BALCOFISH, Final report, 2011

January 31, 2012.

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*NB The department has changed name from January 1, 2012. The old name was Department of Zoology

Content

1. Executive summary

In the BALCOFISH project we have been investigating chemical pollution by developing fish monitoring. The purpose has been to provide science-based input to foster the development of appropriate measures in the management of the Baltic Sea environment to protect it against anthropogenic and hazardous chemicals. This is a very urgent issue because modern society utilizes more man-made chemicals than ever before and hundreds of new chemicals reach the market every year. Many of these end up in the coastal environment as chemical mixtures, impacting the well-being of organisms. In the project scientists from Germany, Denmark and Sweden have been working together and applied an integrated approach i.e. combination of measurements of different biological end points and of analyses of contaminants to develop ideas and techniques in order to improve the tools to assess impact of hazardous chemicals in coastal fish populations. The work has included development of new and application of existing tools and approaches in biological effect monitoring, field sampling campaigns, gene array technology, population genetics and modelling, and contaminants measurement. The viviparous eelpout (*Zoarces viviparus*) has been the selected key fish species.

The BALCOFISH project has been divided into six work packages which have been divided into 24 Tasks. Major key results and activities are summarized below.

- A metadatabase, called BonusHAZ, has been assembled, covering both new and old eelpout monitoring data. The BonusHAZ contains data on biometrics, biomarkers, reproductive success, chemistry data and other. Eelpout data generated in the BALCOFISH project has been submitted to the ICES Data Centre. Moreover, BonusHaz provides an important base for the development of guidelines for biological effects monitoring in eelpout including integration of biomarker responses, levels of contaminants and disturbances on the different level of fish population. A dedicated monitoring guideline will be submitted to ICES working group for biological effects of contaminants.

- For standardising and harmonisation of common methodology, BALCOFISH has arranged several workshops on sampling and dissection of fish, assessment of reproductive success and work with the common data base. One of these workshops was a jointly arranged by the BALCOFISH and BEAST projects. Experiences, data and fish material for different analyses has frequently been exchanged between the two BONUS projects. The BALCOFISH project has arranged two large sampling campaigns. These campaigns covered eight Swedish, five Danish and three German coastal sites.

- Observations at polluted sites have repeatedly shown disorders and malformations among eelpout fry. The findings reveal that pathological disorders of the eelpout broods were more frequent at German and Danish costal sites than in the Swedish sites. In adults, eelpout assessment of the testis has shown higher occurrence of intersex in the eelpout sampled from German and Danish sites compared to Swedish sites. The results show that intersex is relatively common in eelpout from coastal sites at least in some areas of the Baltic Sea. This finding of apparently feminized male eelpout suggests the exposure to endocrine disrupting substances i.e. estrogenic compounds in the coastal environment. Overall the eelpout has proven to be a valuable bio-indicator for the monitoring of different kinds of reproductive disorders in fish.

This feature supports the recommendation of the HELCOM CORESET project to select the eelpout as a core bio-indicator for monitoring in the Baltic Sea.

- Age classed Leslie matrix population models have been developed for four reference sites in the Baltic Sea, representing a gradient in salinity and climate. Results show that, despite depending on different life history characteristics, the survival of early life stages is most important for population growth and persistence. Low survival in early life stages is observed in contaminated sites, with higher frequencies of malformation and dead larvae as compared to reference sites. The range of change in survival of larvae necessary to affect population dynamics (i.e. growth) is well within the range documented at contaminated costal sites. Hence, induced malformation from contamination can have a large effect on population dynamics, and even lead to extinction of local populations depending on the dynamics of the populations.

- Molecular gene markers had been applied to study genetic differentiation, diversity and gene flow among eelpout populations. The three methods applied to describe the genetic population structure in eelpout show different potential to assess the spatial differentiations. The study with isozymes at the Danish coast showed that the eelpout populations must be considered as stationary, whereas the study in a Swedish coastal area using microsatellites suggests that gene flow among the studied populations of eelpout is stronger than usually thought. This apparent discrepancy might however depend on the differing sizes and geographical constraints for migration between the two sampling areas studied. It is important to consider these aspects in planning, and selection of coastal stations, and in assessment of monitoring activities with the eelpout. Concerning the third method (AFLP analyses), the studies indicated that contamination could be correlated to a reduced diversity. However, further analyses are needed to explore the potential of this AFLP method.

- Based on our sequencing of the hepatic transciptome of the eelpout gene expression DNA microarrays have been developed. We have applied DNA microarrays in a very comprehensive study of 160 female eelpout and 50 larvae from 16 different sampling sites. These data has provided us a unique possibility to compare specific gene expression profiles correlating to other endpoints studied during our joint sampling campaigns in the Baltic, including data on chemical exposure, malformations, sublethal biomarkers and reproductive success. Although we have not yet finalize the analyses of the entire dataset, we have evaluated a global gene expression study on eelpout from one of the polluted sites including a reference site. Our explorative analyses revealed over-expressed genes involved in apoptosis and DNA damage, a probable effect of the polluted environment and potential biomarkers for stress. Most interestingly, genes taking part in the innate immune system were suppressed. Biomarkers assessing the status of the immune system are not included in current monitoring studies. This finding could indicate the value of including such assays.

- Brominated dioxins, in a mixture reflecting the concentration found in the Baltic Sea was used in laboratory exposure studies on adult zebrafish. The results indicate that all brominated dioxins were taken up by the female fish and transferred to eggs. Exposure of brominated dioxins reduced spawning success, altered ovarian morphology and reduced hepatic vitellogenin gene expression which indicate that brominated dioxins may impair reproductive function in fish. The studies also include mixtures of brominated flame retardants (BFR). Also BFR exposure to zebrafish resulted in changed ovarian morphology and impaired reproductive functions. Another compound tested in exposure studies on zebrafish was 2, 4, 6- tribromophenol, widely used as industrial chemical. Our results show that dietary exposure to tribromophenol can interfere with reproduction and affects the fertility. Overall, these results provide data for risk analysis of chemicals and contribute to the identification of chemicals possibly affecting reproduction in Baltic fish species.

- The biological effect data provide strong evidences for differences in contaminant loads between the German, Danish and Swedish regions. The analyses of selected groups of contaminants do not however indicate any obvious differences between the regions. The reason for this apparent discrepancy can be that the selected and analyzed chemicals do not accurately reflect the contaminant species composition in the areas. Different sensitivity to contaminants of eelpout populations from different regions in the Baltic Sea, and the possible impact of abiotic differences e.g. salinity and temperature between the areas should be taken into account in future studies.

- These BALCOFISH results fully support that the field monitoring strategy applied here is useful in assessing impact of contaminants in coastal Baltic waters but care must be taken to size of geographic area, selection of biological endpoints as well as to possible genetic and other differences between eelpout populations from different parts of the Baltic Sea.

The immediate perspective beyond the BALCOFISH project are to develop and implement potential biomarkers obtained from the large sets of gene expression profiles of eelpout from polluted regions in the Baltic Sea, to continue the efforts to link especially reproductive performance in the eelpout to contaminants loads in its natural environment, to further investigate gonadal disorders (e.g. intersex) caused by endocrine disruptors, and to further explore and implement the Integrated Biomarker Assessment tool for environmental risk assessment in eelpout. In the longer perspective, the development in omics technology allows in future to find out if the chemical pollution in the Baltic eelpout can drive genetic variation which would have practical implications in for example planning and designing of future monitoring programmes. It is a significant need to make use of new technologies to develop common techniques and assessment criteria for national- and regional -scale assessment of the marine environment. This is necessary in order to establish monitoring programmes and set assessment criteria of the marine environmental statues within the Marine Strategy Framework Directives. The development of such technologies and strategies will in future be an aid in the assessments of environmental impact of **mixtures** of toxic chemicals occurring in the Baltic Sea regions.

2. Gained scientific results during the reporting period.

In the Balcofish project the work is divided in six work packages. Below is reported results subdivided into these WPs:

WP1. Provide a data matrix on contaminant levels, effects and population descriptors in eelpout, and supporting environmental variables from Baltic coastal waters.

Within the project a large data matrix on several biological effects and contaminants measures in eelpout has been compiled. The data are stored in the project database named BonusHaz, which has been developed in cooperation with the BEAST project. The structure of the BonusHaz database has been developed in line with the existing ICES database for environmental data. ICES code lists has been implemented in the BonusHAZ structure, but also additional codes for parameters not present in the ICES database has been added, for instance most codes related to the brood layer. The usage of theICES code lists as a foundation for data exchange and consistency between institutes. A formal arrangement with ICES have been made so their code lists from their RECO database http://www.ices.dk/datacentre/reco/reco.asp can be used as it also will benefit in the submission of BonusHaz data to the ICES database within the project period and also for the future.

The BALCOFISH data submitted to the BonusHaz database have been made available for all BALCOFISH partners for further data analyses from a common share point (http://www2.dmu.dk/1_Viden/2_Miljoe-tilstand/3_vand/4_balcofish/index.html).
In addition to data generated within the BALCOFISH project period, also some already existing data on biological effects in eelpout has been identified and included in BonusHaz . Data includes national and regional monitoring and research data in Sweden, Denmark and Germany. The Danish data include several years monitoring data on biometrics, CYP1A/protein, reproductive success, and some surveys of contaminants and biomarker studies e.g. intersex, PAH-metabolites. Swedish data includes several years monitoring and research data on biometrics, various biomarkers, reproductive success and contaminants. German data includes regional survey and monitoring data on reproductive success and intersex and additional biomarker data from research projects. In addition, also relevant supporting environmental variables such as sea water temperature and salinity was identified if available within the station information.

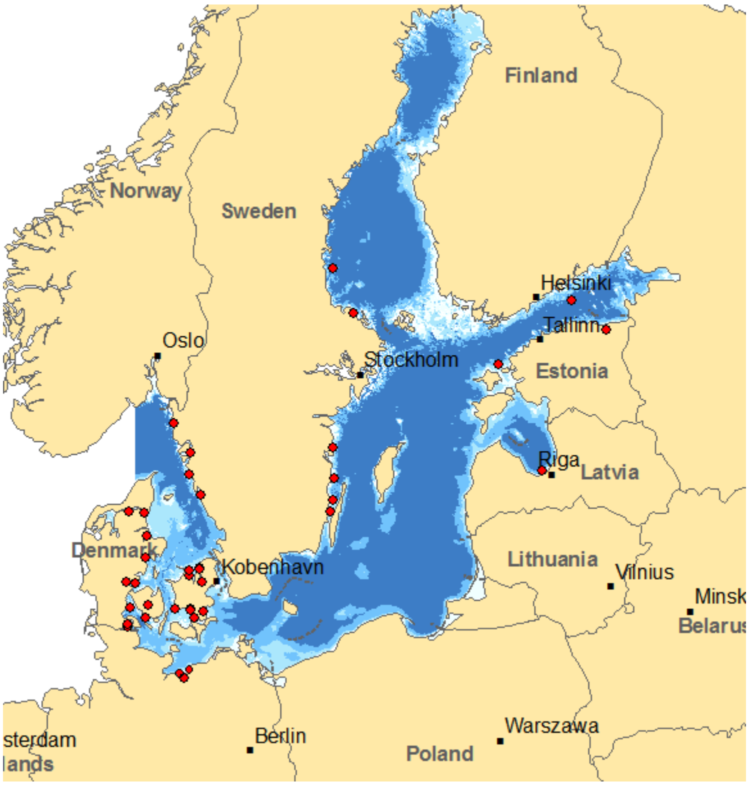
At the moment more than 30000 eelpout results from the period from 2003 to 2010 have been submitted to the BonusHaz database from Swedish west coast (4 stations), Swedish east coast (4 stations), Botnian Bight (2 stations), Danish Belt Sea (20 stations), German Baltic Sea (3 stations), Gulf of Riga (2 stations). The locations on sampling stations with eelpout data in the BonusHaz database has been visualised at a GIS map (Figure 1.), which has been made available from a public web site (http://dmugisweb.dmu.dk/BALCOFISH/Default.aspx). Here the eelpout stations can also be compared to extract of the locations of existing stations with environmental data in the ICES database.

The data of biological effects measures in the BonusHaz database include both data on several contaminant-specific biomarkers, general effects biomarkers, endocrine disruption and reproductive success.

The data on contaminants levels in eelpouts from the Baltic Sea are all generated within the BALCOFISH project and include following groups of important hazardous substances, i.e. Dioxins, Furans, Coplanar PCBs, PBDEs, organotins, trace metals, phenols and PFCs.

There is at the moment some restriction on the use of data, as they are only available for BALCOFISH partners for now, but in 2012 eelpout data for several of the reported parameters are intended to be public available after submission to ICES database. The location of BonusHaz database is Arhus University, Dept. Bioscience, Denmark (E-mail: bonushaz@dmu.dk), and the BALCOFISH contact persons are Jakob Strand, Aarhus University, Dept. Bioscience, Denmark (E-mail: jak@dmu.dk) and Lars Förlin, Göteborg University, Sweden, (E-mail: lars.forlin@zool.gu.se).

Figure 1. GIS-based illustration of the locations on sampling stations with eelpout data in the BonusHaz database.



WP2 Development of new tools for studying contaminants effects in eelpout in the Baltic Sea

In work package 2 the purpose has been to develop new molecular tools to enable an improved ability to monitor, detect and understand effects of contaminants on coastal fish populations in the Baltic region. The work has been divided into three tasks, involving 1) the development of gene expression micorarrays, 2) tools for studying genetic diversity, and 3) a search for sex–specific genetic markers.

Our successful effort to sequence the hepatic transcriptome of the eelpout through 454-pyrosequencing, provided a crucial basis for developing tools for gene expression analyses in this biomonitoring species. This involves both quantitative PCR analyses as well as microarrays. From this sequence data, we developed two different DNA microarrays, using two different technical platforms with up to 135,000 oligonucleotide probes per array. The performance of the DNA microarray performance has been validated through comparisons with quantitative PCR and the excellent correlation provides confidence to gene expression data generated by microarray (Fig 2). Validated, sensitive and specific eelpout mRNA microarrays as well as specific primers for quantitative PCR are therefore now available for field studies of contaminant effects in Baltic coastal fish.

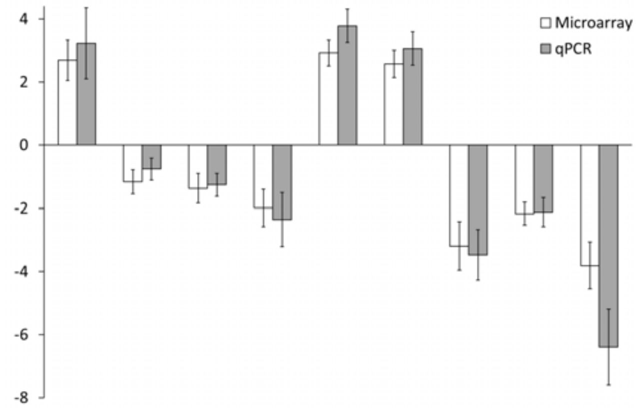


Figure 2. Validation of eelpout microarray-analyses based on a comparison of the fold-change of 9 selected genes between fish from two costal sites. The agreement of array data with quantitative PCR, which is considered the "golden standard methodology", is excellent.

Previous studies on eelpout population genetics has focussed on isozyme variability. Here, four additional methods were developed and evaluated in order to improve our abilities to understand migration and selection pressures from different pollution sources. These addition methods include 1) Amplified Fragment Length Prolymophism (AFLP) – whole genome analysis, 2) development of microsatellite markers (in collaboration with Trier University), 3) analyses of allele-diversity of the phylogenetic gene Cyt b, and 4) analyses of the diversity of the detoxifying gene CYP1A. Clearly the most successful approach of the four was the microsatellite strategy, and this methodology was chosen for further validation and implementation. All in all, ten microsatellite loci were isolated and characterized. All loci were polymorphic. The observed and expected heterozygosities ranged from 0.257 to 0.943 and 0.239 to 0.909 respectively in eelpout form the Baltic. Except for one, all loci were in Hardy-Weinberg equilibrium.The microsatellite loci characterized in this study will contribute to a better knowledge of migration processes and gene flow between different populations of this species in the Baltic. These efforts also provided "ready-to-go" genetic populations tools for the project sampling complementing the earlier developed tools of isozyme variability.

As skewed sex ratios of eelpout embryos have been found frequently at polluted sites in the Baltic, we searched for a genetic basis for sex determination which potentially could be used to investigate if there are indeed are phenotypic males (induced by pollutants) with a female genetic background (sex-reversed individuals). Homology searches for known genetic sex markers as well as explorative attempts using Randomly Amplified Polymorphic DNA did not reveal any genetic sex markers in the eelpout, required for providing conclusive evidence for sex reversal. It should however be stressed that is not unusual among fish that genetic sex markers are not present or found despite decades of searches for such markers, perhaps reflecting the larger sexual plasticity and possibly vulnerability to pollutants of certain fish regarding the processes controlling sexual development.

WP 2 publications and reports

Albertsson E, Rad A, Sturve J, Larsson DGJ, and Förlin L.Carbonyl reductase in eelpout and rainbow trout treated with paraquat and β-naphthoflavone. (Submitted manuscript) (also relevant to WP 3)

Asker N, Kristiansson E, Albertsson E, Larsson DGJ and Förlin L. Hepatic transcriptome profiling indicate altered expression of apoptosis and immune related genes in eelpout (*Zoarces viviparus*), caught near Gothenburg harbor. (Manuscript) (also relevant to WP 3)

Bergek S, Franzén F, Quack M, Hochkich A, Kinitz T, Prestegaard T and Appelberg M. Panmixia in *Zoarces viviparus* L. in the Baltic Proper: Implications for environmental monitoring studies. (Under revision in J. Fish Biol.) (also relevant to WPs 3 and 5)

Kinitz, T, Quack, M, Hochkich A, Hankeln T, Bergek S, Prestegaard T and Veith M. Isolation and characterization of ten polymorphic microsatellite loci for the eelpout, *Zoarces viviparus* (Linnaeus, 1758) Molecular Ecology Recourses (in press). (also relevant to WPs 3 and 5)

Kristiansson E, Asker N, Förlin L and Larsson DG. (2009) Characterization of the *Zoarces viviparus* liver transcriptome using massively parallel pyrosequencing. BMC Genomics. 31;10:345.

Larsson DGJ, Kristiansson E, Asker N, Förlin L (2012) A search for sex specific genetic markers in eelpout (Scientific report, BONUS/BALCOFISH)

WP3 Applying existing and new tools in field studies of eelpout in contaminated coastal sites in the Baltic Sea

The BALCOFISH project has performed two large field sampling campaigns: one in the spring and one in the autumn of 2009. In the field campaigns biomarker and fish health responses were investigated in eelpout from a number of different coastal sites in four different Baltic regions in Sweden, Germany and Denmark. The investigated sites were selected to cover sites with no or low local sources of human activities (used as reference sites) and sites located close to urban activities such as in the vicinity of large cities (harbours) and industries. In work package 3 the current environmental contaminants situation and contaminants effects observed in eelpout in different Baltic coastal regions has been investigated. The work package has been divided into seven tasks. A major effort has been set on exploring biological effects and includes methodology for measurement of biomarker responses, reproductive success and population genetics. In collaboration with other projects e.g. BONUS BEAST, the Danish FORMÅL project and the Swedish Integrated Fish Monitoring project, other sampling sites in the Baltic region and parameters have also been measured on eelpout during these studies.

Monitoring biomarker responses in eelpout from different coastal sites

Research resources were combined to test a broad set of biomarkers in eelpout in differently polluted coastal sites in the Baltic Sea. The results include response of biomarker for detoxifications (CYP1A/EROD), endocrine disruption (e.g vitellogenin), oxidative stress (e.g. the antioxidant defences system and oxidative damages), and levels of retinoids/vitamin A. Results also contain information about PAH content in bile and morphometric data. The results indicate both site specific differences in these biomarker response parameters within each region, especially within the Swedish west coast sites and the Danish sites, but also differences between the regions as well as seasonal differences were observed. For example the results of the within region comparison indicate, as expected, highest EROD activities and content of PAH in eelpout from the contaminated sites in the Swedish west coast and in the Danish regions. The regional differences showed for example a lower AChE and EROD activities in the German fish when compared especially to the Swedish regions. This indicates differences in contamination of chemicals seemingly being neurotoxic and compounds possibly affecting reproduction and/or being mutagenic in these two regions. The health status of eelpout has also been assessed in collaboration with the Swedish Integrated Fish Monitoring project where eelpouts in Swedish costal sites have been studied for more than two decades. Results indicate time trends with for example successively increasing EROD activities, changes in biomarkers indicating oxidative stress and increasing numbers of white blood cells. The latter is possibly indicating immune toxic effects in the fish.

Pathology in eelpout and larvae malformation

Based on earlier findings about high incidence of intersex in male eelpout from German coastal waters it has been a task of the BALCOFISH project to monitor this gonadal disorder in sub-regions of the Baltic Sea as well. In total 488 testes from 4 German, 5 Danish and 4 Swedish stations were microscopically analyzed for the presence of intersex (ovotestis). It became apparent that this pathological alteration was present in samples from every sub-region. On average about 30% of eelpout from German coastal stations showed intersex whereas 39% of Danish eelpout were affected. In contrast at stations from the Swedish west coast on average only 9% of male eelpout possessed intersex. This finding leads to the conclusion that eelpout at German and Danish are more impacted by endocrine active compounds than their fellow species in Swedish waters.

Table 1. Prevalence and severity of intersex in male eelpout from eastern Baltic Sea areas

Country	Station	[n]	Intersex [%]	IS Index	IS-Index [Median]	Mean prev. [%]
Germany	Salzhaff	66	**25,8**	1,32 ± 1,34	0,83	29,7
	Wismar	67	**34,3**	0,67 ± 0,98	0,17	
	Eggers Wiek	42	**28,6**	1,15 ± 1,12	0,65	
	Fehmarnbelt	10	**30,0**	0,11 ± 0,06	0,11	
Denmark	Fredericksvaerk	34	**50,0**	0,95 ± 1,11	0,56	39,1
	Roskilde	33	**24,2**	0,99 ± 1,13	0,67	
	Agersoe	45	**37,8**	0,53 ± 0,60	0,17	
	Karrebaek	24	**54,2**	1,17 ± 1,39	0,39	
	Isefjord	31	**29,0**	0,30 ± 0,45	0,11	
Sweden	Fjällbacka	44	**6,8**	1,22 ± 1,97	0,11	9,0
	Stennungsund	29	**13,8**	1,01 ± 1,70	0,22	
	Göteborg	45	**4,4**	0,08 ± 0,04	0,08	
	Vendelsö	18	**11,1**	0,47 ± 0,43	0,47	

In the course of eelpout male gonad histology it was noticed that macrophage aggregates (MA) were present at varying frequencies in eelpout samples from different stations. MA are distinctive clusters of cells part of the immune defense, which increase in size or frequency in e.g. conditions of environmental stress. On average gonadal MA were more prominent in Danish eelpout compared to German and Swedish specimens. The regional variability of MA leads to the conclusion that gonadal MA are putative tissue biomarker of environmental stress like this is known from MA in liver and spleen.

Reproductive disorder is a valuable biomarker when studying the effects of environmental pollutants. The viviparous eelpout is unique suitable in the studies of reproductive success as effects in individual mother fish can be correlated to the respective broods. In total, 669 female eelpouts were collected during the 2009 autumn campaign and the individual broods were analyzed. The findings revealed that pathological disorders of eelpout broods were more frequent at German and Danish coastal stations. Samples from the Swedish west and east coast showed variable prevalences of malformed larvae in broods. However, on average this pathology was clearly lower compared to German and Danish waters. The BALCOFISH data about eelpout reproductive disorders were complemented by corresponding data of the BEAST project. Overall the eelpout has proven to be as a valuable bio-indicator for the monitoring of different kinds of reproductive disorders in fish. This feature supports the recommendation of the HELCOM CORESET project to select the eelpout as a core bio-indicator for monitoring in the Baltic Sea.

In collaboration with the Danish FORMÅL project, the use of biomarkers to assess the biological mechanisms behind elevated levels of deformities in eelpout larvae in Danish waters was studied. The biomarkers were content of vitamins (A, E and B), steroids, PAH-metabolites and lipid, as well as EROD activity in eelpout. The responses of many of the biomarkers were different at the impacted sites compared to the reference site, especially for the vitamins, steroids, CYP1A and PAH-metabolites. There are indications that the cause of deformities could be related to lower levels of vitamin A, E and B and the most sensitive time-period seems to be from the time that eggs are developed until the fry are hatched.

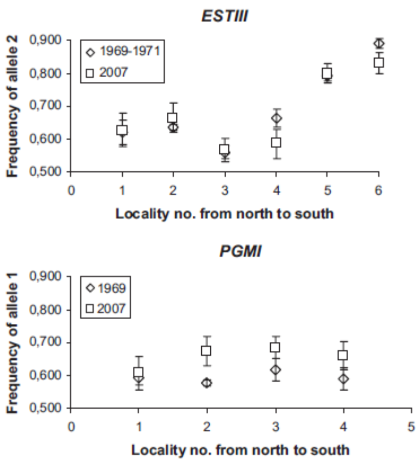
Population genetics in different Baltic eelpout populations

With the purpose to assess population genetics three methods have been applied in different Baltic eelpout populations on different spatial scales. The methods make use of AFLP, isozymes or microsatellites.

AFLP is a DNA fingerprinting technique used in population genetics. In order to study whether contamination can cause a reduced genetic diversity, studies was performed on eelpout sampled during the same two BALCOFISH sampling campaigns. AFLP-analysis was performed on four different populations, two from Denmark (Roskilde fjord and Isefjorden) and two from Germany (Saltzhaff and Wismar bay). The within population diversity was between 44.8 and 48.9 % similar, with Isefjorden having the lower similarity and Wismar bay the highest. Wismar bay is the site presumed to be the most contaminated and Isefjorden the least contaminated, indicating that contamination could be correlated to a reduced diversity. However, further analyses are needed to explore the potential of this method.

In the next population genetic study the frequency and variability of specific isozymes were used to assess the population diversity. The five enzymes used, representing 10 loci showed a high preservation in six Danish eelpout populations. A strong congruence in the genetic composition was found between the samples collected 36–38 years ago and those collected today. In addition, a high homogeneity was observed between the offspring and the mothers as well as between offspring and all adults. Significant shifts in allele frequency were observed for some of the enzymes, but the total analysis showed that the eelpout populations must be considered as stationary.

Figure 3. Frequency of esterase (ESTIII) allele 2 and phosphoglucomutase (PGMI) allele 1, measured in two periods (1969–1971 and today). The bars are standard deviation of the estimated allelic frequency.



In a third study the genetic population structure of the eelpout was investigated by using microsatellites. Samples were collected at ten sites in the Baltic Proper, covering a distance of approximately 90 km. Ten newly developed microsatellite loci were used to infer the population structure. No global spatial genetic differentiation was found (global FST = 0.0001; Dest = -0.0003), indicating a strong gene flow at this scale, neither any clear pattern of isolation by distance was found. The results suggest that gene flow among the studied populations of *Z. viviparus* is stronger than usually thought, which might be caused by environmental homogeneity. This is important for planning and evaluating monitoring activities in this species and for the interpretation of ecotoxicological studies. Strong migration might lead to wrong conclusions concerning the pollution in a given area. Therefore, reference stations in this area should be placed at a larger distance than presently practiced.

The three methods applied to describe the genetic population structure in eelpout show different potential to assess the spatial differentiations. The study with isozymes showed that the eelpout populations must be considered as stationary, whereas the study using microsatellites suggest that gene flow among the studied populations of *Z. viviparus* is stronger than usually thought. This discrepancy might depend on the sampling areas and the spatial scale, which differs between these two studies. Concerning the AFLP analyses, they indicated that contamination could be correlated to a reduced diversity. However, further analyses are needed to explore the potential of the AFLP method.

Application of gene expression studies in eelpout in different Baltic populations

To explore molecular responses to pollutants and search for new potential biomarkers, global hepatic gene expression analysis has been performed. In total, 16 different sampling sites located in Sweden, Denmark and Germany including 160 female eelpout were individually analysed. Gene expression studies were also performed on collected larvae from the same individual mother fish from 5 of the sites and 50 additional analyses were performed (10 larvae/sites). All studies were performed on liver tissues. Preliminary analysis on the whole data set using within-region comparison show that eelpouts from different polluted sites had different gene expression profiles for e.g. cytochrome p450's, a large family of detoxification enzyme, possible reflecting the different types of exposures and stress conditions in these sites. Results also indicate that the gene expression profiles of female eelpout from polluted or pristine site are more similar than the gene expression profiles compared between female and her respective larvae e.g. genes involved in xenobiotic and lipid metabolic processes are found in females and DNA replication and tissue morphogenesis are pronounced in larvae. Despite these differences there are also genes and processes that do have similar expression patterns for female and larvae which are also unique for specific sites. This is of great interest when studies of maternal transfer of e.g. specific pollutants are been performed. Preliminary data also indicate that females with a high reproductive success (i.e. producing large amounts of healthy larvae) and females with low reproductive success (i.e. with low amount of living larvae in her brood) have different gene expression profiles thus these findings can be used in understanding mechanisms behind reproductive success of individual females and how some females can "protect" her brood.

Although we have not finalized the analyse of the entire data set, gene expression study including complete data analysis has been evaluated on female eelpout capture in two of the 16 sites i.e. Göteborg harbour and the reference site on the Swedish west coast, using our eelpout DNA microarray. Studies indicate that the eelpout in Göteborg harbour have an impaired innate immune system which might affect their response to e.g. bacterial infections. Results also showed that genes known to be expressed during apoptosis and in response to

DNA damage were found in a higher level in eelpout from the harbour, probably in response to pollutants. These findings indicate that gene expression analysis constitute an important exploratory complement to traditional environmental monitoring as both the status of the innate immune system and the apoptotic response could be potential biomarkers to be included in future biomonitoring studies.

Gene expression studies on eelpout have also been performed using quantitative PCR. The enzyme carboxyl reductase (CBR) was earlier identified in proteomic studies on fish after exposure to sewage effluent. CBR is known to be involved in the protection of oxidative stress and thus its potential use as a biomarker was evaluated. Expression of CBR was assessed using quantitative PCR performed on eelpout from the 16 sampling sites in Sweden, Denmark and Germany and also assessed in an exposure studies. Results indicate that CBR is as a promising biomarker of exposure, at least in acute exposure studies.

Comparative studies with stickleback in different coastal sites in the Baltic

With the purpose to compare the results from the BALCOFISH eelpout studies with other fish species found in the Baltic Sea, wild adult three-spined stickleback were caught from several locations at the Swedish west and east coast, in Denmark and from several locations on the German coast. The sampling took place during the late spring and in the summer and both male and female were caught. Tissue samples (mainly gonads but also liver and spleen) were taken for histopathological examination. The results obtained so far from this examination does not reveal any major differences in stickleback caught at the different locations. In particular, a special effort was made to examine the testis of male stickleback living at the same stations as eelpout for the presence of intersex. In contrast to findings concerning eelpout no signs of intersexuality were observed in sticklebacks. This result demonstrates that eelpout is particularly susceptible to develop intersex and as such can indicate the impact of endocrine disrupters.

Contaminant /congener patterns in fish from various coastal sites

In this work package it has also been the purpose to explore contaminants exposure by performing chemical analysis on fish. Eelpout adults and larvae were therefore collected from a number of the BALCOFISH coastal sites, including both contaminated and reference sites, as well as coastal sites from the eastern part of the Baltic. These samples were provided by the BONUS BEAST project. The fish samples were analyzed for 7 different contaminant groups ranging from dioxins to phenolic compounds. Results showed no obvious spatial patterns between the areas investigated. However, a higher concentration of the contaminant groups were seen in Danish sites compared to the other sites. With the exception of certain perifluorinated compounds (i.e. PFASs), higher contaminant loads were usually seen in contaminated compared to reference sites, which was expected. Maternal transfer of contaminants from adult female eelpout to larvae was apparent. From the results it can be suggested that this screening of a variety of selected chemicals generally show somewhat higher contaminants levels in costal sites classified as contaminated. Generally these sites also show more indications of biomarkers responses. Because, however, no obvious or small patterns were apparent across the sampled region, and as many of the selected contaminants were below detection limit in the eelpout from some sites, it seems as if eelpout is not an ideal species for regular monitoring for some of the selected and analyzed contaminants in the Baltic region.

As an additional deliverable, plasma samples from 160 individual eelpouts from 16 sampling sites in Sweden, Denmark and Germany have been analysed for 115 pharmaceuticals using an established liquid chromatography tandem mass spectrometry method (LC-MS/MS) in collaboration with excellent analytichal chemistry lab at Umeå University. Final evaluation of the data is on-going but low levels of a few pharmaceuticals have been detected in some individual samples. Even though the levels are low (ng/ml), the fact that they can be measured at all in coastal Baltic Fish is noteworthy, and warrant a further investigation. Additional information on the impact of these anthropogenic emissions on coastal waters and exposed biota is therefore needed.

In conclusion

From the performed activities in work package 3, we can conclude that there are relatively large differences in biomarker signals e.g. EROD/CYP1A activities, reproductive performances including e.g. larvae malformations and gonad disorders e.g. intersex between the four different regions in Germany, Denmark and Sweden. This data provides strong evidences for large differences in contaminants load between these areas. The analyses of the selected groups of contaminants in the eelpout do not however indicate any marked differences in the between the different geographic regions. The reasons for this apparent discrepancy is not known but likely explanations can be that the selected chemicals do not accurately reflect the contaminant composition in the areas and/or different sensitivity to contaminants of eelpout populations from different regions in the Baltic Sea, and/or other abiotic differences e.g. salinity and temperature and biotic differences including different feeding strategies and genetic differences. In fact, the BALCOFISH results seem to indicate genetic population differences, but this needs to be further explored. These BALCOFISH results fully support that the field monitoring strategy applied here is useful in assessing impact of contaminants in coastal Baltic waters but care must be taken to size of geographic area, selection of biological endpoints as well as to possible genetic and other differences between eelpout populations from different parts of the Baltic Sea.

WP 3 publications and reports

Albertsson E, Rad A, Sturve J, Larsson DGJ, and Förlin L.Carbonyl reductase in eelpout and rainbow trout treated with paraquat and β-naphthoflavone. (Submitted manuscript) (also relevant to WP 2)

Albertsson E. From Proteomic Analysis to Biomarkers Application. Studies of Carbonyl Reductase in Fish. ISBN: 978-91-628-8348-5. PhD-thesis from University of Gothenburg. Available on line: http://hdl.handle.net/2077/26664

Albertsson E, Gercken J, Strand J, Asker N, Bergek S, Holmqvist I, Kamman U, Parkkonen J and Förlin L. Biomarker responses in eelpout from different coastal sites in Sweden, Denmark and Germany. (Manuscript)

Asker N, Kristiansson E, Albertsson E, Larsson DGJ and Förlin L. Hepatic transcriptome profiling indicate altered expression of apoptosis and immune related genes in eelpout (*Zoarces viviparus*), caught near Gothenburg harbor. (Manuscript) (also relevant to WP 2)

Asker N, Kristiansson E, Larsson DGJ and Förlin L. Analysis of global hepatic gene expression I wild eelpout (*Zoarces viviparus*): correlation to reproductive output, chemical exposure and biomarkers. (Manuscript)

Asker N, Kristiansson E, Larsson DGJ and Förlin L. Comparison of gene expression patterns between mother fish and embryos of the viviparous eelpout (*Zoarces viviparus*). (Manuscript)

Bergek S, Franzén F, Quack M, Hochkich A, Kinitz T, Prestegaard T and Appelberg M. Panmixia in *Zoarces viviparus* L. in the Baltic Proper: Implications for environmental monitoring studies. (Submitted manuscript to J. Fish Biol) (also relevant to WPs 2 and 5)

Bergek S, Ma Q, Vetemaa M, Franzén F and Appelberg M. From individuals to populations - Impacts of environmental pollution on natural eelpout populations. Ecotoxicology and Environmental Safety (accepted) (also relevant to WP 5)

Dahllöf I and Strand J (2010). Miljøfarlige stoffer i ålekvabbe - Delrapport I. Report the Danish Nature Agency, Ministry of the Environment, Denmark (In Danish).

Dahllöf I and Strand J (2010). Biomarører in ålekvabbe – Delrapport II. Report from the Danish Nature Agency, Ministry of the Environment, Denmark (in Danish).

Dahllöf I, Strand J, Gustavson K, Bjerregaard P. (2011). Miljøfarlige stoffer og ålekvabbe - Samlet analyse. Report from the Danish Nature Agency, Ministry of the Environment, Denmark, in press (In Danish).

Gercken J, Strand J, et al., Widespread occurrence of intersex in eelpout (*Zoarces viviparus*) from German coastal waters and different Baltic Sea sub-regions. (Manuscript)

Gercken J, Strand J, Franzén F, and Parkkonen J, Large-scale assessment of impaired reproductive success of Baltic Sea eelpout (*Zoarces viviparus*). (Manuscript in preparation)

Kinitz, T., Quack, M., Hochkich, A., Hankeln, T., Bergek, S., Prestegaard, T. and M. Veith. Isolation and characterization of ten polymorphic microsatellite loci for the eelpout, *Zoarces viviparus* (Linnaeus, 1758) Molecular Ecology Recourses, (in press). (also relevant to WPs 2 and 5)

Tairova ZM, Strand J, Chevalier J, Andersen O. (2011). PAH biomarkers in common eelpout (*Zoarces viviparus*) from Danish waters. Marine Environmental Research. (In Press, Corrected Proof, Available online 24 September 2011, PS)

Simonsen V and Strand J. (2010). Genetic variation of *Zoarces viviparus*: six populations revisited after about 35 years. Hereditas 147: 250–255

WP4 Confirming laboratory studies and species comparison

The purpose of work packages 4 has been to explain chemically-caused effects observed in field studies on feral fish with laboratory studies using the model fish species zebrafish and the native three-spined stickleback. The work package has been divided into two tasks.

The methodology included exposure to adult zebrafish feed spiked with a mixture of structurally diverse BFRs (brominated flame retardants) to investigate accumulation from feed, maternal transfer, and effects on reproduction and early life-stage development. One of the compounds in the mixture, i.e. 2,4,6-tribromophenol, was tested separately. The BFRs were also tested individually in an embryo toxicity test, to screen for effects of waterborne BFRs on early life stages. To investigate effects of PBDDs (polybrominated dibenzodioxins) on reproduction, early life-stage development, adult zebrafish were exposed to feed spiked with 2,3,7,8-tetraBDD (TBDD), or a mixture of PBDDs that was designed to reflect relative concentrations found in Baltic Sea biota.

Most of the brominated compounds detected in female fish were also found in their offspring, and may thereby affect the fish embryos at a critical period during development. For the BFRs in the mixture, hydrophobic compounds were transferred from the female to the developing eggs to a higher extent than less hydrophobic compounds. For the PBDDs, no relation between transfer and number and position of the bromine atoms could be found, nor could a relation between transfer and hydrophobicity. Exposure to both BFRs and PBDDs significantly changed ovarian morphology, and the histopathological alterations were in general the most sensitive reproductive endpoint. Among the reproductive output parameters, the most sensitive was fertilization success.

Although most of the brominated compounds were maternally transferred, minor effects were in general seen on early life-stage development in offspring. However, the reduced hatching success in offspring from adult zebrafish dietary exposed to the BFR mixture shows that maternal transfer of BFRs may affect early life-stage development (reproductive success) in fish. Overall, the results suggest that maternal transfer is an important exposure route for several of the brominated environmental chemicals and that these compounds may interfere with reproduction and early life-stage development in fish.

Based on the results from the zebrafish, three-spined stickleback were exposed to the same mixture of brominated dioxins used in the zebrafish study. Juvenile three-spined sticklebacks were caught in the Baltic Sea in the vicinity of Öregrund in November 2010. The sticklebacks were then exposed for either 2,3,7,8-tetraBDD (TBDD) or the mixture of 12 PBDD for 2 months, during the period of sexual maturation. The zebrafish were exposed to a low, medium and high concentration of the brominated dioxins and the stickleback were only exposed for the low concentration feed in order to mimic natural conditions. After the exposure period the stickleback were sampled for chemical analysis, histopathology, EROD analysis and gene expression. Preliminary results from the stickleback study indicate increased EROD concentration in the group exposed to TBDD. No change in gene expression was recorded between the control and exposed groups, which may indicate that the stickleback is less sensitive to PDDDs and TBDD compared with the zebrafish.

WP 4 publications and reports

Arnoldsson K, Norman Haldén A, Norrgren L, Haglund P. (2012) Retention and maternal transfer of environmentally relevant PBDD/Fs, PCDD/Fs, and PCBs in zebrafish (Danio rerio) after dietary exposure. Envir. Con. Tox (in press).

Norman Halden A, Rattfelt Nyholm J, Andersson PL, Holbech H and Norrgren L (2010) Oral exposure of adult zebrafish (*Danio rerio*) to 2,4,6-tribromophenol affects reproduction. Aq. Tox. 100(1) 30-37

Norman Halden E. (2010) Exposure of zebrafish to brominated environmental chemicals. ISBN 978-91-576-7542-2, PhD thesis SLU

Norman Halden A, Arnoldsson K, Haglund P, Mattsson A, Ullerås E, Sturve J. and Norrgren L. (2011) Retentation and maternal transfer of brominated dioxins in zebrafish (*Danio rerio*) and effects on reproduction, aryl hydrocarbon receptor mediated genes, and EROD activity Aq.Tox. 201, 150-161

Norman Halden A, Rydh J, Rattfeldt J, Andersson PL and Norrgren L. Reproductive and developmental effects in zebrafish (*Danio rerio*) exposed to structurally diverse brominated flame retardants. (Submitted manuscript)

Näslund, J, Sturve, J, Arnoldsson K, Haglund, P and Norrgren L. Oral exposure to a mixture of brominated dioxins; Effects on juvenile three-spined stickleback. (Manuscript)

Näslund, J, Gercken, J, Strand, J and Norrgren. Gonad morphology in three-spined stickleback from coastal zones of the Baltic Sea. (Manuscript)

Rattfelt Nyholm J, Norman A, Norrgren L, Haglund P and Andersson PL. (2009) Uptake and biotransformation of structurally diverse brominated flame retardants in zebrafish (*Danio rerio*) after dietary exposure. Environmental Toxicology and Chemistry 28(5):1035-1042

WP5 Linking gene to population

The aim of work package 5 has been to develop a model to simulate changes in eelpout population dynamics under different environmental scenarios. The exploration of population-level effects of contaminants is important as the outcomes may differ from those seen at the individual level. Eelpout have been used for several decades as a bioindicator for hazard substances in both the field and laboratory tests, and individual effects on reproduction have been reported. However, the influence of these effects at the population level remains unexplored.

Methods used for simulating the effects of different types of disturbances on population dynamics were based on existing and additionally sampled data on reproduction, growth and mortality to elaborate an individual based model for eelpout population dynamics. An age structured Leslie matrix population model was developed for four, non-polluted reference sites in Skagerrak, western Baltic Proper, eastern Baltic Proper and the Bothnian Sea, representing a gradient in salinity and climate.

To reveal the eelpout population structure and migration processes, ten microsatellite loci for eelpout, were isolated and characterized. All loci were found to be polymorphic and were tested in 35 individuals of one population from Swedish Baltic coastal waters. The observed and expected heterozygosities ranged from 0.257 to 0.943 and 0.239 to 0.909, respectively. The microsatellite loci characterized in this study were then used to reveal the genetic population structure of the eelpout in the Baltic Sea at a small geographical scale. Samples were collected at ten locations in one of the sites used for population model, covering a distance of approximately 90 km. No global spatial genetic differentiation was found (global F_{ST} = 0.0001; D_{est} = -0.0003), indicating strong gene flow at this scale, neither could any clear pattern of isolation by distance be found. The results suggested that the gene flow among the studied populations of *Z. viviparus* was stronger than usually thought, which might be caused by environmental homogeneity.

To simulate changes in eelpout population dynamics under different environmental scenarios, the four Leslie matrix models were parameterized using data from non-polluted eelpout populations in Skagerrak, Baltic Proper, Gulf of Bothnia and Gulf of Finland. The four sites represent an environmental gradient in salinity. Compiled life-history data revealed marked differences among sites in several of the measured variables; growth rate, fecundity, age at maturity and longevity being the most important. The effect of pollution on the eelpout populations was then simulated by combining the outputs from the Leslie matrices with data from laboratory and field studies exploring reproductive impairment in contaminated environments. Our results show that despite differences in life-history characteristics between sites, survival of early life stages (*i.e.* larvae and zero-year-old fish) was the most important factor affecting population growth and persistence in all of the four studied eelpout populations (Fig 4).

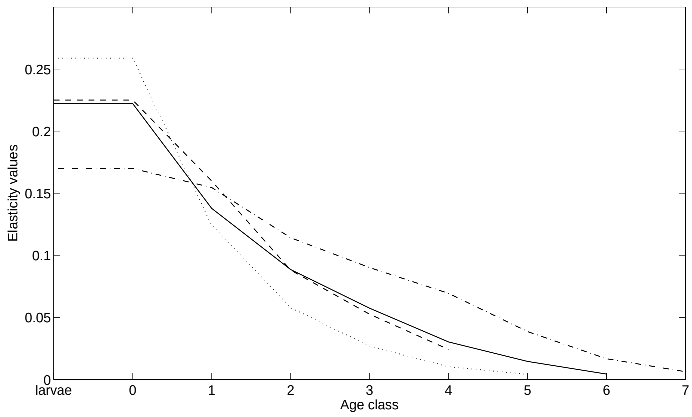


Figure 4. Elasticity analysis showing the relative contribution of the different stages to the population growth rate. Large dashed line refers to Skagerack population, small dashed to Baltic Proper, solid line to Gulf of Bothnia and dashed dot to Gulf of Finland.

The range of change in survival of larvae necessary to change population dynamics (*i.e.* population growth rate) and persistence was well within the range documented in recipient and experimental studies of chemicals and industrial waste waters. Our results suggest that larval malformation resulting from environmental pollution may have large effects on natural populations, leading to population losses and possibly even extinction. By incorporating individual-level pollutant response data, it could therefore be inferred that the documented reproductive impairment due to pollution has serious consequences not only for individuals but also for populations. However, as suggested by the genetic studies, strong migration might lead to wrong conclusions concerning the pollution in a given area.

WP 5 publications and reports

Bergek S, Franzén F, Quack M, Hochkich A, Kinitz T, Prestegaard T and Appelberg M. Panmixia in *Zoarces viviparus* L. in the Baltic Proper: Implications for environmental monitoring studies. (Submitted manuscript to J. Fish Biol.) (also relevant to WPs 2 and 3)

Bergek S, Ma Q, Vetemaa M, Franzén F and Appelberg M. From individuals to populations - Impacts of environmental pollution on natural eelpout populations. Ecotoxicology and Environmental Safety (accepted). (also relevant to WP 3)

Kinitz T, Quack M, Hochkich A, Hankeln T, Bergek S, Prestegaard T and Veith M. Isolation and characterization of ten polymorphic microsatellite loci for the eelpout, *Zoarces viviparus* (Linnaeus, 1758) Molecular Ecology Recourses, (in press). (also relevant to WPs 2 and 3).

WP6 Bridging the gap between scientists, stakeholders and environmental managers

Work package 6 has been divided into nine tasks with the purposes to ensure transfer of research needs and research outcome, and thus to benefit development of both environmental sciences and management of the Baltic Sea. BALCOFISH has a set up a website (www.balcofish.science.gu.se/english). This has regularly been updated by the webmaster Noomi Asker.

Nine BALCOFISH steering and coordination meeting have been held during the project period. Meeting places have alternated between Göteborg and Öregrund in Sweden and Sominestation in Denmark and one meeting in Berlin in Germany. Most of these workshops were "ordinary" coordination and steering meetings. The workshop in Berlin was associated to the UBA organised "Eelpout monitoring workshop" where BALCOFISH and other scientists presented and discussed monitoring activities including eelpout and other organisms to an international audience of environmental scientists and managers. At the very successful practical workshop at Sominstation in Denamrk both BALCOFISH and BEAST partners attended. Important issues discussed in details concerned standardisation of methodology for sampling and dissection of fish, and for assessing reproductive success, and the common databank for BALCOFISH and BEAST. In the section "3. Practical implementations of project outputs" is listed BALCOFISH scientists participation in relevant stakeholder and scientific both national and international committees, different national and international policy documents and action plans work.

In addition, In BALCOFISH Y1 and Y2 reports is listed dissemination activities at scientific meetings, meetings with stakeholders as well as teaching in courses given to undergraduate and graduate students. Also in 2011 the project scientists have attended a number of meetings occasions but we do not present any list for these ordinary "duties" for the BALCOFIH scientists in this final report.

Eelpout review paper

A review paper on the use of eelpout in environmental monitoring in the Baltic Sea and the results thereof has been completed and published. This review was a joint effort between UBA (German Federal Environmental Agency) and BALCOFISH. The review compiles monitoring data about the eelpout in the frame of the need to develop common assessment criteria and methodology standards for the implementation of the Marine Strategy Framework Directive.

Environmental assessment criteria and integrated biomarker assessment tool

Development of Environmental Assessment Criteria

Concerning the development of environmental indices, BALCOFISH has together with the colleagues from the BEAST project contributed to develop and recommend environmental assessment tools (e.g. Background Documents and Assessment Criteria) for suitable biological indicators for the assessment of pollution effects since eelpout is regarded as one of an important marine indicator species for the Baltic Sea. This has relevance for both future assessments in relation to for Baltic Sea Action Plan (BSAP) and the requirements of the

European Union Marine Strategy Framework Directive (MSFD) Descriptor 8 in the Baltic Sea.

A set of “Core Indicators” and “Candidate Indicators” were selected, because they describe either effects caused by mixtures of contaminants or exposure to specific contaminants. They have also been chosen because they indicate adverse effects at different biological levels of responses, i.e. molecular/biochemical/cellular levels (“early warning”), tissue/organism (morphological alterations) or reproduction/population levels (health/diseases, reproductive impairments or population-relevant endpoints) levels. Moreover, for these indicators cause-and-effect relationships have been studied and specific Assessment Criteria for the Baltic Sea, i.e. Background Assessment Criteria (BAC) and/or Environmental Assessment Criteria (EAC) have been established within BEAST and BALCOFISH based on existing and newly generated data for a range of species including eelpout. All together 10 BAC- and 8 EAC-values has been developed for biological effects in eelpout within the following biological effects indicators: PAH-specific effects, general stress caused by a range of contaminants, effects caused by genotoxic contaminants, endocrine disruption, effects of exposure to organic compounds such as PAHs, planar PCBs and dioxins, effects caused by neurotoxic contaminants and reproductive success impairments (embryo aberrations in broods) caused by a range of contaminants.

Development of Integrated Biomarker Assessment Tool (IBAT)
The BALCOFISH and BEAST projects have also cooperated in developing an Integrated Biomarker Assessment Tool (IBAT) in Excel-format (version 1), which allows comparisons of input data for relevant biological effect parameters with the BAC- and EAC-values, and thus can calculate an overall Integrated Biomarker Assessment Score (IBAS). IBAS includes the principle of using factors, i.e. weighted score values, depending on the biological response level of the respective biological effects, i.e. (M/C) molecular/cellular, (T/O): tissue/organism or (R/P): reproduction/population. By this procedure, IBAS considers the biological significance of the effects observed.

The methodology for the assessment share similarities with the principle in the indicator-based integrative assessment tool “CHASE”, which has been developed during the HELCOM “integrated thematic assessment of hazardous substances in the Baltic Sea”. Subsequently, IBAT has the potential to be incorporated as an improvement into the CHASE-tool for future assessments. IBAS has the potential to be used for both , i.e. a single species (e.g. eelpout) as a kind of health index, if enough data for relevant biological effect indicators is available, or for several species as an integrative measure for the all observed biological effects in a particular area. For both possibilities it must be considered that data of effects at all assessment classes are available for the calculations.

IBAT has been tested and evaluated by using a joined dataset obtained from the BonusHaz database covering different indicators for biological effects in eelpout at five stations in the Belt Sea. The dataset comprise data from November 2009 in combination with data for intersex from spring 2009, in total 8-10 biological effects parameters from all biological response levels. It can be assessed from the determined IBAS-values, that there is a high risk for pollution effects in eelpout at two of the tested stations, i.e. at Karrebæk Fjord and Frederiksværk, whereas at Agersø and Roskilde the risk for pollution effects is moderate, indicated as yellow. Only at Isefjord, a low risk for pollution effects is indicated (Figure 5). These risk levels of pollution effects in eelpout are reasonable taken the knowledge on existing contaminant levels at the respective stations into considerations.

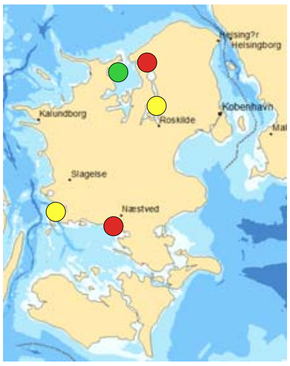


Figure 5. The result of the integrated Biomarker "traffic-light" assessment for five eelpout stations in the Danish part of Belt Sea.

ICES guidelines

During BALCOFISH project workshop and meetings, it was agreed upon that a common guideline for monitoring reproductive success in eelpout was missing, describing the methodology, parameters to be reported and relevant quality assurance issues. Other biological effects indicators like PAH-metabolites, EROD, micronuclei, AChE, intersex etc. should instead be part of the monitoring guidelines for the respective effect indicators as ICES guidelines have the aim to address the specific methodologies, which not necessarily are species-depended. The monitoring guideline for reproductive success in eelpout is based on existing monitoring guidelines in Sweden, Denmark and Germany, and therefore describes the main similarities, but also where differences in monitoring strategies might exist. This also includes description of the sampling, the reported parameters for biometrics of the adult and pregnant eelpout and the broods together with the classification of abnormal development of eelpout fry, i.e. embryo and larvae, see also outline below. However, finishing of guideline has been delayed in the course of the project period, among others because it was not clear to what extent the ICES database office will approve the new proposed codes for parameters for assessing impact on the reproductive success in eelpout.

The following outline of the guideline has been prepared to follow the ICES template for ICES Techniques in Marine Environmental Sciences (TIMES).

Title: Biological effects of contaminants: reproductive success in fish.

1. Introduction
2. Eelpout as indicator species in monitoring
3. General description of reproductive biology of eelpout
4. Collection and Station information
   - 4.1. Sampling
   - 4.2. Transportation
   - 4.3. Ethical issues
5. Laboratory analyses
   - 5.1. Investigation of adult females
   - 5.2. Investigation of broods and development of eelpout fry
6. Quality assurance
7. Data recording and reporting parameters
8. Assessment criteria
9. Relevant ICES codes for submission to the ICES database

10. References
11. Appendix 1. Specific categories for abnormal fry development

The draft guideline is now scheduled to be submitted at the end of February 2012 for Agenda item 10 at the meeting of ICES working group of biological effects of contaminants (WGBEC), March 12 - 16, 2012 in Porto, Portugal.

Educational materials

Besides a significant number of research articles, reports and other types of publications during the BALCOFISH project, a book with the title ”Ecology and Animal Health” has been produced, this as a part of a series including also two other issues ”Sustainable Agriculture” and ”Rural Development and Land use”. A major aim with these books are to describe and compare the environmental situation in the Baltic Sea and the Great Lakes, US. Discharge of nutrients from agriculture and wastewater treatment plants, as well as from industries, transportation and other human activities leads to eutrophication and other forms of pollution with impact on for instance fish health and populations. Target groups are students, teachers, experts and people working in government offices, ministries, and municipalities and as agricultural advisors and managers of different natural resource based activities in rural areas. The issue Ecology and Animal Health (approx. 350 pages) which will be published during spring 2012 includes chapters dealing with the ecosystem health in the Baltic Sea region. The concept to produce these books was presented, well recognized and supported at United Nations, New York, USA, 2009. Norrgren is the editor of the issue "Ecology and Animal Health".

Relevant eelpout data to ICES

A BALCOFISH dataset consisting of parameters for 13884 biometric and 3485 biological effects indicators, where codes exist in the ICES’s RECO database http://www.ices.dk/datacentre/reco/reco.asp, has in the end of the project period been submitted to ICES. This includes eelpout data from two German, five Swedish and eight Danish stations with data from the period 2003 – 2010.

Table 2. Data for following parameters have been submitted to ICES. The numbers of submitted individual data are in brackets ()

PARAM	PARAM_Desc	Count
AChE	acetylcholine esterase activity (Unit example: amount acetylthiocholine (ACTC)/time/amount protein)	(116)
APOPTS	apoptosis	(21)
BASFC	basophilic foci	(21)
CA	calcium	(9)
CAT	catalase activity (Unit example: micromol/min/mg protein)	(170)
CHOLA	cholangioma	(21)
CHOLC	cholangiocarcinoma	(21)
CL	chlorine	(8)
CLCFC	clear cell foci	(21)

DNAAD	DNA adducts	(10)
EOSFC	eosinophilic foci	(21)
EROD	EROD - pmol resorufin/mg-protein/min, substrate: 7-ethoxyresorufin	(619)
FIBINC	fibrillar inclusions	(21)
FIBROS	fibrosis	(21)
GLYCCV	variable glycogen content	(21)
GRANLM	granuloma	(21)
GST	glutathionine transferase (Unit example: micromol/min/mg protein)	(248)
GUTWTMEA	weight - gutted (mean or individual)	(2846)
HEMAGA	hemangioma	(21)
HEMAPS	hemangiopericytic sarcoma	(21)
HEMAS	hemangiosarcoma	(21)
HEMOSD	hemosiderosis	(21)
HEPBCM	mixed hepatobiliary carcinoma	(21)
HEPCA	hepatocellular adenoma	(21)
HEPCC	hepatocellular carcinoma	(21)
HEPCNP	hepatocellular and nuclear polymorphism	(21)
HYVCBE	hydropic vacuolization of biliary epithelial cells and/or hepatocytes	(21)
K	potassium	(9)
LNMEA	length (mean or individual) (in combination with matrix)	(2926)
LYMCINF	lymphocytic/monocytic infiltration	(21)
MELAMC	melanomacrophage centres	(21)
MXDFC	mixed foci of cellular alteration	(21)
NA	sodium	(9)
NAD	no abnormalities detected	(21)
NECRCG	coagulative necrosis	(21)
PANACA	pancreatic acinar cell adenoma	(21)
PANACC	pancreatic acinar carcinoma	(21)
PHOSLD	phospholipidosis	(21)
PROTV	protein concentration (volume) (Unit example: mg protein / ml homogenized matrix)	(341)
PYR1OHEQ	1-hydroxy pyrene equivalent	(15)
REGNR	regeneration	(21)
SPNHEP	spongiosis hepatitis	(21)
VACFC	vacuolated foci	(21)
VTG	vitellogenin (microgram per ml)	(166)
WTMEA	weight (mean or individual) (in combination with matrix)	(8112)

Data for parameters without existing ICES codes could not at the end of the project period become submitted to the ICES database. In addition to codes for some of our biomarkers, it also includes relevant codes for reproductive success in eelpout. However, BALCOFISH has been in communication with ICES during 2010 and 2011 and new codes for these parameters from the BonusHaz database have been forwarded as a proposal for ICES. These new codes await now a formal approval by the ICES expert system, before they can become valid for ICES, probably during 2012. Data for eelpout parameters without existing ICES codes are therefore at the moment only stored in the project database BonusHaz.

WP 6 publications and reports

Hedman JE, Rüdel H, Gercken J, Bergek S, Strand J, Quack M, Appelberg M, Förlin L, Tuvikene A, Bignert A. (2012) Eelpout (*Zoarces viviparus*) in marine environmental monitoring. Mar. Pollut. Bull. 62(10):2015-29.

HELCOM 2010. Hazardous substances in the Baltic Sea: An integrated thematic assessment of hazardous substances in the Baltic Sea. Korpinen, Samuli and Laamanen, Maria (Eds.) ; Andersen, Jesper H. ; Asplund, Lillemor ; Berger, Urs ; Bignert, Anders ; Boalt, Elin ; Broeg, Katja ; Brzozowska, Anna ; Cato, Ingemar ; Durkin, Mikhail ; Gamaga, Galina ; Gustavson, Kim ; Haarich, Michael ; Hedlund, Britta ; Köngäs, Petriina ; Lang, Thomas ; Larsen, Martin M. ; Lehtonen, Kari ; Mannio, Jaakko ; Mehtonen, Jukka ; Murray, Ciarán ; Nielsen, Sven ; Nyström, Bo ; Pazdro, Ksenia ; Ringeltaube, Petra ; Schiedek, Doris ; Schneider, Rolf ; Stankiewicz, Monika ; Strand, Jakob ; Sundelin, Brita ; Söderström, Martin ; Vallius, Henry ; Vanninen, Paula ; Verta, Matti ; Vieno, Niina ; Vuorinen, Pekka J. ; Zahharov, Andre. Helsinki Commission, 2010. 116 s. (Baltic Sea Environment Proceedings; 120B).

Lehtonen, Kari ; Broeg, Katja ; Strand, Jakob ; Schiedek, Doris ; Sundelin, Brita ; Lang, Thomas ; Vuorinen, Pekka J. (2010). Biological effects of hazardous substances: status and trends. In: Hazardous substances in the Baltic Sea : An integrated thematic assessment of hazardous substances in the Baltic Sea. red. / HELCOM. Helsinki Commission, 2010. s. 54-60 (Baltic Sea Environment Proceedings; 120B).

"Ecology and Animal Health" Norrgren L. editor. In a series of book issues aimed to describe and compare the environmental situation in the Baltic Sea and the Great Lake, US. To be published in spring 2012.

Jakob Strand (2011). Reproductive success in fish: eelpout (*Zoarces viviparus*),
ICES SGEH Biological Effects methods Background Documents for the Baltic Sea region
In: PRELIMINARY CORE INDICATORS FOR BIOLOGICAL EFFECTS, Agenda Item 5
Progress in the HELCOM CORESET project, presented at HELCOM JOINT ADVISORY BOARD (JAB) meeting, Third Meeting, Warsaw, Poland, 28-29 June 2011 (RT)

HELCOM 2011. Core indicators and Indicator Fact Sheets, PROPOSED CORE INDICATORS WITH BACKGROUND DOCUMENTATION AND GES BOUNDARIES concerning eelpout as indicator species for contaminant and contaminant effects. Agenda Item 4, HELCOM Monitoring and Assessment Group (MONAS), 15th Meeting, Vilnius, Lithuania, 4-7 October, 2011.

Strand J. and Broeg K. Integrated Biomarker Assessment Tool (IBAT). Excel-format for determine an Integrated Biomarker Assessment Score, version 1, January 2012. Aarhus University, Dept. Bioscience, Denmark. Software

Strand J, Appelberg M, Gercken J, Franzén F, Örn S, Förlin L et. al. Biological effects of contaminants: reproductive success in fish. ICES Techniques in Marine Environmental Sciences (TIMES). (Manuscript in prepration)

Strand 2012. BALCOFISH technical report on environmental assessment criteria and integrative biomarker assessment tools for eelpout monitoring. Aarhus University, Dept. Bioscience, Denmark.

D. Schiedek, K. Lehtonen, B. Sundelin, , T. Lang, J. Strand, U. Kamman, K. Broeg, J. Barsiene, H. Dabrowska, Application of Biological Effects Indicators for assessing Good Environmental Status in accordance with Baltic Sea Action Plan and Marine Strategy Framework Directive. (Manuscript)

3. Practical implementation of project outputs (performance statistics 1-4)

In this section is listed each BALCOFISH scientists participation in relevant stakeholder and scientific both national and international committees and different national and international policy documents and action plans work and is related to the performance statistics 1-4.

(1) List of contributions carried out to consultations by European Commission.

Anders Bignert
- CMA, (Chemical Monitoring Activity), Biota guidelines, 3 meeting occassions
- MSFD (MARINE STRATEGY FRAMEWORK DIRECTIVE) Task Group
 Descriptor 8: Contaminants and pollution effects, 3 meeting occasions
- MSFD task group Descriptor 9: Contaminants in fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards, 1 meeting occasions

Joakim Larsson
- Antibiotics in the external environment – a driver of resistance? 2012, Invited report to the EEA, to be included in an upcoming publication from the EEA on Emerging Chemicals.

(2) List of stakeholder and scientific committees where BALCOFISH scientists have served as members or observers

Magnus Appelberg
- Participating (chair) in three HELCOM FISH-PRO meetings (Tallinn, Jan 28-30, 2009; Tallinn Feb 1-3, 2010; Helsinki, Feb 7-9 2011).
- Participating in three HELCOM CORESET meetings (Helsinki Nov 3-4, 2010; Helsinki Feb 16-18, 2011; Riga June 15-17, 2011) aiming at developing indicators for the MSFD

Anders Bignert
- HELCOM CORESET Expert Workshop on hazardous substances indicators, 4 meeting occasions

Lars Förlin
- Swedish EPA and county Västra Götalands Workshop on Effect based monitoring tools and assessment criteria, Göteborg, Sweden 25-26 January 2011.
- ICES Marine Chemistry Working Group: 33rd meeting. Swedish Meteorological and Hydrological Institute (SMHI), Gothenburg, Sweden. March 3, 2011. Presented Integrated fish monitoring in coastal areas.
- EEA Workshop on Aquatic Ecotoxicology, May 5-6 2010 aiming at identifying the most urgent issues in aquaic ecotoxicology.
- Swedish EPA and Havsmiljöinstitutet, Havsmiljöseminariet 2011 in Costa Boda, May 2-3, 2011. The purpose is to present national Swedish environmental monitoring to regional stakeholders and policymakers (in Swedish).
- Serve as member of the European Research Council LS9 Panel for Starting Grants evaluation 2011

Jens Gercken
- German North Sea and Baltic Sea Monitoring Programme, Ad Hoc Working Group on Pollutants and Biological Effects, Hamburg, 29. June and December 16, 2011
- Association of German Engineers (VDI), Working Group for the development of a standard for using biological effects methods in fish. (DIN, the German Institute for Standardization) Frankfurt 24.-25. March 2011 and Heidelberg 22.-23. September 2011

Joakim Larsson
- Serve as member of Swedish reference group for JPI-AMR (Joint Programming Initiative on Anti Microbial Resistance), 2010 and 2011.
- Serve as member of FORMAS evaluation board for environmental pollutants, 2011.
- Serve as steering board member of the Centre for Marine Research at the University of Gothenburg, 2011.

Jakob Strand
- HELCOM Project for Expert Network on Monitoring and Protecting of Coastal Fish and Lamprey Species, HELCOM FISH meeting 2/2009, Tallinn, Estonia, 28-30 January 2009
- ICES WGBEC meeting, 16-20 March 2009, Weymouth Laboratory, UK
- ICES WGBEC meeting, 16-20 March 2009, Weymouth Laboratory, UK
- National stakeholder committee with Fishery organisations and local EPAs. Pollution effects in the Great Belt. 9. September 2009.
- Second HELCOM HOLAS Expert Workshop on Thematic Assessment of Hazardous - Substances, Copenhagen, Denmark, 28-29 September 2009
- Third HELCOM HOLAS Expert Workshop on Thematic Assessment of Hazardous Substances, Copenhagen, Denmark, 25-26 November 2009
- ICES Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC), ICES Headquarters, Denmark, 25–29 January 2010
- HELCOM CORESET Expert Workshop on hazardous substances indicators, Second Meeting, SYKE, Helsinki, Finland, 2-3 February 2011
- ICES Study Group of Ecosystem health (SGEH), Riga, Latvia, 11 -15. April, 2011.
- Danish EPA, The Marine Topic Center in Denmark, National monitoring of contaminants and biological effects of contaminants, NOVANA, 2009 – 2011.
- Swedish EPA and county Västra Götalands Workshop on Effect based monitoring tools and assessment criteria, Göteborg, Sweden 25-26 January 2011.

(3) List of relevant policy documents and action plans where BALCOFISH scientists has resulted in modifications.

Jakob Strand
- HELCOM CORESET/JAB/MONAS, Input to Agenda Item 4: Core indicators and Indicator Fact Sheets, PROPOSED CORE INDICATORS WITH BACKGROUND DOCUMENTATION AND GES BOUNDARIES concerning eelpout as indicator species for contaminant and contaminant effects. HELCOM Monitoring and Assessment Group, 15th Meeting, Vilnius, Lithuania, 4-7 October, 2011.

Leif Norrgren
- Swedish expert in Validation Management Group for Fish-OECD (in Paris) and Nordic Council of Ministries (Nord-Utte)

(4) List of suggestions for designing, implementing and evaluating the efficacy of pertinent public policies and governance by BALCOFISH scientists

Jakob Strand
- Danish EPA. Revision of nationwide monitoring program for nature and environment (NOVANA) 2011- 2015 concerning marine monitoring of contaminants and Pollution effects.
- Danish EPA. Miljøfarlige stoffer og ålekvabbe – samlet analyse. MIU alm. del Bilag 150, Miljøudvalget 2011-12.
- Danish EPA. Danish initial assessment for MSFD concerning contaminants and Pollution effects, 2011 - 2012.

4. Comparison with the original research and financial plan

The BALCOFISH project follows the original research and financial plan. However, the analyses of gene expression data (task 3.6) are not yet completed. The original microarray platform was not developing as fast as other competing platforms and we decided to change to the NimbleGen microarray platform. This change has now made it possible for us to study 135,000 genes per array instead of 15,000, and this for the same price. The switch to a new technology has by necessity involved extra work to optimise analytical steps, delaying us by about 9 months. We have now successfully completed the actual microarray analyses set forth, but the bioinformatic analyses and correlations of about 27,000,0000 gene expression data points with other endpoints is not yet completed. It is still our aim that the planned analyses will be done, and to reach this ambition the University of Gothenburg have dedicated internal funding to employ a scientists to work on these analyses during 2012.

In the task dedicated to search for sex specific genetic markers (task 2.3) such markers were not discovered (which was a quite possible outcome, based on studies on other species). It was therefore not possible to gather conclusive evidence for the presence of sex-reversed eelpout at contaminated sites.

In the task 3.8 we aimed to study contaminant responses in flounder caught in the same field sites as eelpouts. This task has not worked as planned. The purpose was to compare what we observed in the eelpout (our model fish species) with other fish species (the flounder) in the same coastal sites we were visiting. However, we have not been successful to get sufficient flounder material in these coastal sites enabling any relevant comparison, although we did large fishing attempts in some of the coastal sites i.e. we put large resources to get the fish material. The material has relatively little news values. Therefore we change Deliverable 3.8 from a scientific report (PS) to a scientific report (RS). In future studies other fish catching strategies are required and also careful selection of suitable coastal sites. We have applied gill and fyke nets in our fish catching activities. For flounder it seems as if trawling is the choice, but many of our visited eelpout sites are not suitable for trawling activities.

Finalising of the ICES guideline (task 6.8) has been delayed in the course of the project period. The reasons for this is among others things that it was not clear to what extent the ICES database office would approve the new proposed codes for parameters for assessing impact on the reproductive success in eelpout.

5. Statement if the research plan and schedule of deliverables had to be adapted

Since the work in Task 2.3 aiming to identify sex specific genetic markers did not reveal any such markers, this has less news value and deliverable D2.3 has been changed from a scientific publication (PS) to a scientific report (RS). Also deliverable 3.8 has been changed from a PS to a RS

Deliverables 3.1, 3.3, 3.7 and 4.1. are in the state of manuscript and it is not yet certain that they will be published as scientific reports/publications before the closer of BALCOFISH. Updates of these statuses will eventually be done, and when appropriate technical reports will be delivered. We have however in most work packages produced many more deliverables than we listed in BALCOFISH table of deliverables.

6. Further research and exploitation of the results

There is a continuous need to improve monitoring of biological effects caused by contaminants in the environment. The immediate perspectives beyond the BALCOFISH project are to develop and implement potential biomarkers obtained from the large sets of gene expression profiles of eelpout from polluted regions in the Baltic Sea in environmental monitoring programmes. The next step is also to continue the efforts to link molecular responses to pollutants with reproductive performance in the eelpout in its natural environment, to further investigate gonadal disorders (e.g. intersex) possibly caused by endocrine disruptors, to further investigate the noteworthy finding of detectable concentrations of some pharmaceuticals in plasma from Baltic coastal fish, and to further explore and implement the Integrated Biomarker Assessment tool for environmental risk assessment in eelpout. The development in omics technology will in future make it possible to find out if the chemical pollution in the Baltic eelpout can drive genetic variation which is not only of scientific interest but also has practical implications in for example planning and designing of future regional monitoring programmes. It is also a significant need to make use of new technologies to develop common methods and assessment criteria for national- and regional-scale assessment of the marine environment. This is necessary in order to establish monitoring programmes and set assessment criteria of the marine environmental statues within the Marine Strategy Framework Directives. The development of such technologies and strategies will in future be an aid in assessments of environmental impact of mixtures of toxic chemicals occurring in the Baltic Sea regions.