Reproductive disorders in amphipods as indicators of effects of hazardous substances in Danish waters

Scientific note from DCE – Danish Centre for Environment and Energy

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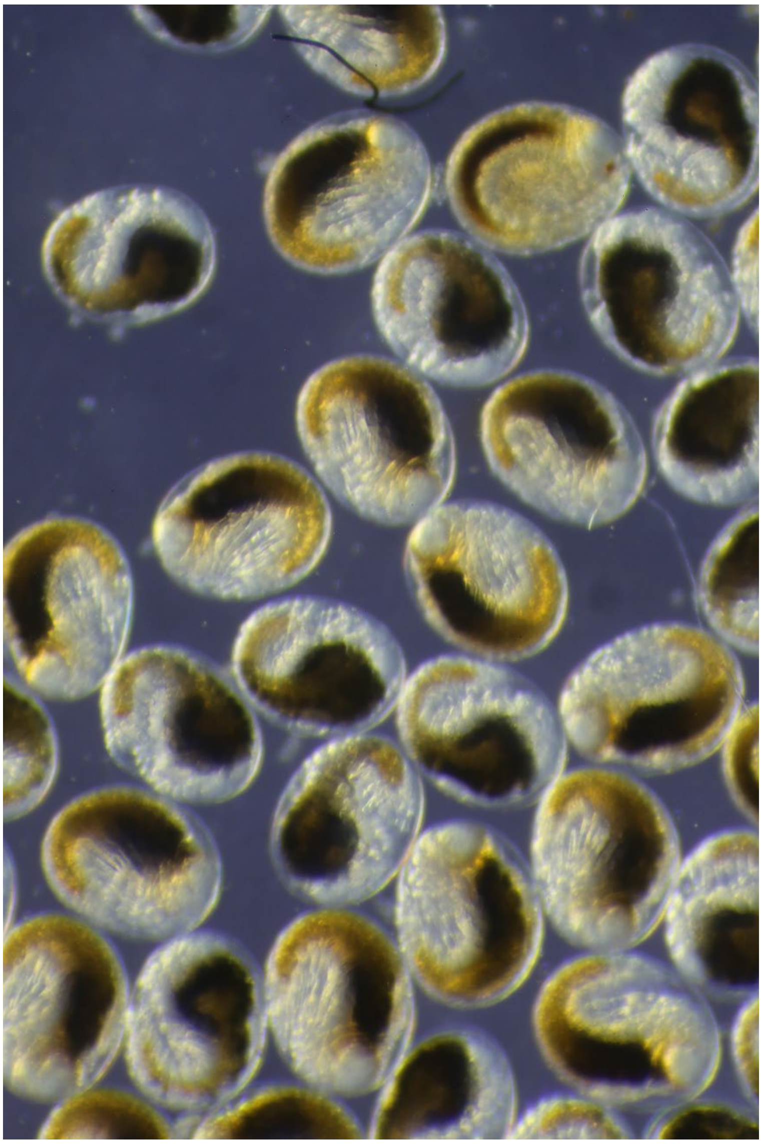

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Data sheet

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Contents

Preface/Summary		**5**
1	**Introduction**	**6**
2	**Materials and Methods**	**7**
	2.1 Amphipods	7
	2.2 Fish	8
	2.3 Data analysis	9
3	**Results and Discussion**	**10**
	3.1 Reproductive disorders in amphipods *Gammarus spp.*	10
	3.2 Reproductive success in eelpout *Zoarces viviparus.*	13
4	**Conclusions**	**15**
5	**Suggestions for future studies**	**16**
6	**Acknowledgments**	**17**
7	**References**	**18**
8	**Annex**	**19**

Preface/Summary

This scientific briefing has been prepared in response to a request from the Danish Environmental Protection Agency, Ministry of Environment, with the assignment of assessing the utility of sub-lethal effect measurements, i.e. reproductive disorders, in amphipods *Gammarus* spp., as an indicator for toxic stress from environmentally hazardous substances in Danish coastal waters. HELCOM EG HZ working group has recommended this indicator: "Reproductive disorders: malformed embryos of amphipods" as a supplementary indicator for toxic stress (HELCOM, 2018). Sub-lethal effects detected in amphipods were compared with sub-lethal effects detected in fish species eelpout (*Zoarces viviparus*), sampled at the same field locations as the amphipods.

This study was done by conducting field studies combined with laboratory microscopy analyses during the period of 2022-2023. Results are evaluated in relation to existing national and regional assessment criteria. It concluded that *Gammarus spp*. has the potential to be used as a bioindicator for Danish coastal waters due to the apparent sensitivity of its reproductive end-points and other qualities, such as abundance and ease of sampling.

1 Introduction

The current project aimed: 1) to evaluate the application of reproductive disorder measurements in amphipods as an indicator for toxic stress from environmentally hazardous substances in coastal waters and 2) to compare the results with corresponding indicators for toxic stress in coastal fish.

The project is related to the integrated environmental monitoring strategy employed under the National Monitoring Programme for the Aquatic and Terrestrial Environment (NOVANA) in Denmark and The Baltic Marine Environment Protection Commission – also known as the Helsinki Commission (HELCOM). In integrated environmental monitoring, chemical concentrations in marine biota and sediment are measured in parallel with biological effects/responses in biota. Biological responses are measured in selected species of organisms, which can be used as bioindicators for monitored areas. These bioindicator species are selected according to several parameters, which include geographical distribution, migration patterns and sensitivity of biological response to exposure for hazardous substances.

In this project, we investigated the utility of the amphipod *Gammarus spp*. as a bioindicator of toxic effects of hazardous substances in Danish coastal waters. The occurrence of embryo aberrations in amphipods was compared with embryo aberrations in fish, eelpout (*Zoarces viviparus*), sampled at the same sites.

Previously, a Danish pilot study has suggested that the amphipod *Gammarus spp*. has a potential to be used as a bioindicator for toxic effects of hazardous substances in coastal waters (Z. M. Tairova & Strand, 2022). In the current study, this method has been explored further by collecting more empirical data at polluted and reference sites.

2 Materials and Methods

2.1 Amphipods

Amphipods of the genus *Gammarus spp*. were collected in May-June in the period of 2020-2023. The results of the first two years, 2020 and 2021, have been presented earlier in Tairova & Strand (2022).

In 2022, amphipods were collected at Kalvebodløbet, Risø and Vellerup Vig, and in 2023 at two stations, Risø and Vellerup Vig. Vellerup Vig is considered as a reference site, as it is less impacted, where the other stations represent more impacted sites, which are regularly monitored under NOVANA program.

All the field sites for amphipod and fish sampling campaigns, together with NOVANA stations ("MFS biota" – sampling sites for hazardous substances measurements in biota), are presented in Figure 2.1.

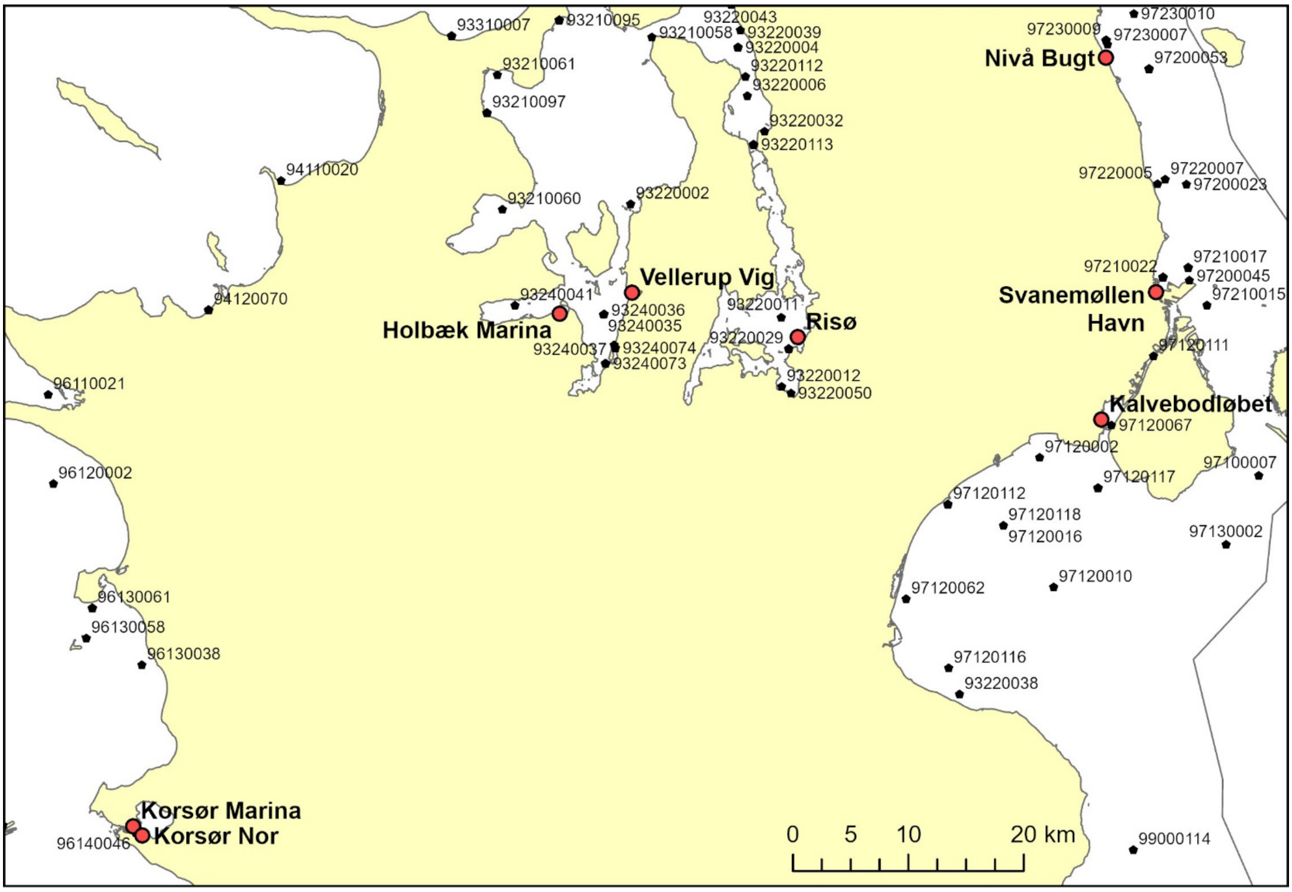


Figure 2.1. Field sites for sampling campaigns used in the period 2020-2023 (names in bold) and NOVANA MFSbiota stations (ID number/"ObservationsstedNr").

All the collected embryos were analyzed per hatch from individual gravid females from all stations, and the results were used to calculate three variables, i.e. the proportion of malformed embryos (embryos with malformations, membrane damaged embryos and undifferentiated embryos whose development halted before gastrulation), the proportion of embryos with all types of aberrations (including embryos with malformations and underdeveloped embryos, i.e. embryos at two developmental stages earlier than main stage per brood and dead embryos) and the proportion of females with more than one malformed embryo (Table 8.2). The two variables, i.e. the proportion of malformed embryos and the proportion of females with more than one malformed embryo are recommended for application and used as assessment criteria in supplementary indicators, according to the HELCOM indicator report (HELCOM, 2018). Microscopy analyses of embryo-stages and embryo aberrations were conducted as described in Fischer 2009, with some modifications (Annex: Tables 8.1-8.2).

2.2 Fish

In 2022-2023, gravid females of eelpout, *Zoarces viviparus* were collected in October-November at NOVANA stations, Kalvebodløbet in Køge Bugt and Risø in Roskilde Fjord, and the brood of each female was analysed for reproductive success. Numbers of gravid females per station/year: Kalvebodløbet in Køge Bugt in 2022 n= 46 and in 2023 n = 26; while in Risø in Roskilde Fjord the number in 2022 was n = 28 and in 2023 n = 49.

Reproductive success was measured as proportion of malformed fry (types B-G), proportion of late dead fry (type A), proportion of early dead fry (type 0) and proportion of total abnormal fry (all types) according to Technical instructions (Jakob Strand, 2013).

The eelpout (*Zoarces viviparus*) is widely used as a bioindicator for monitoring of toxic stress from hazardous substances in Danish coastal waters. This is partly because eelpout is relatively stationary in the coastal environment and because the development of embryos takes place in the female fish. Both factors make the species well-suited as a bioindicator for monitoring of environmentally hazardous substances and their effects (Jakob Strand, 2013; Z. M. Tairova et al., 2012). The hazardous substances that can affect embryo and larval development in fish include organochlorines, pesticides, polycyclic aromatic hydrocarbons (PAHs), heavy metals and organometals (Davies & Vethaak, 2012). Monitoring data for biological effect indicators can be used for both state and impact assessments and they can also be evaluated using environmentally relevant assessment criteria (ACs) developed within international scientific fora working with monitoring and assessment. The background assessment criteria (BAC) are analogous to background assessment concentrations or a natural response level. The environmental assessment criteria (EAC) represent levels of response below which unacceptable responses at higher levels, e.g. organism or population, would not be expected (Davies & Vethaak, 2012; OSPAR, 2013). These ACs have also been developed for the indicator organisms and the respective biological effect measurements deployed within the NOVANA-programme (Z. Tairova & Strand, 2021). The ACs for eelpout reported in ICES WGBEC report (2013) and OSPAR (2013) are listed in Table 2.1.

Table 2.1. Background assessment criteria (BAC) and environmental assessment criteria (EAC) for biological effect indicators in eelpout for “Reproductive success in eelpout” used in the Danish monitoring programme NOVANA (ICES WGBEC, 2013; OSPAR, 2013).

Effect indicator	BAC	EAC
Eelpout		
Mean prevalence of malformed fry (type B-G)	1%	2%
Mean prevalence of late dead fry (type A)	2%	4%
Mean prevalence of early dead fry (type 0)	2.5%	5%
Mean prevalence of total abnormal fry (all types)	5%	10%

2.3 Data analysis

Calculations of percentile, mean, median, frequency distributions and bootstrapping (100 000 runs, according to recommendation in HELCOM, 2018) were done using R© and Excel©. The map (Figure 2.1) was prepared based on stations for MSF biota extracted from OdaV2 . For data extraction R Studio 2023.06.1 and for Map production ArcGIS Pro 3.2.0 was used.

3 Results and Discussion

3.1 Reproductive disorders in amphipods *Gammarus spp.*

The embryo malformation indicator for amphipods is a multimetric indicator based on two variables measured in the sampled population: (1) the proportion of malformed embryos and (2) the proportion of females with more than one malformed embryo. In order to achieve a "good status" for an area under investigation, both variables must be below or equal to their respective threshold values (HELCOM, 2018). A third variable "proportion of embryos with all types of aberrations" was measured in this study in order to make a closer comparison with the similar variable in fish, i.e. "mean prevalence of total abnormal fry (all types)" (Table 3.4). All three variables are measured in the same pool of field-collected gravid females. As an addition to a variable "the proportion females with more than one malformation", a variable "the proportion of females with more than one aberration" was measured to explore the various variables for this indicator.

The results of reproductive disorders – mean proportions of all types of aberrations and malformations - and females with more than one type of aberration and malformation from the period 2020-2023 are shown in Figures 1 and 2.

The results from 2020 and 2021 were presented per station in an earlier technical report (Tairova & Strand, 2022), and in this scientific briefing these are presented together with data from 2022-2023 and used for calculation of threshold values only.

All amphipods underwent taxonomy analysis in the period of 2022-2023, and the results on gammarid species were 64.5% *Gammarus locusta*, 14.5% *Gammarus zaddachi*, 14.5% *Gammarus tigrinus*, 4.3% *Gammarus oceanicus* and 2.2% *Gammarus salinus* (n=138). The taxonomy analysis of amphipods from two stations in 2020 (Nivå Bugt (n=15) and Holbæk Marina (n=28)) demonstrated that 88.4% were *Gammarus locusta*, while 11.6% were *Gammarus zaddachi* (Z. M. Tairova & Strand, 2022).

In the previous study of the results from the period of 2020-2021 (Tairova & Strand, 2022), the mean and median values and the 90th percentile (which represents the threshold value) were calculated. For this calculation, two frequency distributions of variable of proportion of malformed embryos per brood for reference and impacted areas were used (Table 3.1).

Table 3.1. Threshold (90th percentiles) and mean and median values for the gammaridean amphi-pods Gammarus spp. (from Tairova & Strand, 2022).

Assessment criteria	Mean	Median	90% percentile
Proportion of malformed embryos, reference stations	0.014	0	0.041
Proportion of malformed embryos, impacted stations	0.11	0.018	0.4

Both distributions for the reference stations (Nivå Bugt and Vellerup Vig) and for all impacted stations were not normally distributed, therefore, median values and mean values were calculated and compared between impacted and reference stations (Table 3.1) and compared to thresholds for gammaridean amphipods in the HELCOM supplementary indicator report (Table 3.2).

Table 3.2. Secondary thresholds* for the gammaridean amphipods Gmelinoides fasciatus, Pontogammarus robustoides and Gammarus tigrinus (based on Gulf of Finland monitoring data, Russia) (HELCOM, 2018).

Assessment criteria	Mean	BAC	EAC	Threshold value
Proportion of malformed embryos	0.02	<0.05	>0.05	0.05
Proportion of females with >1 malformed embryo	0.15	<0.2	>0.2	0.2

* - In areas where *Monoporeia affinis*, the species used for monitoring in the Swedish National Marine Monitoring Program, does not occur naturally or is found sporadically and/or at low abundances, other amphipods with a similar life cycle and reproduction biology can be used to derive the embryo malformation indicator to establish the, so-called, "secondary thresholds" for other amphipod species belonging to gammarids (HELCOM 2018).

Resulting mean values and threshold values (Table 3.1) for the variable "proportion of malformed embryos" from baseline data (i.e. based on natural variation from reference stations, i.e. Vellerup Vig and Nivå Bugt) were below the mean values and threshold values presented in the HELCOM indicator report (see Table 3.2). Median values are lower than mean values due to not normal distribution. Mean values and threshold values from the distribution based on data from impacted stations (Table 3.1) were above the mean values and threshold values presented in the HELCOM indicator report (see Table 3.2).

In the previous study, the mean and median values and the 90th percentile were calculated using raw/untreated data on individual broods. In this study, due to the larger data set available, mean and the 90th percentile were calculated in two different ways:

- The values of mean and the 90th percentile were calculated for reference and impacted sites per station/per year, and then the median value for the area, i.e. "reference area" and "impacted area", were calculated based on the values per station/per year.
- The values of mean and the 90th percentile were calculated for reference and impacted areas in the same way as abovementioned values, while using the results of distributions from bootstrapping. The application of bootstrapping to derive distributions on data for reproductive disorders is suggested in the HELCOM indicator report (HELCOM, 2018). Bootstrapping data treatment is recommended for distribution normalization with a limited number of data-points.

The results are presented in the Table 3.3.

Table 3.3. Median of mean values (means calculated per station) and thresholds (the 90th percentile) with and without the bootstrapping of the data for the period of 2020-2023.

Assessment criteria	Median of Means	90% percentile	Median of Means (bootstrapping)	90% percentile (bootstrapping)
Proportion of malformed embryos, reference stations	0.018	0.027	0.019	0.028
Proportion of embryos with all types of aberrations, reference stations	0.018	0.0299	0.019	0.028
Proportion of females with >1 malformed embryo	0.2		0.13	0.23

The calculated threshold values (Table 3.3) are comparable with the threshold values calculated for gammaridean species in the HELCOM supplementary indicator report (Table 3.2). The threshold values for two variables, proportions of malformed and aberrated embryos (both at 0.028), are lower than the threshold value for "proportion of malformed embryos" presented in the HELCOM supplementary indicator report (Table 3.2). The threshold value for the variable, "proportion of females with more than one malformed embryo" is slightly higher, i.e. 0.23, than the threshold value for the same variable presented in the HELCOM supplementary indicator report (Table 3.2).

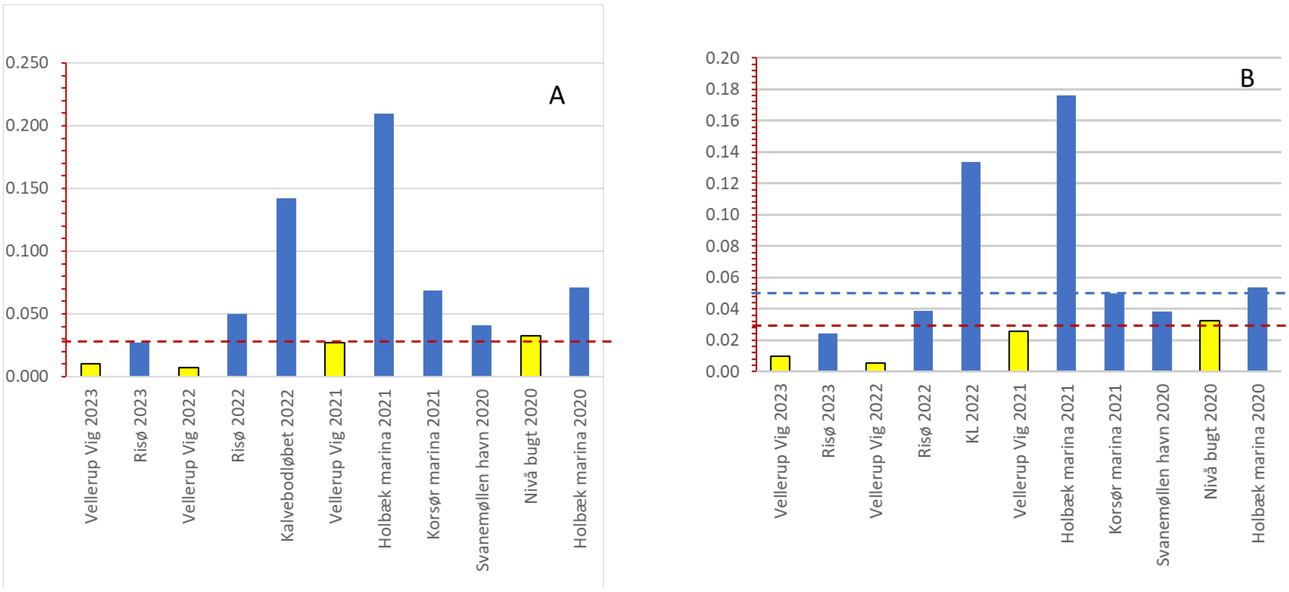


Figure 3.1. Mean proportion of all types of aberrations in embryos (A) – underdeveloped, i.e. embryos at two developmental stages earlier than main stage per brood; membrane aberrations; malformations; undifferentiated embryos whose development halted before gastrulation and dead embryos in the brood; and a mean proportion of all types of malformed embryos (B) – embryos with malformations, membrane damaged embryos and undifferentiated embryos. Vellerup Vig og Nivå Bugt (yellow columns) are the reference stations. Mean proportions were calculated by dividing the sum of all aberrations/malformations by the total number of embryos per brood). Red line: threshold value, corresponding to 90th percentile after bootstrapping, found in this study. Blue line: threshold value from HELCOM indicator report (2018).

The results demonstrate a higher response of both variables, mean proportion of aberrations and malformations in amphipods from the stations that are considered to be impacted compared to the reference stations (Figure 3.1). The response for both variables from Nivå Bugt is higher than the calculated threshold value, unlike at other reference stations, which leads to call into question the relevance of this station as a reference site.

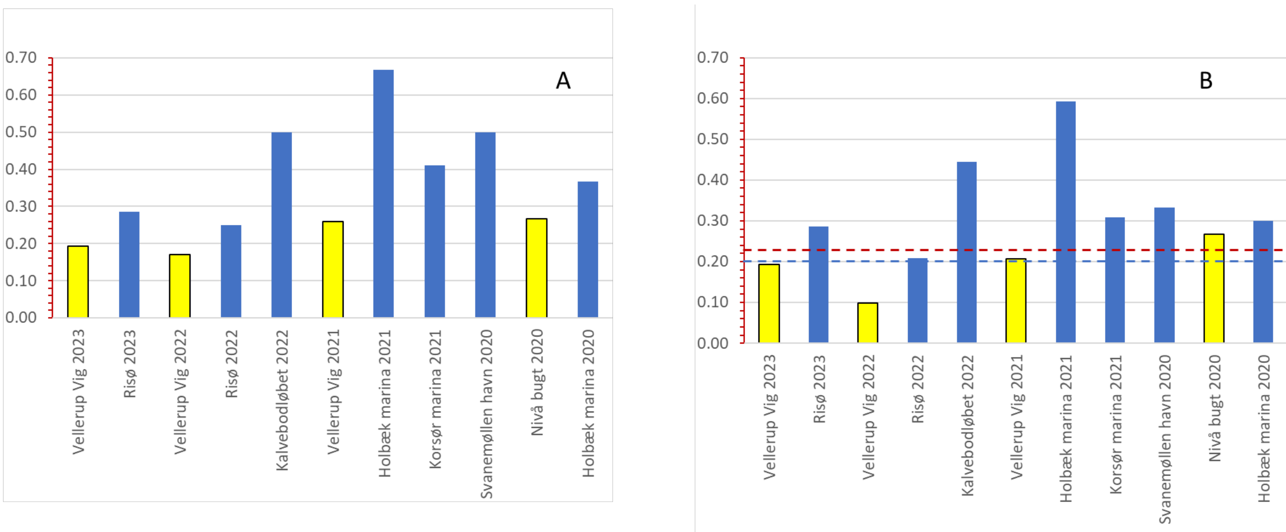


Figure 3.2. Proportion of females with more than one aberrated embryo (A) and with more than one malformed embryo (B). Proportions were calculated by diving the sum of all females with more than one aberrated/malformed embryo by the total number of embryos per brood. Vellerup Vig and NivåBugt (yellow columns) are the reference stations. Red line: threshold value, corresponding to 90th percentile after bootstrapping, found in this study. Blue line: threshold value from HELCOM indicator report (2018).

The results demonstrate a higher response of both variables, proportion of females with more than one aberration and females with more than one malformation, in amphipods from the stations that are impacted compared to the reference stations. The response for both variables from Nivå Bugt is higher than the response from Vellerup Vig measured in 2021, 2022 and 2023. Also, the response of females with more than one malformed embryo is higher than both threshold values, which leads to speculate whether this station is relevant as a reference site.

Table 3.4. Proportions of abnormally developed embryos in broods of eelpout from Roskilde Fjord (Risø) and Køge Bugt (Kalvebodløbet) in 2022 and 2023.

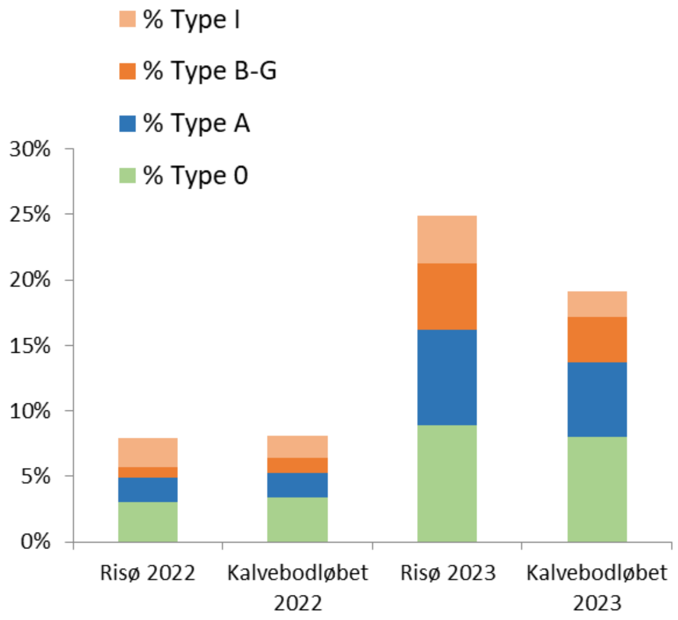
	Risø 2022	Kalvebodløbet 2022	Risø 2023	Kalvebodløbet 2023
Mean prevalence of early dead fry (type 0)	3.0%	3.4%	8.9%	8.0%
Mean prevalence of late dead fry (type A)	1.9%	1.9%	7.3%	5.7%
Mean prevalence of malformed fry (type B-G)	0.8%	1.1%	5.1%	3.5%
Dwarfs (significantly smaller embryos than the mean of the brood, Type I)	2.2%	1.7%	3.6%	1.9%
Mean prevalence of total abnormal fry (all types)	7.6%	7.9%	22.8%	17.7%

3.2 Reproductive success in eelpout *Zoarces viviparus*.

All the collected embryos were analyzed per hatch from individual gravid females from both stations. The results were used to calculate different types of abnormally developed embryos in broods of eelpout (Table 3.4).

Comparison of the values for different types of abnormal development in eelpout embryo with existing assessment criteria (Table 2.1) showed that several types of abnormal development were over BAC value at both stations in 2022, while all the types of abnormal development, including malformations (type B-G), were over EAC values at both stations in 2023 (Table 3.4). There are several potential reasons for this increase in abnormalities of all types in eelpout in 2023. A type 0 (early dead embryo) is also a sensitive indicator for low oxygen levels. Higher numbers in 2023 could be a result of higher temperature and occurrence of oxygen depletion in Danish coastal waters in the late summer-fall 2023 (Hjorth & Josefson, 2010; J Strand et al., 2004). Prevalence of malformations (types B-G) is a sensitive indicator for pollution with hazardous substances. In 2023, the levels of hazardous substances could be increased in Danish coastal waters, including at these sites, due to the higher amount of rainfall and, consequently, increase in the inflow of the hazardous substances from land this year (Rubek, 2024). Upon analyses for reproductive success, eelpouts were collected for chemical analyses under the NOVANA program, and the results of the chemical analyses will reveal levels of hazardous substances in female fish.

Figure 3.3. Proportions of abnormally developed embryos in broods of eelpout from Roskilde Fjord (Risø) and Køge Bugt (Kalvebodløbet) in 2022 and 2023, presented as mean values for different categories of aberrated development.



Comparison of the results of reproductive disorders in amphipods and fish revealed elevated responses in reproductive disorders in eelpout analyzed at two NOVANA stations, Risø in Roskilde Fjord and Kalvebodløbet in Køge Bugt (Figure 3.3), as was observed with amphipods.

4 Conclusions

Reproductive disorders are a HELCOM recommended indicator for biological effect monitoring of environmental pollution with hazardous substances. The results of this study indicate that reproductive disorders in the amphipods *Gammarus spp* are a sensitive end-point for detecting sub-lethal toxicity of hazardous substances in coastal waters. Therefore, this indicator can be viewed as a potential indicator for Danish waters for environmental assessment of areas impacted with chemical pollution. Reproductive disorders as an indicator for environmental pollution have an advantage of linking the sub-lethal response to a higher organizational level in the ecosystem – observed effects in reproduction in an indicator organism can be linked to potential effects in populations, which increases the ecological relevance of this indicator.

It was possible to derive a threshold value consistent with threshold values for gammarids presented in HELCOM (2018) based on the data collected in the period 2020-2023. Results from a pilot study and the present study allowed to calculate the threshold values (Table 3.3), which are comparable with the threshold values calculated for gammaridean species in the HELCOM supplementary indicator report (Table 3.2). The threshold values for the two indicators, proportions of malformed and aberrated embryos, both at 0.028, are lower than the threshold value for "proportion of malformed embryos" at 0.05, presented in the HELCOM supplementary indicator report (Table 3.2). The threshold value for the indicator, "proportion of females with more than one malformed embryo" is slightly higher, i.e. 0.23, than the threshold value for the same indicator presented in the HELCOM supplementary indicator report (Table 3.2). Although the interspecies differences between the threshold values for three gammaridean species from the Gulf of Finland (Table 3.2) and *Gammarus spp*. from Danish waters could be expected, the threshold values observed in this study are comparable to those presented in the HELCOM supplementary indicator report. These threshold values can be applied for assessment of environmental conditions, which can affect the reproduction in coastal amphipods.

Elevated levels of reproductive responses in amphipods from Kalvebod in Køge Bugt and Risø in Roskilde Fjord are consistent with elevated levels in reproductive responses observed in fish studied in the same two NOVANA stations.

Gammarus spp. in Danish coastal waters fulfills the criteria as a bioindicator organism due to such qualities as:

- - Ecological relevance for Danish coastal areas;
- - Relative abundance and ease of sampling in most of the areas that were studied in this project;
- - Sensitivity of this indicator was comparable to the HELCOM supplementary indicator thresholds;
- - Sensitivity of this indicator was comparable to routinely used reproductive responses in fish in Danish monitoring programme.

Due to abovementioned reasons, it is possible to conclude that *Gammarus spp*. has the potential to be used as a bioindicator for environmental pollution in Danish coastal waters.

5 Suggestions for future studies

Based on the experience with establishing indicators of biological responses to pollution with hazardous substances in Denmark and other countries (e.g. Ronisz et al., 2005), we recommend conducting further baseline studies to determine spatial and temporal variability of the suggested indicator – reproductive disorders in *Gammarus spp*.

Baseline studies that are comprised of repetitive and seasonal field sampling of amphipods at sites with various degrees of pollution are recommended. Additionally, the laboratory studies, comprised of various analyses of other biomarkers, e.g. enzymatic activity, which also can be used as biological responses to chemical pollution in amphipods in order to investigate specificity to pollutants, are also recommended.

6 Acknowledgments

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8 Annex

Table 8.1. Embryo-stages for Gammarus spp. (Amphipoda)

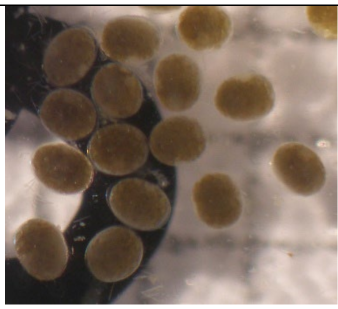
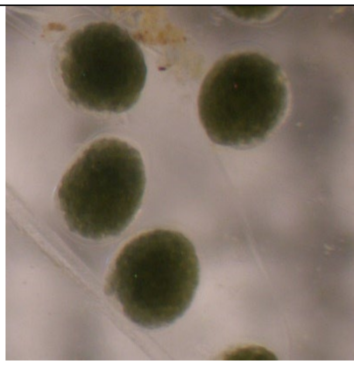
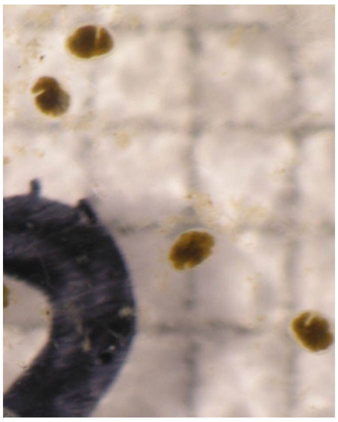
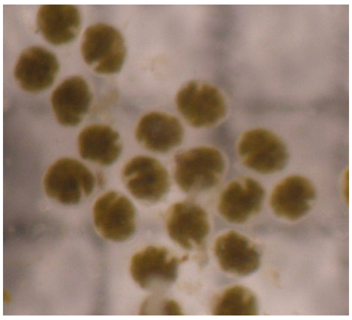
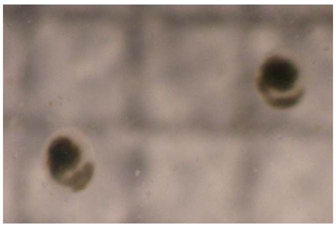
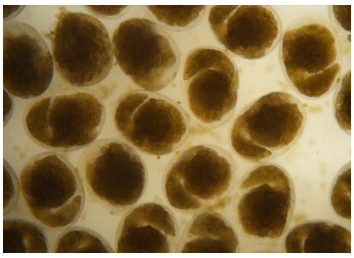
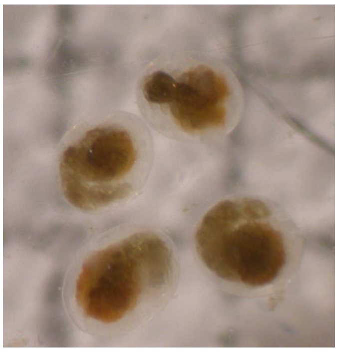
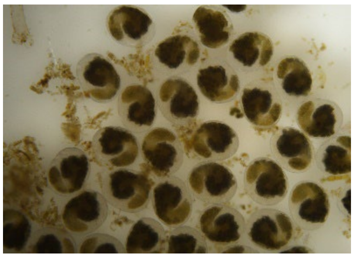
Stage	Distinguishing marks	Description	Illustrations
1	Cluster of cells, single cells more visible	Cluster of cells with no clear structure, single cells more visible	 
2	Small fracture/fractures in cell structure	Small fracture/fractures in cell structure.	 
2B	Fracture widens, 'head' is shaped.	Fracture is wider; shape is like a 'keyhole'.	 
3	"Comma"-shaped. The "comma" takes up most of the space in the embryo. The "tail" part is made of different type of cells	"Comma"-shaped. Legs are not clearly visible yet, but more like white 'foam'. The "comma" takes up most of the space in the embryo.	 

Table 8.1. Embryo-stages for Gammarus spp. (Amphipoda)

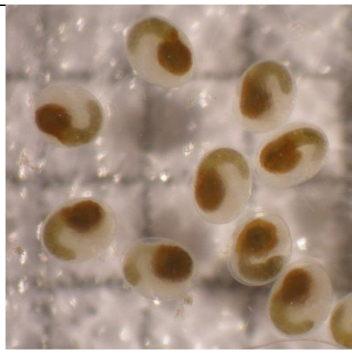
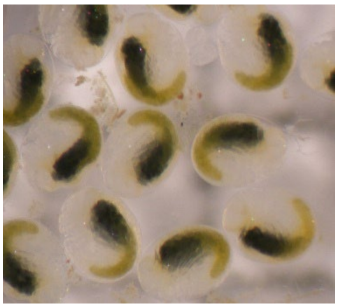
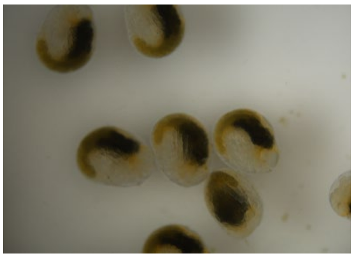
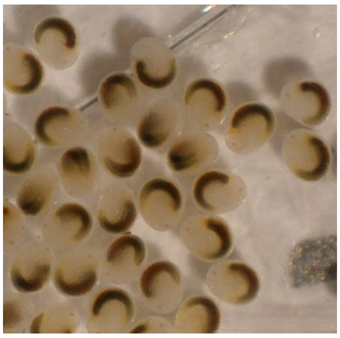
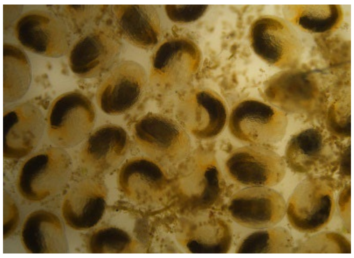
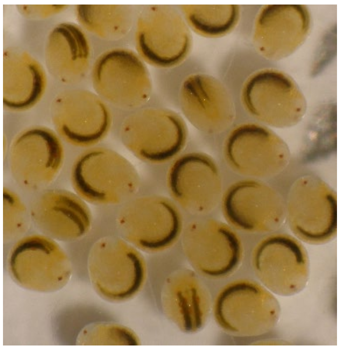
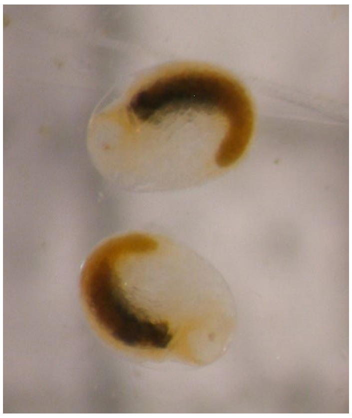
Stage	Distinguishing marks	Description	Illustrations	
4	"Comma"-shaped. The comma takes up less space and the legs more in the embryo	Still "Comma"-shaped, legs are now clearly visible and look more like legs. The "comma" takes up less space and the legs more in the embryo.		
5	Less "comma"-shaped, with distinguishable legs. No eyes yet.	Less "comma"-shaped, with clear legs and a white see-through "head". No eyes yet.		
5B	Similar to Stage 6 – two stripes, eyes however are not existing or only a suggestion for an eye (not red but a light spot).			
6	"Banana"-shaped, two stripes, with clear red eyes.	"Banana"-shaped, with clear red eyes. Shape of juvenile is clear in embryo.		

Table 8.1. Embryo-stages for Gammarus spp. (Amphipoda)

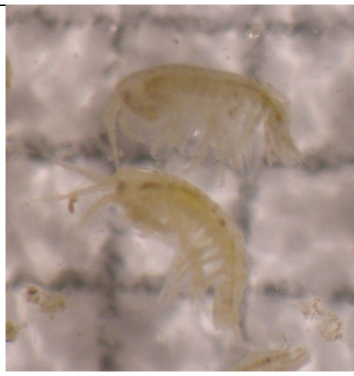
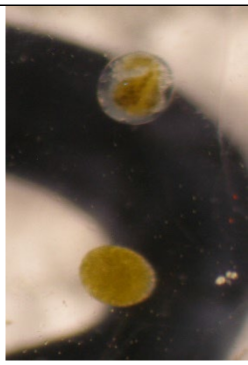
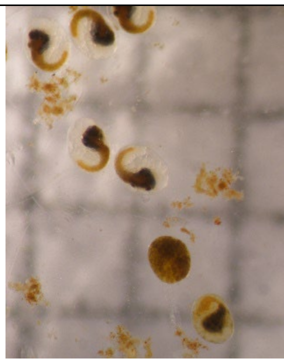
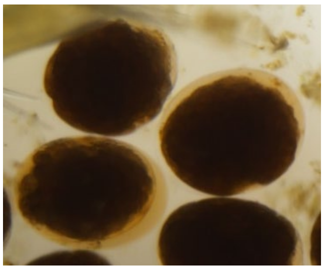
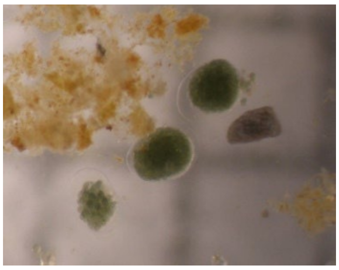
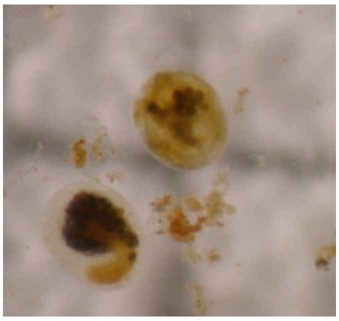
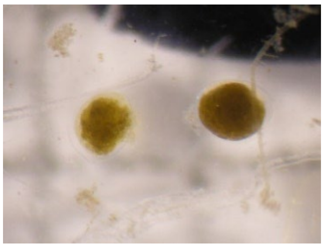
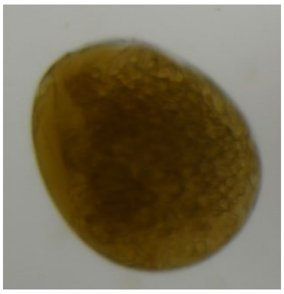
Stage	Distinguishing marks	Description	Illustrations	
7	Small copy of adults.	Juveniles, still curved and/or hatching from the egg and/or potentially free swimming.		

Table 8.2. Embryo aberration forms Gammarus spp. (Amphipoda)

	Name	Description	Illustrations	
1	Underdeveloped	Embryos in other developmental stage than rest of the brood		
2	Membrane abberations	Enlarged membranes and/or leakage between inner and outer membrane.		
3	Malformations	Malformed embryos		

4	Undifferentiated embryos	Embryos where the features of the cell structure is no longer clear		
5	Dead	Embryo is dead and looks milky and white.