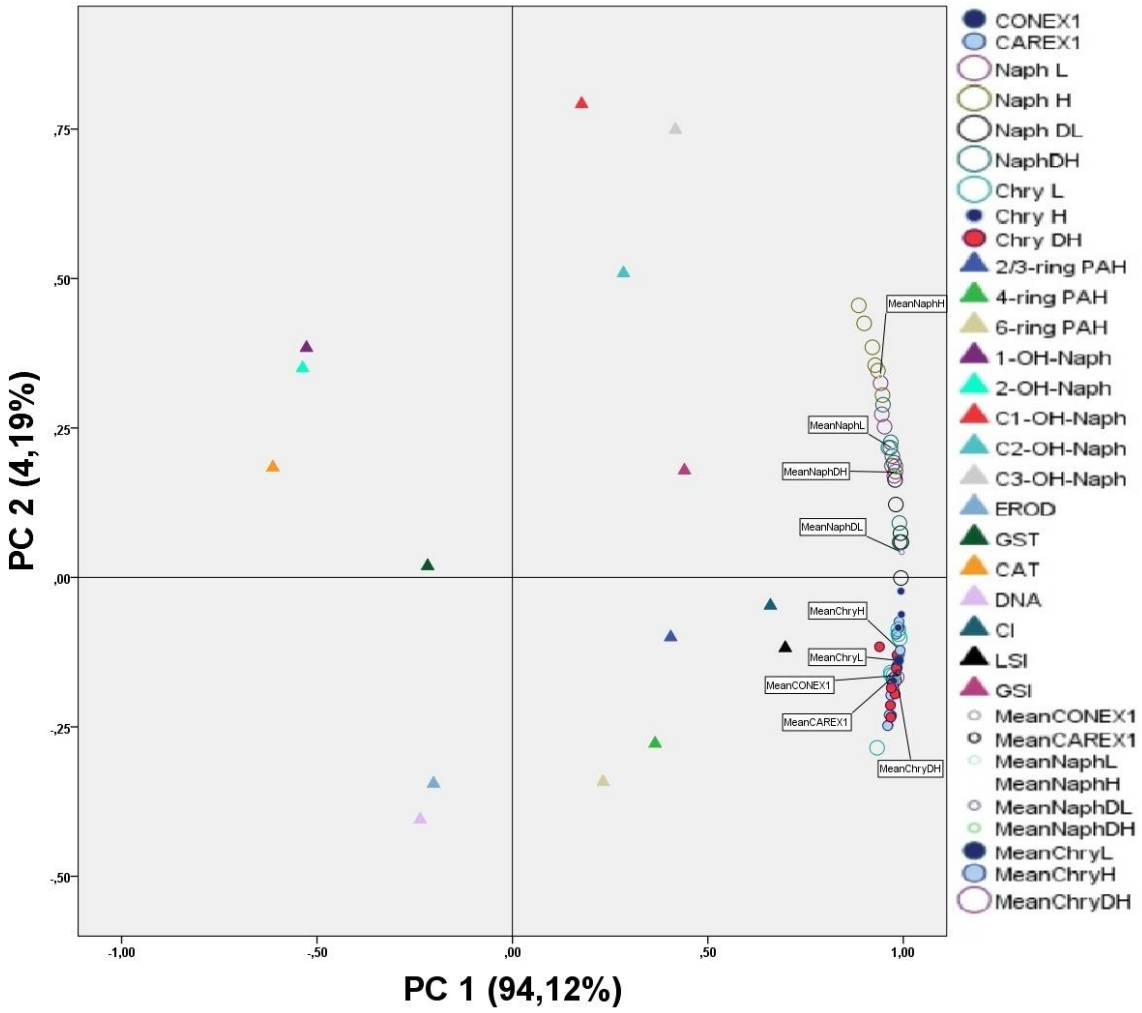

Figure 5. Dataset A: Scores and loadings plot with the first two principal components (PC 1: 94.12%, PC 2: 4.19% of the total variance). Labelled markers are the mean values of each group (scale: 675 x 806.25, size in points).

The distinction of the naphthalene exposed groups among all the groups can be attributed to the sensitivity of the PAH metabolite biomarker, but considering that it has been used in studies before [10] showing that the method is more sensitive for PAH metabolite detection than the parent compound, it didn't show in the PCA at all (Figure 4A and 4C), even in the combination (Figure 4A) where only biomarkers for PAH metabolites were used.

In Figure 5, the good correlation observed between DNA adduct levels and PAH metabolites (using FF) in bile demonstrates the coherence between these two biomarkers. This shows that to

evaluate the influence of crude oil pollution, the use of DNA adducts and PAH metabolites in bile are effective as complementary methods, with the detection of metabolites in bile as a first line of screening [9].

Table 8 shows that Naph H has the highest biomarker response with an IBR value of 2.47 and its corresponding metabolite (Naph DH) has the lowest (0.41) using all the biomarkers in the dataset. The Naph DH IBR value is lower than the IBR calculated for the control (1.28) and carrier (0.76) groups. In different combinations of biomarkers, Naph DH has the lowest IBR value (0.41, 0.11 and 0.29 for IBR B, C and D, respectively). The sets of biomarkers are the same as used in generating the PCA plots [A – PAH metabolites (FF and GC-MS), B – EROD, GST, CAT, DNA adduct and general health indices (CI, LSI and GSI), C – without 6-ring (FF) and C1-C3-OH-Naph (GC-MS) from the PAH metabolite biomarkers, D – biochemical (GST, CAT and DNA adduct) and general health indices (CI, LSI and GSI)]. The carrier group has the lowest IBR (0.25) for set E. Naph H can be noted to have the highest IBR for the biomarker combinations with PAH metabolites, emphasizing that only naphthalene metabolites were analyzed by GC-MS method (IBR A, B and D). Chry DH has the highest IBR (0.65) in IBR E. Naph L has the highest (0.91) for the other combinations without the PAH metabolite biomarkers. Despite the different combinations of biomarker data used, the IBR star plots doesn't seem to correspond to the PAH gradient of the exposure groups.

Table 8. IBR values for each group in dataset A using different sets of biomarkers.

IBR	CONEX1	CAREX1	Naph L	Naph H	Naph DL	Naph DH	Chry L	Chry H	Chry DH
IBR A	1.28	0.76	0.98	2.47	0.70	0.41	1.53	0.91	1.19
IBR B	0.75	0.39	0.22	2.44	0.22	0.11	0.85	0.43	0.45
IBR C	0.49	0.57	0.91	0.04	0.49	0.29	0.76	0.52	0.87
IBR D	0.74	0.82	1.02	2.25	0.64	0.39	1.21	0.89	1.28
IBR E	0.45	0.25	0.52	0.51	0.46	0.29	0.53	0.35	0.65

Beliaeff and Burgeot [16] noted that the biomarker position in the star plot should be arranged according to their response to levels of exposure and this should be able to discriminate between different pollutant concentration. In this study the biomarkers are positioned according to their response when the score calculations were made. But the graphic representations using the biomarker arrangement in the study failed to discriminate between the different dose groups.

In the original publication the authors only included biochemical biomarkers such as GST, AChE, catalase enzyme activities and DNA adducts. But in this study, specific biomarkers (PAH metabolites in bile measured using two methods, FF and GC-MS, and EROD), general health biomarkers (histochemical biomarkers of toxic effects for the 30-day exposure) and general health

index (CI, LSI and GSI) were added to the evaluation. The effects of their inclusion is shown in Figure 6.

The biomarker data scores in Appendix G were used to make the IBR star plots in Figure 6. The star plots show the effects of different biomarkers used in calculating the IBR values. Compared to Figures 6A and 6B, where all the PAH metabolite values were used, Figure 6D still shows a similar trend where Naph H has the highest biomarker response among the groups. Without the results from the PAH metabolite biomarkers (Figure 6C and 6E), Chry DH has the highest response from the biomarkers but still smaller in value compared to the highest IBR value of Naph H in Figures 6A, 6B and 6D.

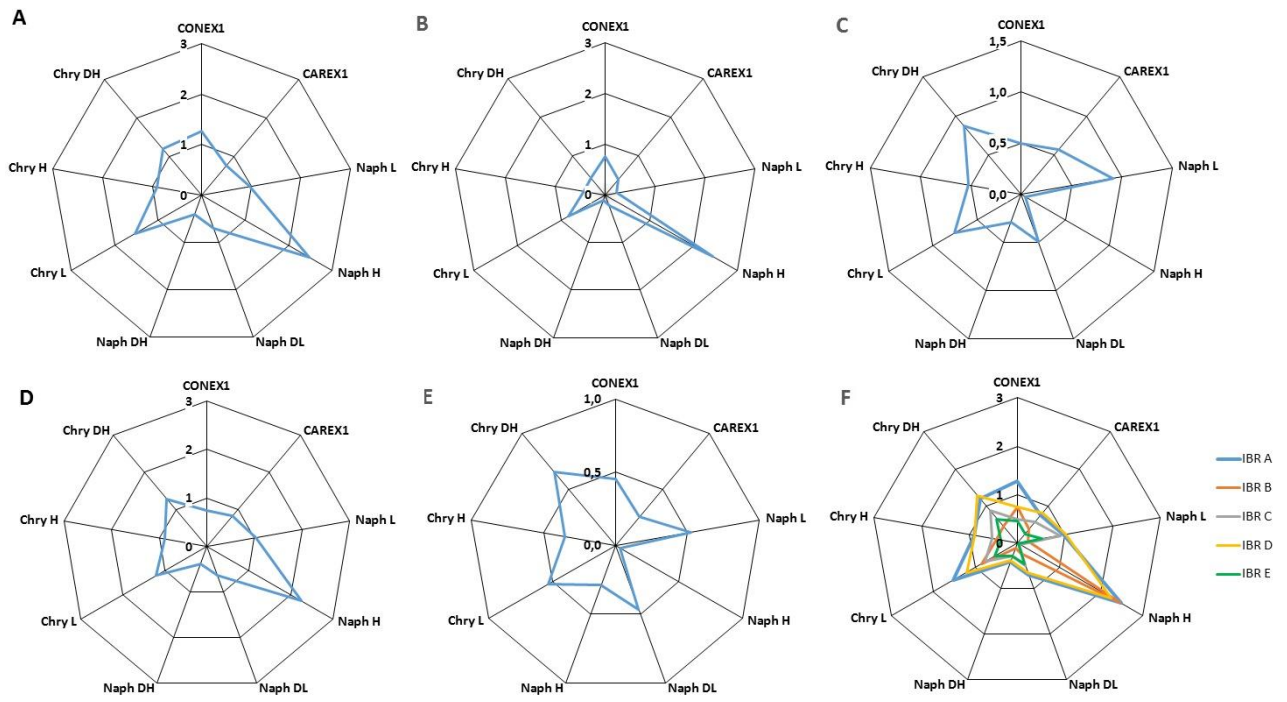

Figure 6. IBR star plots for the different exposure groups using different sets of biomarkers. A – all biomarkers, B – PAH metabolites (FF and GC-MS), C – EROD, GST, CAT, DNA adduct and general health index (CI, LSI and GSI), D – all biomarkers excluding results from 6-ring (FF) and C1-C3-OH-Naph (GC-MS) due to high number of values below the detection limit, E – biochemical biomarkers (GST, CAT, DNA adduct) and general health indices (CI, LSI, GSI), F – A-E for comparison.

After comparing the IBR star plots, there is no reasonable agreement between the exposure gradients and IBR variation. This result has also been observed in the study by Beliaeff and Burgeot [16]. In addition, the IBR index did not also appear to be related to the difference in parent and metabolite exposures. The large variability observed for Naph H and the rest of the exposure groups

in three of the combinations with PAH metabolite biomarkers is most likely related to the results from the GC-MS method accounted for the naphthalene responses only.

To examine the influence of a particular biomarker type in the overall response of the sample to PAH exposure, the IBR/n was calculated and presented graphically in Figure 7. This was calculated since the results of the IBR depends on the number of biomarkers used in the plot. High index values are observed for Naph H in sets using PAH metabolite biomarkers (Figure 7) compared to its metabolite. The IBR index of the same group decreased considerably when the analysis was performed without using the PAH metabolite biomarker results. Chry L and the control groups remain consistent in all sets, despite the change in number of biomarkers.

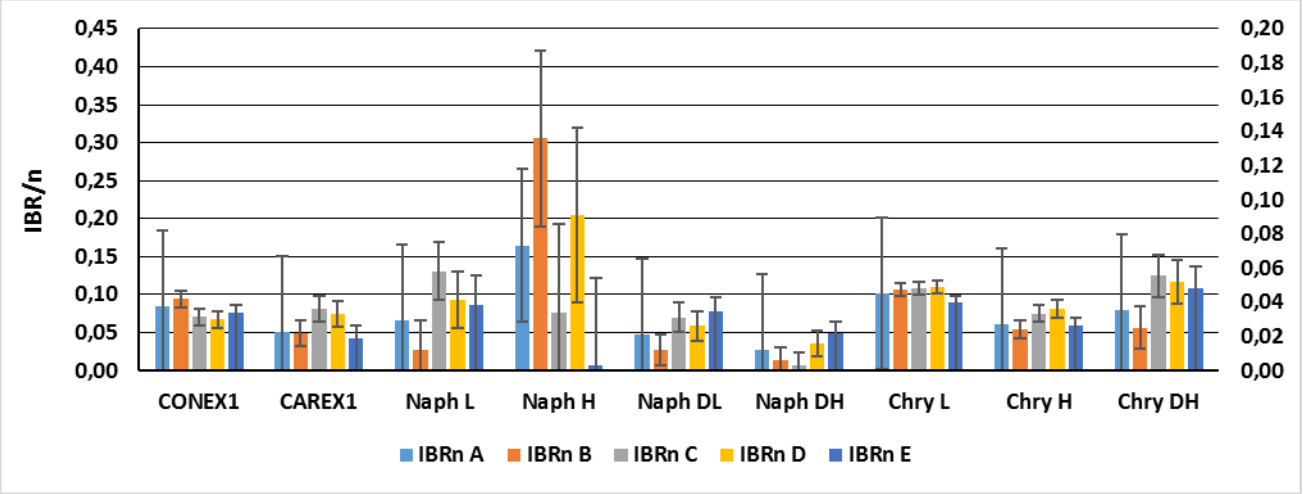

Figure 7. IBR/n of each group comparing different sets of biomarkers. Mean and standard deviation for different sets of biomarkers. A – all biomarkers, B – PAH metabolites (FF and GC-MS), C – EROD, GST, CAT, DNA adduct and CI (CI, LSI and GSI), D – all biomarkers excluding results from 6-ring (FF) and C1-C3-OH-Naph (GC-MS) due to high number of values below the detection limit, E – biochemical biomarkers (GST, CAT, DNA adduct) and general health indices (CI, LSI, GSI).

The axes of each star plot in Figure 8 represent different numbers of biomarkers, and the line in each axis depends on the response of the biomarker to exposure and corresponds to the relative response of the biomarker within that exposure group. These star plots are created to compare each exposure group in every set of biomarkers. The calculated scores for each biomarker specific for each group in Appendix G were used to make the star plots. The biomarkers 1-OH-Naph and 2-OH-Naph which has the highest influence in three of the sets with PAH metabolite biomarkers in the set for Naph H (Figure 8A, 8B and 8D). When the PAH metabolite data (FF and GC-MS) is not used, the degree of response for DNA adduct and EROD dominates and significantly decreases for Naph H. Standardized biomarker value for EROD is also dominating for Naph L. DNA adduct also has a prominently higher value in Chry H when only the biochemical biomarkers and general health

indices were used (Figure 8E). CAT activity and CI has the highest degree of response for Naph DH, the biomarkers which the corresponding parent compound has the lowest response (Figure 8E).

In most calculations using IBR [36], the number of biomarkers used has been limited to 4-6. It has been the first time in this study to use more biomarkers with the varying response to exposure in calculating the IBR value. The result has shown that the index takes into consideration the variations contained in the different combinations of biomarkers as the number of biomarkers increase. The index has also clearly represented the biomarkers with the biggest response for a specific group (Figure 8). Due to that, there was a clear difference between the metabolites and the parent compound in both naphthalene and chrysene exposed groups, but not the dose-specific differences, as usually determined in previous IBR applications [16,36,37]. But it should be noted that, in this method the index can be biased and the 'zero' value of one biomarker causes the IBR index to be low regardless of whether the other biomarker values are high [36].

A visual overview of the biomarker evaluation given by the star plots reveal that the results for the PAH metabolite data contributed considerably to high IBR values. In comparing this behavior of the biomarkers when combined with biomarkers affecting other systems, the induction responses of phase II enzymes are generally less pronounced as also seen in other studies [55]. This weak response of GST activity may be masked by natural variability factors (such as sex and maturity) [12]. With regards to the result for hepatic CAT activity, in previous studies its increase was only observed in some experiments with fish exposed to polychlorinated biphenyls (PCBs), bleached craft mill effluents (BKMEs) or PAH-containing sediments in the field, but most laboratory studies could not demonstrate any significant alterations [12]. It has been reported [15] that the standard deviation of mean becomes expectedly smaller as the number of biomarkers increase leading to the insignificant difference in IBR values when the number of biomarkers is sufficiently large, as also shown in Figure 8 for most of the groups.

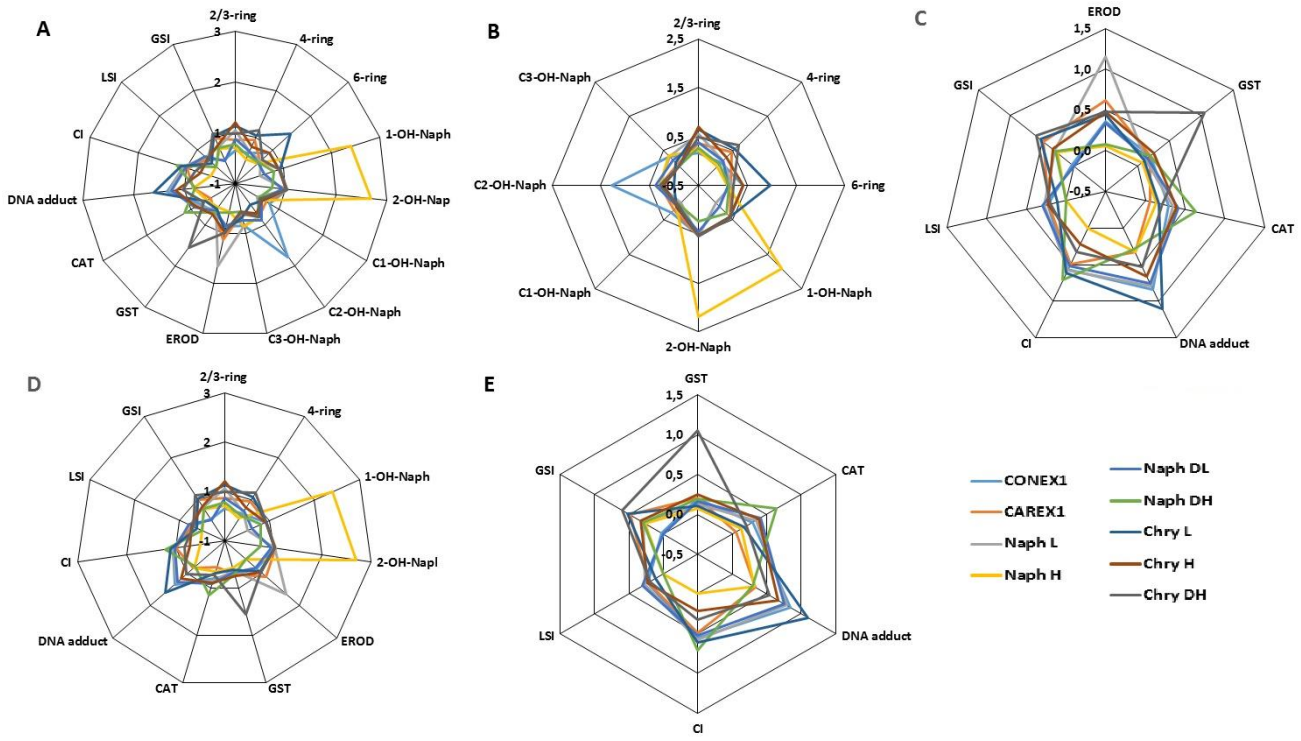

Figure 8. Star plots representing each biomarker in each set for the different groups.

All samples from the nine exposure groups displayed mean BAI values in the range of 20-30 (Table 4) indicating that there is no significant difference between the exposure groups in terms of health condition of the individual samples. Only one individual in Chry L (fish no. 63) was calculated to have a mean BAI value of 30, the highest from the dataset.

As shown in Table 4, between exposed groups, the highest mean BAI value is from Chry L (25.92) indicating the worst condition among the groups according to the BAI value characterized in the previous studies [15]. The group with the lowest mean BAI value is Chry H (23.27), with a value that has a small difference from the mean BAI values of CONEX1 and CAREX1 (23.67). Between the naphthalene exposed groups and their metabolites, the groups exposed to the parent compound has a higher mean BAI value. The same can be observed with the chrysene and chrysene-diol exposed groups. Although, Chry L has a higher mean BAI value (25.92) compared to its metabolite, Chry DH has a higher mean BAI value (25.10) than Chry H (23.27).

The graphical representation in Figure 9 does not discriminate between the control groups and the exposed groups. There is also no clear difference between the parent compound groups and the metabolites. And there is no clear evidence of a dose-response relationship. All groups seem to be in the same state when the grading for the different biomarkers are used. Mean BAI values for CONEX1 and CAREX1 are the same and Naph L seem to have a narrow range as opposed to values

in Chry L. This is calculated using all biomarkers during the exposure setup (PAH metabolites: FF, GC-MS, EROD, GST, CAT, DNA adducts, and general health indices: CI, LSI and GSI).

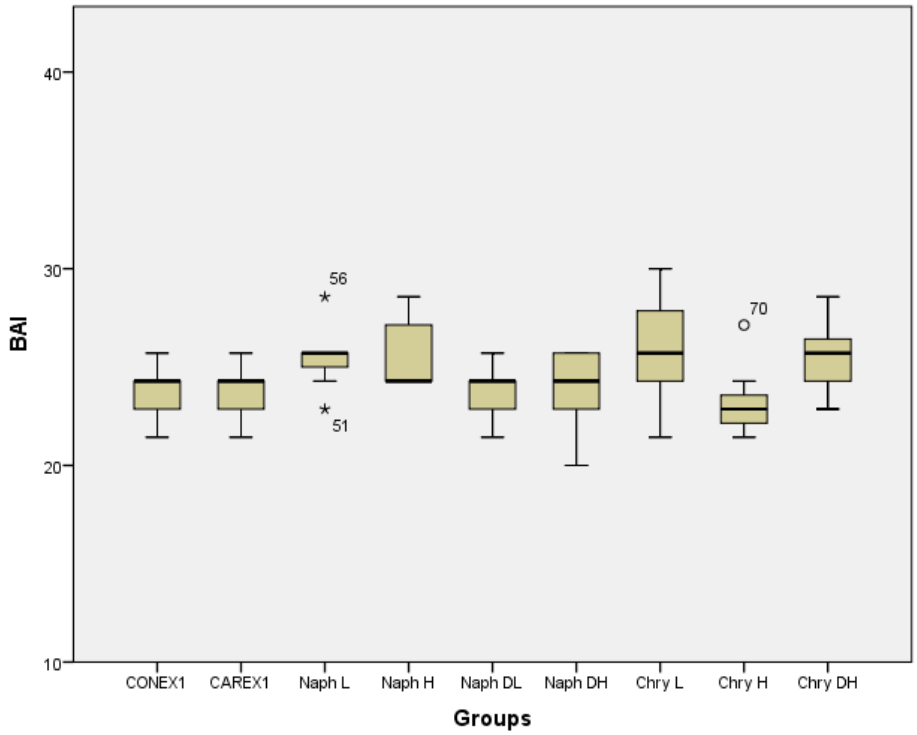

Figure 9. BAI values (25 and 75 percentiles, min and max values, median) of each group in dataset A. * - extremes, ○ – outliers (numbers beside the symbol indicate the fish number).

Figure 10 also shows that there are calculated values for the individual samples in dataset A that is lower than the lowest initial BAI value established by Broeg et al. [15]. This values are found in BAI 5, as a result of using the least number of biomarkers among the other combinations of biomarkers. If the critical value of 25 as used by Broeg. et al [15], would have been applied in this study, all individuals in all exposure groups would have been close to critical condition due to the concentration of PAHs they are exposed to, according to the number of biomarker analysis included in BAI 1. But at the same time, decreasing the number of biomarkers (BAI 5) results into BAI values that indicate good condition for the same individual samples and groups with critical condition in BAI 1. Figure 10 allows the comparison of using different sets of biomarkers to adjust numerical values reflecting the progression of toxicity (BAI value of 10 - 40 for stages 1 - 4), as well as the setting of critical values for a specific purpose when using BAI in environmental assessments.

The sets of biomarkers in BAI 1-5 is similar to the sets A-E used in PCA and IBR. Changing the types of biomarkers included in the BAI calculation doesn't change the trend among the exposure groups from one set to another. The mean BAI values for each sample only decrease or

increase relative to the number of biomarkers (Figure 10). Although BAI 2 has a deviating trend compared to the rest of the other sets, the result still doesn't discriminate the higher dose groups from the lower, or the control from the exposed groups. Unlike the results in IBR and PCA, changing the number and what type of biomarker to include in the calculation of the index changes the overall picture.

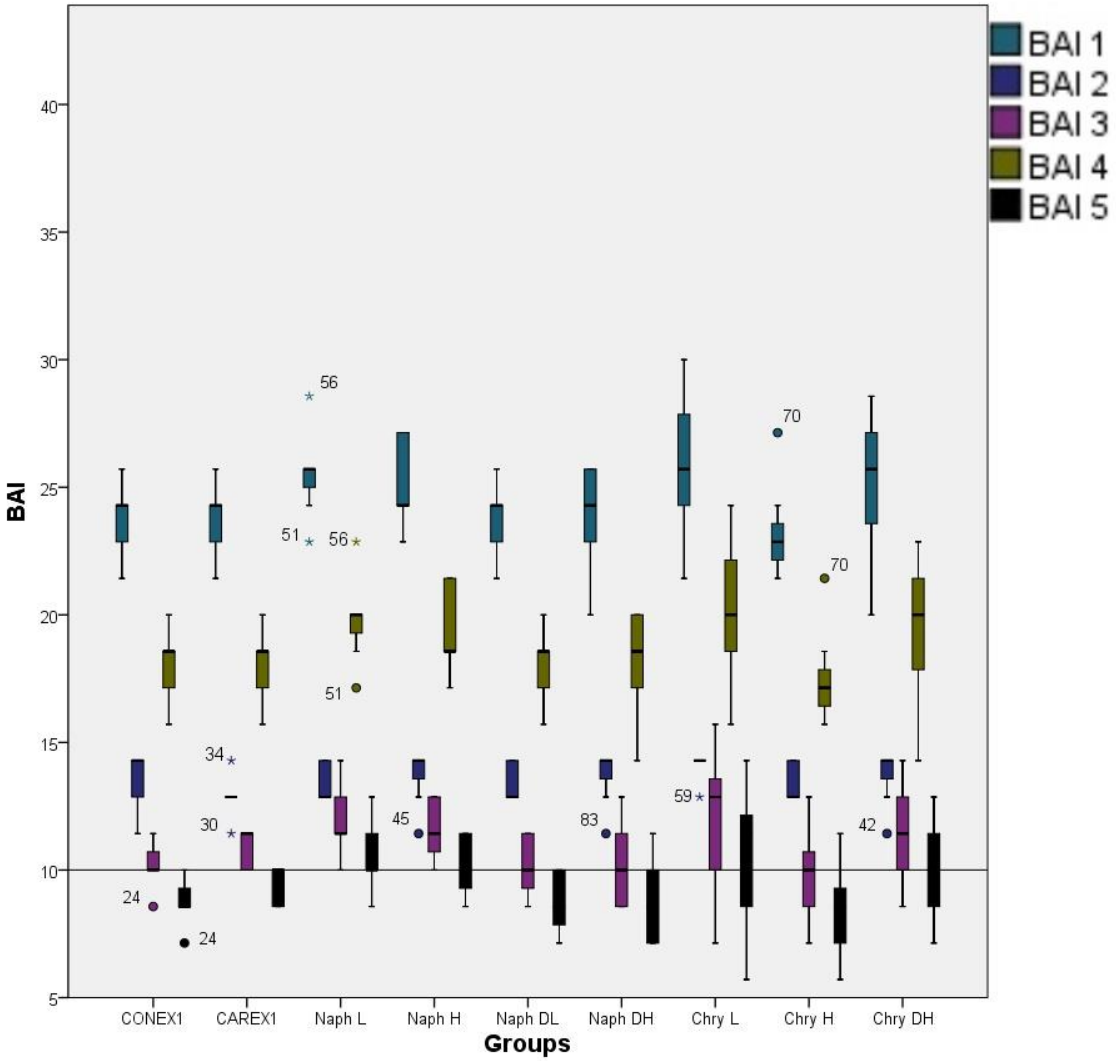

Figure 10. BAI values (25 and 75 percentiles, min and max values, median) of each group in dataset A, using different combinations of biomarkers. BAI 1 – all biomarkers, BAI 2 – PAH metabolites (FF and GC-MS), BAI 3 – EROD, GST, CAT, DNA adduct and CI (CI, LSI and GSI), BAI 4 – all biomarkers excluding results from 6-ring (FF) and C1-C3-OH-Naph (GC-MS) due to high number of values below the detection limit, BAI 5 – biochemical biomarkers (GST, CAT, DNA adduct) and general health indices (CI, LSI, GSI). * - extremes, ○ – outliers (numbers beside the symbol indicate the fish number).

In this case, the BAI's discrimination power was limited as it failed to differentiate between different exposure groups, parent compounds and metabolites. Established reference values and

ranges used to define the health stages for some parameters (i.e. general health parameters) are needed to improve the index. The results in this study states that IBR and BAI gave different results in terms of the status of the organism.

4.1.2 Second exposure (7 days)

The plot of scores and loadings represented in this section are obtained using the individual samples and biomarker analysis outlined in Chapter 2.1.2.

PCA generated using the 32 samples and 16 variables of dataset B shows that two principal components account for 96.25% of the original variance (Figure 11). Results for LOW7 in the original dataset was reduced to 7 fish due to the absence of GC-MS data for fish no. 17 in the group. The rest of the exposure groups included the original 8 individuals in each group. The examination of score plot using the two principal components is shown in Figure 11. The figure shows the correlation of CON7 and LOW7 and their negative correlation to MED7 and HIGH7. This is shown by the separation of most of the members in the group to the upper and lower side of the plot. The health condition of the individual fish has been differentiated by the PCA because there is a clear distinction between high and low doses. And the clustering of the individual samples in the same group indicates that the index is able to discriminate between the groups and the close relationship between CON7 and LOW7 compared to MED7 and HIGH7 (Figure 11), using the responses of all the individual biomarkers. Although some of the individuals in the LOW7 are more related to the higher exposed groups than the control.

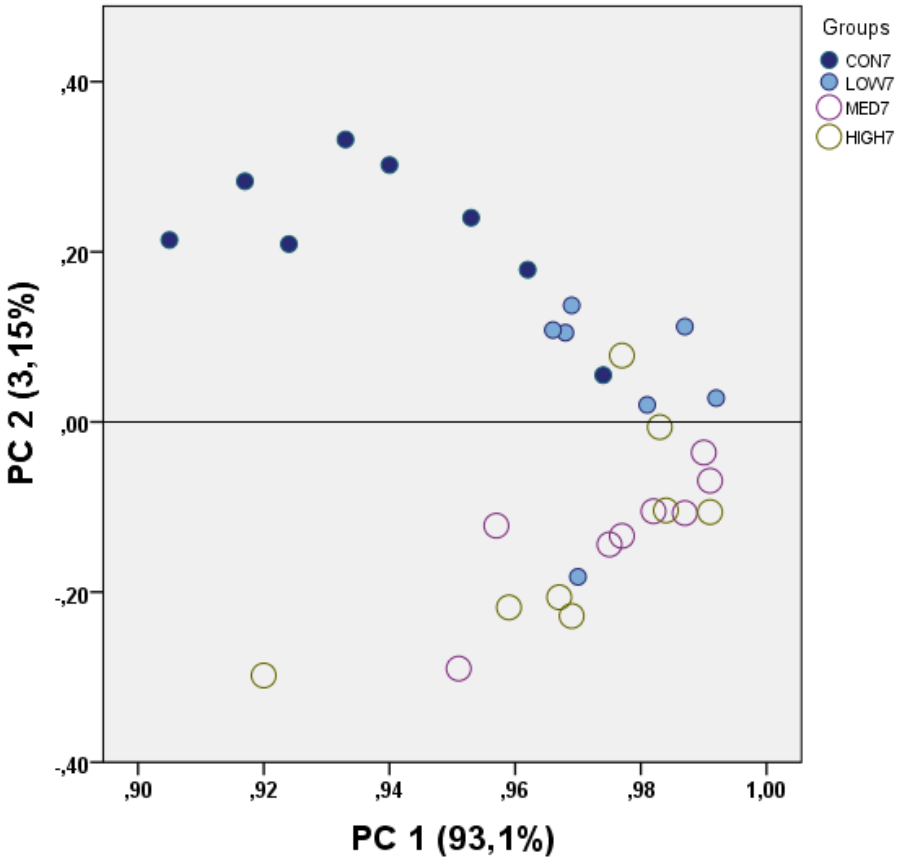

Figure 11. Plot of scores with principal components 1 (93.1%) and 2 (3.15%) of dataset B. n=7 (LOW7) and n=8 (CON7, MED7, HIGH7). Scale: 405 x 506.25, size in points.

As was seen in Figure 4, PCA was also able to distinguish the importance of the different sets of biomarkers even though the biomarkers used in dataset A are not all the same as the ones used in dataset B. In the plots shown in Figure 12, there's a clear separation between two groups of biomarker responses using the analysis from PAH metabolites data (Figure 12A) but not the same can be said for the PCA using biomarker responses for EROD, GST, CAT, CI and LSI (Figure 12B). In Figure 12A, each group is divided depending on the degree of exposure similar to the result in Figure 11, using all biomarkers. The first two principal components of both the score plots using the different suites of biomarkers explain more than 90% of the total variance (Figure 12, A-B). PCA was also able to discriminate CON7 from MED7 and HIGH7. The percentage of the total variance of the two principal components using the results from PAH metabolite biomarkers is lower compared to the combination without it. In Figure 12A, the individuals using only the results from the PAH metabolite biomarkers has a total variance of 94.21% for the two components (PC 1: 89.01%, PC 2: 5.2%). Without the PAH metabolite biomarkers (Figure 12B), the two components explain 88.86% and 11.14% of the total variance for PC 1 and 2, respectively. There are less combinations of biomarkers in this exposure setup due to fewer biomarkers analyzed. As for Figure

12B, the resulting score plot using the biomarker results of EROD, GST, CAT and condition indices (CI and LSI) show no distinct clustering of the individual samples within the same group and separation between different groups.

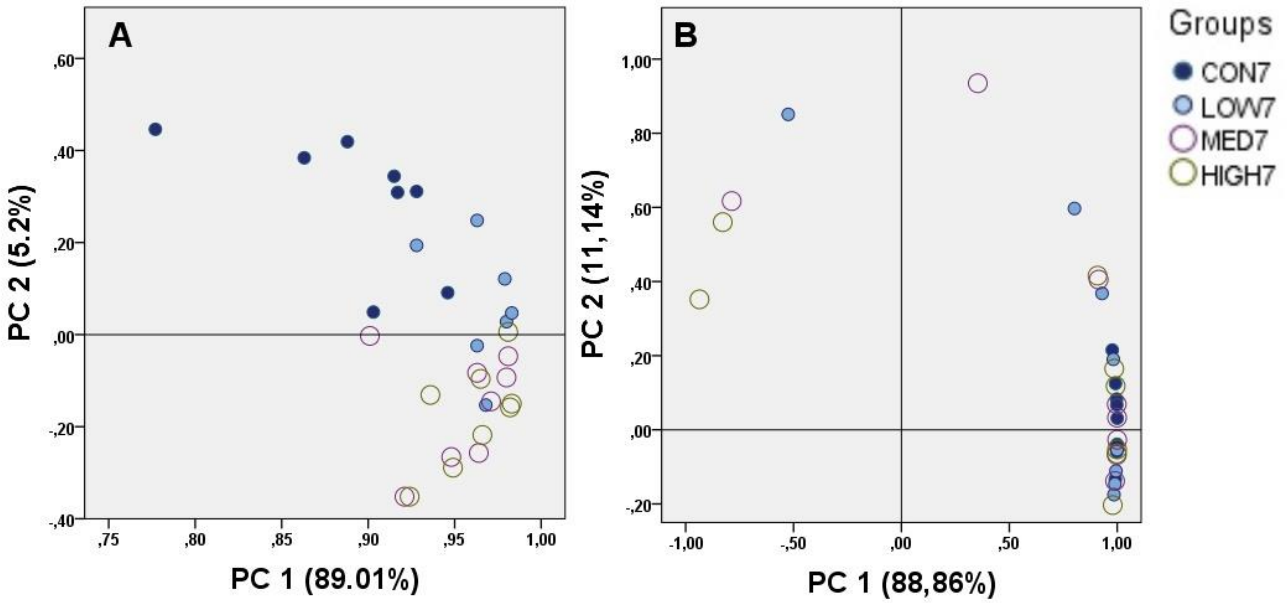

Figure 12. Dataset B: score plots of the individual samples using different combinations of biomarkers, according to the level of organization affected by the exposure. A – PAH metabolites (FF and GC-MS), B – EROD, GST, CAT and general health indices (CI and LSI). Scale: 405 x 506.25, size in points.

The score plot without the results from the PAH metabolite data (Figure 12A) shows less correlation among the individual samples compared to the score plot using all the biomarker data (Figure 11) and only the results from PAH metabolite biomarkers (Figure 12A). This again accounts to the sensitivity of the biomarker to distinguish between the groups, as previously shown in Figure 3 and 4. Figures 12A and 12B also show that the type of biomarker affects the correlation between the exposure groups, as opposed to the results obtained for the first exposure setup (Figures 3 and 4). The grouping of the individuals from different exposure groups are more distinguished for MED7 and HIGH7, being separated from the remaining groups (Figure 12A).

The positons of the biomarkers using their respective response in the 32 samples used for dataset B are shown in Figure 13. The distinction of MED7 and HIGH7 among all the groups can be attributed to the sensitivity of the PAH metabolite biomarkers, considering that it has been used in studies before [9] showing that PAH metabolites is a sensitive biomarker in such exposures. As indicated, the analysis for PAH metabolites are more closely related compared to the other biomarkers (EROD, GST, CAT, CI and LSI) at the far left side of the plot. Comparing the positions of the loadings and scores in the plot, the separation of the first group (CON7 and LOW7) from the

second group (MED7 and HIGH7) can be attributed to their low values for PAH metabolite data and high value in EROD, GST, CAT, CI and LSI.

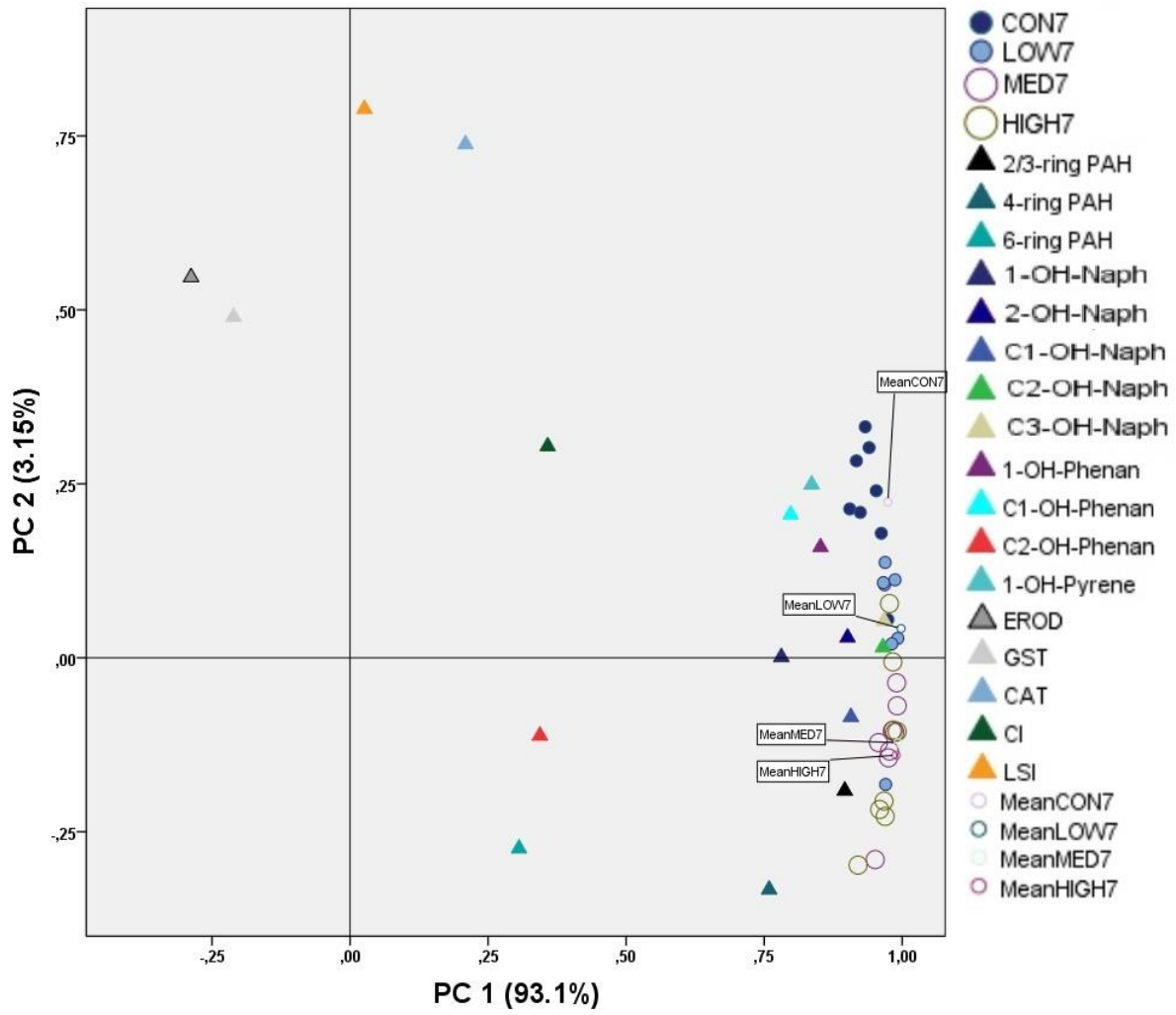

Figure 13. Dataset B: Scores and loadings plot of the first two principal components (PC 1: 93.1%, PC 2: 3.15% of the total variance). Scale: 645 x 806.25, size in points.

As shown in Table 9, HIGH7 has the highest biomarker response with an IBR value of 16.27 (IBR A) and 15.06 (IBR B). The lowest IBR values in these calculations are from CON7 (0.51 and 0.04) on both IBR A and B. For IBR C, MED7 has the lowest IBR value (0.24) and LOW7 resulted to 1.89, which is the highest IBR value in the set without the PAH metabolite biomarkers. This increase in IBR value for the LOW7 in IBR C may be attributed to the high score calculated for the group's general health indices (CI and LSI). The sets of biomarkers are the same as used in PCA [A – all biomarkers, B - PAH metabolites (FF and GC-MS), C – EROD, GST, CAT and general health indices (CI and LSI)].

Table 9. IBR values for each group in dataset B using different sets of biomarkers.

Groups	CON7	LOW7	MED7	HIGH7
IBR A	0.51	1.50	6.49	16.27
IBR B	0.04	0.31	6.25	15.06
IBR C	0.54	1.89	0.24	1.16

Star plots revealed details about the biological responses obtained for each group. As observed in Figure 14, the differences in the numerical values can easily be seen compared to the results presented in Table 9. In the star plots, the trend from lowest IBR value to highest (CON7 to HIGH7) is observed in IBR A and B (Figures 14A, 14B and 14D), but not for IBR C. The star plots focus on the effects of different biomarkers used in calculating the IBR values. Compared to Figures 14A and 14B, where all the PAH metabolite data were used, Figure 14C does not show a similar trend because LOW7 has the highest biomarker response among the groups. But compared to the other high BAI values of HIGH7 in IBR A and B, the value of LOW7 in IBR C is significantly lower (Figure 14D).

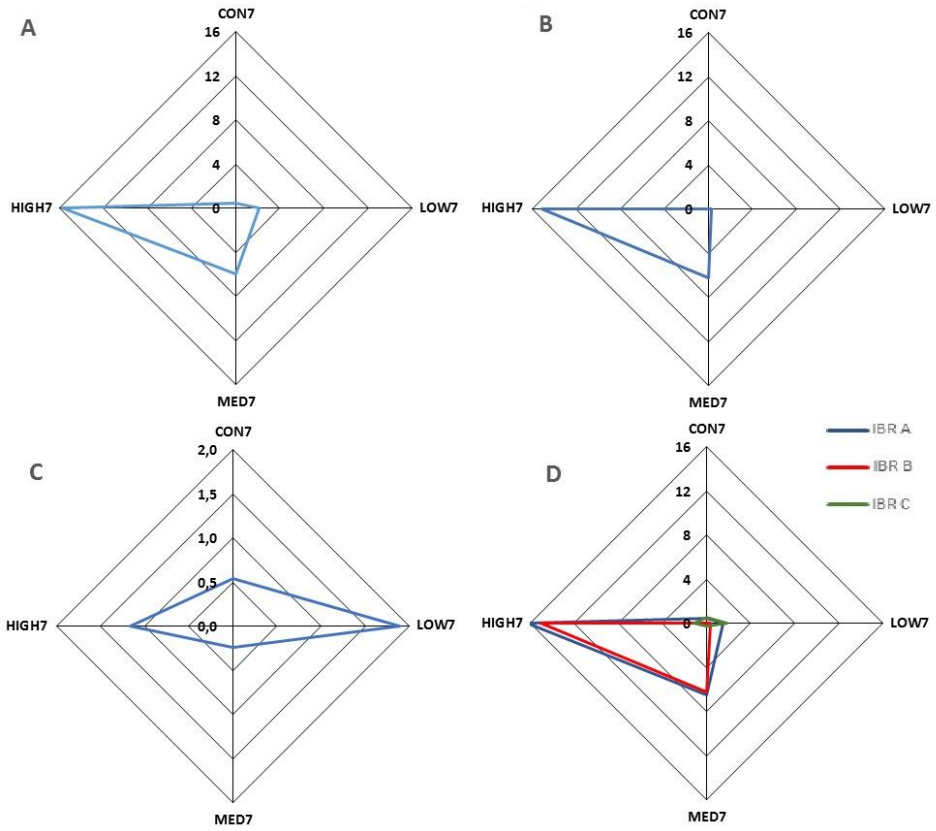

Figure 14. IBR star plots for the different exposure groups using different sets of biomarkers. A – all biomarkers, B – PAH metabolites (FF and GC-MS), C – EROD, GST, CAT and general health indices (CI and LSI), D – A-C for comparison.

The graphic representations were able to discriminate between the different concentration groups although the difference of IBR values in IBR C does not follow the corresponding concentration gradients of the groups. Similar to the IBR calculations done for the groups in the first exposure, this calculation used different biomarkers than the ones used by Beliaeff and Burgeot [16]. Specific biomarkers (PAH metabolites in bile measured using two methods, FF and GC-MS, and EROD), general health indices (CI and LSI) were added to GST and CAT analysis for the evaluation.

Similar to the results for the first exposure setup, to examine the influence of a particular biomarker type in the overall response of the sample to PAH exposure, the IBR/n was also calculated for this exposure setup and presented graphically in Figure 15. This was calculated since dataset B includes a different number of biomarkers than the biomarkers in dataset A. Overall, highest IBR/n values were calculated for HIGH7 in sets using PAH metabolite biomarkers (Figure 15) compared to CON7, and MED7 which showed moderately high-to-high IBR/n values in these sets.

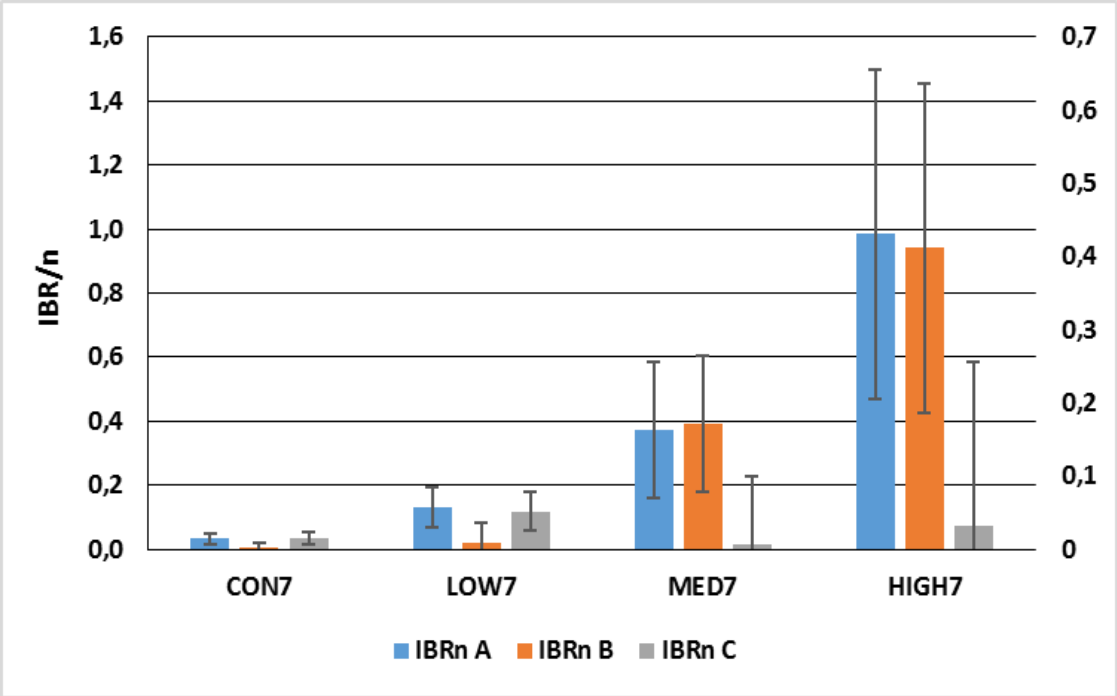

Figure 15. IBR/n for the different sets of biomarkers used for dataset B. Mean and standard deviation for different sets of biomarkers. A – all biomarkers (PAH metabolite: FF and GC-MS, EROD, GST, CAT, CI and LSI), B – PAH metabolites (FF and GC-MS), C – EROD, GST, CAT and general health indices (CI and LSI).

As opposed to the IBR results presented in Figure 6, there is a reasonable agreement between the star plots of IBR and biomarker responses for dataset B as shown in Figure 16. The star plots coincide with the exposure gradients of the different exposure groups. Star plots also revealed that

the PAH metabolite biomarkers contributed to high IBR values, especially for HIGH7. Figure 16 shows that high index values are calculated for HIGH7, but the IBR value of the same group decreased considerably in sets without the PAH metabolite biomarkers. Although CI and LSI scores for HIGH7 increased in IBR C. The control group remains consistent in all sets despite the change in number of biomarkers except for the group's GST results in IBR C. The PAH metabolite data also showed its sensitivity in the IBR calculation for the first exposure setup, as can also be observed in the results in Figure 16. There is a clear dose-specific differences in the calculations using this biomarker.

Different biomarker arrangements on the star plots produce different IBR/n values [36], therefore biomarkers were orderly represented in the axes of start plots from the less (FF and GC-MS) to the most complex (CI) biological level, as suggested by Marigómez et al. [37]. Axes of each star plot in Figure 16 represents different biomarkers used in dataset B, combined in different sets. Each axis represents the response of the biomarker to exposure and corresponds to the relative response of the biomarker within that exposure group. These star plots are created to compare each exposure group in every set of biomarkers. Graphically, it is obvious that for the HIGH7, the PAH metabolite biomarkers have the highest influence in the sets including that biomarker (Figure 16A and 16B). When the PAH metabolite data (FF and GC-MS) is not used, the degree of response for CI dominates for the exposed groups, but not for the control group. IBR score for LOW7 has the highest value (1.37) for CI using this set of biomarkers (IBR C). In Figure 16A, it is also observed that the LOW7 has the highest response in CI and CON7 in GST.

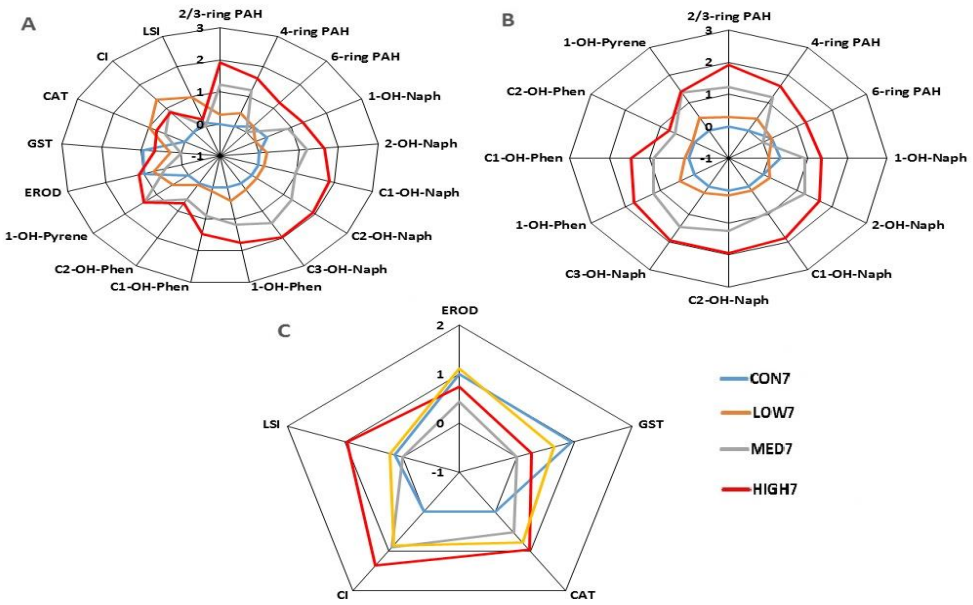

Figure 16. Star plots representing each biomarker in each set for the different groups.

All individual samples from the 4 groups displayed mean BAI values in the range of 25-35 (Table 5). Graphically represented, the increase of mean BAI value for each group can be seen from CON7 to HIGH7 (Figure 17). In between exposed groups, the highest mean BAI value is from HIGH7 (34.22) indicating the worst condition among the groups according to the BAI value characterized in the previous studies [15]. The group with the lowest mean BAI value (27.81) is CON7, followed by the mean BAI value of LOW7 (29.06). The graphical representation in Figure 17 clearly discriminates between the control groups and the exposed groups, indicating a clear evidence of a dose-response relationship. This is calculated using all biomarkers during the exposure setup (PAH metabolites: FF, GC-MS, EROD, GST, CAT, CI and LSI).

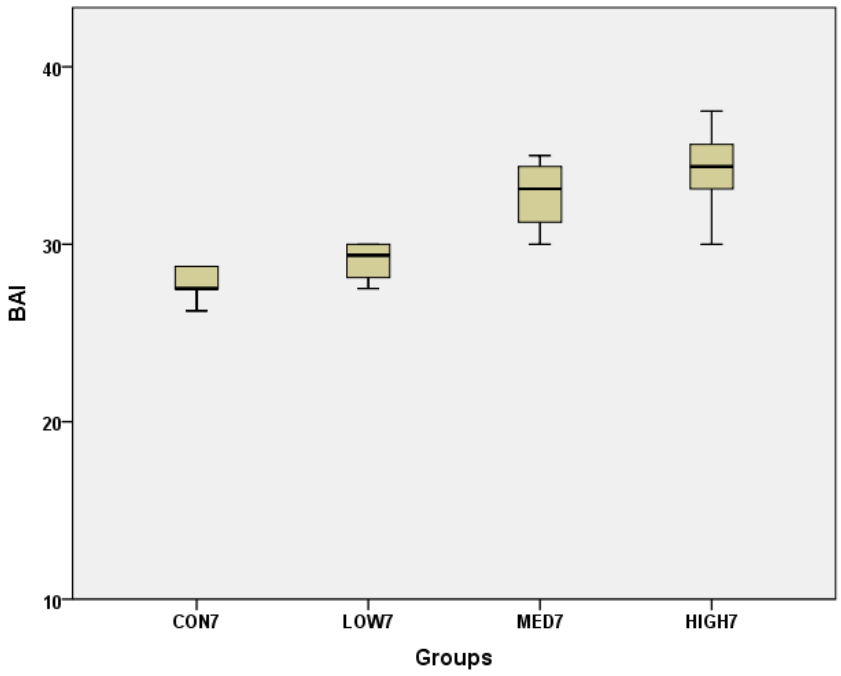

Figure 17. BAI values (25 and 75 percentiles, min and max values, median) of each group 7 days after the second exposure.

Considering that BAI was designed as an index for the assessment of the multifactorial contamination situation of coastal areas and originally included only biomarkers of general toxicity [15], it was able to identify the quality of contamination using the second exposure setup. The biomarker responses used in this study are also generally used for the assessment of specific PAH exposure, not as a screening test as used in previous studies. That may have had an effect why the results are not very discriminating in terms of the exposed groups and the degree of exposure in the first exposure setup.

The sets of biomarkers in BAI 1-3 is similar to the sets A-C used in PCA and IBR. Changing the types of biomarkers included in the BAI calculation changes the trend among the exposure groups from one set to another, in this case. The mean BAI values for each individual only decrease or increase relative to the number of biomarkers used in the calculation (Figure 18). Although BAI 3 deviates from the other two sets including the analysis for PAH metabolite biomarkers. Compared to the first two BAI calculations (BAI 1 and 2) the result doesn't discriminate the higher dose groups from the lower, or the control from the exposed groups in BAI 3. Like the results in IBR and PCA for this exposure setup and period, changing the number and what type of biomarker to include in the calculation of the index changes the end result. The range of BAI values for each group using different sets of biomarkers are within the 10-40 range except for one individual (8.75) in LOW7. Overall, Figure 18 has provided reliable information for the assessment of condition of the individual samples by using the combined application of different suites of biomarkers in individual organisms.

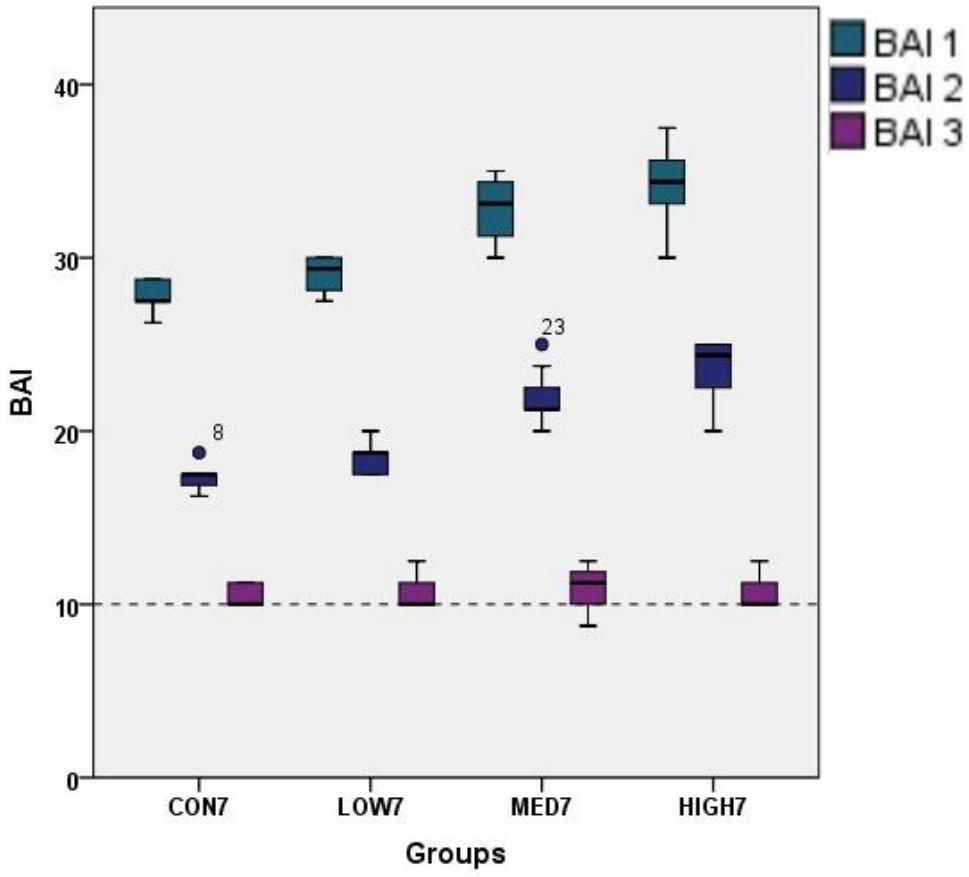

Figure 18. BAI values (25 and 75 percentiles, min and max values, median) of each group 7 days into exposure in the second exposure setup, using different combinations of biomarkers. BAI 1 – all biomarkers (PAH metabolite: FF and GC-MS, EROD, GST, CAT, CI and LSI), BAI 2 – PAH metabolites (FF and GC-MS), BAI 3 – EROD, GST, CAT and general health indices (CI and LSI). ○ – outliers (numbers beside the symbol indicate the fish number).

The results of the BAI calculation were useful for dataset B to determine the different exposure groups and provided an output which is easy to understand. But solid reference values and optimization of the ranges used to define the health status stages for some parameters (similar for the first exposure setup) are also needed to improve it [36]. It must also be considered that large suites of biomarkers would give every biomarker a similar weight and not all of them involve equal environmental relevance [37], like the general health index in different exposure setups.

The IBR and BAI in this case (Figure 14 and 18) produced similar results wherein most cases agreed with the known contamination levels in different exposure groups, as was previously demonstrated in a similar study comparing the two indices [36]. As seen in Figures 17 and 18, there is good accordance with the level of exposure in the second exposure, from control to high exposure groups.

4.1.3 Intraperitoneal and CFS exposure

The statistical analyses done in this section used the combined results of the first exposure and the results of the individual samples analyzed after 7 days of the second exposure. The biomarker results used are the results of the common biomarkers between the two exposures.

Plots for PCA were generated using the 12 biomarker responses as variables and 108 individual samples as cases. Figure 19 shows that the first two principal components extracted from the analysis account for 96.66% of the original variance. Each group in the first exposure is comprised of 7 individual A. cod, and the rest of the exposure groups in the second exposure included the original 8 individual samples. Figure 19 shows the positive correlation of all the groups in the second exposure (CON7, LOW7, MED7 and HIGH7), control groups (CONEX1 and CAREX1) and chrysene and its metabolite, and the negative correlation of the aforementioned groups with naphthalene group and its metabolite. This difference is shown by the separation between two clumps of the exposure groups according to the results of the PAH metabolite data (FF and GC-MS), EROD, GST, CAT, CI and LSI results. It can also be observed in Figure 19 that MED7 and HIGH7 are more correlated to the groups in the first exposure compared to CON7 and LOW7.

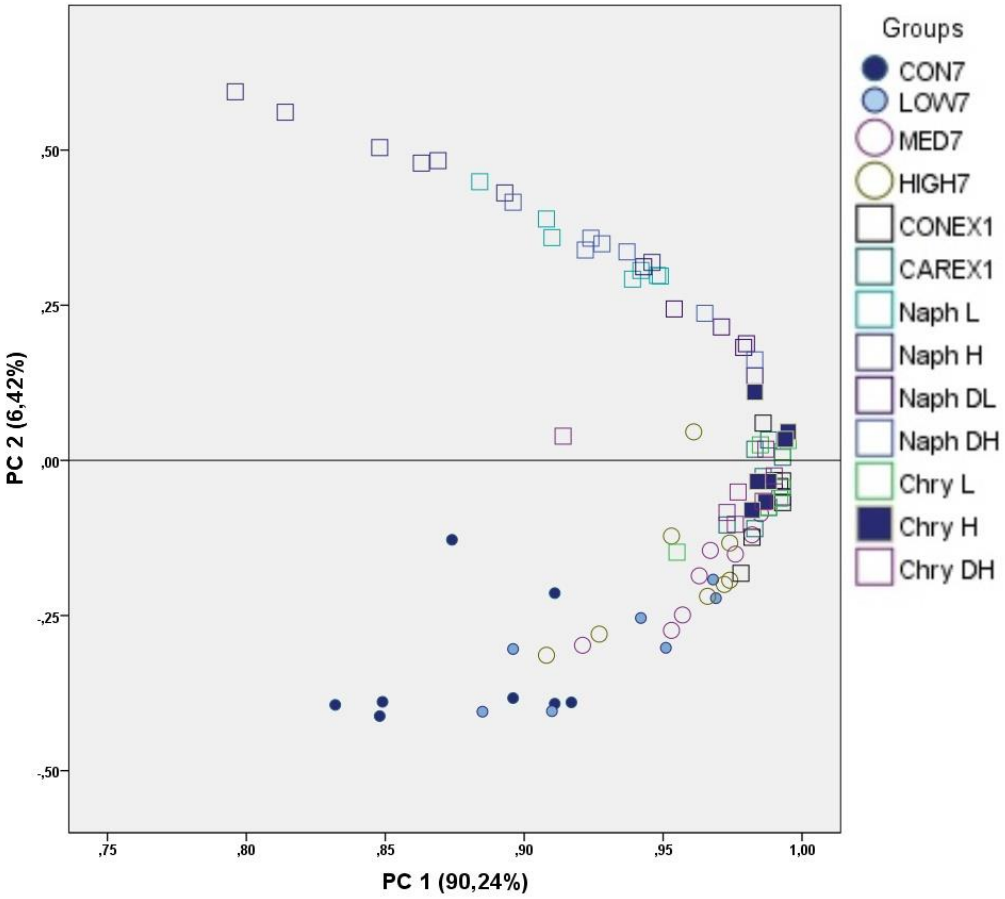

Figure 19. Plot of scores with principal components 1 (90.24%) and 2 (6.42%) of dataset D (scale: 645 x 746.25, size in points).

The first two principal components of the score plots using the different suites of biomarkers explain more than 90% of the total variance (Figure 20, A-B). In Figure 20A, the total variance for PC 1 is 85.2% and for PC 2 is 12.11%. Without the PAH metabolite biomarkers (Figure 20B), the two components explain 76.08% and 23.92% of the total variance for PC 1 and 2, respectively. As has been observed on all the plot of scores using different sets of biomarkers for the previous datasets, the set using PAH metabolite data shows a similar correlation between the groups (Figure 20A) with the plot of scores using all biomarkers in dataset D (Figure 19). This shows the consistent influence of the PAH metabolite biomarkers in determining the coordinates of the individual samples in the group in the PCA. But unlike in Figure 20A, the dividing of groups between exposures is more distinct in the set without the PAH metabolite data (Figure 20B). Groups in the second exposure are clumped on the left side of the plot, and the groups of the first exposure are more scattered on the right side, with an exception of some outliers for CONEX1 and Naph L. The individual samples within CON7 and LOW7 are also more clumped in Figure 20B than in 20A. The percentage of the total variance of the two principal components using the results from PAH

metabolite biomarkers is lower compared to the combination without it. There are less combinations of biomarkers in this exposure setup due to fewer biomarkers analyzed, compared to the sets of biomarkers used for dataset A. Both Figure 19 and 20A show that the clustering of the individual samples within each group is more pronounced using the PAH metabolite biomarkers especially for the groups within the second exposure. But Figure 20B shows that without the PAH biomarker data, the separation of individual samples between exposures are more pronounced.

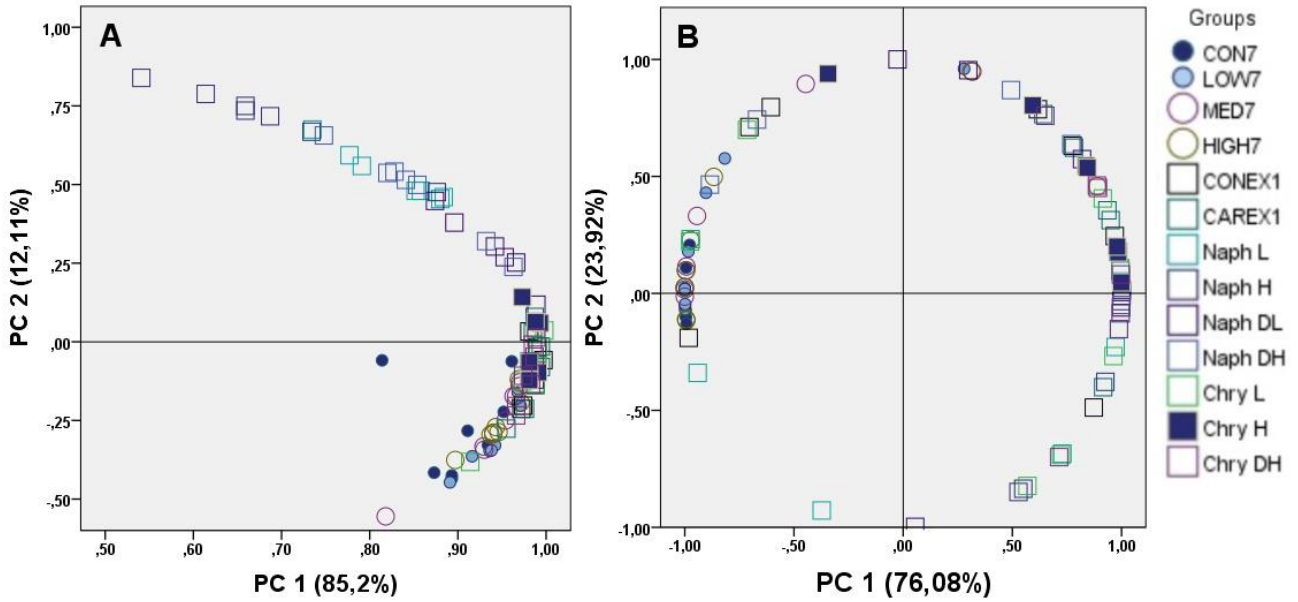

Figure 20. Dataset D: score plots of the individual samples using different combinations of biomarkers, according to the level of organization affected by the exposure. A – PAH metabolites (FF and GC-MS), B – EROD, GST, CAT and general health indices (CI and LSI). Scale: 405 x 506.25, size in points.

Figure 21 shows the loading and score plot of dataset D, with the selected biomarker responses from each exposure used, namely: PAH metabolite analysis (FF and GC-MS), EROD, GST, CAT, CI and LSI for general health indices. As shown in Figure 21, all the control groups from both exposures, Chry L and H, Chry DH and LOW7, MED7 and HIGH7 are separated from Naph L and H and their metabolites (Naph DL and DH). This is also evident in the plot of scores in Figure 19. The positions of the individual samples in the coordinates of PC 1 and 2 show that the relationship between the exposure groups and which method for PAH metabolite determination played a role in groups' clustering. It shows in Figure 21 that results for 6-ring, EROD and CAT contributed to the clustering of the groups from the second exposure to some of the groups in the first exposure. Not all the PAH biomarker data seemed to have influenced clustering of groups towards the first principal component, as was observed in the previous loading plots where the

biomarker is very dominant (Figure 5 and 13). The biomarker data were not able to show a certain distinction in detecting the biotransformation of PAH metabolites than the presence of the parent compound, as seen in Figure 21.

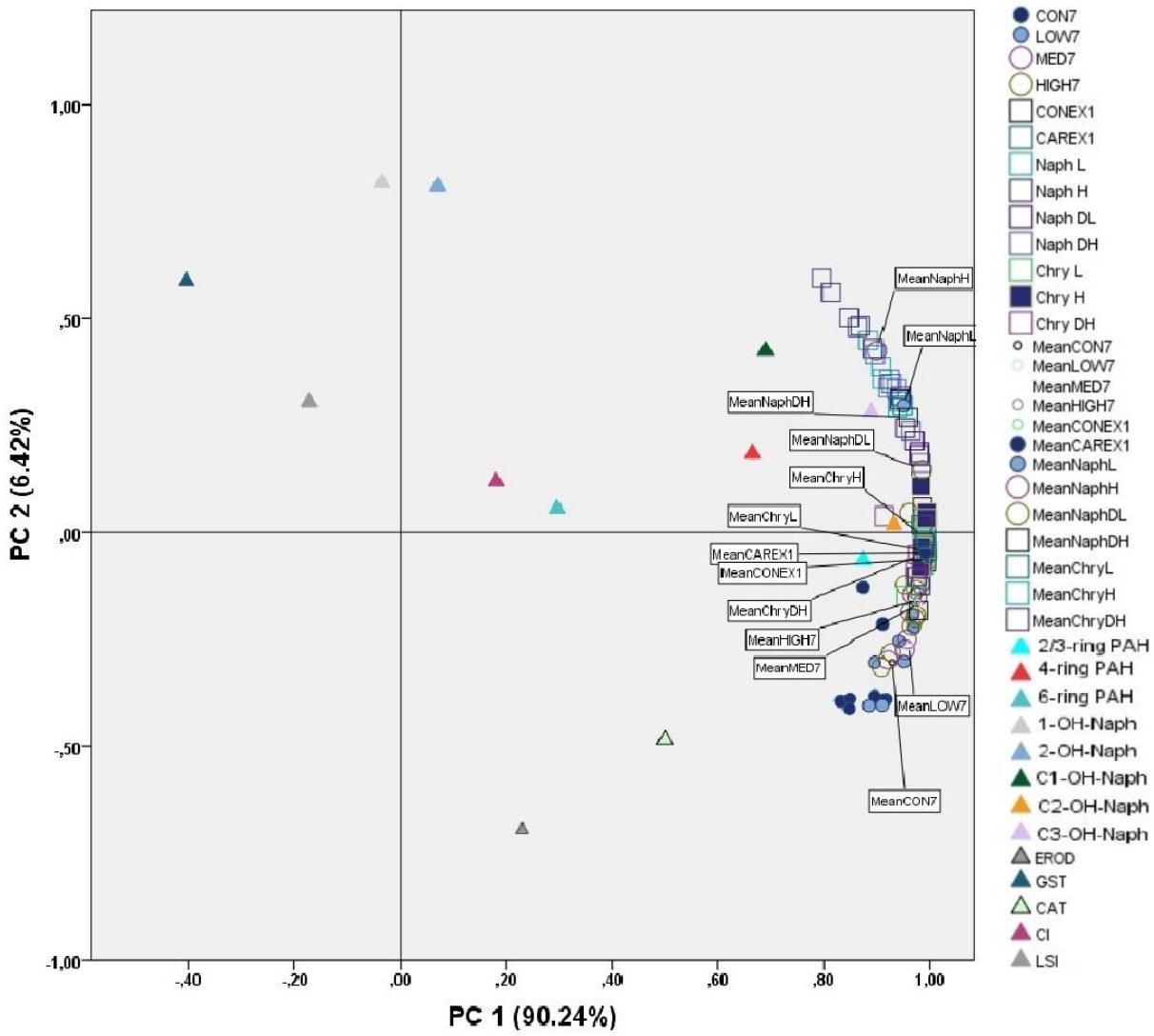

Figure 21. Dataset D: score and loading plot of the first two principal components (PC 1: 90.24%, PC 2: 6.42% of the total variance). Labelled markers are the mean values of each group (scale: 645 x 806.25, size in points).

The health status may not have been emphasized by the PCA using the selected biomarker results in dataset D but the sensitivity of the biomarkers was able to differentiate between some groups in the two exposures.

As shown in Table 10, HIGH7 has the highest biomarker response with an IBR value of 12.34 and 12.55 for IBR A and B, respectively. Chry DH has the highest IBR value (1.13) in IBR C. The lowest IBR value (0.87 and 0.52) in IBR A and B calculations is from the Naph DL, and Naph

H (0.17) for IBR C. CON7 has an IBR value of 2.10 in IBR A which is higher than the IBR values of the exposed groups from the first exposure, except for Naph H (4.46). There is no difference between the exposure groups in the first exposure, but there is an increasing trend in the second exposure from LOW7 to HIGH7 in both IBR A and B, CON7 has a higher IBR value (2.10 and 1.53) than LOW7 in these IBR sets. For IBR C, MED7 has a lower IBR value (0.51) than LOW7 (0.78). None of the control groups from both exposures had the lowest score in any of the biomarker sets.

Table 10. IBR values for each group in dataset D using different sets of biomarkers.

Group	IBR A	IBR B	IBR C
CONEX1	0.97	0.72	0.26
CAREX1	1.10	0.84	0.23
Naph L	1.41	0.68	0.75
Naph H	4.46	4.27	0.17
Naph DL	0.87	0.52	0.31
Naph DH	0.95	0.53	0.40
Chry L	1.05	0.77	0.22
Chry H	1.02	0.84	0.18
Chry DH	1.85	0.72	1.13
CON7	2.10	1.53	0.25
LOW7	1.50	0.62	0.78
MED7	4.07	4.46	0.51
HIGH7	12.34	12.55	0.98

The IBR values in Table 10 are represented in the star plots in Figure 22. As was seen in Table 10, the highest IBR value is from the HIGH7 (Figure 22A and 22B) considering all the groups in both exposures. The next highest biomarker response is from Naph H for IBR calculations using PAH biomarker data. Without the values for PAH biomarker results, the IBR values decrease considerably for all exposure groups and the groups display no pattern corresponding to their degree of exposure. Although it was observed that the results from the different sets of biomarkers were consistent in the PCA result (Figure 19 and 20), this is not observed in Figure 22 for IBR, especially the difference in IBR of the groups using PAH metabolite biomarkers and without it., in the calculation

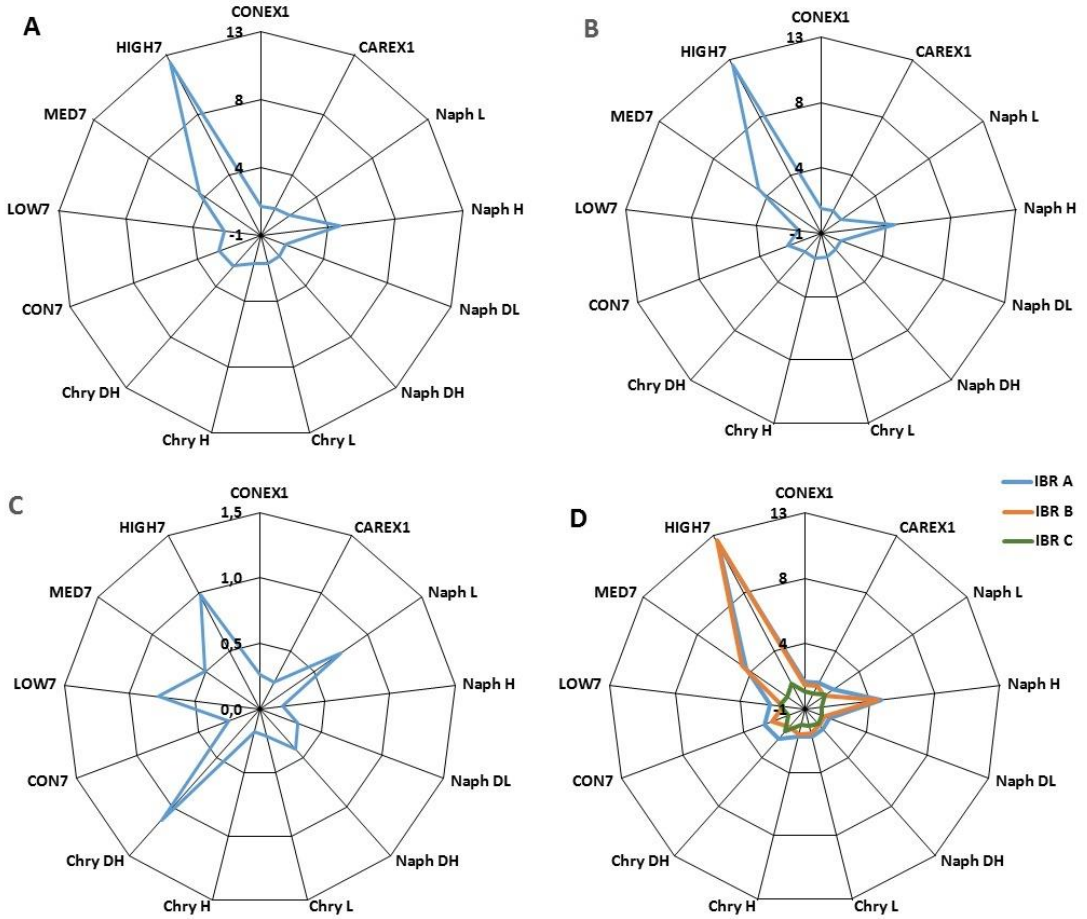

Figure 22. IBR star plots for the different exposure groups in dataset D using different sets of biomarkers. A – all biomarkers (PAH metabolite: FF and GC-MS, EROD, GST, CAT, CI and LSI), B – PAH metabolites (FF and GC-MS), C –EROD, GST, CAT and CI (CI and LSI).

The graphic representations were consistently able to discriminate between the different dose groups of the second exposure but not in the first exposure. Similar to the IBR calculations done for datasets A and B, this calculation used different biomarkers than the ones used by Beliaeff and Burgeot [16], specific biomarkers (PAH metabolites in bile measured using two methods, FF and GC-MS), EROD and general health index (CI and LSI) were added to the biochemical analysis of GST and CAT analysis.

The values for the calculated IBR/n for dataset D is presented graphically in Figure 23. This was calculated since dataset D uses a different number of biomarkers than the biomarkers in datasets A and B. Despite the mode of exposure which is more specific for naphthalene and chrysene in the first exposure, exposure to crude oil still influenced the higher IBR value using the GC-MS method for only naphthalene compounds (Figure 23). The number of biomarkers used in the IBR/n calculation did not have a significant effect in the trend for the different groups in the second exposure, compared to its effects to the groups in the first exposure (Figure 23).

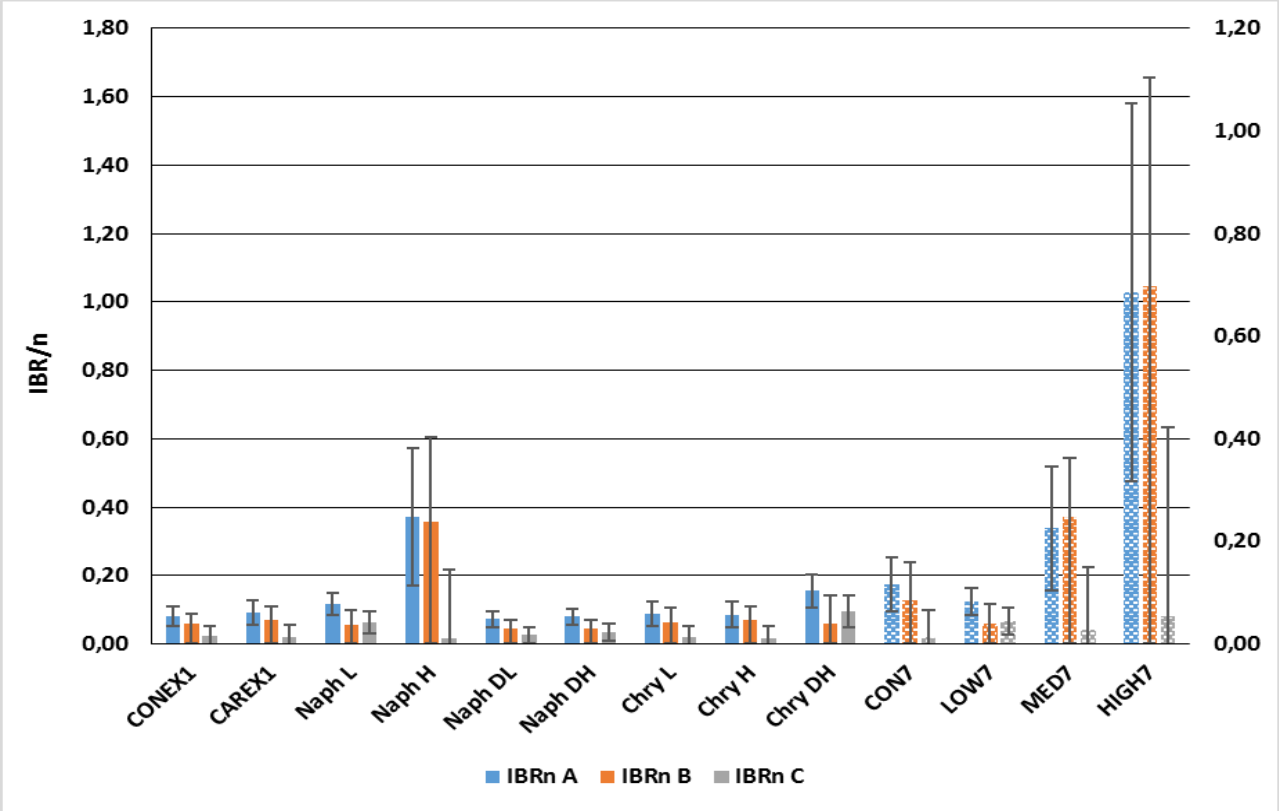

Figure 23. IBR/n for dataset D using different sets of biomarkers. A – all biomarkers (PAH metabolite: FF and GC-MS, EROD, GST, CAT, CI and LSI), B – PAH metabolites (FF and GC-MS), C –EROD, GST, CAT, CI and LSI. Mean and standard deviation.

IBR index does serve as a useful indicator for stress in the individual, although not very clear in the first exposure setup. The index produces satisfactory discrimination between groups in the second exposure whatever the combination of biomarkers is. From the star plot, the difference in biomarker responses from the most exposed groups in the different exposure setups are emphasized.

The axes of each star plot in Figure 24 represents different biomarkers used in dataset D, in different combinations. For Naph H, the GC-MS results are obviously influencing the high IBR value for the group. Note that in the analysis where HIGH7 has the highest response, Naph H has a low response, and vice versa (Figure 24A and 24B). Without the PAH metabolite biomarker data, EROD dominates the high IBR for HIGH7 (Figure 24C). The other biomarkers which dominate the other exposure groups are GST for the Chry DH, CAT for LOW7 and LSI for Naph L. The group exposed to high concentration of PAH and the lesser exposed groups were distinguished in the second exposure, and there is a causal relationship observed for the biomarker and specific pollutant level.

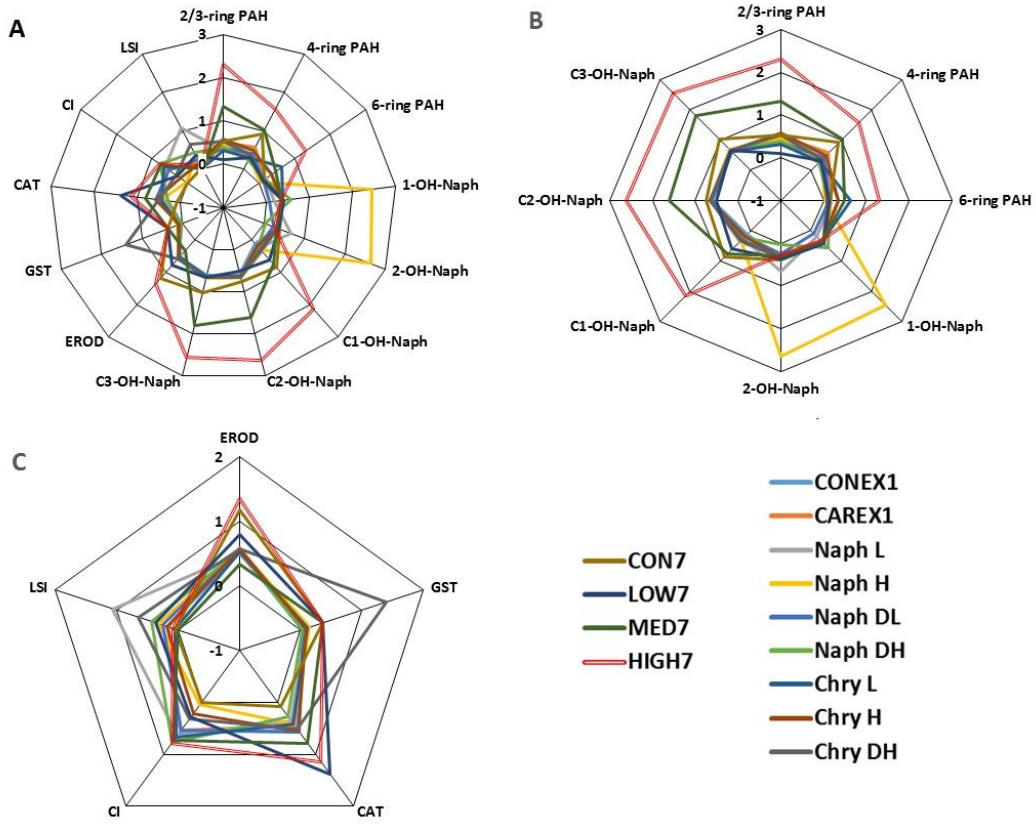

Figure 24. Star plots representing the biomarkers used in the index analysis of dataset D.

All individual samples from all exposure groups displayed mean BAI values in the range of 10-30 (Table 7), indicating that the number of biomarkers used is sufficient to recognize the degree of exposure among the groups within the limits set in previous BAI applications which is 10-40 [15,16,36-38]. According to the BAI values calculated for each group, the scores can discriminate the groups within the second exposure but not similar can be said for the groups in the first exposure (Figure 25). As the values indicate in Table 7 in Section 3.1.3, the highest BAI value (28.33) is from the HIGH7, together with Naph L and H, Chry L and H and Chry DH. The only dose-related difference that can be observed among the groups in the first exposure is between the Chry L and H, but contradictingly, Chry L has a higher BAI value than the more exposed counterpart. Despite the strong biomarker responses for HIGH7 (Figure 25), the group still has a lower BAI value compared to the high dose groups of the first exposure.

The graphical representation in Figure 25 does not discriminate between the control groups and the exposed groups for the first exposure, but deviates the different groups from each other in the second exposure. There is also no clear difference between the parent compound groups and the metabolites despite the different biomarkers used in datasets A and D. These results are all based on the biomarker analysis of PAH metabolites, EROD, GST, CAT, CI and LSI.

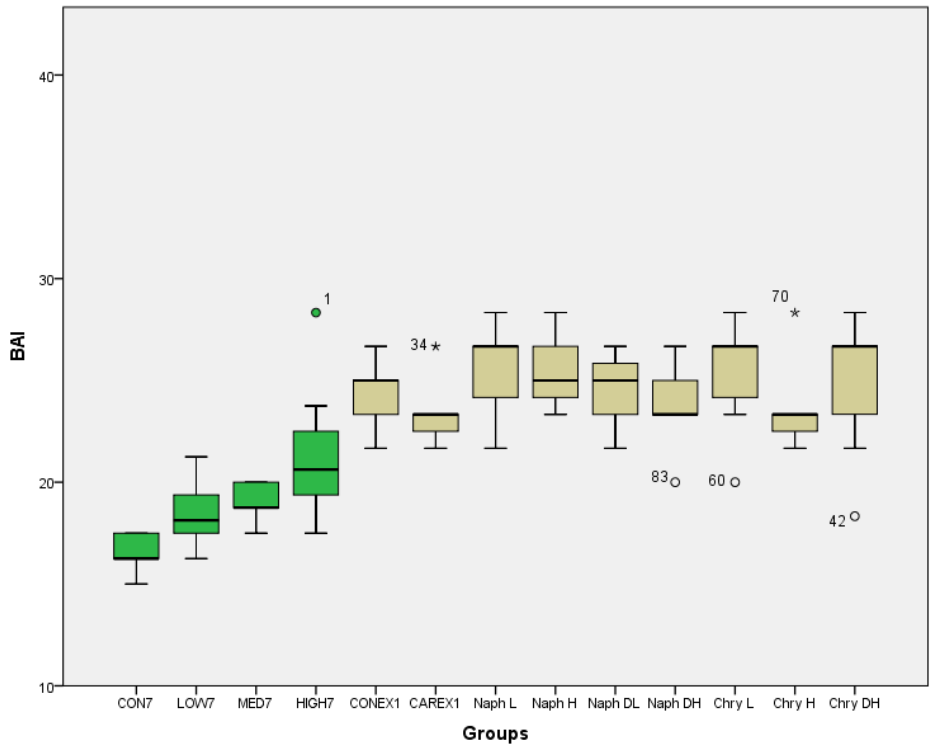

Figure 25. BAI values (25 and 75 percentiles, min and max values, median) of each group for dataset D. * - extremes, ○ – outliers (numbers beside the symbol indicate the fish number).

The sets of biomarkers in BAI 1-3 is similar to the sets A-C used in PCA and IBR. Changing the types of biomarkers included in the BAI calculation does not change the trend among the exposure groups from one set to another, in this case (Figure 26). The mean BAI values for each individual only decrease or increase relative to the number of biomarkers used in the calculation as seen in Figure 18. Compared to the first two BAI calculations (BAI 1 and 2), the result for BAI 3 does not have a dose-response pattern for the groups in the second exposure. Like the results in IBR and PCA for this exposure setup, changing the number and what type of biomarkers to include in the calculation of the index changes the end result. The range of BAI values for each group using different sets of biomarkers exceeded the minimum of 10 for the BAI 3 calculations. Overall, Figure 26 has provided reliable information for the assessment of condition of the individual samples by using the combined application of different suites of biomarkers in individual organisms in the second exposure group, but no variation among the groups in the first exposure.

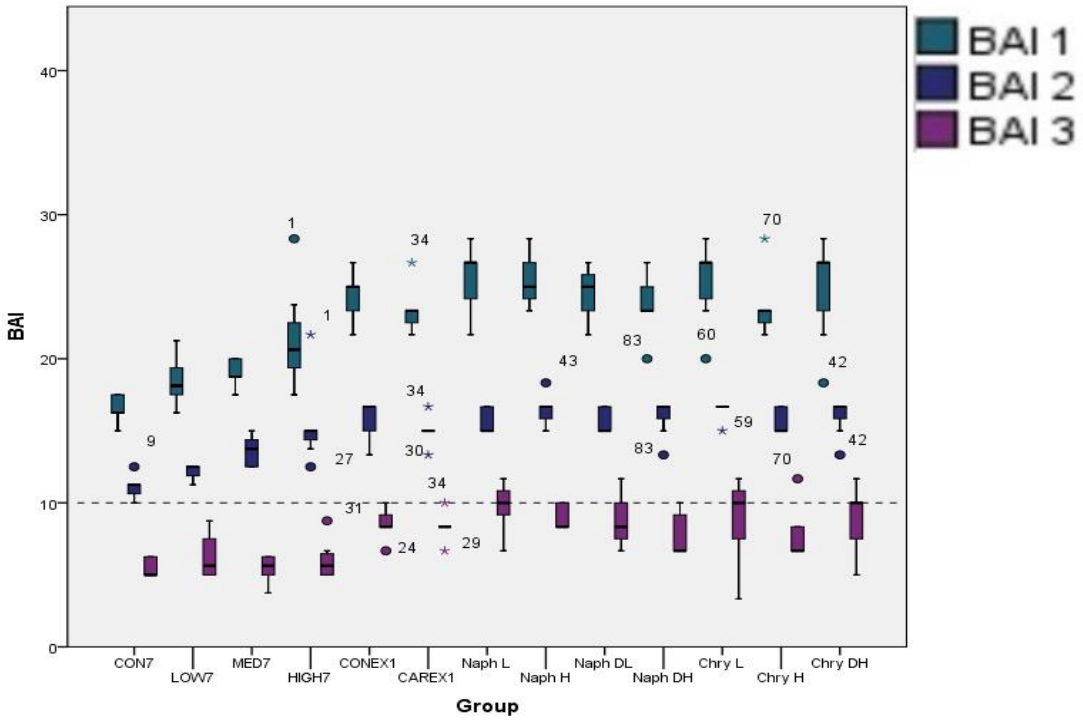

Figure 26. BAI values (25 and 75 percentile, min and max values, median) of each group 7 days into exposure in the second exposure setup, using different combinations of biomarkers. BAI 1 – all biomarkers (PAH metabolite: FF and GC-MS, EROD, GST, CAT, CI and LSI), BAI 2 – PAH metabolites (FF and GC-MS), BAI 3 – EROD, GST, CAT and general health indices (CI and LSI). * - extremes, ○ – outliers (numbers beside the symbol indicate the fish number).

According to the results presented for dataset D in all indices, PCA and IBR were not able to clearly discriminate between the groups within and between the two exposures, except for the PCA obtained for individual samples using the biomarker data of EROD, GST, CAT, CI and LSI for the analysis. BAI results were able to discriminate between the groups in the second exposure but not the first exposure. Difference in BAI values were also clear between the two exposures as seen in Figure 26. IBR star plots for the biomarkers in Figure 24 were able to present something about the biomarkers involved which is an information not clearly evident in using the BAI values.

4.1.4 Second exposure (30 days)

All results for the indices represented in this section are obtained using the individual samples and biomarker analyses after 30 days into the second exposure, as explained in Section 2.1.2. The results used for dataset C represents the degree of damage caused after 30 days into exposure to crude oil in different concentrations. The effects that are observed through the biomarker responses used in this dataset are considered the ‘late’ effects of the contaminant as opposed to the ‘early’ signals resulting from the analysis of the previous datasets.

The PC 1 and 2 explains 97.03% of the total variance. Figure 27 shows the correlation of CON30 and LOW30, and their negative correlation to MED30 and HIGH30, as shown by the separation of the groups to the upper and lower side of the plot. The clumping of the individual samples of CON30 and LOW30 is more pronounced after 30 days of exposure, compared to the PCA results for dataset B (Figure 11). It is also shown in Figure 27 that LOW30 is more clumped with the rest of the exposed groups emphasizing the separation of the control group from the exposed groups.

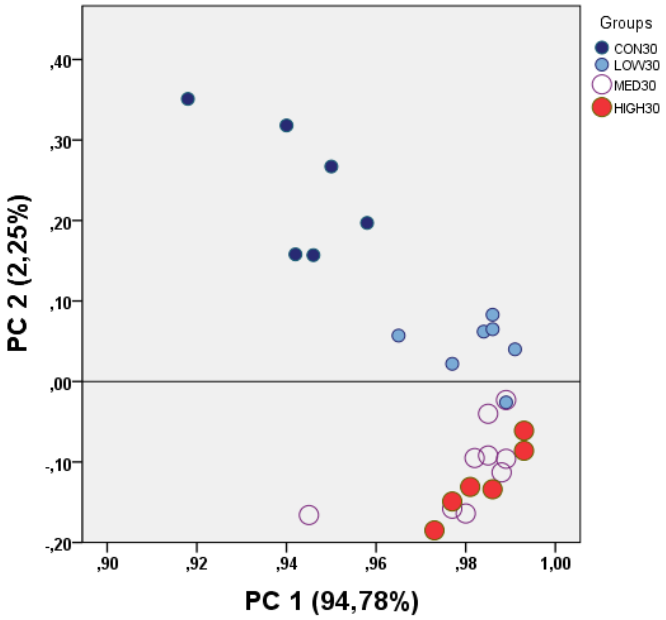

Figure 27. Plot of scores with principal components 1 (94.78%) and 2 (2.25%) of dataset C (scale: 375 x 468.75, size in points). CON30: n = 6 (control 39 has no GC-MS results) and LOW30: n = 7 (low 47 has no histopathology results).

The percentage of the total variance explained by the first two principal components in the score plots using different sets of biomarkers vary depending on the type of biomarker used (Figure 28). It is observed in Figure 28 that using the results from the PAH metabolite biomarkers give a much clearer separation of the groups compared to the separation of groups in PCA without the PAH metabolite (Figures 28B and 28C). This indicates that without the PAH metabolite biomarkers, the correlation among the groups decrease compared to the combinations without that biomarker, accounting to the sensitivity of the biomarker to distinguish between the groups. This sensitivity was already observed in the results for datasets A, B and D. The percentage of the total variance explained by the two principal components in the set with the PAH metabolite biomarkers is higher compared to the combination without it. As can be seen in the figure, the individual results from the PAH metabolite biomarkers only has a total variance of 97.23% for the two components (PC 1:

92.87%, PC 2: 4.36%). Figure 28 emphasizes the distinction between the exposed groups and the control, as explained by the first principal factor. Without the PAH metabolite biomarkers, (Figure 28B), the two components equal to 53.41% and 22.82% of the total variance for PC 1 and 2, respectively. This decrease in percentage between different suites of biomarkers can also be observed in the first exposure, where the number of principal components extracted from the results increase in number as the results of the PAH metabolite is removed. But using the results from histopathology only increases the percentage again but with only 2.58% (Figure 26C). The total variance explained by the first two components increases even more with the use of EROD, GST, CAT, CI and LSI data (Figure 28D). For this PCA, the percentage of the total variance increases to 96.61% (PC 1: 71.4%, PC 2: 25.21%) from the previous percentage. This indicates that the PAH metabolite results influences the distribution of the components dominantly, consistent with the previous results from the other datasets. Although, there is still no clear separation between the HIGH30 and MED30, but separation is evident for CON30 and LOW30.

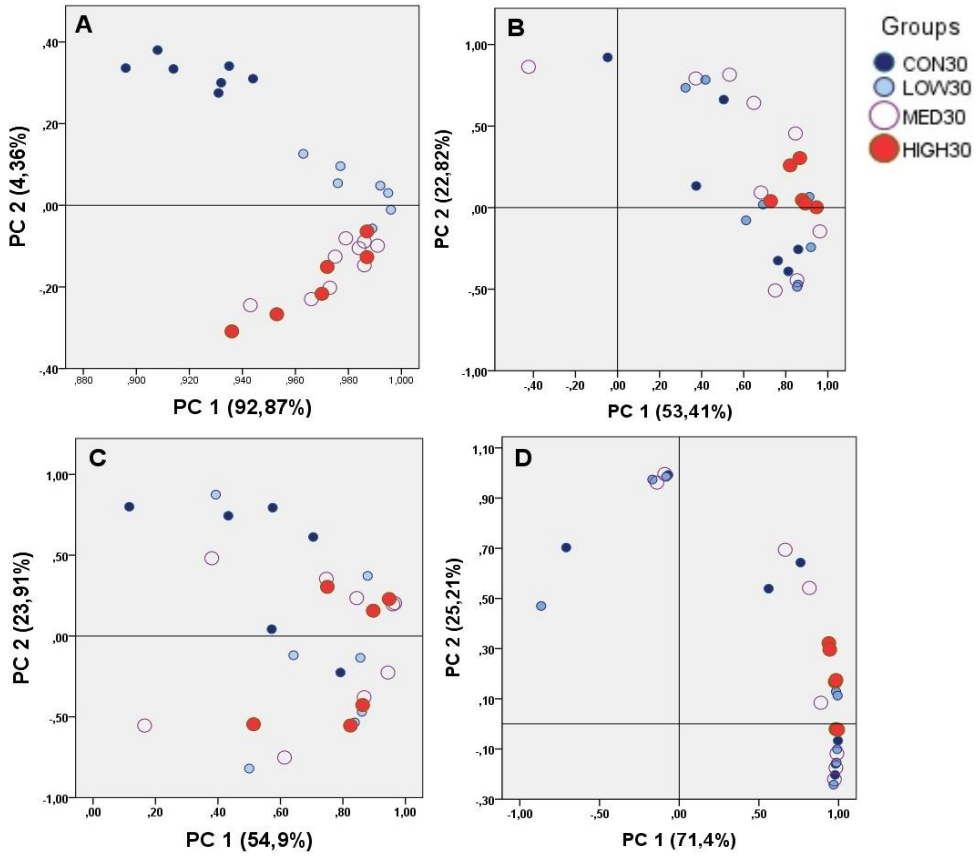

Figure 28. Dataset C: score plots of the individual samples using different combinations of biomarkers, according to the level of organization affected by the exposure. A – PAH metabolites (FF and GC-MS), B – EROD, GST, CAT, general health indices (CI and LSI) and gill and liver histopathology, C – gill and liver histopathology, D – EROD, GST, CAT, CI and LSI (scale: 645 x 746.25, size in points).

Figure 29 shows the loading and score plot using the results of all the biomarkers in the second exposure setup, including the gill and liver histopathology. As shown in the figure, CON30 and LOW30 is separated from MED30 and HIGH30, due to their PAH metabolites data, in both methods. The clumping of CON30 can be attributed to their high values in GST, CAT, LSI and CI (Figure 29). This was also evident in the plot of scores of dataset C in Figure 27. It shows in Figure 29 that the GC-MS method determined the response of the samples for MED30 and HIGH30, causing the exposed groups to separate from the other groups. For the PCA of dataset C, LOW30 is also more related to the other exposed groups than CON30, as was the case just after 7 days of exposure.

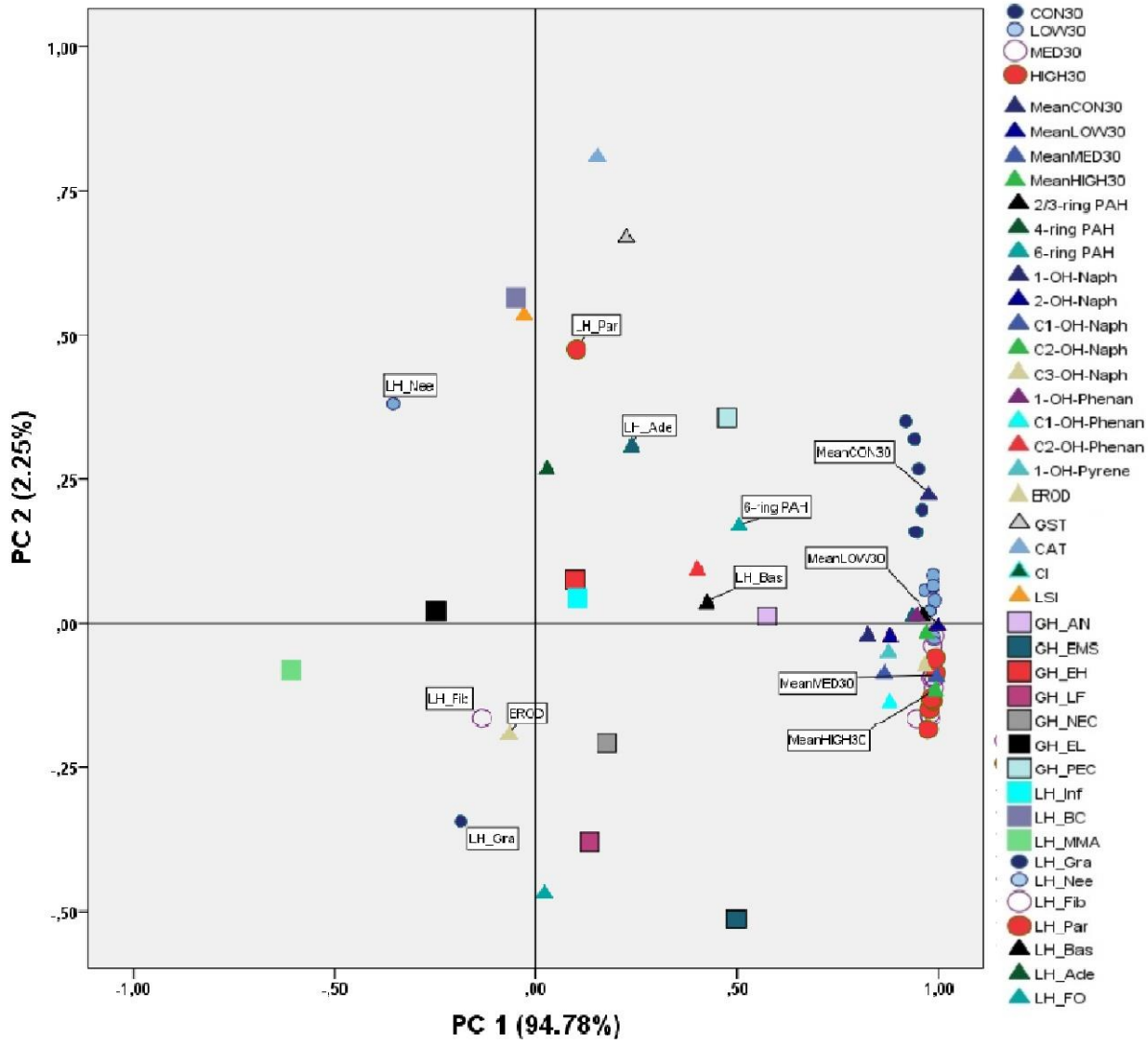

Figure 29. Dataset C: score and loading plot of the first two principal components (PC 1: 95.99%, PC 2: 2.18% of the total variance). Labelled markers are the means of each group and some biomarkers with the same symbol as the different group. LH – liver histopathology and GH – gill histopathology (scale: 645 x 806.25, size in points).

Table 11 shows that HIGH30 has the highest biomarker response with an IBR value of 24.84 and CON30 has the lowest (0.00) using the comparison of all the biomarker sets (IBR A-E). Among the different combinations of biomarkers, CON30 has also the lowest IBR values (1.59, 0.00 and 1.59 for IBR A, B and C, respectively). IBR A and B are the sets using PAH metabolite biomarkers for the calculation of the IBR value and IBR C uses all excluding the PAH biomarker data (EROD, GST, CAT, CI, LSI, gill and liver histopathology). IBR D uses the biomarker data for gill and liver histopathology only, and IBR E used EROD, GST, CAT, CI and LSI data for IBR calculation. In sets not using the PAH metabolite biomarkers in the calculation, the LOW30 has the lowest value (0.55) for IBR D and HIGH30 in IBR E (0.03). The same can be observed with the highest BAI value comparing the different sets where HIGH30 has the highest value (24.84 and 24.16) in sets using the PAH metabolite data, but MED30 has the highest value (3.27, 2.88 and 0.70) for the sets without the biomarker (IBR C, D and E).

Table 11. IBR values for each group in dataset C using results from different suites of biomarkers.

Groups	CON30	LOW30	MED30	HIGH30
IBR A	1.59	2.72	14.02	24.84
IBR B	0.00	0.82	10.88	24.16
IBR C	1.59	1.88	3.57	2.17
IBR D	1.56	1.55	2.88	2.14
IBR E	0.46	0.19	0.70	0.03

The biomarker scores presented in Appendix G were used to make the IBR star plots in Figure 30. Compared to Figures 30A and 30B, where all the PAH metabolite data were used, Figure 30C, 30D and 30E shows a different trend where MED30 has the highest biomarker response among the groups, instead of HIGH30.

As Beliaeff and Burgeot [16] mentioned, the biomarker position in the star plot according to their response to levels of exposure should be able to discriminate between different levels of exposure. In addition to the biomarkers used in the previous studies and datasets in this report, histochemical biomarkers of toxic effects for the 30-day exposure were added to the evaluation. The effects of their inclusion is shown in Figure 30C and 30D. The high IBR values calculated for the HIGH30 in the first two biomarker combinations with PAH metabolite data is most likely related to sensitivity of the biomarker. The results of EROD, GST, CAT, CI and LSI have a very minimal effect in the calculation of IBR values as in Figure 30C and 30D, they are both almost similar despite the different biomarkers. Figure 30E shows the low IBR score for EROD, GST, CAT, CI and LSI of HIGH30.

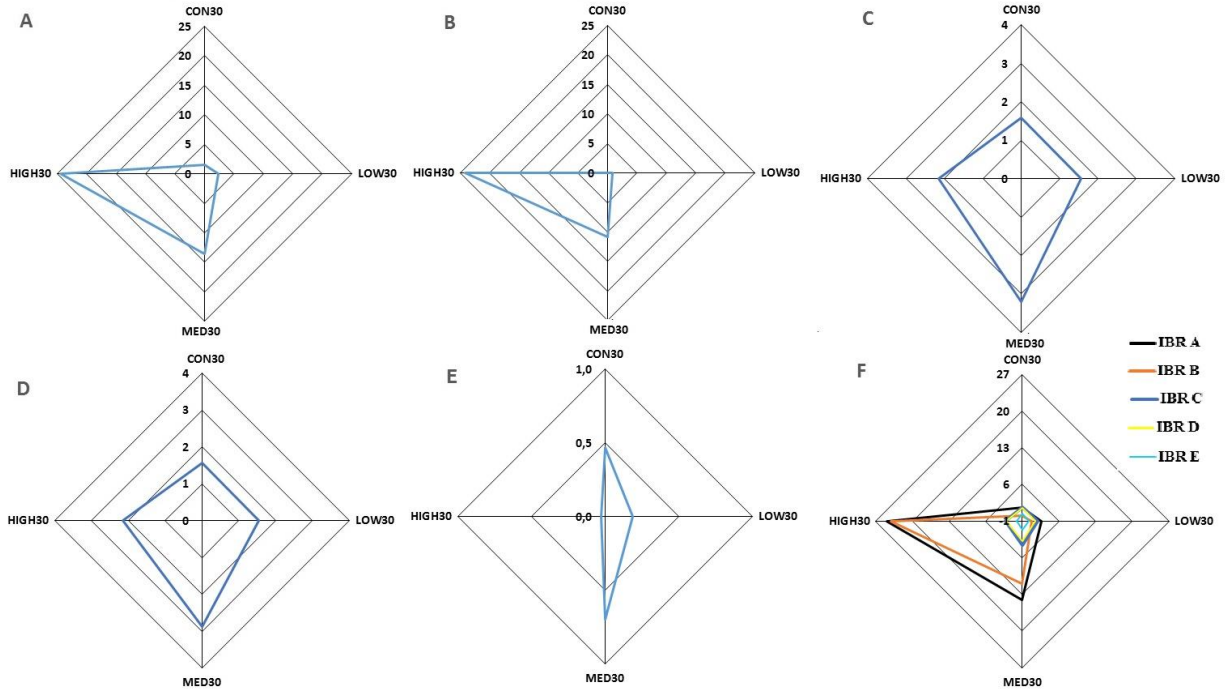

Figure 30. IBR star plots for the different exposure groups using different sets of biomarkers. A – all biomarkers, B – PAH metabolites (FF and GC-MS), C – EROD, GST, CAT, general health index (CI and LSI) and gill and liver histopathology, D – gill and liver histopathology, E – EROD, GST, CAT, CI and LSI, F – A-E for comparison.

As was done in the IBR/n results for datasets A, B and D presented in the previous sections, the influence of a particular biomarker type in the overall response of the individual sample to PAH exposure was calculated and presented graphically in Figure 31. The calculation of IBR/n for dataset C used 7 biomarkers in different combinations. High index values are observed for HIGH30 in IBRn A-C. The IBRn index of the same group decreased considerably in sets without the PAH metabolite biomarker. In IBRn C and D, the index values for all the groups are not obviously differentiated graphically, and does not follow a dose-response variation. IBRn E shows a clear dose-response relationship among the groups.

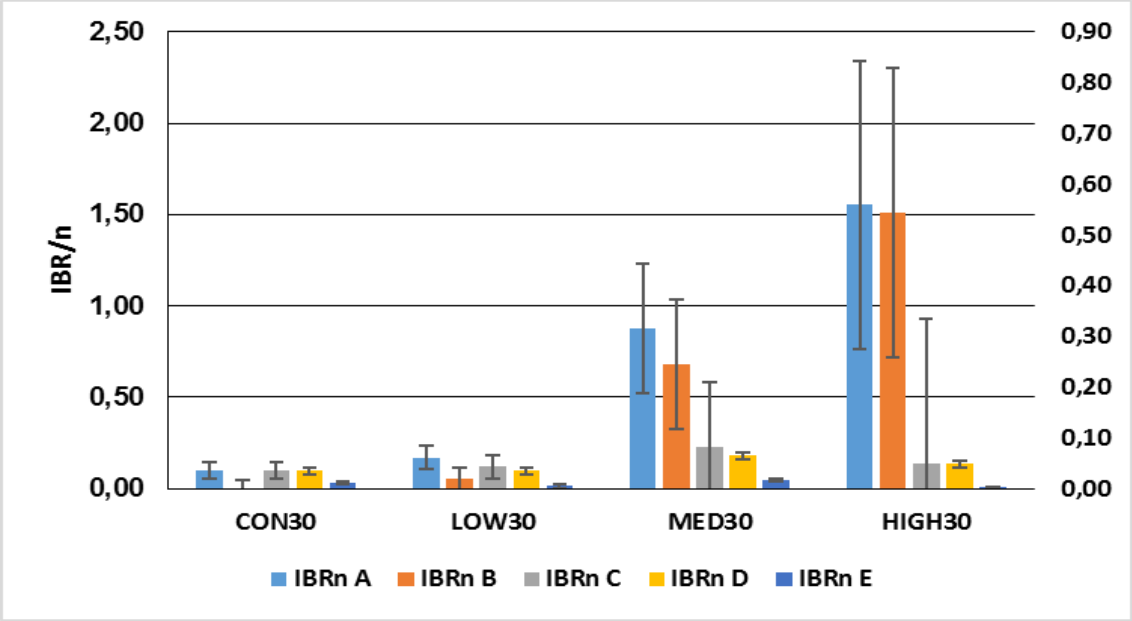

Figure 31. IBR/n for the different sets of biomarkers used for dataset C. Mean and standard deviation for different sets of biomarkers. A – all biomarkers, B – PAH metabolites (FF and GC-MS), C – EROD, GST, CAT, general health index (CI and LSI) and gill and liver histopathology, D – gill and liver histopathology, E – EROD, GST, CAT, CI and LSI.

These star plots are created to compare each exposure group in every set of biomarkers, as was done in the previous datasets. The calculated scores for each biomarker specific for each group in Appendix G was used to make the star plots. Graphically, it is cognizable that for HIGH30 the PAH metabolites data using the GC-MS method has the highest influence in two of the sets with that biomarker analysis in the set (Figure 32A and 32B). When the PAH metabolites data (FF and GC-MS) is not used, the degree of response for the other biomarkers varies in the different groups (Figure 32, C-E). Scores for EMS is markedly high for the HIGH30, Nec and MMA (histopathology) for CON30, EROD and CI for LOW30 in Figure 32D.

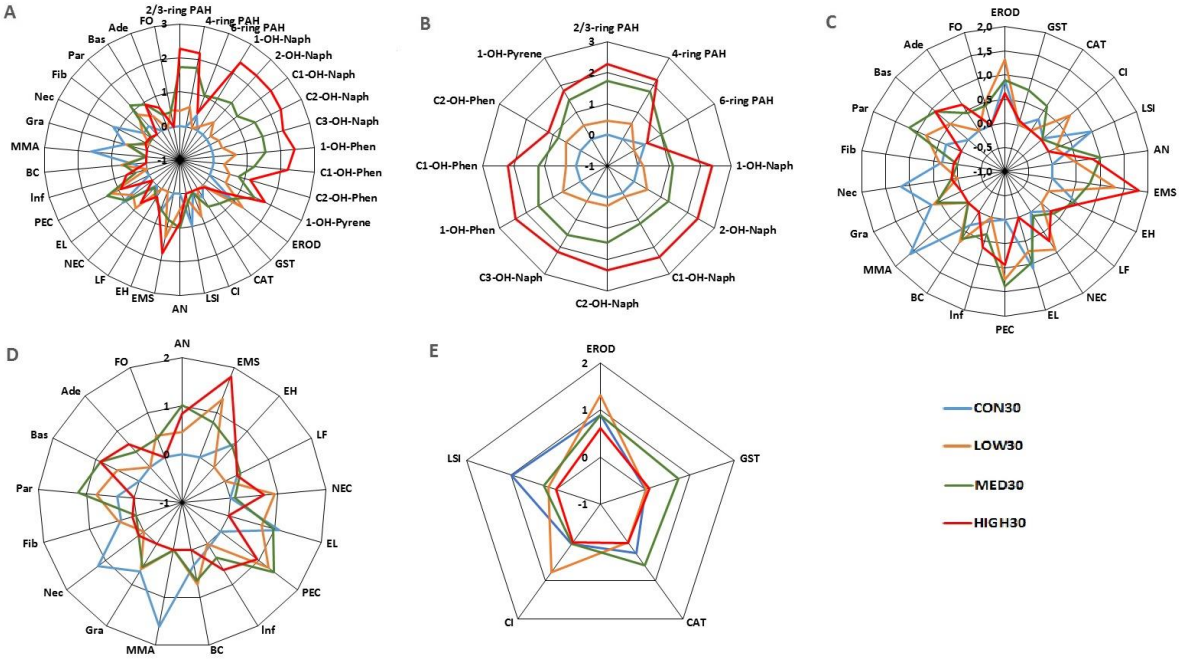

Figure 32. Star plots representing each biomarker in each set for the different groups.

All samples from the four exposure groups displayed mean BAI values in the range of 45-100 (Table 6) which are all higher than the designated scoring based on the previous studies done using the index [15]. Despite that, the average BAI values are very high. The differences in mean BAI values between the groups are evident, exposing the health condition of the samples that were exposed to different concentrations of crude oil.

Between exposed groups, the highest mean BAI value is from the HIGH30 (92.78) indicating the worst condition among the groups according to the BAI value characterized previously [15]. The group with the lowest (63.88) mean BAI value is CON30, with a value that has a small difference from the mean BAI value of LOW30 (64.84). The graphical representation in Figure 33 shows the difference in health condition of the individuals between the control groups and the exposed groups.

There is a clear evidence of a dose-response relationship except for one individual sample in LOW30 which had a lower mean BAI value than the samples from the control group. This is calculated using all biomarkers during the exposure setup (PAH metabolites: FF and GC-MS, EROD, GST, CAT, gill and liver histopathology, and general health indices: CI and LSI). As shown in Figure 33, there is good accordance with the level of exposure in the second exposure, from control to high exposure groups.

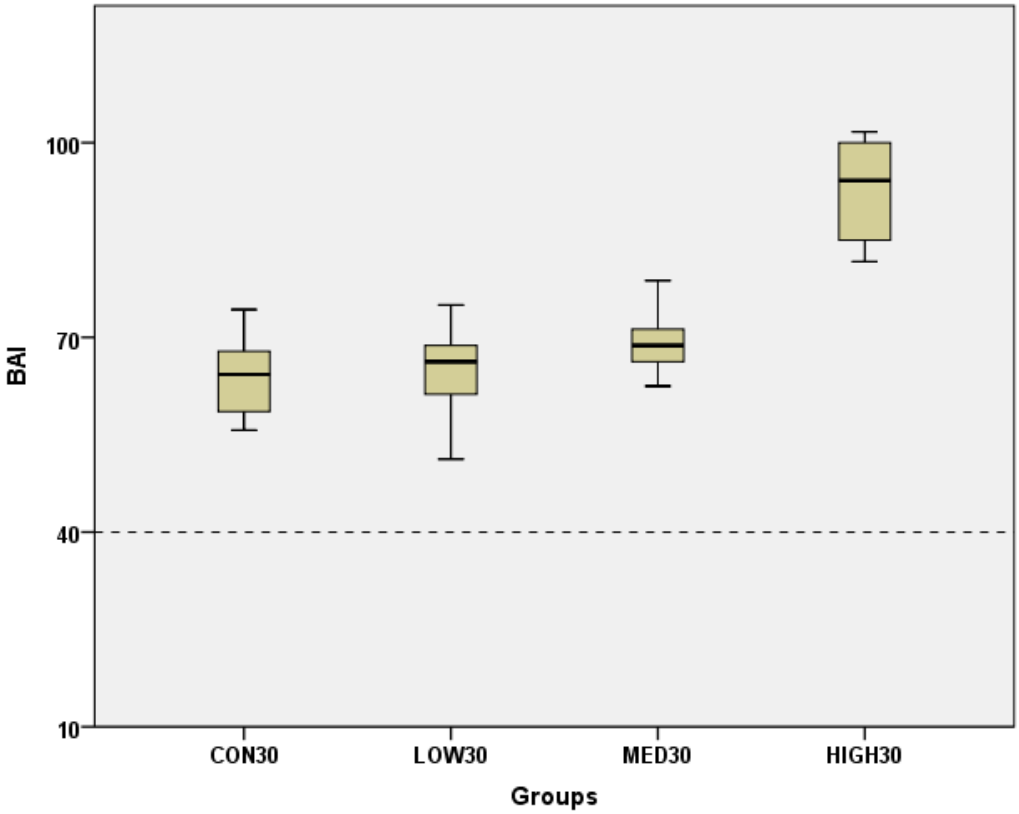

Figure 33. BAI values (25 and 75 percentiles, min and max values, median) of each group in dataset C.

The sets of biomarkers in BAI 1-5 is similar to the sets A-E used in PCA and IBR. Changing the types of biomarkers included in the BAI calculation changes the trend among the exposure groups from one set to another, as observed in BAI 3 and BAI 5 where there is no dose-response relationship in the results. Although the mean BAI values for each individual only decrease or increase relative to the number of biomarkers (Figure 34). HIGH30 still has the highest BAI value in all combinations, except for BAI 3 where MED30 has a higher average BAI value.

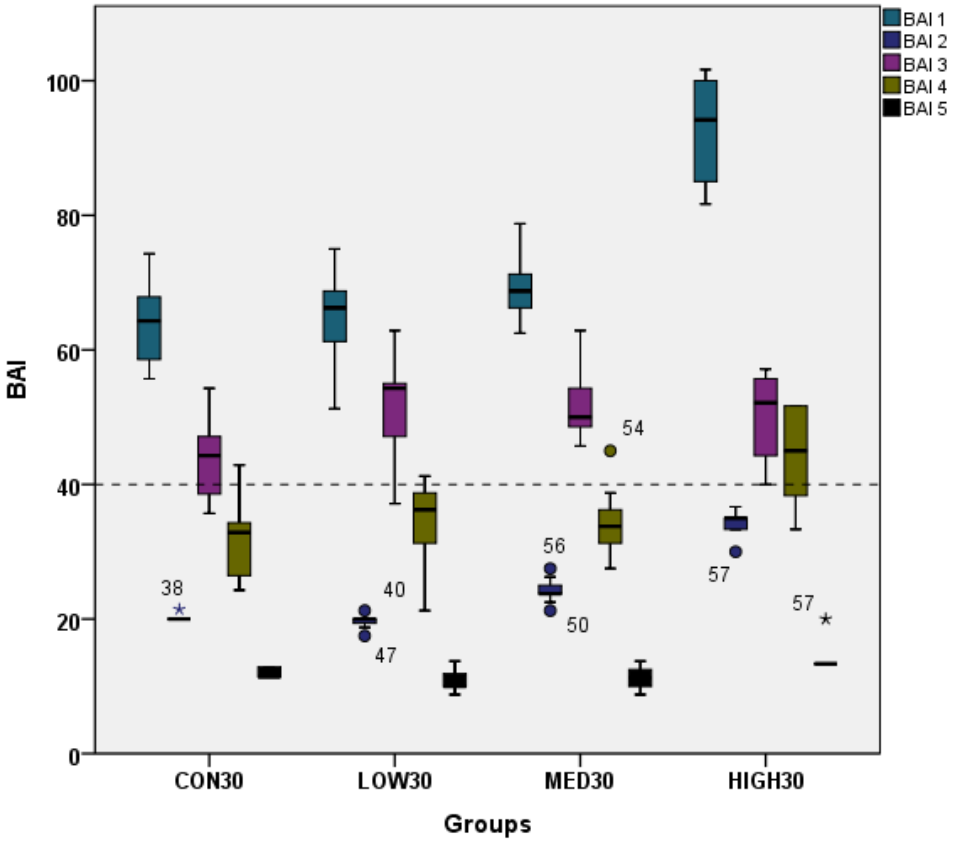

Figure 34. BAI values (25 and 75 percentiles, min and max values, median) of each group in exposure 1, using different combinations of biomarkers. BAI 1 – all biomarkers, BAI 2 – PAH metabolites (FF and GC-MS), BAI 3 – EROD, GST, CAT, general health index (CI and LSI) and gill and liver histopathology, BAI 4 – gill and liver histopathology, BAI 5 – EROD, GST, CAT, CI and LSI. * - extremes, ○ – outliers (numbers beside the symbol indicate the fish number).

Unlike the results in IBR and PCA, changing the number and what type of biomarker to include in the calculation for BAI doesn't change the overall picture. According to Van der Oost et al. [12], the levels of bile metabolites are generally indicative of short-term exposure of a day up to one week providing information on recent exposure only, but results as used in the analysis for dataset C obtained for the 30-day exposure shows that the biomarker still has a strong influence on the overall assessment of the individual health condition of the fish.

4.2 Data communication

It is possible to define the physiological status of the exposure related alterations using a suite of validated biomarkers at different levels of organization on an individual organism. The progression of these alterations were detected by the biomarker response as demonstrated in the indices especially for the results in the second exposure setup. The different biomarker responses

were successfully integrated and combined to compare different datasets. As was already demonstrated in previous studies mentioned in this report, and the representations presented in Chapter 3, the multi-biomarker approach has clearly allowed the attainment of information that cannot be obtained from just measuring the amount of pollutant alone in the tissue (i.e. by measuring body burden). However, in some cases (Section 3.2.1) there is still the need to demonstrate that biomarkers respond in a regular and predictable manner to increasing exposures and in by what means of being exposed to the pollutant. The characterization of the different exposure groups in different exposure setups by means of the multivariate techniques allowed the distinguishing of the prevalence of the parent PAH compound against the synthetic metabolite and between different exposure concentrations. The selected indices have indeed provided a wide array of information about biological effects of PAH in Atlantic cod and may therefore be used as a capable tool in determining the health condition of the organism.

The PCA was also able to distinguish the importance of the different sets of biomarkers (Figures 4, 12, 20 and 28) in extracting the principal components. It shows that careful selection of biomarkers incorporated in an evaluation using the multi-biomarker approach can have an effect in the overall environmental assessment. The health status may not have been emphasized by the PCA because there was no clear distinction between high and low doses using the data from the first exposure, but the biomarker involved in extracting the components are pointed out (Figure 5, 13, 21 and 29). And the clustering of the individual samples in the same group indicates that the index was able to discriminate between the groups using the responses of the individual biomarkers.

IBR has also succeeded in providing the information needed in this study, identifying differences between exposure groups and different biomarker responses, and can in this sense be regarded as a very useful tool. PAH metabolite biomarkers showed to be a very suitable biomarker because it was able to differentiate the exposed groups from the control and the high sensitivity it displayed in the biomarker analysis (Figures 8, 16, 24 and 32). It is also conclusive that BAI can also be applied using biomarkers with reference and critical values that are not previously known, but this may cause the overall resolution to be limited and does not provide information about the mechanisms of biological response. For both BAI and IBR, they showed a good accordance with the level of exposure established for the different groups for the second exposure because it is evident in these indices that the BAI and IBR values increase from control to high dose group. PAH metabolite biomarkers still had a strong influence on differentiating the degrees of damage to the individuals using these indices. In all the PCA, according to the respective biomarker results used to obtain the principal components, only one principal component can be extracted to explain the division of the

groups because the first principal component explains almost always 85% of the total variance, except for the different combinations of biomarkers in the first exposure set up (Figure 4B and 4D), for dataset D (Figure 20B) and all the sets without the use of PAH biomarker data in the 30-day second exposure (Figure 28B, 28C and 28D).

Considering all the results obtained, one of the many advantages of incorporating biomarker responses in a statistical analysis tool is to be able to simplify the information that the biomarkers contain into figures that can easily be interpreted. The simplification of data has the purpose of being able to communicate the results in a much wider field than only to those working closely with environmental ecotoxicology. The following figures are summary accounts of such simplifications to relay various information from the biomarker data.

Figure 35 gives an overview of the biomarkers that were used in the study, according to the level of biological organization affected by the pollutant. The sentinel organism is also presented in the figure, although not the same species.

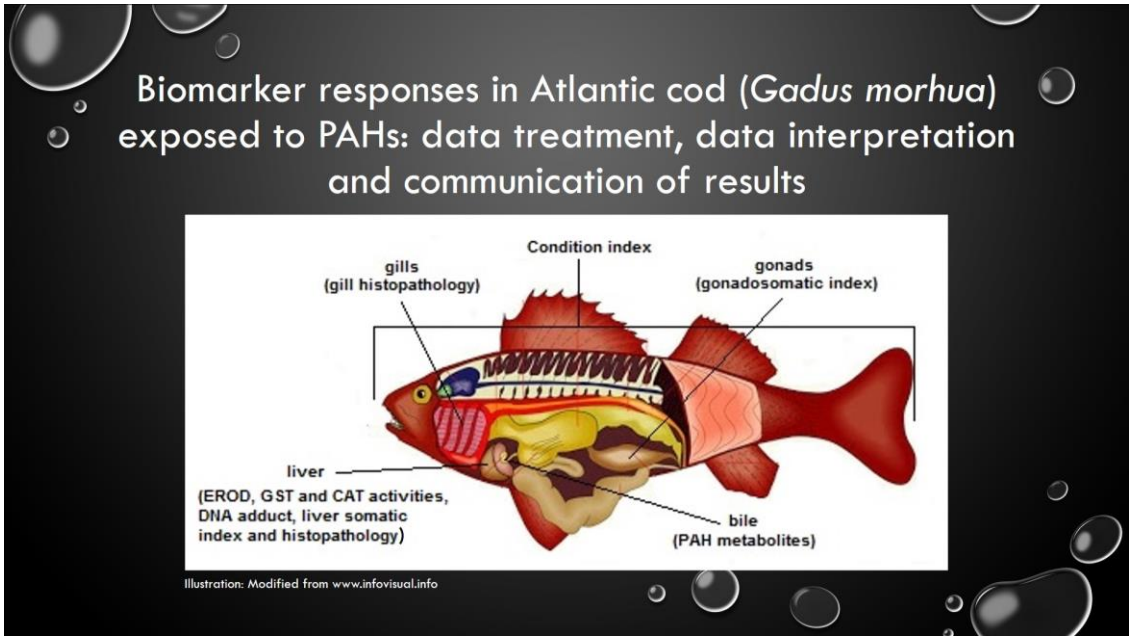

Figure 35. Presentation of the study title and analyzed biomarkers in the sentinel organism.

All the groups in the first exposure are defined in the presentation in Figure 36, along with the graphical representations obtained from the different multivariate statistical analyses used. The emphasis on this presentation is to see how the different statistical tool was able to discriminate between the exposure groups and the biomarkers involved in the differentiation. There is also specific attention turned towards the differentiation between the groups exposed to the parent

compound and their synthetic metabolite counterparts, as well as the dose-response relationship of the different groups in the first exposure.

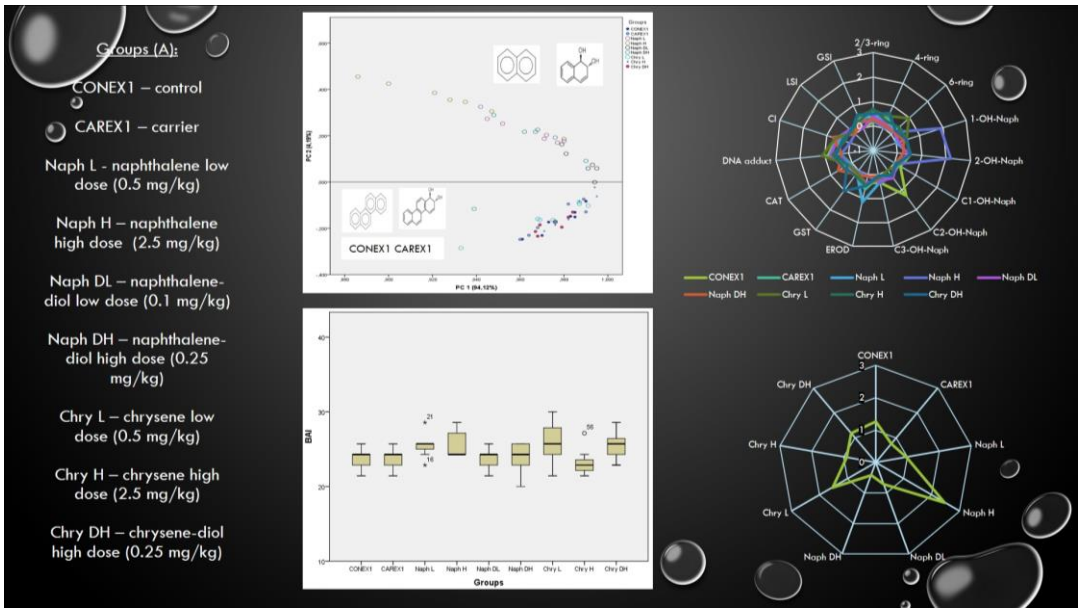

Figure 36. Presentation of results for the first exposure.

Similar to the previous figures in this section, Figure 37 summarizes the interpretations from the indices used to integrate the biomarker responses from the second exposure, with sampling done after 7 days. The different exposure groups are designed to discern between the effects of mixed PAH to fish exposed in different concentrations.

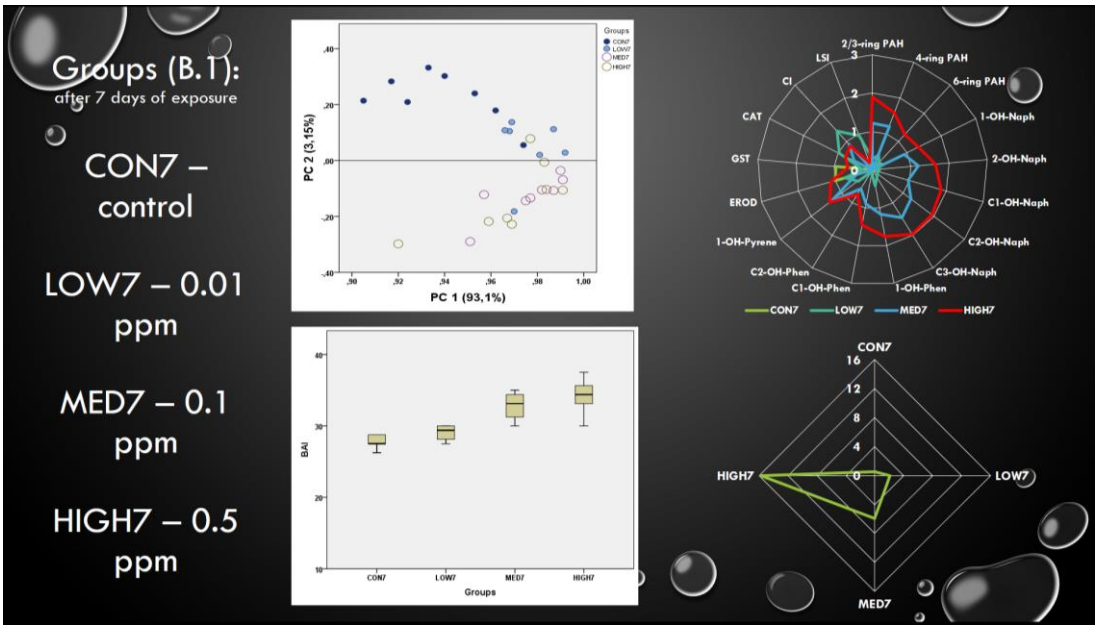

Figure 37. Presentation of results of the biomarker data obtained 7 days after the second exposure.

In addition to the purpose of differentiating between the exposure groups within the specific exposure, the difference in effects of PAH in fish using different exposure methods is also given emphasis in Figure 38. Biomarker data used in the indices are obtained 7 days after exposure, for both methods, as explained thoroughly in Chapter 2.

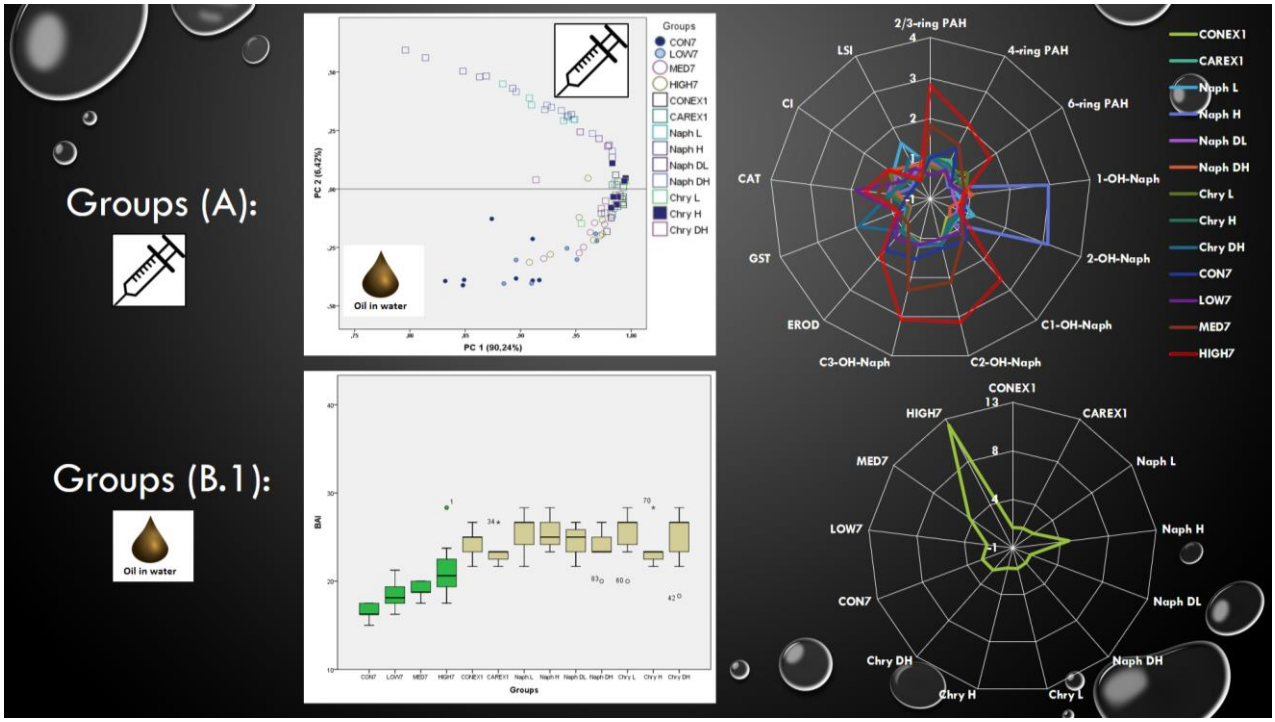

Figure 38. Presentation of results using biomarker data from the first and second exposures.

To obtain the results presented in Figure 39, biomarkers were analyzed at the end of the exposure (30 days) in the second setup. The exposure groups in Figure 39 have the same concentrations as the groups in Figure 37, although there is an additional biomarker used for the biomarker analysis in this case. Gill and liver histopathological data was also incorporated to obtain the results presented in Figure 39.

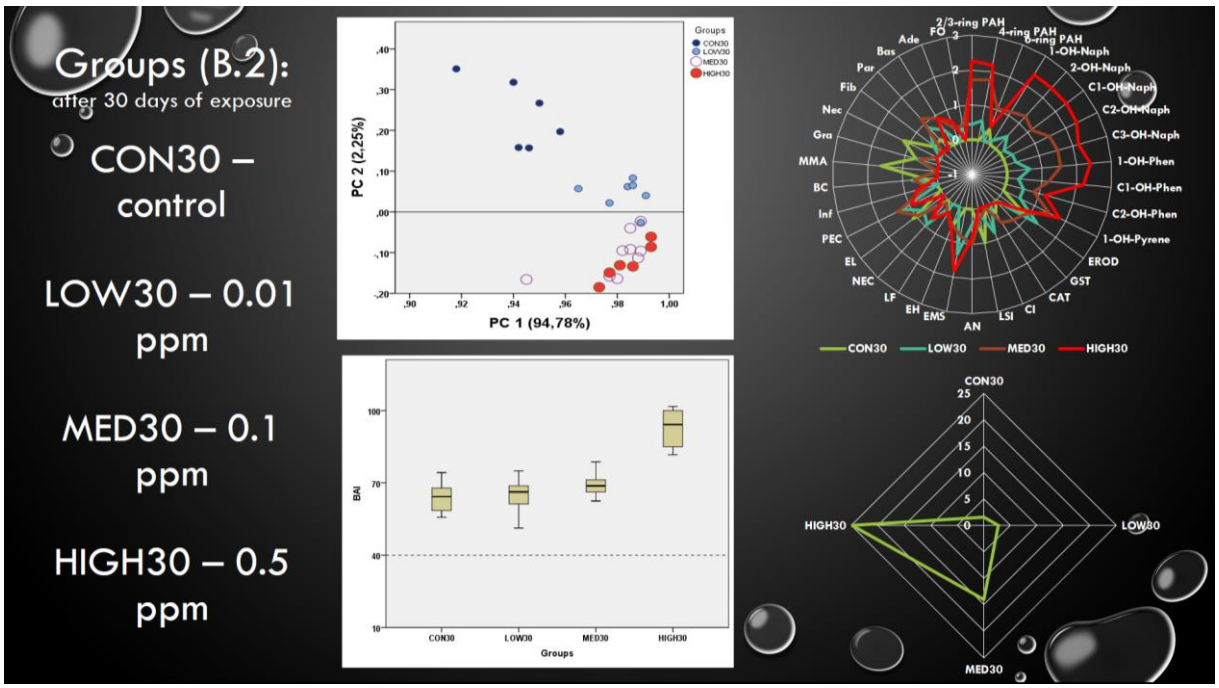

Figure 39. Presentation of results of the biomarker data obtained 30 days after the second exposure.

4. CONCLUSION

Referring to the results presented in the previous chapter, it can be concluded that the integrative indices based on biomarker data were able to indicate the health condition of individual samples and groups despite their difference in graphical representations, resolution and sensitivity. The integration of biomarker data to statistical analysis tools were also successful in simplifying the interpretation of biological effects of PAH in Atlantic cod. The indices were also able to distinguish between different methods of exposure and the degree of damage to the organism in different concentrations of PAH. As was already done in previous index comparisons, all these integrative indices provided a general overview about the degree of biological effects of pollution in marine organisms and may therefore serve as a useful tool in presenting simplified results. In addition, it can also be concluded based on the results that, after all the multivariate analysis were carried out with different pollutant doses and after different exposure times, the most prominent of the reported effects are evidently presented using the second exposure setup, being able to discriminate between groups after 7 days and 30 days of exposure. PCA and IBR analyses were able to acquire information about the biomarkers involved in the differentiation of groups. The graphic representations obtained using IBR and BAI showed that the response of the biomarker is correlated to the degree of exposure especially in the results from the second exposure setup.

7. FURTHER RECOMMENDATION

Future research studies should be done to achieve a proper calibration between the different indices. Selection of biomarkers that should be included in each index should be established, as well as using indices that complement each other (i.e. BAI to obtain information on general stress and IBR for both effect and exposure biomarkers). Baseline and reference values for the different biomarkers specifically for Atlantic cod should also be established to elucidate the mechanism behind the effects on selected fish species. BAI stages have not been established specifically for Atlantic cod, and there is a possibility that there is no benchmark parameter for this purpose yet. Since it is not in the scope of this report to elaborate on this, it will be a good continuation for further research, for parameter reference purposes, baseline establishment or concrete thresholds.

REFERENCES:

1. McCarthy, J.F., and L.R. Shugart, *Biomarkers of Environmental Contamination*. United States: Chelsea, MI (US); Lewis Publishers, 1990. Print.
2. Viarengo, A., et al., *The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms.* Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 2007. **146**(3): p. 281-300.
3. Sundt, R.C., et al., *Biomarker responses in Atlantic cod (Gadus morhua) exposed to produced water from a North Sea oil field: Laboratory and field assessments.* Marine Pollution Bulletin, 2012. **64**(1): p. 144-152.
4. Nahrgang, J., et al., *Seasonal variation in biomarkers in blue mussel (Mytilus edulis), Icelandic scallop (Chlamys islandica) and Atlantic cod (Gadus morhua)-Implications for environmental monitoring in the Barents Sea.* Aquatic Toxicology, 2013. **127**: p. 21-35.
5. Marshall, A.G. and R.P. Rodgers, *Petroleomics: the next grand challenge for chemical analysis.* Accounts of Chemical Research, 2004. **37**(1): p. 53-59.
6. Feng, X., W. Pisula, and K. Müllen, *Large polycyclic aromatic hydrocarbons: synthesis and discotic organization.* Pure and Applied Chemistry, 2009. **81**(12): p. 2203-2224.
7. Pampanin, D.M. and M.O. Sydnes, *Polycyclic aromatic hydrocarbons a constituent of petroleum: presence and influence in the aquatic environment*. 2013. INTECH Open Access Publisher.
8. Varanasi, U. and J.E. Stein, *Disposition of xenobiotic chemicals and metabolites in marine organisms.* Environmental Health Perspectives, 1991. **90**: p. 93.
9. Aas, E., et al., *PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod.* Aquatic Toxicology, 2000. **51**(2): p. 241-258.
10. Directive, Water Framework, *EC of the European Parliament and of the Council of 23.1*. Directive 2000/60, 2000.
11. Peakall, D.B., *The role of biomarkers in environmental assessment (1). Introduction.* Ecotoxicology, 1994. **3**(3): p. 157-160.
12. Van der Oost, R., J. Beyer, and N.P. Vermeulen, *Fish bioaccumulation and biomarkers in environmental risk assessment: a review.* Environmental Toxicology and Pharmacology, 2003. **13**(2): p. 57-149.

13. Huggett, R.J., and R.J. Huggett, *Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress*. (No. Sirsi) i9780873715058. 1992.

14. Schmolke, S., et al., *Multivariate statistical approach to the temporal and spatial patterns of selected bioindicators observed in the North Sea during the years 1995–1997.* Helgoland Marine Research, 1999. **53**(3-4): p. 257-266.

15. Broeg, K., et al., *The "bioeffect assessment index"(BAI): A concept for the quantification of effects of marine pollution by an integrated biomarker approach.* Marine Pollution Bulletin, 2005. **50**(5): p. 495-503.

16. Beliaeff, B. and T. Burgeot, *Integrated biomarker response: A useful tool for ecological risk assessment.* Environmental Toxicology and Chemistry, 2002. **21**(6): p. 1316-1322.

17. Adams, S.M., A.M. Brown, and R.W. Goede, *A quantitative health assessment index for rapid evaluation of fish condition in the field.* Transactions of the American Fisheries Society, 1993. **122**(1): p. 63-73.

18. Bayne, B.L., *The Effects of Stress and Pollution on Marine Animals*. New York: Praeger, 1985. Print.

19. McCarty, L.S. and K.R. Munkittrick, *Environmental biomarkers in aquatic toxicology: Fiction, fantasy, or functional?* Human and Ecological Risk Assessment: An International Journal, 1996. **2**(2): p. 268-274.

20. Beyer, J., *Handbook: Biomarker Interpretation Basis*. RF-Akvamiljø: Stavanger, Norway, 2004. p. 26 + appendix.

21. Hylland, K., et al., *Water column monitoring near oil installations in the North Sea 2001–2004.* Marine Pollution Bulletin, 2008. **56**(3): p. 414-429.

22. Den Besten, P., *Concepts for the implementation of biomarkers in environmental monitoring.* Marine Environmental Research, 1998. **46**(1): p. 253-256.

23. Kim, W.-K., S.-K. Lee, and J. Jung, *Integrated assessment of biomarker responses in common carp (Cyprinus carpio) exposed to perfluorinated organic compounds.* Journal of Hazardous Materials, 2010. **180**(1): p. 395-400.

24. Dupuy, C., et al., *A multibiomarker approach on the Atlantic tomcod (Microgadus tomcod) in the St. Lawrence Estuary.* Environmental Science and Pollution Research, 2013. **20**(2): p. 749-760.

25. Goksøyr, A. and L. Förlin, *The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring.* Aquatic Toxicology, 1992. **22**(4): p. 287-311.

26. Whyte, J.J., et al., *Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure.* Critical Reviews in Toxicology, 2000. **30**(4): p. 347-570.

27. Hylland, K., et al., *Biomarkers in monitoring – a review*. Norwegian Institute for Water Research (NIVA): Oslo, Norway, 2006. p. 106.

28. Lin, E.L., S.M. Cormier, and J.A. Torsella, *Fish biliary polycyclic aromatic hydrocarbon metabolites estimated by fixed-wavelength fluorescence: comparison with HPLC-fluorescent detection.* Ecotoxicology and Environmental Safety, 1996. **35**(1): p. 16-23.

29. Sundt, R.C., et al., *High matrix interference affecting detection of PAH metabolites in bile of Atlantic hagfish (Myxine glutinosa) used for biomonitoring of deep-water oil production.* Marine Environmental Research, 2011. **71**(5): p. 369-374.

30. Jonsson, G., et al., *The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials.* Journal of Environmental Monitoring, 2003. **5**(3): p. 513-520.

31. Amat, A., et al., *DNA adducts in fish following an oil spill exposure.* Environmental Chemistry Letters, 2006. **4**(2): p. 93-99.

32. Camargo, M.M. and C.B. Martinez, *Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream.* Neotropical Ichthyology, 2007. **5**(3): p. 327-336.

33. Webster, L., et al., *A proposal for the assessment criteria to be used for the assessment of monitoring data for the concentrations of hazardous substances in marine sediments and biota in the context of the OSPAR QSR 2010.* FRS Collaborative Report, 2009. **2**(09).

34. Celander, M., et al., *Temporal aspects of induction of hepatic cytochrome P450-1A and conjugating enzymes in the viviparous blenny (Zoarces viviparus) treated with petroleum hydrocarbons.* Aquatic Toxicology, 1994. **29**(3-4): p. 183-196.

35. Davies, I.M., D. Vethaak, and C. International Council for the Exploration of the Sea, *Integrated marine environmental monitoring of chemicals and their effects.* 2012.

36. Broeg, K. and K.K. Lehtonen, *Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach.* Marine Pollution Bulletin, 2006. **53**(8): p. 508-522.

37. Marigómez, I., et al., *Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige oil spill "Mussel Watch".* Ecotoxicology, 2013. **22**(3): p. 486-505.

38. Serafim, A., et al., *Application of an integrated biomarker response index (IBR) to assess temporal variation of environmental quality in two Portuguese aquatic systems.* Ecological Indicators, 2012. **19**: p. 215-225.

39. Abdi, H. and L.J. Williams, *Principal component analysis.* Wiley Interdisciplinary Reviews: Computational Statistics, 2010. **2**(4): p. 433-459.

40. Pampanin, D.M., et al., *Biological effects of Polycyclic Aromatic Hydrocarbons (PAH) and Their First Metabolic Products In In Vivo Exposed Atlantic Cod (Gadus morhua).* Journal of Toxicology and Environmental Health (Part A: Current Issues), Unpublished manuscript, 2016. 10.1080/15287394.2016.1171993: p. 14.

41. Jonsson, G., et al., *Quantitative determination of de-conjugated chrysene metabolites in fish bile by HPLC-fluorescence and GC–MS.* Chemosphere, 2004. **54**(8): p. 1085-1097.

42. Sanni, S., et al., *A continuous flow system (CFS) for chronic exposure of aquatic organisms.* Marine Environmental Research, 1998. **46**(1): p. 97-101.

43. M. Nilsen, B., K. Berg, and A. Goksøyr, *Induction of Cytochrome P4501A (CYP1A) in Fish A Biomarker for Environmental Pollution.* Cytochrome P450 Protocols, 1998: p. 423-438.

44. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Analytical Biochemistry, 1976. **72**(1-2): p. 248-254.

45. Habig, W.H., M.J. Pabst, and W.B. Jakoby, *Glutathione S-transferases the first enzymatic step in mercapturic acid formation.* Journal of Biological Chemistry, 1974. **249**(22): p. 7130-7139.

46. Claiborne, A., *Catalase activity.* CRC Handbook of Methods for Oxygen Radical Research, 1985. **1**: p. 283-284.

47. Jonsson, G., et al., *An evaluation of two fluorescence screening methods for the determination of chrysene metabolites in fish bile.* Chemosphere, 2004. **56**(8): p. 81-90.

48. Reichert, W.L. and B. French, *The ^{32}P-Postlabeling protocols for assaying levels of hydrophobic DNA adducts in fish*. US Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northwest Fisheries Science Center, 1994.

49. Goede, R.W. and B.A. Barton, *Organismic indices and an autopsy-based assessment as indicators of health and condition of fish*. American Fisheries Society Symposium, 1990. **8**(93): p. 108.

50. Nash, R.D., A.H. Valencia, and A.J. Geffen, *The origin of Fulton's condition factor—setting the record straight.* Fisheries, 2006. **31**(5): p. 236-238.

51. Hinton, D.E., et al., *Histopathologic biomarkers.* Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress. Lewis Publ., Boca Raton, Fl, USA, 1992. p. 157-209.

52. Bernet, D., et al., *Histopathology in fish: proposal for a protocol to assess aquatic pollution.* Journal of Fish Diseases, 1999. **22**(1): p. 25-34.

53. Zitko, V., *Principal component analysis in the evaluation of environmental data.* Marine Pollution Bulletin, 1994. **28**(12): p. 718-722.

54. ICES, M., *Report of the working group on biological effects of contaminants (WGBEC).* ICES Ostend, Belgium, 2004: p. 1-86.

55. Beyer, J., et al., *Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: a review.* Environmental Toxicology and Pharmacology, 2010. **30**(3): p. 224-244.

APPENDIX

A	Dataset A: biomarker data from first exposure
B	Dataset B: biomarker data from second exposure (sampling after 7 days)
C	Dataset C: biomarker data from second exposure (sampling after 30 days)
D	Stages of toxically-induced alterations of the biomarkers and corresponding BAI values
E	Standardized values used for PCA calculations (datasets A-C)
F	Score calculations in IBR (datasets A-D)
G	Individual scores for all biomarkers in each group (datasets A-D)

Appendix A

Dataset A: biomarker data from first exposure

Fish #	Group	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
		2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
22	CONEX1	8,20	2,10	0,30	21,82	30,77	452,53	456,63	938,76	0,00	0,41	0,04	0,01	0,80	3,02	0,42
23	CONEX1	9,70	4,60	0,80	15,01	13,51	324,30	200,00	659,80	0,60	1,27	0,03	0,01	0,78	2,27	1,40
24	CONEX1	13,70	6,50	1,40	29,81	27,08	477,16	374,57	830,98	0,18	0,38	0,11	0,01	0,77	1,10	0,33
25	CONEX1	9,50	6,00	0,40	27,77	69,81	702,81	200,00	952,21	0,00	1,01	0,02	0,01	1,00	4,01	12,67
26	CONEX1	3,30	3,70	0,40	16,49	25,82	302,87	200,00	500,00	0,82	0,78	0,09	0,01	0,80	0,87	0,29
27	CONEX1	5,80	4,00	0,20	11,20	9,52	210,58	200,00	500,00	1,50	0,72	0,06	0,01	0,80	1,44	3,59
28	CONEX1	6,90	3,80	0,20	22,47	16,37	219,77	220,22	500,00	0,93	0,80	0,03	0,01	0,94	6,41	0,37
29	CAREX1	3,80	5,30	0,70	20,60	20,45	396,92	200,00	730,51	0,00	0,47	0,07	1,72	0,93	2,42	7,44
30	CAREX1	2,90	2,80	0,00	21,15	40,37	394,76	200,00	500,00	0,64	0,93	0,19	0,01	0,76	1,03	0,40
31	CAREX1	8,70	0,50	0,10	27,13	23,28	224,32	200,00	712,71	0,01	0,73	0,05	0,43	0,82	1,09	0,11
32	CAREX1	6,80	3,80	1,00	26,19	30,28	429,23	200,00	865,14	0,18	0,91	0,03	0,01	0,68	2,79	0,72
33	CAREX1	5,00	1,70	0,30	9,14	22,55	276,61	200,00	500,00	0,36	0,54	0,06	0,43	0,86	5,28	0,54
34	CAREX1	13,50	8,90	1,60	12,19	17,57	304,10	200,00	611,65	0,00	1,02	0,04	0,01	0,83	1,59	0,68
35	CAREX1	10,00	3,20	1,50	11,06	12,85	200,00	200,00	500,00	0,00	0,61	0,05	0,09	0,72	4,99	0,55
50	Naph L	4,80	3,30	0,90	454,64	1249,88	418,14	200,00	818,30	0,60	0,88	0,09	0,82	0,74	0,86	0,33
51	Naph L	12,00	1,80	0,30	146,07	1157,40	437,84	200,00	734,33	0,98	0,61	0,03	0,65	0,77	5,53	0,65
52	Naph L	4,70	2,70	0,00	112,25	277,51	381,35	200,00	528,60	0,64	0,96	0,34	0,01	0,80	0,76	0,27
53	Naph L	15,10	9,30	2,30	128,66	479,98	555,37	200,00	500,00	1,72	0,87	0,04	0,87	0,80	2,84	0,12
54	Naph L	13,40	2,90	0,03	125,66	304,61	200,00	200,00	544,39	1,13	1,07	0,04	0,60	0,82	1,70	0,46
55	Naph L	16,30	5,40	1,50	124,63	260,17	224,33	200,00	523,76	0,63	1,03	0,04	0,01	0,94	3,96	0,24
56	Naph L	8,90	4,70	0,10	141,82	731,72	200,00	200,00	500,00	0,76	1,25	0,02	4,01	0,79	2,87	0,38
43	Naph H	5,70	4,60	1,90	2338,04	3427,63	304,97	200,00	638,21	0,00	1,26	0,14	0,56	0,77	0,91	0,30
44	Naph H	8,70	4,80	1,20	465,82	1710,00	394,62	200,00	610,15	0,15	1,14	0,06	0,63	0,72	1,13	0,35
45	Naph H	2,20	1,90	0,10	1641,44	4042,46	531,62	200,00	745,66	0,00	0,92	0,11	0,36	0,62	0,98	0,30
46	Naph H	6,00	2,60	0,10	869,97	1885,24	434,55	200,00	606,36	0,83	0,85	0,08	0,55	0,82	1,20	0,34
47	Naph H	9,40	5,30	0,60	132,47	239,00	213,42	200,00	500,00	0,18	0,86	0,04	0,50	0,73	1,30	0,05
48	Naph H	6,80	7,20	2,30	319,02	608,30	200,00	200,00	500,00	0,76	0,92	0,07	0,93	0,82	2,31	0,06
49	Naph H	16,80	6,10	0,00	478,79	1135,56	210,64	200,00	519,18	1,00	1,74	0,02	1,04	0,84	2,19	0,40
71	Naph DL	7,70	4,30	0,30	159,52	122,40	781,98	262,70	987,71	0,23	1,36	0,03	0,01	1,04	1,92	14,86
72	Naph DL	14,00	3,60	0,70	337,40	190,30	410,96	227,09	969,58	0,07	1,31	0,03	0,01	0,90	3,69	0,14
73	Naph DL	11,60	4,60	0,00	99,29	115,04	452,06	285,79	713,25	0,00	0,45	0,05	0,01	0,76	2,49	0,66
74	Naph DL	8,50	3,30	1,50	92,79	63,42	200,00	200,00	500,00	0,47	0,47	0,04	0,01	0,86	2,94	3,17
75	Naph DL	11,60	3,00	1,90	20,16	17,14	200,00	200,00	500,00	0,32	0,15	0,03	0,33	0,80	3,31	0,04
76	Naph DL	4,50	3,10	0,50	186,68	64,85	200,00	200,00	500,00	0,98	1,10	0,05	0,12	0,78	2,46	0,09
77	Naph DL	11,30	8,00	0,60	61,28	57,97	269,83	217,47	588,77	0,00	1,20	0,10	0,01	0,78	1,39	0,51

78	Naph DH	8,10	4,40	0,50	154,74	87,95	481,30	200,00	617,09	0,01	1,36	0,11	0,39	0,81	1,08	0,07
79	Naph DH	11,90	5,20	1,30	383,76	269,55	506,15	200,00	1079,39	0,00	0,81	0,13	0,53	0,72	1,32	0,45
80	Naph DH	4,90	6,50	2,20	567,38	707,73	496,46	200,00	747,43	1,14	0,49	0,03	0,01	0,89	2,96	0,63
81	Naph DH	5,20	2,00	1,30	272,50	155,21	200,00	200,00	500,00	0,24	1,08	0,12	0,99	0,77	1,00	0,36
82	Naph DH	11,00	6,30	0,01	103,91	77,17	378,46	200,00	610,56	0,89	0,62	0,21	0,53	0,90	1,07	0,40
83	Naph DH	3,40	2,20	0,02	277,14	184,54	200,00	200,00	500,00	0,32	0,58	0,05	0,01	0,83	1,78	0,26
84	Naph DH	21,40	7,50	0,10	298,49	214,70	200,00	200,00	500,00	0,26	0,38	0,05	0,43	0,82	2,12	0,09
57	Chry L	10,10	9,00	3,70	14,41	41,11	429,88	200,00	669,98	1,47	0,40	0,10	0,58	0,83	0,86	3,60
58	Chry L	9,90	4,70	2,20	24,87	74,83	452,74	218,04	918,39	0,79	0,41	0,04	1,29	0,80	4,81	0,10
59	Chry L	6,20	1,20	0,03	21,67	36,02	328,48	263,89	669,11	0,11	1,82	0,13	0,15	0,79	1,13	0,07
60	Chry L	9,10	5,40	0,10	31,48	30,95	458,13	200,00	678,96	0,16	0,57	-	0,59	0,70	1,16	0,45
61	Chry L	5,60	5,30	2,90	15,05	26,62	215,10	200,00	500,00	0,76	1,12	0,10	2,89	0,79	1,02	0,31
62	Chry L	23,10	9,90	0,60	11,65	12,65	200,00	200,00	729,92	0,00	1,12	0,02	0,31	0,92	9,86	1,72
63	Chry L	13,70	5,40	1,80	16,56	28,60	200,00	200,00	500,00	0,97	1,55	0,08	2,77	0,85	1,65	0,39
64	Chry H	7,60	3,80	0,00	30,30	51,49	449,54	200,00	839,02	0,50	0,85	0,07	0,15	0,79	1,25	0,36
65	Chry H	4,90	4,70	0,30	27,91	50,31	471,95	220,47	623,95	0,77	0,44	0,01	0,01	1,04	5,85	0,08
66	Chry H	3,60	4,40	0,05	28,64	69,54	460,17	200,00	512,69	0,05	0,86	0,10	0,01	0,79	1,20	0,38
67	Chry H	12,20	4,80	1,30	28,33	21,56	481,36	200,00	1006,84	0,00	0,27	0,02	0,01	0,92	5,64	0,66
68	Chry H	4,90	1,90	0,50	15,90	11,86	211,94	200,00	500,00	0,04	0,50	0,06	0,78	0,82	0,75	0,53
69	Chry H	2,50	4,50	0,30	13,07	18,73	200,00	200,00	500,00	0,00	0,49	0,04	0,01	0,79	1,22	0,66
70	Chry H	4,10	4,40	1,30	8,92	21,65	254,97	200,00	500,00	0,29	1,00	0,03	0,01	0,96	3,09	0,40
36	Chry DH	3,80	4,70	1,20	18,55	22,94	329,29	200,00	601,48	0,22	0,46	0,09	0,89	0,82	1,03	0,41
37	Chry DH	7,40	6,10	1,00	16,47	21,43	339,82	200,00	731,31	0,36	1,29	0,08	1,16	0,96	1,82	-
38	Chry DH	5,10	4,80	0,60	27,63	33,73	476,95	200,00	677,08	0,69	21,72	0,10	0,49	0,82	0,95	0,37
39	Chry DH	7,40	5,30	0,70	19,91	52,27	561,77	200,00	971,11	0,14	1,20	0,02	1,19	0,93	5,00	0,36
40	Chry DH	16,10	11,50	2,60	19,79	22,22	229,04	200,00	579,36	0,07	0,69	0,03	0,85	0,95	2,27	0,06
41	Chry DH	5,30	6,60	0,60	12,43	12,33	200,00	200,00	500,00	0,16	1,35	0,07	0,69	0,79	1,79	0,33
42	Chry DH	1,50	3,60	0,20	10,26	11,20	213,62	200,00	500,00	0,00	0,54	0,03	0,38	0,76	0,91	5,70

Appendix B

Dataset B: biomarker data from second exposure (sampling after 7 days)

Fish #	Group	FF			GC-MS									EROD	GST	CAT	CI	LSI
		2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
2	CON7	0,40	0,27	0,09	0,67	1,12	2,35	1,92	2,00	1,23	2,33	2,47	1,96	6,22	0,01	0,07	0,25	0,29
3	CON7	0,69	0,42	0,11	0,54	1,05	2,26	2,06	2,16	1,99	2,33	2,25	2,02	27,29	0,02	0,02	0,24	0,26
4	CON7	0,82	0,48	0,17	1,89	0,47	2,31	2,09	2,05	1,89	2,35	2,48	2,09	4,99	0,05	0,04	0,24	0,31
5	CON7	0,88	0,52	0,19	0,93	0,64	2,47	1,82	2,22	1,84	1,92	2,11	1,95	18,64	0,02	0,11	0,18	0,41
6	CON7	0,90	0,62	0,19	0,50	0,68	2,05	1,97	2,08	1,81	1,57	1,51	1,85	2,84	0,01	0,06	0,26	0,28
7	CON7	0,96	0,64	0,28	0,87	0,78	2,03	1,82	2,10	1,17	2,26	1,26	2,05	20,89	0,01	0,09	0,26	0,33
8	CON7	1,00	0,65	0,38	0,61	0,68	1,98	1,80	2,36	1,99	2,37	2,06	2,00	4,17	0,02	0,06	0,26	0,27
9	CON7	1,15	0,71	0,44	0,70	0,81	2,26	1,77	2,13	1,83	2,32	2,27	2,10	6,81	0,02	0,04	0,26	0,25
10	LOW7	1,00	0,21	0,01	0,90	1,27	2,29	2,64	2,64	2,10	2,21	2,04	2,00	20,81	0,02	0,23	0,27	0,42
11	LOW7	1,02	0,65	0,06	0,93	0,87	2,26	2,37	2,59	2,05	2,34	1,72	2,04	3,56	0,01	0,06	0,28	0,25
12	LOW7	1,12	0,75	0,12	0,67	1,11	2,12	2,49	2,78	2,06	2,64	1,66	2,26	17,56	0,03	0,27	0,28	0,61
13	LOW7	1,17	0,76	0,17	1,03	1,50	2,54	2,55	2,77	1,90	2,53	2,36	2,16	14,46	0,00	0,10	0,25	0,26
14	LOW7	1,23	0,80	0,21	1,06	1,01	2,36	2,46	2,71	1,93	2,42	2,53	2,25	1,10	0,00	0,06	0,26	0,23
15	LOW7	1,25	0,82	0,33	0,97	1,15	2,54	2,72	2,97	2,10	2,27	1,52	2,25	0,00	0,00	0,06	0,27	0,29
16	LOW7	1,29	0,85	0,34	0,87	0,79	2,42	2,54	2,97	2,13	1,88	2,55	2,32	7,93	0,02	0,21	0,27	0,59
17	LOW7	1,41	0,87	0,54	-	-	-	-	-	-	-	-	-	1,68	0,00	0,11	0,28	0,36
18	MED7	1,36	0,65	0,01	1,41	1,91	2,87	3,50	3,50	2,03	2,78	2,62	2,32	0,55	0,00	0,02	0,28	-
19	MED7	1,41	0,75	0,01	1,20	1,55	2,70	3,24	3,49	2,13	2,60	1,70	2,32	8,21	0,00	0,16	0,26	0,28
20	MED7	1,50	0,84	0,01	1,44	1,86	2,76	3,38	3,39	2,32	2,78	2,62	2,43	4,87	0,00	0,24	0,27	0,23
21	MED7	1,50	0,86	0,07	1,14	1,37	2,61	3,20	3,34	2,11	2,18	1,66	2,38	5,59	0,01	0,12	0,23	0,23
22	MED7	1,56	0,86	0,16	1,62	1,77	2,76	3,35	3,54	2,33	2,91	2,50	2,34	12,72	0,01	0,03	0,28	0,26
23	MED7	1,60	0,97	0,21	1,66	2,07	2,95	3,58	3,85	2,75	3,36	2,34	2,80	4,77	0,01	0,11	0,28	0,50
24	MED7	1,63	1,04	0,25	1,29	1,47	2,77	3,17	3,18	2,14	2,48	1,43	2,11	0,00	0,01	0,14	0,26	0,22
25	MED7	2,03	1,36	0,29	1,11	1,22	2,72	3,26	3,43	2,01	2,27	2,81	2,28	1,58	0,02	0,01	0,24	0,20
26	HIGH7	1,54	0,76	0,18	1,53	1,76	3,10	3,56	3,74	2,40	3,10	1,92	2,61	7,29	0,02	0,18	0,27	0,40
27	HIGH7	1,57	0,90	0,25	1,33	1,52	2,75	3,23	3,34	2,17	2,59	1,38	2,26	2,80	0,00	0,10	0,27	0,24
28	HIGH7	1,71	0,91	0,27	1,37	1,63	2,86	3,46	3,67	2,53	3,12	2,20	2,42	4,46	0,01	0,05	0,26	0,28
29	HIGH7	1,73	1,00	0,36	1,80	2,24	3,24	3,76	3,60	2,56	2,93	2,83	2,15	-0,85	0,01	0,06	0,26	0,40
30	HIGH7	1,73	1,02	0,41	1,38	1,68	2,76	3,31	3,54	2,40	2,84	2,83	2,38	50,61	0,00	0,01	0,25	0,26
31	HIGH7	1,78	1,03	0,57	1,51	1,76	2,84	3,44	3,64	2,44	2,96	2,40	2,37	37,86	0,04	0,30	0,27	0,36
32	HIGH7	2,00	1,23	1,00	1,37	1,75	2,72	3,29	3,39	2,28	2,57	2,61	2,35	0,00	0,01	0,16	0,26	0,26
1	HIGH7	2,01	1,29	-	1,64	2,03	3,29	3,84	3,86	2,51	3,32	2,10	2,65	1,14	0,02	0,12	0,24	0,29

Appendix C

Dataset C: biomarker data from second exposure (sampling after 30 days)

Fish #	Group	FF			GC-MS									EROD	GST	CAT	CI	LSI
		2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
33	CON30	1,91	1,18	0,34	8,64	5,63	273,31	68,00	58,00	22,51	110,00	175,00	57,36	16,18	0,00	0,10	0,74	0,90
34	CON30	2,10	1,68	0,38	9,12	1,81	227,32	56,00	84,00	35,60	202,96	78,00	37,21	0,58	0,02	0,13	0,71	0,93
35	CON30	2,39	1,91	0,44	5,96	1,72	209,93	94,00	93,00	48,57	84,00	157,00	77,11	-0,62	0,02	0,09	0,82	0,91
36	CON30	2,45	2,12	0,53	2,57	1,19	235,41	54,00	90,00	29,00	98,00	157,00	43,23	5,64	0,01	0,10	0,87	0,74
37	CON30	3,83	2,44	0,80	1,02	6,55	323,91	48,00	91,00	74,18	33,00	163,00	47,59	0,00	0,02	0,11	0,72	0,76
38	CON30	3,84	2,59	1,22	9,74	1,75	189,00	106,00	101,00	63,14	150,00	120,00	134,06	7,43	0,07	0,59	0,83	4,62
39	CON30	6,65	3,56	2,24	3,59	2,05	241,74	55,00	61,00	61,49	99,00	101,00	82,05	0,78	0,06	0,79	0,83	8,30
40	LOW30	10,77	2,84	0,01	10,93	15,20	320,61	752,74	1547,02	162,97	201,02	665,05	282,41	5,40	0,05	0,08	0,78	1,34
41	LOW30	11,90	3,11	0,18	5,38	8,65	288,09	557,12	461,00	70,40	279,31	242,00	71,35	9,62	0,02	0,14	0,81	0,34
42	LOW30	12,55	3,37	0,37	11,18	11,05	375,28	692,12	941,42	87,84	265,61	233,00	94,78	0,00	0,04	0,11	0,86	0,67
43	LOW30	13,34	5,16	0,43	8,05	16,33	279,70	403,82	526,98	88,27	372,52	259,00	137,06	2,08	0,02	0,06	0,81	0,96
44	LOW30	17,05	5,37	0,57	12,98	19,93	325,19	649,07	557,99	145,36	163,00	136,00	110,39	39,01	0,02	0,52	0,89	0,90
45	LOW30	23,76	6,17	0,93	3,93	8,66	254,36	193,00	435,00	82,94	329,20	259,00	87,49	3,69	0,01	0,03	0,76	3,38
46	LOW30	28,10	8,02	0,95	4,00	4,95	219,20	195,00	176,00	86,68	181,00	221,00	55,01	2,02	0,04	0,24	0,79	0,40
47	LOW30	32,98	8,69	1,06	-	-	-	-	-	-	-	-	-	0,00	0,02	0,37	1,04	1,11
48	MED30	37,66	8,82	0,25	29,41	56,75	462,37	1652,61	1829,81	162,31	372,22	472,00	178,33	-0,90	0,06	0,95	0,75	2,31
49	MED30	47,50	9,07	0,39	14,53	28,21	671,56	2267,47	2720,77	197,47	522,60	516,74	189,30	10,25	0,05	0,49	0,85	0,46
50	MED30	48,94	9,10	0,50	23,58	29,97	905,70	2440,19	2510,94	138,26	353,00	141,00	167,50	7,49	0,01	0,05	0,82	2,54
51	MED30	57,77	11,50	0,57	22,39	20,78	565,78	2118,32	4067,86	244,09	863,37	335,00	178,84	18,26	0,02	0,10	0,69	0,47
52	MED30	65,69	11,68	1,27	21,13	21,52	525,12	1923,65	3216,94	185,16	817,15	111,00	147,39	0,00	0,09	0,09	0,74	0,94
53	MED30	68,88	11,71	1,73	21,04	18,85	755,07	3115,07	4071,66	138,80	1089,79	60,00	381,87	0,84	0,03	0,06	0,85	1,34
54	MED30	71,82	13,60	1,92	15,42	9,08	448,87	1384,70	2220,81	142,97	412,58	346,00	242,87	0,00	0,01	0,16	0,74	0,54
55	MED30	78,15	13,96	1,95	19,48	19,80	704,24	2147,41	1894,65	156,31	513,08	539,61	145,27	1,86	0,13	0,91	0,83	2,12
56	MED30	89,83	16,99	3,18	20,01	32,75	710,63	3362,97	4552,81	253,66	880,37	809,10	274,28	0,64	0,05	0,43	0,83	1,10
57	HIGH30	54,33	8,73	0,20	24,35	39,72	722,78	2197,98	2261,54	209,07	573,78	71,00	121,96	3,58	0,06	0,63	0,89	-
58	HIGH30	59,47	10,87	0,58	28,84	60,11	1147,04	3711,67	4533,14	243,87	1029,85	664,03	238,17	1,83	0,03	0,09	0,80	0,95
59	HIGH30	79,26	13,42	0,90	48,09	63,41	1025,08	4793,91	6441,96	328,63	1098,37	75,00	318,00	3,12	0,01	0,05	0,68	0,78
60	HIGH30	80,91	14,54	0,99	34,56	45,08	1135,91	5051,96	5675,20	316,29	1872,11	179,00	378,53	1,24	0,04	0,12	0,81	0,86
61	HIGH30	84,83	14,80	1,09	37,66	29,80	899,12	3233,12	3921,43	257,26	1332,52	1351,72	251,42	1,60	0,04	0,23	0,76	0,85
62	HIGH30	131,32	22,71	1,66	52,21	41,49	1535,32	2539,76	2304,19	160,00	607,35	467,00	163,08	1,30	0,01	0,06	0,78	0,91

Fish #	Group	Gill histopatholgy							Liver histopathology									
		AN	EMS	EH	LF	NEC	EL	PEC	Inf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
33	CON30	0	0	2	1	0	4	1	1	0	1	1	1	0	0	0	0	0
34	CON30	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0
35	CON30	0	0	2	1	0	2	1	0	0	1	0	1	0	0	0	0	0
36	CON30	1	1	3	2	2	1	1	0	0	0	3	1	2	1	0	0	0
37	CON30	0	0	4	0	0	0	1	0	0	0	0	0	0	0	0	0	0
38	CON30	1	0	2	1	1	3	1	0	1	0	0	2	0	0	0	0	0
39	CON30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	LOW30	2	1	2	1	3	0	2	0	0	0	2	0	0	0	0	0	0
41	LOW30	0	1	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0
42	LOW30	0	2	1	1	0	2	3	0	1	0	0	0	0	1	0	0	0
43	LOW30	0	2	1	2	1	3	0	0	0	0	3	0	0	0	0	0	2
44	LOW30	3	2	3	0	4	1	4	0	0	0	0	1	0	1	2	0	0
45	LOW30	0	1	2	0	2	3	2	0	2	0	0	0	0	0	0	0	0
46	LOW30	2	1	1	1	1	0	4	1	0	0	0	0	3	1	0	0	0
47	LOW30	2	1	2	0	2	1	3	0	0	0	0	1	0	0	0	0	0
48	MED30	0	0	1	1	0	3	2	0	0	0	0	1	0	1	0	1	0
49	MED30	0	0	1	0	0	1	3	0	1	0	1	2	0	1	0	0	0
50	MED30	1	2	2	0	0	1	4	2	1	0	1	0	0	0	0	0	0
51	MED30	2	1	1	1	1	2	2	0	0	0	0	0	0	0	0	0	2
52	MED30	1	0	4	1	1	2	2	0	0	0	0	0	0	0	0	0	0
53	MED30	3	1	2	1	0	1	1	0	0	0	3	0	0	0	2	0	0
54	MED30	2	3	3	1	2	4	2	0	0	0	0	0	1	1	2	0	0
55	MED30	4	0	2	0	1	0	3	0	1	0	0	0	0	1	1	0	0
56	MED30	4	1	4	3	2	1	4	1	0	0	0	0	0	1	1	0	0
57	HIGH30	0	1	4	0	1	0	3	2	0	0	0	0	0	0	2	0	0
58	HIGH30	1	1	1	1	4	1	1	0	0	0	0	0	0	0	0	0	0
59	HIGH30	3	2	2	1	2	0	4	0	0	0	0	0	0	0	1	0	0
60	HIGH30	4	2	2	0	0	1	1	1	0	0	0	1	0	0	1	1	0
61	HIGH30	2	3	4	2	2	1	1	0	0	0	0	0	0	0	0	0	0
62	HIGH30	0	2	1	1	0	0	2	0	0	0	0	1	1	0	0	0	0

Appendix D

Stages of toxically-induced alterations of the biomarkers and corresponding BAI values

Biomarkers		Stage 1	Stage 2	Stage 3	Stage 4
FF (PFE µg/ml)	**2/3-ring**	< 3,9	< 29,25	< 92,43	> 92,43
	Numerical value BAI	10	20	30	40
	4-ring	< 3,9	< 29,25	< 92,43	> 92,43
	Numerical value BAI	10	20	30	40
	6-ring	< 3,9	< 29,25	< 92,43	> 92,43
	Numerical value BAI	10	20	30	40
GC-MS (ng/g)	**1-OH-Naph**	< 2549	< 11725	< 34156	> 34156
	Numerical value BAI	10	20	30	40
	2-OH-Naph	< 2549	< 11725	< 34156	> 34156
	Numerical value BAI	10	20	30	40
	C1-OH-Naph	< 2549	< 11725	< 34156	> 34156
	Numerical value BAI	10	20	30	40
	C2-OH-Naph	< 2549	< 11725	< 34156	> 34156
	Numerical value BAI	10	20	30	40
	C3-OH-Naph	< 2549	< 11725	< 34156	> 34156
	Numerical value BAI	10	20	30	40
	1-OH-Phenan	< 691	< 5320,7	< 15 823,9	> 15 823,9
	Numerical value BAI	10	20	30	40
	C1-OH-Phenan	< 691	< 5320,7	< 15 823,9	> 15 823,9
	Numerical value BAI	10	20	30	40
	C2-OH-Phenan	< 691	< 5320,7	< 15 823,9	> 15 823,9
	Numerical value BAI	10	20	30	40
	1-OH-Pyrene	< 27	< 197,7	< 437,4	> 437,4
	Numerical value BAI	10	20	30	40
EROD activity		<=9,2	< 145	< 151	> 151
	Numerical value BAI	10	20	30	40
GST activity		< ,645	< ,950	< 1,5	> 1,5
	Numerical value BAI	10	20	30	40
CAT activity		<= 0,03	< 0,34	< 0,95	> 0,95
	Numerical value BAI	10	20	30	40
DNA adduct		0	< 1,6	< 6	> 6
	Numerical value BAI	10	20	30	40
CI		> 1,5	1,5 - 0,51	0,51 - 0,1	< 0,1
	Numerical value BAI	10	20	30	40
LSI		0 - 2,5	2,5 - 0,5	0,5 - 0,05	< 0,05
	Numerical value BAI	10	20	30	40
GSI		> 0,25	> 0,05	> 0,01	< 0,01
	Numerical value BAI	10	20	30	40

Gill histopathology	AN	1	2	3	4
	Numerical value BAI	10	20	30	40
	EMS	1	2	3	4
	Numerical value BAI	10	20	30	40
	EH	1	2	3	4
	Numerical value BAI	10	20	30	40
	LF	1	2	3	4
	Numerical value BAI	10	20	30	40
	NEC	1	2	3	4
	Numerical value BAI	10	20	30	40
	EL	1	2	3	4
	Numerical value BAI	10	20	30	40
	PEC	1	2	3	4
	Numerical value BAI	10	20	30	40
Liver histopathology	lnf	1	2	3	4
	Numerical value BAI	10	20	30	40
	BC	1	2	3	4
	Numerical value BAI	10	20	30	40
	MMA	1	2	3	4
	Numerical value BAI	10	20	30	40
	Gra	1	2	3	4
	Numerical value BAI	10	20	30	40
	Nee	1	2	3	4
	Numerical value BAI	10	20	30	40
	Fib	1	2	3	4
	Numerical value BAI	10	20	30	40
	Par	1	2	3	4
	Numerical value BAI	10	20	30	40
	Bas	1	2	3	4
	Numerical value BAI	10	20	30	40
	Ade	1	2	3	4
	Numerical value BAI	10	20	30	40
	FO	1	2	3	4
	Numerical value BAI	10	20	30	40

Appendix E

Standardized values used for PCA calculations

Dataset A

Fish #	Group	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
		2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
22	CONEX1	0,96	0,49	0,11	1,36	1,50	2,66	2,66	2,97	0,00	0,15	0,02	0,00	0,26	0,60	0,15
23	CONEX1	1,03	0,75	0,26	1,20	1,16	2,51	2,30	2,82	0,20	0,36	0,01	0,00	0,25	0,51	0,38
24	CONEX1	1,17	0,88	0,38	1,49	1,45	2,68	2,57	2,92	0,07	0,14	0,04	0,00	0,25	0,32	0,12
25	CONEX1	1,02	0,85	0,15	1,46	1,85	2,85	2,30	2,98	0,00	0,30	0,01	0,00	0,30	0,70	1,14
26	CONEX1	0,63	0,67	0,15	1,24	1,43	2,48	2,30	2,70	0,26	0,25	0,04	0,00	0,26	0,27	0,11
27	CONEX1	0,83	0,70	0,08	1,09	1,02	2,33	2,30	2,70	0,40	0,24	0,03	0,00	0,25	0,39	0,66
28	CONEX1	0,90	0,68	0,08	1,37	1,24	2,34	2,34	2,70	0,29	0,26	0,01	0,00	0,29	0,87	0,14
29	CAREX1	0,68	0,80	0,23	1,33	1,33	2,60	2,30	2,86	0,00	0,17	0,03	0,43	0,29	0,53	0,93
30	CAREX1	0,59	0,58	0,00	1,35	1,62	2,60	2,30	2,70	0,22	0,28	0,08	0,00	0,24	0,31	0,15
31	CAREX1	0,99	0,18	0,04	1,45	1,39	2,35	2,30	2,85	0,00	0,24	0,02	0,16	0,26	0,32	0,05
32	CAREX1	0,89	0,68	0,30	1,43	1,50	2,63	2,30	2,94	0,07	0,28	0,01	0,00	0,23	0,58	0,23
33	CAREX1	0,78	0,43	0,11	1,01	1,37	2,44	2,30	2,70	0,13	0,19	0,02	0,16	0,27	0,80	0,19
34	CAREX1	1,16	1,00	0,41	1,12	1,27	2,48	2,30	2,79	0,00	0,31	0,02	0,00	0,26	0,41	0,22
35	CAREX1	1,04	0,62	0,40	1,08	1,14	2,30	2,30	2,70	0,00	0,21	0,02	0,04	0,24	0,78	0,19
50	Naph L	0,76	0,63	0,28	2,66	3,10	2,62	2,30	2,91	0,20	0,27	0,04	0,26	0,24	0,27	0,12
51	Naph L	1,11	0,45	0,11	2,17	3,06	2,64	2,30	2,87	0,30	0,21	0,01	0,22	0,25	0,81	0,22
52	Naph L	0,76	0,57	0,00	2,05	2,44	2,58	2,30	2,72	0,21	0,29	0,13	0,00	0,26	0,25	0,10
53	Naph L	1,21	1,01	0,52	2,11	2,68	2,75	2,30	2,70	0,43	0,27	0,02	0,27	0,25	0,58	0,05
54	Naph L	1,16	0,59	0,01	2,10	2,49	2,30	2,30	2,74	0,33	0,32	0,02	0,20	0,26	0,43	0,16
55	Naph L	1,24	0,81	0,40	2,10	2,42	2,35	2,30	2,72	0,21	0,31	0,02	0,00	0,29	0,70	0,09
56	Naph L	1,00	0,76	0,04	2,15	2,86	2,30	2,30	2,70	0,24	0,35	0,01	0,70	0,25	0,59	0,14
43	Naph H	0,83	0,75	0,46	3,37	3,54	2,49	2,30	2,81	0,00	0,35	0,06	0,19	0,25	0,28	0,12
44	Naph H	0,99	0,76	0,34	2,67	3,23	2,60	2,30	2,79	0,06	0,33	0,03	0,21	0,24	0,33	0,13
45	Naph H	0,51	0,46	0,04	3,22	3,61	2,73	2,30	2,87	0,00	0,28	0,04	0,13	0,21	0,30	0,11
46	Naph H	0,85	0,56	0,04	2,94	3,28	2,64	2,30	2,78	0,26	0,27	0,03	0,19	0,26	0,34	0,13
47	Naph H	1,02	0,80	0,20	2,13	2,38	2,33	2,30	2,70	0,07	0,27	0,02	0,18	0,24	0,36	0,02
48	Naph H	0,89	0,91	0,52	2,51	2,78	2,30	2,30	2,70	0,25	0,28	0,03	0,29	0,26	0,52	0,03
49	Naph H	1,25	0,85	0,00	2,68	3,06	2,33	2,30	2,72	0,30	0,44	0,01	0,31	0,26	0,50	0,15
71	Naph DL	0,94	0,72	0,11	2,21	2,09	2,89	2,42	3,00	0,09	0,37	0,01	0,00	0,31	0,46	1,20
72	Naph DL	1,18	0,66	0,23	2,53	2,28	2,61	2,36	2,99	0,03	0,36	0,01	0,00	0,28	0,67	0,06
73	Naph DL	1,10	0,75	0,00	2,00	2,06	2,66	2,46	2,85	0,00	0,16	0,02	0,00	0,25	0,54	0,22
74	Naph DL	0,98	0,63	0,40	1,97	1,81	2,30	2,30	2,70	0,17	0,17	0,02	0,00	0,27	0,60	0,62
75	Naph DL	1,10	0,60	0,46	1,33	1,26	2,30	2,30	2,70	0,12	0,06	0,01	0,12	0,26	0,63	0,02
76	Naph DL	0,74	0,61	0,18	2,27	1,82	2,30	2,30	2,70	0,30	0,32	0,02	0,05	0,25	0,54	0,04
77	Naph DL	1,09	0,95	0,20	1,79	1,77	2,43	2,34	2,77	0,00	0,34	0,04	0,00	0,25	0,38	0,18
78	Naph DH	0,96	0,73	0,18	2,19	1,95	2,68	2,30	2,79	0,00	0,37	0,05	0,14	0,26	0,32	0,03
79	Naph DH	1,11	0,79	0,36	2,59	2,43	2,71	2,30	3,03	0,00	0,26	0,05	0,18	0,24	0,37	0,16
80	Naph DH	0,77	0,88	0,51	2,75	2,85	2,70	2,30	2,87	0,33	0,17	0,01	0,00	0,28	0,60	0,21
81	Naph DH	0,79	0,48	0,36	2,44	2,19	2,30	2,30	2,70	0,09	0,32	0,05	0,30	0,25	0,30	0,13
82	Naph DH	1,08	0,86	0,01	2,02	1,89	2,58	2,30	2,79	0,28	0,21	0,08	0,18	0,28	0,32	0,15
83	Naph DH	0,64	0,51	0,01	2,44	2,27	2,30	2,30	2,70	0,12	0,20	0,02	0,00	0,26	0,44	0,10
84	Naph DH	1,35	0,93	0,04	2,48	2,33	2,30	2,30	2,70	0,10	0,14	0,02	0,16	0,26	0,49	0,04

57	Chry L	1,05	1,00	0,67	1,19	1,62	2,63	2,30	2,83	0,39	0,15	0,04	0,20	0,26	0,27	0,66
58	Chry L	1,04	0,76	0,51	1,41	1,88	2,66	2,34	2,96	0,25	0,15	0,02	0,36	0,25	0,76	0,04
59	Chry L	0,86	0,34	0,01	1,36	1,57	2,52	2,42	2,83	0,05	0,45	0,05	0,06	0,25	0,33	0,03
60	Chry L	1,00	0,81	0,04	1,51	1,50	2,66	2,30	2,83	0,06	0,20	0,00	0,20	0,23	0,33	0,16
61	Chry L	0,82	0,80	0,59	1,21	1,44	2,33	2,30	2,70	0,25	0,33	0,04	0,59	0,25	0,31	0,12
62	Chry L	1,38	1,04	0,20	1,10	1,14	2,30	2,30	2,86	0,00	0,33	0,01	0,12	0,28	1,04	0,44
63	Chry L	1,17	0,81	0,45	1,24	1,47	2,30	2,30	2,70	0,29	0,41	0,03	0,58	0,27	0,42	0,14
64	Chry H	0,93	0,68	0,00	1,50	1,72	2,65	2,30	2,92	0,17	0,27	0,03	0,06	0,25	0,35	0,13
65	Chry H	0,77	0,76	0,11	1,46	1,71	2,67	2,35	2,80	0,25	0,16	0,01	0,00	0,31	0,84	0,03
66	Chry H	0,66	0,73	0,02	1,47	1,85	2,66	2,30	2,71	0,02	0,27	0,04	0,00	0,25	0,34	0,14
67	Chry H	1,12	0,76	0,36	1,47	1,35	2,68	2,30	3,00	0,00	0,10	0,01	0,00	0,28	0,82	0,22
68	Chry H	0,77	0,46	0,18	1,23	1,11	2,33	2,30	2,70	0,02	0,18	0,03	0,25	0,26	0,24	0,19
69	Chry H	0,54	0,74	0,11	1,15	1,30	2,30	2,30	2,70	0,00	0,17	0,02	0,00	0,25	0,35	0,22
70	Chry H	0,71	0,73	0,36	1,00	1,36	2,41	2,30	2,70	0,11	0,30	0,01	0,00	0,29	0,61	0,15
85	Chry DH	0,81	0,88	0,46	1,36	1,09	2,30	2,30	2,70	0,33	0,24	0,01	0,11	0,28	0,59	0,81
86	Chry DH	1,10	0,75	0,36	1,21	1,53	2,33	2,30	2,70	0,23	0,35	0,01	0,13	0,24	0,80	0,08
87	Chry DH	0,86	0,79	0,04	1,20	1,10	2,30	2,30	2,70	0,07	0,37	0,07	0,05	0,26	0,32	0,06
36	Chry DH	0,68	0,76	0,34	1,29	1,38	2,52	2,30	2,78	0,09	0,16	0,04	0,28	0,26	0,31	0,15
37	Chry DH	0,92	0,85	0,30	1,24	1,35	2,53	2,30	2,86	0,13	0,36	0,03	0,33	0,29	0,45	0,00
38	Chry DH	0,79	0,76	0,20	1,46	1,54	2,68	2,30	2,83	0,23	1,36	0,04	0,17	0,26	0,29	0,14
39	Chry DH	0,92	0,80	0,23	1,32	1,73	2,75	2,30	2,99	0,06	0,34	0,01	0,34	0,29	0,78	0,13
40	Chry DH	1,23	1,10	0,56	1,32	1,37	2,36	2,30	2,76	0,03	0,23	0,01	0,27	0,29	0,51	0,02
41	Chry DH	0,80	0,88	0,20	1,13	1,12	2,30	2,30	2,70	0,06	0,37	0,03	0,23	0,25	0,45	0,12
42	Chry DH	0,40	0,66	0,08	1,05	1,09	2,33	2,30	2,70	0,00	0,19	0,01	0,14	0,25	0,28	0,83

Dataset B

Fish #	Group	FF			GC-MS									EROD	GST	CAT	CI	LSI
		2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
2	CON7	0,40	0,27	0,09	0,67	1,12	2,35	1,92	2,00	1,23	2,33	2,47	1,96	0,86	0,01	0,07	0,25	0,29
3	CON7	0,69	0,42	0,11	0,54	1,05	2,26	2,06	2,16	1,99	2,33	2,25	2,02	1,45	0,02	0,02	0,24	0,26
4	CON7	0,82	0,48	0,17	1,89	0,47	2,31	2,09	2,05	1,89	2,35	2,48	2,09	0,78	0,05	0,04	0,24	0,31
5	CON7	0,88	0,52	0,19	0,93	0,64	2,47	1,82	2,22	1,84	1,92	2,11	1,95	1,29	0,02	0,11	0,18	0,41
6	CON7	0,90	0,62	0,19	0,50	0,68	2,05	1,97	2,08	1,81	1,57	1,51	1,85	0,58	0,01	0,06	0,26	0,28
7	CON7	0,96	0,64	0,28	0,87	0,78	2,03	1,82	2,10	1,17	2,26	1,26	2,05	1,34	0,01	0,09	0,26	0,33
8	CON7	1,00	0,65	0,38	0,61	0,68	1,98	1,80	2,36	1,99	2,37	2,06	2,00	0,71	0,02	0,06	0,26	0,27
9	CON7	1,15	0,71	0,44	0,70	0,81	2,26	1,77	2,13	1,83	2,32	2,27	2,10	0,89	0,02	0,04	0,26	0,25
10	LOW7	1,00	0,21	0,01	0,90	1,27	2,29	2,64	2,64	2,10	2,21	2,04	2,00	1,34	0,02	0,23	0,27	0,42
11	LOW7	1,02	0,65	0,06	0,93	0,87	2,26	2,37	2,59	2,05	2,34	1,72	2,04	0,66	0,01	0,06	0,28	0,25
12	LOW7	1,12	0,75	0,12	0,67	1,11	2,12	2,49	2,78	2,06	2,64	1,66	2,26	1,27	0,03	0,27	0,28	0,61
13	LOW7	1,17	0,76	0,17	1,03	1,50	2,54	2,55	2,77	1,90	2,53	2,36	2,16	1,19	0,00	0,10	0,25	0,26
14	LOW7	1,23	0,80	0,21	1,06	1,01	2,36	2,46	2,71	1,93	2,42	2,53	2,25	0,32	0,00	0,06	0,26	0,23
15	LOW7	1,25	0,82	0,33	0,97	1,15	2,54	2,72	2,97	2,10	2,27	1,52	2,25	0,00	0,00	0,06	0,27	0,29
16	LOW7	1,29	0,85	0,34	0,87	0,79	2,42	2,54	2,97	2,13	1,88	2,55	2,32	0,95	0,02	0,21	0,27	0,59
17	LOW7	1,41	0,87	0,54	-	-	-	-	-	-	-	-	-	0,43	0,00	0,11	0,28	0,36

18	MED7	1,36	0,65	0,01	1,41	1,91	2,87	3,50	3,50	2,03	2,78	2,62	2,32	0,19	0,00	0,02	0,28	-
19	MED7	1,41	0,75	0,01	1,20	1,55	2,70	3,24	3,49	2,13	2,60	1,70	2,32	0,96	0,00	0,16	0,26	0,28
20	MED7	1,50	0,84	0,01	1,44	1,86	2,76	3,38	3,39	2,32	2,78	2,62	2,43	0,77	0,00	0,24	0,27	0,23
21	MED7	1,50	0,86	0,07	1,14	1,37	2,61	3,20	3,34	2,11	2,18	1,66	2,38	0,82	0,01	0,12	0,23	0,23
22	MED7	1,56	0,86	0,16	1,62	1,77	2,76	3,35	3,54	2,33	2,91	2,50	2,34	1,14	0,01	0,03	0,28	0,26
23	MED7	1,60	0,97	0,21	1,66	2,07	2,95	3,58	3,85	2,75	3,36	2,34	2,80	0,76	0,01	0,11	0,28	0,50
24	MED7	1,63	1,04	0,25	1,29	1,47	2,77	3,17	3,18	2,14	2,48	1,43	2,11	0,00	0,01	0,14	0,26	0,22
25	MED7	2,03	1,36	0,29	1,11	1,22	2,72	3,26	3,43	2,01	2,27	2,81	2,28	0,41	0,02	0,01	0,24	0,20
26	HIGH7	1,54	0,76	0,18	1,53	1,76	3,10	3,56	3,74	2,40	3,10	1,92	2,61	0,92	0,02	0,18	0,27	0,40
27	HIGH7	1,57	0,90	0,25	1,33	1,52	2,75	3,23	3,34	2,17	2,59	1,38	2,26	0,58	0,00	0,10	0,27	0,24
28	HIGH7	1,71	0,91	0,27	1,37	1,63	2,86	3,46	3,67	2,53	3,12	2,20	2,42	0,74	0,01	0,05	0,26	0,28
29	HIGH7	1,73	1,00	0,36	1,80	2,24	3,24	3,76	3,60	2,56	2,93	2,83	2,15	-0,82	0,01	0,06	0,26	0,40
30	HIGH7	1,73	1,02	0,41	1,38	1,68	2,76	3,31	3,54	2,40	2,84	2,83	2,38	1,71	0,00	0,01	0,25	0,26
31	HIGH7	1,78	1,03	0,57	1,51	1,76	2,84	3,44	3,64	2,44	2,96	2,40	2,37	1,59	0,04	0,30	0,27	0,36
32	HIGH7	2,00	1,23	1,00	1,37	1,75	2,72	3,29	3,39	2,28	2,57	2,61	2,35	0,00	0,01	0,16	0,26	0,26
1	HIGH7	2,01	1,29	-	1,64	2,03	3,29	3,84	3,86	2,51	3,32	2,10	2,65	0,33	0,02	0,12	0,24	0,29

Dataset C

Fish #	Group	FF			GC-MS									EROD	GST	CAT	CI	LSI
		2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
33	CON30	0,46	0,34	0,13	0,98	0,82	2,44	1,84	1,77	1,37	2,05	2,25	1,77	1,24	0,00	0,04	0,24	0,28
34	CON30	0,49	0,43	0,14	1,01	0,45	2,36	1,76	1,93	1,56	2,31	1,90	1,58	0,20	0,01	0,05	0,23	0,29
35	CON30	0,53	0,46	0,16	0,84	0,44	2,32	1,98	1,97	1,70	1,93	2,20	1,89	-0,42	0,01	0,04	0,26	0,28
36	CON30	0,54	0,49	0,18	0,55	0,34	2,37	1,74	1,96	1,48	2,00	2,20	1,65	0,82	0,00	0,04	0,27	0,24
37	CON30	0,68	0,54	0,25	0,31	0,88	2,51	1,69	1,96	1,88	1,53	2,21	1,69	0,00	0,01	0,05	0,24	0,24
38	CON30	0,69	0,55	0,35	1,03	0,44	2,28	2,03	2,01	1,81	2,18	2,08	2,13	0,93	0,03	0,20	0,26	0,75
39	CON30	0,88	0,66	0,51	0,66	0,48	2,39	1,75	1,79	1,80	2,00	2,01	1,92	0,25	0,02	0,25	0,26	0,97
40	LOW30	1,07	0,58	0,00	1,08	1,21	2,51	2,88	3,19	2,21	2,31	2,82	2,45	0,81	0,02	0,03	0,25	0,37
41	LOW30	1,11	0,61	0,07	0,80	0,98	2,46	2,75	2,66	1,85	2,45	2,39	1,86	1,03	0,01	0,06	0,26	0,13
42	LOW30	1,13	0,64	0,14	1,09	1,08	2,58	2,84	2,97	1,95	2,43	2,37	1,98	0,00	0,02	0,05	0,27	0,22
43	LOW30	1,16	0,79	0,16	0,96	1,24	2,45	2,61	2,72	1,95	2,57	2,41	2,14	0,49	0,01	0,03	0,26	0,29
44	LOW30	1,26	0,80	0,19	1,15	1,32	2,51	2,81	2,75	2,17	2,21	2,14	2,05	1,60	0,01	0,18	0,28	0,28
45	LOW30	1,39	0,86	0,28	0,69	0,99	2,41	2,29	2,64	1,92	2,52	2,41	1,95	0,67	0,01	0,01	0,25	0,64
46	LOW30	1,46	0,96	0,29	0,70	0,77	2,34	2,29	2,25	1,94	2,26	2,35	1,75	0,48	0,02	0,09	0,25	0,14
47	LOW30	1,53	0,99	0,31	-	-	-	-	-	-	-	-	-	0,00	0,01	0,14	0,31	0,32
48	MED30	1,59	0,99	0,10	1,48	1,76	2,67	3,22	3,26	2,21	2,57	2,67	2,25	-1,02	0,02	0,29	0,24	0,52
49	MED30	1,69	1,00	0,14	1,19	1,47	2,83	3,36	3,43	2,30	2,72	2,71	2,28	1,05	0,02	0,17	0,27	0,16
50	MED30	1,70	1,00	0,18	1,39	1,49	2,96	3,39	3,40	2,14	2,55	2,15	2,23	0,93	0,01	0,02	0,26	0,55
51	MED30	1,77	1,10	0,20	1,37	1,34	2,75	3,33	3,61	2,39	2,94	2,53	2,25	1,28	0,01	0,04	0,23	0,17
52	MED30	1,82	1,10	0,36	1,34	1,35	2,72	3,28	3,51	2,27	2,91	2,05	2,17	0,00	0,04	0,04	0,24	0,29
53	MED30	1,84	1,10	0,44	1,34	1,30	2,88	3,49	3,61	2,15	3,04	1,79	2,58	0,26	0,01	0,03	0,27	0,37
54	MED30	1,86	1,16	0,47	1,22	1,00	2,65	3,14	3,35	2,16	2,62	2,54	2,39	0,00	0,00	0,07	0,24	0,19
55	MED30	1,90	1,17	0,47	1,31	1,32	2,85	3,33	3,28	2,20	2,71	2,73	2,17	0,46	0,05	0,28	0,26	0,49
56	MED30	1,96	1,26	0,62	1,32	1,53	2,85	3,53	3,66	2,41	2,95	2,91	2,44	0,22	0,02	0,15	0,26	0,32
57	HIGH30	1,74	0,99	0,08	1,40	1,61	2,86	3,34	3,35	2,32	2,76	1,86	2,09	0,66	0,02	0,21	0,28	-
58	HIGH30	1,78	1,07	0,20	1,47	1,79	3,06	3,57	3,66	2,39	3,01	2,82	2,38	0,45	0,01	0,04	0,26	0,29
59	HIGH30	1,90	1,16	0,28	1,69	1,81	3,01	3,68	3,81	2,52	3,04	1,88	2,50	0,61	0,00	0,02	0,23	0,25
60	HIGH30	1,91	1,19	0,30	1,55	1,66	3,06	3,70	3,75	2,50	3,27	2,26	2,58	0,35	0,02	0,05	0,26	0,27
61	HIGH30	1,93	1,20	0,32	1,59	1,49	2,95	3,51	3,59	2,41	3,12	3,13	2,40	0,42	0,02	0,09	0,25	0,27
62	HIGH30	2,12	1,37	0,43	1,73	1,63	3,19	3,40	3,36	2,21	2,78	2,67	2,22	0,36	0,00	0,03	0,25	0,28

Fish #	Group	Gill histopatholgy							Liver histopathology									
		AN	EMS	EH	LF	NEC	EL	PEC	Inf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
33	CON30	0,00	0,00	0,48	0,30	0,00	0,70	0,30	0,30	0,00	0,30	0,30	0,30	0,00	0,00	0,00	0,00	0,00
34	CON30	0,30	0,00	0,30	0,00	0,30	0,30	0,00	0,00	0,00	0,30	0,00	0,30	0,00	0,00	0,00	0,00	0,00
35	CON30	0,00	0,00	0,48	0,30	0,00	0,48	0,30	0,00	0,00	0,30	0,00	0,30	0,00	0,00	0,00	0,00	0,00
36	CON30	0,30	0,30	0,60	0,48	0,48	0,30	0,30	0,00	0,00	0,00	0,60	0,30	0,48	0,30	0,00	0,00	0,00
37	CON30	0,00	0,00	0,70	0,00	0,00	0,00	0,30	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
38	CON30	0,30	0,00	0,48	0,30	0,30	0,60	0,30	0,00	0,30	0,00	0,00	0,48	0,00	0,00	0,00	0,00	0,00
39	CON30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	LOW30	0,48	0,30	0,48	0,30	0,60	0,00	0,48	0,00	0,00	0,00	0,48	0,00	0,00	0,00	0,00	0,00	0,00
41	LOW30	0,00	0,30	0,30	0,00	0,30	0,30	0,30	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,30	0,00	0,00
42	LOW30	0,00	0,48	0,30	0,30	0,00	0,48	0,60	0,00	0,30	0,00	0,00	0,00	0,00	0,30	0,00	0,00	0,00
43	LOW30	0,00	0,48	0,30	0,48	0,30	0,60	0,00	0,00	0,00	0,00	0,60	0,00	0,00	0,00	0,00	0,00	0,48
44	LOW30	0,60	0,48	0,60	0,00	0,70	0,30	0,70	0,00	0,00	0,00	0,00	0,30	0,00	0,30	0,48	0,00	0,00
45	LOW30	0,00	0,30	0,48	0,00	0,48	0,60	0,48	0,00	0,48	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
46	LOW30	0,48	0,30	0,30	0,30	0,30	0,00	0,70	0,30	0,00	0,00	0,00	0,00	0,60	0,30	0,00	0,00	0,00
47	LOW30	0,48	0,30	0,48	0,00	0,48	0,30	0,60	0,00	0,00	0,00	0,00	0,30	0,00	0,00	0,00	0,00	0,00
48	MED30	0,00	0,00	0,30	0,30	0,00	0,60	0,48	0,00	0,00	0,00	0,00	0,30	0,00	0,30	0,00	0,30	0,00
49	MED30	0,00	0,00	0,30	0,00	0,00	0,30	0,60	0,00	0,30	0,00	0,30	0,48	0,00	0,30	0,00	0,00	0,00
50	MED30	0,30	0,48	0,48	0,00	0,00	0,30	0,70	0,48	0,30	0,00	0,30	0,00	0,00	0,00	0,00	0,00	0,00
51	MED30	0,48	0,30	0,30	0,30	0,30	0,48	0,48	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,48
52	MED30	0,30	0,00	0,70	0,30	0,30	0,48	0,48	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
53	MED30	0,60	0,30	0,48	0,30	0,00	0,30	0,30	0,00	0,00	0,00	0,60	0,00	0,00	0,00	0,48	0,00	0,00
54	MED30	0,48	0,60	0,60	0,30	0,48	0,70	0,48	0,00	0,00	0,00	0,00	0,00	0,30	0,30	0,48	0,00	0,00
55	MED30	0,70	0,00	0,48	0,00	0,30	0,00	0,60	0,00	0,30	0,00	0,00	0,00	0,00	0,30	0,30	0,00	0,00
56	MED30	0,70	0,30	0,70	0,60	0,48	0,30	0,70	0,30	0,00	0,00	0,00	0,00	0,00	0,30	0,30	0,00	0,00
57	HIGH30	0,00	0,30	0,70	0,00	0,30	0,00	0,60	0,48	0,00	0,00	0,00	0,00	0,00	0,00	0,48	0,00	0,00
58	HIGH30	0,30	0,30	0,30	0,30	0,70	0,30	0,30	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
59	HIGH30	0,60	0,48	0,48	0,30	0,48	0,00	0,70	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,30	0,00	0,00
60	HIGH30	0,70	0,48	0,48	0,00	0,00	0,30	0,30	0,30	0,00	0,00	0,00	0,30	0,00	0,00	0,30	0,30	0,00
61	HIGH30	0,48	0,60	0,70	0,48	0,48	0,30	0,30	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
62	HIGH30	0,00	0,48	0,30	0,30	0,00	0,00	0,48	0,00	0,00	0,00	0,00	0,30	0,30	0,00	0,00	0,00	0,00

Appendix F

Score calculations in IBR

Dataset A

CONEX1	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	8,16	4,39	0,53	20,65	27,55	384,29	264,49	697,39	0,58	0,77	0,05	0,01	0,84	2,73	2,72
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	-0,08	-0,14	-0,37	-0,42	-0,41	0,28	1,28	0,32	0,33	-0,16	-0,26	-0,69	0,21	0,20	0,58

CAREX1	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	7,24	3,74	0,74	18,21	23,91	317,99	200,00	631,43	0,17	0,74	0,07	0,39	0,80	2,74	1,49
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	-0,28	-0,44	-0,12	-0,42	-0,41	-0,20	0,00	-0,08	-0,59	-0,17	0,03	-0,18	-0,28	0,21	0,12

Naph L	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	10,74	4,30	0,73	176,25	637,33	345,29	200,00	592,77	0,92	0,95	0,09	1,00	0,81	2,65	0,35
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	0,48	-0,18	-0,13	0,00	0,41	0,00	0,00	-0,31	1,12	-0,09	0,32	0,64	-0,20	0,15	-0,31

Naph H	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	7,94	4,64	0,89	892,22	1864,03	327,12	200,00	588,51	0,42	1,10	0,07	0,65	0,76	1,43	0,26
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	-0,13	-0,02	0,05	1,90	2,07	-0,13	0,00	-0,34	-0,03	-0,03	0,11	0,18	-0,77	-0,53	-0,34

Naph DL	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	9,89	4,27	0,79	136,73	90,16	359,26	227,58	679,90	0,29	0,86	0,05	0,07	0,85	2,60	2,78
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	0,29	-0,19	-0,07	-0,11	-0,33	0,10	0,38	0,22	-0,31	-0,12	-0,38	-0,61	0,25	0,13	0,60

Naph DH	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	9,41	4,87	0,78	293,99	242,41	351,77	200,00	650,64	0,41	0,76	0,10	0,41	0,82	1,62	0,32
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	0,19	0,08	-0,08	0,31	-0,12	0,05	0,00	0,04	-0,05	-0,16	0,61	-0,15	-0,06	-0,43	-0,32

Chry L	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	11,10	5,84	1,62	19,38	35,82	326,33	211,70	666,62	0,61	1,00	0,08	1,23	0,81	2,93	0,95
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	0,56	0,53	0,92	-0,42	-0,40	-0,14	0,00	0,13	0,41	-0,07	0,14	0,95	-0,16	0,31	-0,08

Chry H	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	5,69	4,07	0,54	21,87	35,02	361,42	202,92	640,36	0,23	0,63	0,05	0,14	0,87	2,71	0,44
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	-0,62	-0,28	-0,36	-0,41	-0,40	0,12	-0,22	-0,02	-0,44	-0,21	-0,36	-0,52	0,56	0,19	-0,27

Chry DH	FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
X	6,66	6,09	0,99	17,86	25,16	335,79	200,00	651,48	0,23	3,89	0,06	0,81	0,86	1,97	1,20
m	8,54	4,69	0,84	177,46	331,26	345,47	211,86	644,34	0,43	1,19	0,07	0,52	0,82	2,38	1,17
s	4,57	2,18	0,84	375,96	741,46	138,35	41,16	165,06	0,44	2,65	0,05	0,74	0,08	1,77	2,67
Y	-0,41	0,64	0,17	-0,42	-0,41	-0,07	0,00	0,04	-0,44	1,02	-0,18	0,38	0,45	-0,23	0,01

Z-values		FF			GC-MS					EROD	GST	CAT	DNA adduct	CI	LSI	GSI
Fish #	Group	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph							
22-28	CONEX1	0,08	0,14	0,37	0,42	0,41	0,28	1,28	0,32	0,33	0,16	0,26	0,69	-0,21	-0,20	-0,58
29-35	CAREX1	0,28	0,44	0,12	0,42	0,41	0,20	0,29	0,08	0,59	0,17	0,03	0,18	-0,28	-0,21	-0,12
50-56	Naph L	0,48	0,18	0,13	0,00	0,41	0,00	0,29	0,31	1,12	0,09	0,32	0,64	-0,20	-0,15	-0,31
43-49	Naph H	0,13	0,02	0,05	1,90	2,07	0,13	0,29	0,34	0,03	0,03	0,11	0,18	-0,77	-0,53	-0,34
71-77	Naph DL	0,29	0,19	0,07	0,11	0,33	0,10	0,38	0,22	0,31	0,12	0,38	0,61	-0,25	-0,13	-0,60
78-84	Naph DH	0,19	0,08	0,08	0,31	0,12	0,05	0,29	0,04	0,05	0,16	0,61	0,15	-0,06	-0,43	-0,32
57-63	Chry L	0,56	0,53	0,92	0,42	0,40	0,14	0,00	0,13	0,41	0,07	0,14	0,95	-0,16	-0,31	-0,08
64-70	Chry H	0,62	0,28	0,36	0,41	0,40	0,12	0,22	0,02	0,44	0,21	0,36	0,52	-0,56	-0,19	-0,27
36-42	Chry DH	0,41	0,64	0,17	0,42	0,41	0,07	0,29	0,04	0,44	1,02	0,18	0,38	-0,45	-0,23	-0,01
	min	0,08	0,02	0,05	0,00	0,12	0,00	0,00	0,02	0,03	0,03	0,03	0,15	-0,77	-0,53	-0,60

Dataset B

CON7	FF			GC-MS									EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	6,86	2,61	0,77	13,34	5,73	173,49	82,63	141,11	62,05	174,08	155,88	101,05	11,48	0,04	0,15	0,76	1,01
m	30,88	6,42	1,04	19,74	36,58	511,55	1581,35	2100,16	160,93	524,59	223,41	198,13	9,39	0,03	0,30	0,82	1,14
s	28,15	4,74	1,61	18,30	39,60	442,30	1741,92	2098,68	116,58	551,80	194,30	118,14	11,72	0,03	0,25	0,08	0,61
Y	-0,85	-0,80	-0,17	-0,35	-0,78	-0,76	-0,86	-0,93	-0,85	-0,64	-0,35	-0,82	0,18	0,49	-0,57	-0,78	-0,21

LOW7	FF			GC-MS									EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	14,97	4,62	0,79	7,63	13,40	241,53	357,03	626,05	110,27	239,33	164,57	157,02	8,39	0,02	0,40	0,87	1,52
m	30,88	6,42	1,04	19,74	36,58	511,55	1581,35	2100,16	160,93	524,59	223,41	198,13	9,39	0,03	0,30	0,82	1,14
s	28,15	4,74	1,61	18,30	39,60	442,30	1741,92	2098,68	116,58	551,80	194,30	118,14	11,72	0,03	0,25	0,08	0,61
Y	-0,56	-0,38	-0,16	-0,66	-0,59	-0,61	-0,70	-0,70	-0,43	-0,52	-0,30	-0,35	-0,09	-0,21	0,39	0,59	0,62

MED7	FF			GC-MS									EROD	GST	CAT	CI	LSI
C	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	41,11	8,32	0,38	24,42	53,48	598,42	2273,16	3204,79	198,82	668,64	266,60	262,45	4,79	0,02	0,29	0,83	0,94
m	30,88	6,42	1,04	19,74	36,58	511,55	1581,35	2100,16	160,93	524,59	223,41	198,13	9,39	0,03	0,30	0,82	1,14
s	28,15	4,74	1,61	18,30	39,60	442,30	1741,92	2098,68	116,58	551,80	194,30	118,14	11,72	0,03	0,25	0,08	0,61
Y	0,36	0,40	-0,41	0,26	0,43	0,20	0,40	0,53	0,33	0,26	0,22	0,54	-0,39	-0,46	-0,04	0,12	-0,34
HIGH7	FF			GC-MS									EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	60,56	10,12	2,39	32,06	70,83	998,99	3459,53	4244,42	266,25	980,65	299,25	266,88	12,91	0,03	0,35	0,82	1,07
m	30,88	6,42	1,04	19,74	36,58	511,55	1581,35	2100,16	160,93	524,59	223,41	198,13	9,39	0,03	0,30	0,82	1,14
s	28,15	4,74	1,61	18,30	39,60	442,30	1741,92	2098,68	116,58	551,80	194,30	118,14	11,72	0,03	0,25	0,08	0,61
Y	1,05	0,78	0,84	0,67	0,86	1,10	1,08	1,02	0,90	0,83	0,39	0,58	0,30	0,18	0,22	0,07	-0,12

Z-values																	
Fish #	Group	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	-OH-Pyren	EROD	GST	CAT	CI	LSI
2-9	CON7	-0,85	-0,80	-0,17	-0,35	-0,78	-0,76	-0,86	-0,93	-0,85	-0,64	-0,35	-0,82	0,18	0,49	-0,57	-0,78	-0,21
11-17	LOW7	-0,56	-0,38	-0,16	-0,66	-0,59	-0,61	-0,70	-0,70	-0,43	-0,52	-0,30	-0,35	-0,09	-0,21	0,39	0,59	0,62
18-25	MED7	0,36	0,40	-0,41	0,26	0,43	0,20	0,40	0,53	0,33	0,26	0,22	0,54	-0,39	-0,46	-0,04	0,12	-0,34
26-32, 1	HIGH7	1,05	0,78	0,84	0,67	0,86	1,10	1,08	1,02	0,90	0,83	0,39	0,58	0,30	0,18	0,22	0,07	-0,12
	min	-0,85	-0,80	-0,41	-0,66	-0,78	-0,76	-0,86	-0,93	-0,85	-0,64	-0,35	-0,82	-0,39	-0,46	-0,57	-0,78	-0,34

Dataset C

CON30	FF			GC-MS									EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	3,31	2,21	0,85	5,81	2,96	242,95	68,71	82,57	47,79	110,99	135,86	68,37	4,28	0,03	0,27	0,79	2,45
m	41,00	8,32	0,92	17,58	21,48	550,95	1581,54	1980,93	144,59	514,03	313,94	161,86	4,76	0,04	0,26	0,80	1,46
s	34,52	5,44	0,72	13,37	18,39	350,70	1501,03	1893,50	85,42	444,31	284,60	98,48	8,02	0,03	0,27	0,07	1,62
Y	-1,09	-1,12	-0,10	-0,88	-1,01	-0,88	-1,01	-1,00	-1,13	-0,91	-0,63	-0,95	-0,06	-0,25	0,03	-0,18	0,61
	Gill histopatholgy						Liver histopathology										
	AN	EMS	EH	LF	NEC	EL	PEC	Inf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
X	0,50	0,17	2,33	0,83	0,67	1,83	0,83	0,17	0,17	0,50	0,67	1,00	0,33	0,17	0,00	0,00	0,00
m	1,34	1,07	2,10	0,79	1,17	1,38	2,03	0,28	0,24	0,10	0,48	0,45	0,24	0,31	0,45	0,07	0,14
s	1,37	0,92	1,08	0,77	1,17	1,21	1,24	0,59	0,51	0,31	0,99	0,63	0,69	0,47	0,74	0,26	0,52
Y	-0,62	-0,98	0,21	0,05	-0,43	0,38	-0,97	-0,18	-0,15	1,28	0,19	0,87	0,13	-0,31	-0,61	-0,27	-0,27
LOW30	FF			GC-MS									EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	18,81	5,34	0,56	8,06	12,11	294,63	491,84	663,63	103,50	255,95	287,86	119,79	7,73	0,03	0,19	0,84	1,14
m	41,00	8,32	0,92	17,58	21,48	550,95	1581,54	1980,93	144,59	514,03	313,94	161,86	4,76	0,04	0,26	0,80	1,46
s	34,52	5,44	0,72	13,37	18,39	350,70	1501,03	1893,50	85,42	444,31	284,60	98,48	8,02	0,03	0,27	0,07	1,62
Y	-0,64	-0,55	-0,50	-0,71	-0,51	-0,73	-0,73	-0,70	-0,48	-0,58	-0,09	-0,43	0,37	-0,23	-0,25	0,55	-0,20
	Gill histopatholgy							Liver histopathology									
	AN	EMS	EH	LF	NEC	EL	PEC	Inf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
X	1,13	1,38	1,63	0,63	1,75	1,38	2,38	0,13	0,38	0,00	0,63	0,25	0,38	0,38	0,38	0,00	0,25
m	1,34	1,07	2,10	0,79	1,17	1,38	2,03	0,28	0,24	0,10	0,48	0,45	0,24	0,31	0,45	0,07	0,14
s	1,37	0,92	1,08	0,77	1,17	1,21	1,24	0,59	0,51	0,31	0,99	0,63	0,69	0,47	0,74	0,26	0,52
Y	-0,16	0,33	-0,44	-0,22	0,49	0,00	0,27	-0,26	0,26	-0,33	0,14	-0,31	0,19	0,14	-0,10	-0,27	0,22

MED30	FF			GC-MS									EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	62,92	11,82	1,30	20,78	26,41	638,81	2268,04	3009,58	179,89	647,13	370,05	211,74	4,27	0,05	0,36	0,79	1,31
m	41,00	8,32	0,92	17,58	21,48	550,95	1581,54	1980,93	144,59	514,03	313,94	161,86	4,76	0,04	0,26	0,80	1,46
s	34,52	5,44	0,72	13,37	18,39	350,70	1501,03	1893,50	85,42	444,31	284,60	98,48	8,02	0,03	0,27	0,07	1,62
Y	0,63	0,64	0,53	0,24	0,27	0,25	0,46	0,54	0,41	0,30	0,20	0,51	-0,06	0,50	0,36	-0,20	-0,09
	Gill histopatholgy							Liver histopathology									
	AN	EMS	EH	LF	NEC	EL	PEC	lnf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
X	1,89	0,89	2,22	0,89	0,78	1,67	2,56	0,33	0,33	0,00	0,56	0,33	0,11	0,56	0,67	0,11	0,22
m	1,34	1,07	2,10	0,79	1,17	1,38	2,03	0,28	0,24	0,10	0,48	0,45	0,24	0,31	0,45	0,07	0,14
s	1,37	0,92	1,08	0,77	1,17	1,21	1,24	0,59	0,51	0,31	0,99	0,63	0,69	0,47	0,74	0,26	0,52
Y	0,40	-0,20	0,11	0,12	-0,34	0,24	0,42	0,10	0,18	-0,33	0,07	-0,18	-0,19	0,52	0,30	0,16	0,16

HIGH30	FF			GC-MS									EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
X	81,68	14,18	0,91	37,62	46,60	1077,54	3588,07	4189,58	252,52	1085,66	467,96	245,20	2,11	0,03	0,20	0,79	0,87
m	41,00	8,32	0,92	17,58	21,48	550,95	1581,54	1980,93	144,59	514,03	313,94	161,86	4,76	0,04	0,26	0,80	1,46
s	34,52	5,44	0,72	13,37	18,39	350,70	1501,03	1893,50	85,42	444,31	284,60	98,48	8,02	0,03	0,27	0,07	1,62
Y	1,18	1,08	-0,02	1,50	1,37	1,50	1,34	1,17	1,26	1,29	0,54	0,85	-0,33	-0,16	-0,24	-0,23	-0,37
	Gill histopatholgy							Liver histopathology									
	AN	EMS	EH	LF	NEC	EL	PEC	lnf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
X	1,67	1,83	2,33	0,83	1,50	0,50	2,00	0,50	0,00	0,00	0,00	0,33	0,17	0,00	0,67	0,17	0,00
m	1,34	1,07	2,10	0,79	1,17	1,38	2,03	0,28	0,24	0,10	0,48	0,45	0,24	0,31	0,45	0,07	0,14
s	1,37	0,92	1,08	0,77	1,17	1,21	1,24	0,59	0,51	0,31	0,99	0,63	0,69	0,47	0,74	0,26	0,52
Y	0,23	0,83	0,21	0,05	0,28	-0,73	-0,03	0,38	-0,47	-0,33	-0,49	-0,18	-0,11	-0,66	0,30	0,38	-0,27

Z-values		FF			GC-MS									EROD	GST	CAT	CI	LSI
Fish #	Group	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene					
2-9	CON30	-1,09	-1,12	-0,10	-0,88	-1,01	-0,88	-1,01	-1,00	-1,13	-0,91	-0,63	-0,95	-0,06	-0,25	0,03	-0,18	0,61
11-17	LOW30	-0,64	-0,55	-0,50	-0,71	-0,51	-0,73	-0,73	-0,70	-0,48	-0,58	-0,09	-0,43	0,37	-0,23	-0,25	0,55	-0,20
18-25	MED30	0,63	0,64	0,53	0,24	0,27	0,25	0,46	0,54	0,41	0,30	0,20	0,51	-0,06	0,50	0,36	-0,20	-0,09
26-32, 1	HIGH30	1,18	1,08	-0,02	1,50	1,37	1,50	1,34	1,17	1,26	1,29	0,54	0,85	-0,33	-0,16	-0,24	-0,23	-0,37
	min	-1,09	-1,12	-0,50	-0,88	-1,01	-0,88	-1,01	-1,00	-1,13	-0,91	-0,63	-0,95	-0,33	-0,25	-0,25	-0,23	-0,37
		Gill histopatholgy							Liver histopathology									
Fish #	Group	AN	EMS	EH	LF	NEC	EL	PEC	lnf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
2-9	CON30	-0,62	-0,98	0,21	0,05	-0,43	0,38	-0,97	-0,18	-0,15	1,28	0,19	0,87	0,13	-0,31	-0,61	-0,27	-0,27
11-17	LOW30	-0,16	0,33	-0,44	-0,22	0,49	0,00	0,27	-0,26	0,26	-0,33	0,14	-0,31	0,19	0,14	-0,10	-0,27	0,22
18-25	MED30	0,40	-0,20	0,11	0,12	-0,34	0,24	0,42	0,10	0,18	-0,33	0,07	-0,18	-0,19	0,52	0,30	0,16	0,16
26-32, 1	HIGH30	0,23	0,83	0,21	0,05	0,28	-0,73	-0,03	0,38	-0,47	-0,33	-0,49	-0,18	-0,11	-0,66	0,30	0,38	-0,27
	min	-0,62	-0,98	-0,44	-0,22	-0,43	-0,73	-0,97	-0,26	-0,47	-0,33	-0,49	-0,31	-0,19	-0,66	-0,61	-0,27	-0,27

Dataset D

CONEX1	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	8,16	4,39	0,53	20,65	27,55	384,29	264,49	697,39	0,58	0,77	0,05	0,84	2,73
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,40	-0,27	-0,33	-0,33	-0,33	-0,06	-0,34	-0,31	-0,36	-0,01	-0,49	0,24	0,48

CAREX1	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	7,24	3,74	0,74	18,21	23,91	317,99	200,00	631,43	0,17	0,74	0,07	0,80	2,74
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,45	-0,46	-0,14	-0,34	-0,34	-0,29	0,00	-0,36	-0,41	-0,02	-0,41	-0,26	0,48

Naph L	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	10,74	4,30	0,73	176,25	637,33	345,29	200,00	592,77	0,92	0,95	0,09	0,81	2,65
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,27	-0,29	-0,15	0,16	0,65	-0,19	0,00	-0,38	-0,32	0,07	-0,33	-0,18	0,43

Naph H	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	7,94	4,64	0,89	892,22	1864,03	327,12	200,00	588,51	0,42	1,10	0,07	0,76	1,43
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,41	-0,19	-0,02	2,43	2,62	-0,26	0,00	-0,39	-0,38	0,13	-0,39	-0,76	-0,34

Naph DL	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	9,89	4,27	0,79	136,73	90,16	359,26	227,58	679,90	0,29	0,86	0,05	0,85	2,60
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,31	-0,30	-0,11	0,04	-0,23	-0,14	-0,37	-0,32	-0,40	0,03	-0,53	0,28	0,40

Naph DH	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	9,41	4,87	0,78	293,99	242,41	351,77	200,00	650,64	0,41	0,76	0,10	0,82	1,62
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,34	-0,12	-0,12	0,53	0,01	-0,17	0,00	-0,34	-0,38	-0,02	-0,24	-0,04	-0,22

Chry L	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	11,10	5,84	1,62	19,38	35,82	326,33	211,70	666,62	0,61	1,00	0,08	0,81	2,93
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,25	0,17	0,62	-0,34	-0,32	-0,26	-0,38	-0,33	-0,36	0,09	-0,38	-0,14	0,60

ChryH	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	5,69	4,07	0,54	21,87	35,02	361,42	202,92	640,36	0,23	0,63	0,05	0,87	2,71
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,53	-0,36	-0,32	-0,33	-0,32	-0,14	-0,39	-0,35	-0,40	-0,08	-0,52	0,59	0,47

Chry DH	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	6,66	6,09	0,99	17,86	25,16	335,79	200,00	651,48	0,23	3,89	0,06	0,86	1,97
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,48	0,24	0,07	-0,34	-0,34	-0,23	0,00	-0,34	-0,40	1,39	-0,47	0,48	0,00

CON7	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	6,86	2,61	0,77	13,34	5,73	173,49	82,63	141,11	11,48	0,04	0,15	0,76	1,01
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,47	-0,80	-0,12	-0,35	-0,37	-0,79	-0,49	-0,71	1,01	-0,34	0,04	-0,81	-0,60

LOW7	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	14,97	4,62	0,79	7,63	13,40	241,53	357,03	626,05	8,39	0,02	0,40	0,87	1,52
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	-0,06	-0,20	-0,10	-0,37	-0,36	-0,55	-0,26	-0,36	0,62	-0,35	1,34	0,53	-0,28

MED7	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	41,11	8,32	0,38	24,42	53,48	598,42	2273,16	3204,79	4,79	0,02	0,29	0,83	0,94
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	1,27	0,91	-0,46	-0,32	-0,29	0,69	1,36	1,50	0,17	-0,35	0,76	0,07	-0,65

HIGH7	FF			GC-MS					EROD	GST	CAT	CI	LSI
	2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph					
X	60,56	10,12	2,39	32,06	70,83	998,99	3459,53	4244,42	12,91	0,03	0,35	0,82	1,07
m	16,06	5,27	0,91	125,45	234,08	400,24	663,50	1124,45	3,45	0,80	0,15	0,82	1,97
s	19,69	3,35	1,15	316,07	621,62	286,41	1182,76	1382,91	7,97	2,22	0,19	0,08	1,60
Y	2,26	1,45	1,29	-0,30	-0,26	2,09	2,36	2,26	1,19	-0,34	1,10	0,02	-0,56

Z-values		FF			GC-MS					EROD	GST	CAT	CI	LSI
Fish #	**Group**	**2/3-ring PAH**	**4-ring PAH**	**6-ring PAH**	**1-OH-Naph**	**2-OH-Naph**	**C1-OH-Naph**	**C2-OH-Naph**	**C3-OH-Naph**					
22-28	CONEX1	0,40	0,27	0,33	0,33	0,33	0,06	0,34	0,31	0,36	0,01	0,49	-0,24	-0,48
29-35	CAREX1	0,45	0,46	0,14	0,34	0,34	0,29	0,39	0,36	0,41	0,02	0,41	-0,26	-0,48
50-56	Naph L	0,27	0,29	0,15	0,16	0,65	0,19	0,39	0,38	0,32	0,07	0,33	-0,18	0,43
43-49	Naph H	0,41	0,19	0,02	2,43	2,62	0,26	0,39	0,39	0,38	0,13	0,39	-0,76	-0,34
71-77	Naph DL	0,31	0,30	0,11	0,04	0,23	0,14	0,37	0,32	0,40	0,03	0,53	-0,28	-0,40
78-84	Naph DH	0,34	0,12	0,12	0,53	0,01	0,17	0,39	0,34	0,38	0,02	0,24	-0,04	-0,22
57-63	Chry L	0,25	0,17	0,62	0,34	0,32	0,26	0,38	0,33	0,36	0,09	0,38	-0,14	-0,60
64-70	Chry H	0,53	0,36	0,32	0,33	0,32	0,14	0,39	0,35	0,40	0,08	0,52	-0,59	-0,47
36-42	Chry DH	0,48	0,24	0,07	0,34	0,34	0,23	0,39	0,34	0,40	1,39	0,47	-0,48	0,00
2-9	CON7	0,47	0,80	0,12	0,35	0,37	0,79	0,49	0,71	1,01	0,34	0,04	-0,81	-0,60
11-17	LOW7	0,06	0,20	0,10	0,37	0,36	0,55	0,26	0,36	0,62	0,35	1,34	-0,53	-0,28
18-25	MED7	1,27	0,91	0,46	0,32	0,29	0,69	1,36	1,50	0,17	0,35	0,76	-0,07	-0,65
26-32, 1	HIGH7	2,26	1,45	1,29	0,30	0,26	2,09	2,36	2,26	1,19	0,34	1,10	-0,02	-0,56
	min	0,06	0,12	0,02	0,04	0,01	0,06	0,26	0,31	0,17	0,01	0,04	-0,81	-0,65

Appendix G

Individual scores for all biomarkers in each group

Dataset	Fish no.	Group	Biomarkers																	
			FF			GC-MS									EROD	GST	CAT	DNA adduct	CI	LSI
			2/3-ring PAH	4-ring PAH	6-ring PAH	1-OH-Naph	2-OH-Naph	C1-OH-Naph	C2-OH-Naph	C3-OH-Naph	1-OH-Phenan	C1-OH-Phenan	C2-OH-Phenan	1-OH-Pyrene						
A	22-28	CONEX1	0,54	0,30	0,00	0,01	0,00	0,48	1,57	0,66	-	-	-	-	0,92	0,05	0,12	0,00	0,98	0,73
	29-35	CAREX1	0,34	0,00	0,25	0,00	0,00	0,00	0,00	0,26	-	-	-	-	0,00	0,04	0,41	0,51	0,49	0,74
	50-56	Naph L	1,11	0,26	0,24	0,42	0,83	0,20	0,00	0,03	-	-	-	-	1,71	0,12	0,70	1,33	0,58	0,69
	43-49	Naph H	0,49	0,41	0,42	2,33	2,48	0,07	0,00	0,00	-	-	-	-	0,56	0,18	0,49	0,87	0,00	0,00
	71-77	Naph DL	0,92	0,24	0,31	0,32	0,09	0,30	0,67	0,55	-	-	-	-	0,28	0,09	0,00	0,08	1,02	0,66
	78-84	Naph DH	0,82	0,52	0,29	0,73	0,29	0,24	0,00	0,38	-	-	-	-	0,54	0,05	0,99	0,54	0,71	0,11
	57-63	Chry L	1,18	0,96	1,29	0,00	0,02	0,06	0,28	0,47	-	-	-	-	1,00	0,14	0,53	1,64	0,61	0,85
	64-70	Chry H	0,00	0,15	0,01	0,01	0,01	0,31	0,07	0,31	-	-	-	-	0,15	0,00	0,03	0,18	1,33	0,72
	36-42	Chry DH	0,21	1,08	0,54	0,00	0,00	0,13	0,00	0,38	-	-	-	-	0,15	1,23	0,20	1,08	1,22	0,30
B	2-9	CON7	0,00	0,00	0,24	0,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	1,00	0,96	0,00	-	0,00	0,12
	11-17	LOW7	0,29	0,42	0,25	0,00	0,19	0,15	0,16	0,23	0,41	0,12	0,04	0,47	0,74	0,25	0,97	-	1,37	0,96
	18-25	MED7	1,22	1,21	0,00	0,92	1,21	0,96	1,26	1,46	1,17	0,90	0,57	1,37	0,43	0,00	0,53	-	0,90	0,00
	26-32, 1	HIGH7	1,91	1,59	1,25	1,34	1,64	1,87	1,94	1,96	1,75	1,46	0,74	1,40	1,12	0,64	0,79	-	0,86	0,22
C	2-9	CON30	0,00	0,00	0,40	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,89	0,00	0,29	-	0,05	0,98
	11-17	LOW30	0,45	0,58	0,00	0,17	0,50	0,15	0,28	0,31	0,65	0,33	0,53	0,52	1,32	0,02	0,00	-	0,78	0,17
	18-25	MED30	1,73	1,77	1,03	1,12	1,28	1,13	1,47	1,55	1,55	1,21	0,82	1,46	0,89	0,75	0,61	-	0,03	0,27
	26-32, 1	HIGH30	2,27	2,20	0,47	2,38	2,37	2,38	2,34	2,17	2,40	2,19	1,17	1,80	0,62	0,09	0,01	-	0,00	0,00
D	22-28	CONEX1	1,05	0,53	0,13	0,04	0,04	0,74	0,15	0,40	-	-	-	-	0,05	0,34	0,03	-	1,05	1,12
	29-35	CAREX1	0,08	0,34	0,31	0,03	0,03	0,50	0,10	0,35	-	-	-	-	0,00	0,33	0,12	-	0,55	1,13
	50-56	Naph L	0,26	0,51	0,30	0,53	1,02	0,60	0,10	0,33	-	-	-	-	0,09	0,42	0,20	-	0,63	1,07
	43-49	Naph H	0,11	0,61	0,44	2,80	2,99	0,54	0,10	0,32	-	-	-	-	0,03	0,49	0,14	-	0,04	0,31
	71-77	Naph DL	0,21	0,50	0,35	0,41	0,14	0,65	0,12	0,39	-	-	-	-	0,02	0,38	0,00	-	1,09	1,04
	78-84	Naph DH	0,19	0,68	0,34	0,91	0,38	0,62	0,10	0,37	-	-	-	-	0,03	0,33	0,29	-	0,77	0,43
	57-63	Chry L	0,27	0,97	1,08	0,04	0,05	0,53	0,11	0,38	-	-	-	-	0,06	0,44	0,15	-	0,67	1,25
	64-70	Chry H	0,00	0,44	0,13	0,05	0,05	0,66	0,10	0,36	-	-	-	-	0,01	0,28	0,01	-	1,40	1,11
	36-42	Chry DH	0,05	1,04	0,52	0,03	0,03	0,57	0,10	0,37	-	-	-	-	0,01	1,74	0,06	-	1,29	0,64
	2-9	CON7	0,06	0,00	0,33	0,02	0,00	0,00	0,00	0,00	-	-	-	-	1,42	0,01	0,57	-	0,00	0,05
	11-17	LOW7	0,47	0,60	0,35	0,00	0,01	0,24	0,23	0,35	-	-	-	-	1,03	0,00	1,87	-	1,34	0,36
	18-25	MED7	1,80	1,71	0,00	0,05	0,08	1,48	1,85	2,22	-	-	-	-	0,58	0,00	1,29	-	0,87	0,00
	26-32, 1	HIGH7	2,79	2,25	1,75	0,08	0,10	2,88	2,86	2,97	-	-	-	-	1,60	0,01	1,63	-	0,83	0,08

Dataset	Fish no.	Group	Biomarkers																
			GSI	Gill histopatholgy							Liver histopathology								
				AN	EMS	EH	LF	NEC	EL	PEC	Inf	BC	MMA	Gra	Nee	Fib	Par	Bas	Ade	FO
A	22-28	CONEX1	0,92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	29-35	CAREX1	0,46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50-56	Naph L	0,03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	43-49	Naph H	0,00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	71-77	Naph DL	0,95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	78-84	Naph DH	0,02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	57-63	Chry L	0,26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	64-70	Chry H	0,07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	36-42	Chry DH	0,35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	2-9	CON7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	11-17	LOW7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18-25	MED7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26-32, 1	HIGH7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	2-9	CON30	-	0,00	0,00	0,66	0,27	0,00	1,10	0,00	0,07	0,33	1,61	0,68	1,19	0,32	0,35	0,00	0,00	0,00	
	11-17	LOW30	-	0,46	1,31	0,00	0,00	0,93	0,72	1,24	0,00	0,73	0,00	0,63	0,00	0,38	0,80	0,51	0,00	0,48	
	18-25	MED30	-	1,01	0,78	0,55	0,34	0,10	0,97	1,39	0,35	0,65	0,00	0,56	0,13	0,00	1,18	0,91	0,43	0,43	
	26-32, 1	HIGH30	-	0,85	1,81	0,66	0,27	0,71	0,00	0,94	0,63	0,00	0,00	0,00	0,13	0,08	0,00	0,91	0,65	0,00	
D	22-28	CONEX1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	29-35	CAREX1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50-56	Naph L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	43-49	Naph H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	71-77	Naph DL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	78-84	Naph DH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	57-63	Chry L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	64-70	Chry H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	36-42	Chry DH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2-9	CON7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	11-17	LOW7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18-25	MED7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26-32, 1	HIGH7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-