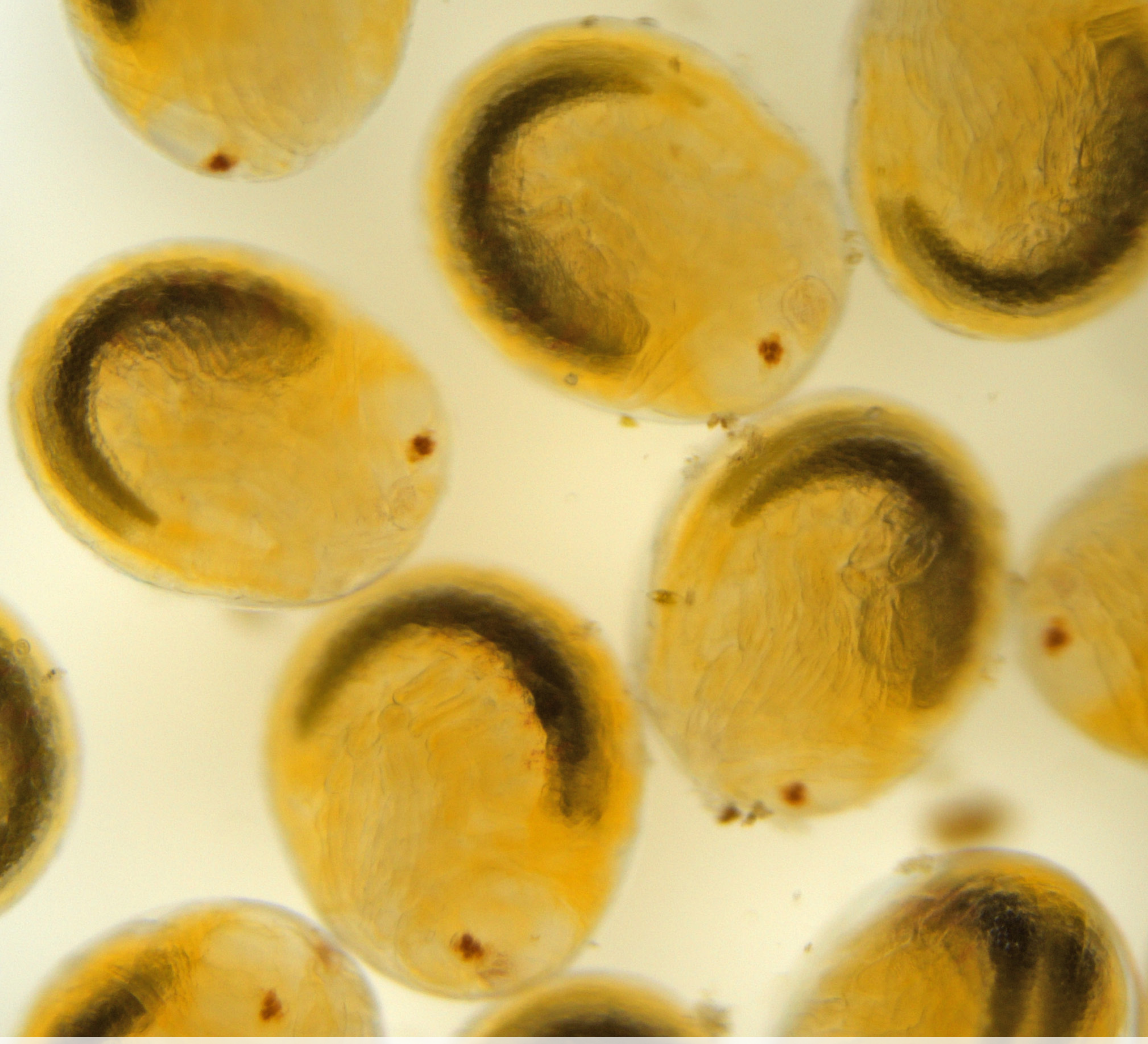

BIOLOGICAL EFFECT MEASUREMENTS IN *GAMMARUS* SPP. AND *COROPHIUM VOLUTATOR* AS INDICATORS OF TOXIC EFFECTS OF HAZARDOUS SUBSTANCES IN DANISH COASTAL WATERS

Technical Report from DCE – Danish Centre for Environment and Energy No. 237 2022

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Abstract: The current project aims to investigate the utility of lethal and sub-lethal toxic effect measurements in two species of amphipods *Gammarus spp* (tangloppe) and *Corophium volutator* (slikkrebsen) as indicators for toxic stress of environmentally hazardous substances in Danish coastal waters.
The study should be seen in the context of the monitoring strategy employed under NOVANA and HELCOM for integrated environmental monitoring, where chemical measurements in marine biota and sediment are measured in parallel with biological effects/responses.
The study includes both lethal 10 day acute-toxicity tests and sub-lethal responses in reproduction. *Corophium volutator* was used in lethal tests and. was used to test sub-lethal response study.
The sub-lethal response end-points in reproduction showed the highest sensitivity and are recommended as surveillance indicators for monitoring of spatial and temporal trends in contaminated areas.

Keywords: Indicator, bioindicator, lethal, sub-lethal, toxicity response.

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Contents

1 Preface/Summary

This technical report has been prepared based on an order from the Danish Environmental Protection Agency, Ministry of Environment, with the assignment of assessing the utility of lethal and sub-lethal toxic effect measurements in two amphipods, *Gammarus spp.* and *Corophium volutator*, as indicators for toxic stress from environmentally hazardous substances in Danish coastal waters.

This has been done by conducting field and laboratory studies supported by chemical analyses. Results are evaluated in relation to existing national and regional assessment criteria and environmental quality standards.

2 Introduction

The current project aimed to investigate the application of two methods used to monitor biological toxic effects of hazardous substances (HS) in coastal areas. 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 measurements 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 contamination.

In this project, we investigated the utility of the amphipods *Corophium volutator* and *Gammarus spp.* as bioindicators of toxic effects of hazardous substances in Danish coastal waters.

The two methods/indicators proposed measure: 1) sub-lethal toxicity responses, i.e. reproduction and embryo development in *Gammarus spp.* and 2) lethal toxicity responses in acute-toxicity test using *Corophium volutator.* Chemical measurements of hazardous substances in sediments, i.e. organotins and heavy metals, from polluted and reference areas were conducted as a supportive parameter for acute biological effect measurement. These groups of contaminants were chosen because they often are the main groups of substances that cause acute toxicity in harbor sediments.

Both methods have shown an indicative biological response due to exposure to various contaminants, including their mixtures. The frequency of malformed embryos has been demonstrated to be significantly higher due to exposure to heavy metals, chlorinated organic compounds, pulp mill effluents, and contaminated sediments in bioassays as well as in field studies than those of control microcosms and reference areas (Sundelin et al, 2008). Sediment bioassays used to study lethal toxicity in *Corophium* as a method is appropriate for the assessment of the effects of unknown mixtures and aim to provide information concerning the immediate effects of sediment-sorbed contaminants during short-term exposure (Roddie & Thain, 2001).

Previously, a Danish pilot study has been conducted to elucidate whether *Corophium spp*. and *Gammarus spp*. have potential to be used as bioindicators for toxic effects of hazardous substances in coastal waters (Fischer, 2009). In this study, animals were collected at different sites (contaminated and reference sites) in Roskilde Fjord and Præstø Fjord. Gravid females were examined for embryo aberrations according to ICES TIMES guidelines (Sundelin et al, 2008) in amphipods from different sites. The study concluded that *Gammarus spp.* seems to be more sensitive than *Corophium spp.* to the reproductive end-points and that both amphipod species have potential to be used as bioindicators for Danish coastal waters due to their capacity to be assessed for several reproductive end-points, i.e. fecundity, embryo aberrations and intersex, and also due to their abundancy and that the animals are reasonably easy to sample.

In another study in Greenland, the benthic amphipod, *Orchomenella pinguis*, was studied. *O. pinguis* was collected at contaminated and reference sites in a fjord adjacent to Sisimiut, West Greenland. The contaminants in the sediments included polycyclic aromatic hydrocarbons (PAHs), heavy metals and tributyltin (TBT). The individuals collected at the most contaminated site had a higher frequency of embryo aberrations, resulting in lower fertility (i.e. actual reproductive success) compared to the reference site individuals (Bach et al., 2010).

2.1 Sub-lethal toxicity response: Reproductive disorders-malformed embryos of amphipods *Gammarus spp.*

Amphipod embryos are sensitive to sediment toxicity during the embryogenesis (Sundelin et al., 2008), and a high rate of malformations may develop due to exposure of females to toxic substances in the sediment. Reproduction in amphipods is assumed to be a suitable indicator of pollution exposure to hazardous substances. This indicator is a HELCOM supplementary indicator and is applicable in assessment units shared by Finland and Sweden. The HELCOM supplementary indicator for reproductive disorders, malformed embryos of amphipods, is mainly based on measurements in *Monoporeia affinis*, which is a keystone species in the Baltic Sea (HELCOM, 2018). The amphipod *Monoporeia affinis* and the marine amphipod *Pontoporeia femorata* are included in the national monitoring program in Sweden (SNMMP) (HELCOM, 2018). However, *Monoporeia affinis*, formerly referred to as *Pontoporeia affinis*, closely resembles another benthic amphipod, *Pontoporeia femorata*, which can be distinguished from *M. affinis* by its light red eyes (Vattenkikaren, 2020). *Pontoporeia femorata* exists in Danish waters, mainly at the soft bottom in deeper areas in the western Baltic Sea and the southern part of the Belt Sea (Database NOVANA, 2005). Gammarus species (*Gammas tigrinus*) are also mentioned in the HELCOM indicators fact sheets (HELCOM, 2018).

This indicator, "*Reproductive disorders: malformed embryos of amphipods*", is not included in the OSPAR pre-CEMP list of "General biological effects" and JAMP Guidelines for general biological effects monitoring (OSPAR, 2007).

There are no available Danish data using the HELCOM primary recommended amphipod species *Monoporeia affinis,* since this species is not found in Danish waters. However, alternative amphipod species of genus *Corophium* and *Gammarus* species may be employed as indicator species. This project was aimed at ensuring that Denmark can report data to the HELCOM core indicator program for malformed embryos in amphipods.

2.2 Lethal response in acute-toxicity test on *Corophium volutator*

Bioassay for sediment toxicity testing and its rationale has been described in detail previously (Roddie & Thain, 2001). In brief, adult *Corophium* are exposed to contaminated field sediments (bioassay) or chemically spiked sediments (toxicity test) for ten days. During this period, burrowing behavior may be assessed by counting the number of amphipods on the sediment surface or actively swimming. At the end of the experiment, the amphipods are sieved from the sediment and the number of surviving animals is recorded. Mortality is assessed by comparing with the internationally proposed assessment criteria used for e.g. toxicity assessments of dredged materials or by the use of an appropriate analysis of variance techniques to compare treatments with controls.

The method as described by Roddie & Thain (2001) may be used to:

a) assess the relative toxicity of field-collected sediments;

b) assess the toxicity of highly contaminated sediments by serial dilution with clean sediment;

c) assess the toxicity of sediments to which chemicals have been added in controlled amounts;

d) map the spatial or temporal distribution of toxicity in a study area.

Such testing of the toxicity of allegedly contaminated sediments is essential for several reasons, as described in Lehtonen et al. (2018):

- assessment of the degree of contamination of impacted marine coastal areas (e.g, for marine spatial planning)
- deciding whether deposition (of dredged materials) to sea is possible
- monitoring/testing the development of sediment quality in an acutely/accidentally polluted site (including the effectiveness of remediation actions)
- detecting chronic pollution (e.g. diffuse pollution from land, leaking municipal or industrial waste water, harbours)
- periodical checking whether routine chemical monitoring of contamination is adequate, i.e. change in toxicity may require a new chemical screening.

3 Materials and Methods

3.1 Sub-lethal toxicity response: Reproductive disorders-malformed embryos of amphipods *Gammarus spp.*

3.1.1 Collection

The sampling strategy was as follows:

Amphipods of the genus *Gammarus spp*. were collected in May-June in 2020 at Svanemøllen Havn (n=5), Nivå Bugt (n=15), Holbæk Marina (n=30) and, in 2021, at Holbæk Marina (n=55), Vellerup Vig (n=59), Korsør Marina (n=39) and Korsør Nor (n=4) (Korsør Nor data were only used in the total distribution of malformed embryos for all impacted stations and not as an individual station due to very few data points).

The locations Nivå Bugt and Vellerup Vig are considered as reference areas in 2020 and 2021. Other abovementioned stations represent sites with certain levels of assumed contamination, being harbours and marinas. Additionally, Korsør Nor was chosen, since analyses of sediments in 2020 from that site have shown high levels of PFAS (Slagelse Kommune, 2021).

Amphipods of the genus *Gammarus spp*. were identified as predominantly *Gammarus locusta*. The taxonomy analysis of amphipods from two stations in 2020 (Nivå Bugt (n=15) and Holbæk Marina (n=28)) demonstrated 88.4% being *Gammarus locusta*, while 11.6% were *Gammarus zaddachi* (n=5). All five individuals of *Gammarus zaddachi* were identified at Nivå Bugt, indicating that species composition possibly is more homogenous in Holbæk Marina.

3.1.2 Microscopy analysis

Embryo hatch were gently collected from individual gravid female (pacified in carbonized native seawater) by blowing a stream of water onto the hatch. All embryo from the hatch were analyzed under microscope to identify embryo stage development (Annex: Table 1) and abnormal embryo development, embryo aberrations (Annex: Table 2). Microscopy analyses of developmental stages and embryo aberrations were conducted as described in Fischer 2009, with some modifications (Annex: Tables 1-2).

3.1.3 Statistical analysis

Calculations of percentile, mean, median and frequency distributions were done using Python© programming language and Excel©.

3.2 Lethal response in acute-toxicity test on *Corophium volutator*

3.2.1 Collection

Sediment-dwelling amphipods of the species *Corophium volutator* were collected at shallow waters in Roskilde Bredning at the site north from Risø, which is considered as a reference station.

The sediments for exposure studies were collected at ten different locations and included both smaller, big and small marinas (x 14 sites), and they were also analysed for TBT and heavy metals for characterizing the pollution levels.

The 14 stations were: Bogense Marina, Assens Marina, Horsens Marina, Marselisborg Lystbådehavn, Egå Marina, Aalborg Sejlklub, Lemvig Marina, Rømø Havn, Holbæk Marina, Nordhavn Fiskerihavn, Sydhavnen, Københavns Havn and two reference sites, Nivå Bugt in the Sound and Nissum Bredning in the Limfjord.

In the test phase, the native sediment from Roskilde Bredning was also used as a reference sample for comparison with contaminated sediments.

3.2.2 Laboratory exposure and analysis

The toxicity test was based on an ICES guideline for sediment bioassays with the sediment dwelling amphipod *Corophium volutator* (Roddie & Thain, 2001). Clean jars of approximately 500 ml were in replicas of two filled with 200 g sediment from test stations and compared with sediment from the reference location in Roskilde Bredning. Test conditions were performed at temperature of 10 C° and a salinity of 15-16‰. After 1 day of acclimatizing of both the collected amphipods and aerated sediment, 20 amphipods were added to each jar and their survival was recorded after 10 days. Each sample was tested with two replicates.

Sediment samples were collected in the inner parts of harbours and marinas using a hand-held HAPS corer attached to a 4 m long stick. Top 5 cm layer of each corer was sliced off from 5 different subsamples and pooled into one sample for each site. Sediment samples from reference sites were collected in shallow waters using a shovel.

3.3 Chemical analyses of sediments

All sediment samples were freeze dried prior analysis for total contents of various metals using Total digestion of 0.2 g sample in Berghof bombs using a $HF/HNO_3/HCl$ mixture in a heating oven following the Loring and Rantala (1992) method and subsequent analyses using ICP-MS (Agilent 7900).

The content of TBT and other organotins were analysed on wet sediments according to method as described in Strand et al., (2003) using in situ ethylation and tripropyltin as internal standard before extraction with pentane. Both methods are accredited methods at Aarhus University and are also used for e.g. NOVANA monitoring in marine areas.

4 Results

4.1 Sub-lethal toxicity response: Reproductive disorders-malformed embryos of amphipods *Gammarus spp.*

All the collected embryos were analyzed per hatch from individual gravid females from all stations, and the results were used to calculate two variables, i.e. the proportion of malformed embryos and the proportion of females with more than one malformed embryo. These two variables are recommended for application and used as assessment criteria in supplementary indicator, according to the HELCOM indicator report (HELCOM, 2018).

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 (Table 4.1). Both variables are measured in the same pool of field-collected gravid females. 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). For this indicator, as for a range of biological effect indicators, background levels have been set for the biological responses, which correspond to the upper limit for the variation in a background assessment criteria (BAC), and the environmental assessment criteria (EAC) have been established. BAC is analogous to background assessment concentrations, or a natural response level, and EAC represents levels of response below which unacceptable responses at higher, e.g. organism or population, levels would not be expected (Davies & Vethaak, 2012; OSPAR, 2013).

Tabel 4.1. Threshold and mean values for the proportion of malformed embryos and the proportion of females with more than one malformed embryo, for *Monoporeia affinis*. Assessment criteria (BAC and EAC) are adopted from Davies & Vethaak, 2012 (HELCOM, 2018).

Assessment criteria	Mean	BAC	EAC	Threshold value
Proportion of malformed embryos	0.041	<0.059	>0.059	0.059
Proportion of females with >1 malformed embryo	0.23	<0.3	>0.3	0.3

In the Gulf of Finland and the Gdansk Bay, secondary thresholds were established for gammaridean amphipod species (Table 4.2). As with *M. affinis*, these thresholds involve two values: one for the percentage of malformed embryos and another for the percentage of females carrying more than one malformed embryo (HELCOM, 2018).

Tabel 4.2. 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 this study, we applied threshold values for *Gammarus* species (Table 4.2), since *M. affinis* does not occur naturally in Denmark and therefore other amphipods with a similar life cycle and reproduction biology are recommended to be used as bioindicators.

In this study, the category of malformed embryos included: underdeveloped, membrane aberrations, malformations and undifferentiated types. Guidelines for identification are presented in Annex Table 2.

In order to compare the results of the present study, the mean and median values and 90% percentile (which represents the threshold value), two frequency distributions using variable of proportion of malformed embryos per brood for reference and impacted areas were calculated. Both distributions for reference stations (Nivå Bugt and Vellerup Vig) and for all impacted stations were not normally distributed. Therefore, in this project median values and mean values, were calculated and compared between impacted and reference stations (Table 4.3), and to compare results with HELCOM supplementary indicator report, where "mean values" are used (2018).

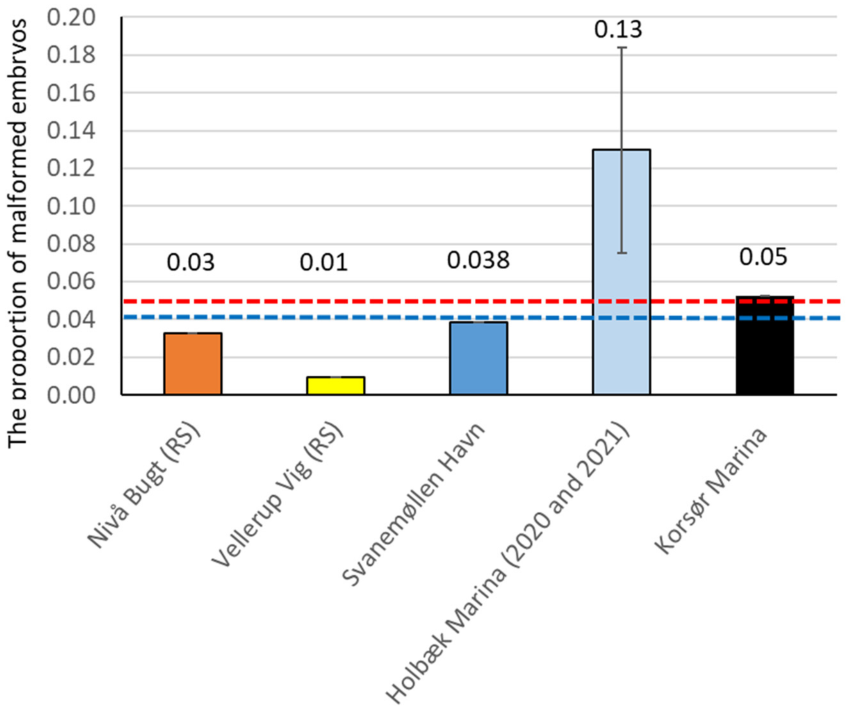
Tabel 4.3. Threshold (90th percentiles) and mean and median values for the gammaridean amphipods *Gammarus spp.*

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

Resulting mean values and threshold values (Table 4.3) 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), are below the mean values and threshold values presented in HELCOM indicator report (see Table 4.2). Median values are lower than mean values due to not normal distribution.

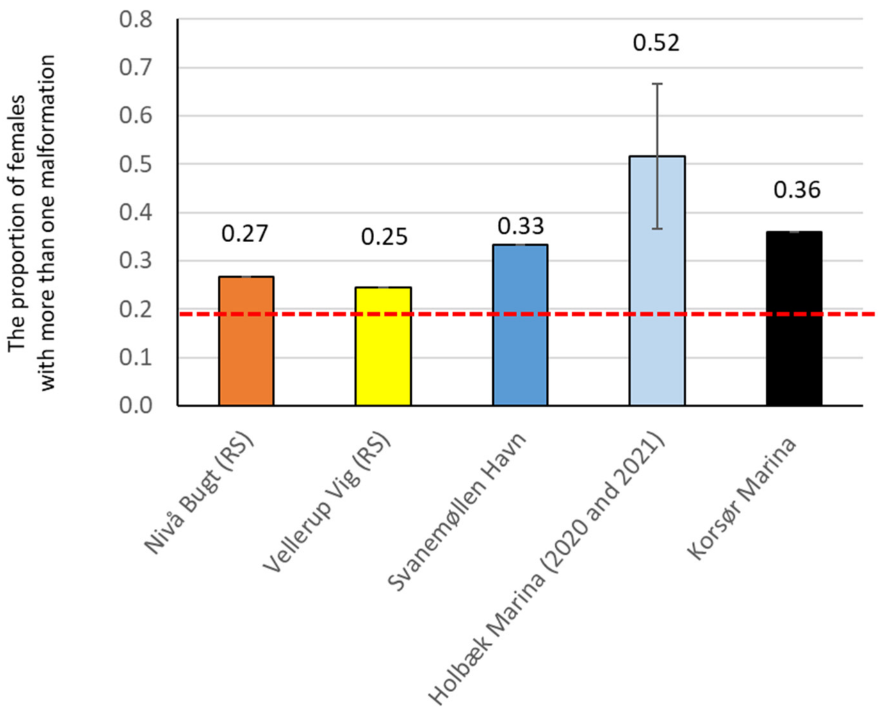
Mean values and threshold values from the distribution based on data from impacted stations (Table 4.3) are above the mean values and threshold values presented in HELCOM indicator report (see Table 4.2).

Figure 4.1. Mean values of the proportion of malformed embryos from all stations (RS: reference station). Red line – threshold value from HELCOM indicator report, blue line – threshold value, corresponding to 90th percentile, found in this study. Holbæk Marina plot: mean value for two data sets, i.e. 2020 and 2021, deviation lines maximum and minimum values.



Mean values from all impacted stations, apart from Svanemøllen Havn(0.038),are above the threshold value described in this study (0.041) and in the HELCOM indicator report (0.05). However, there is a clear general tendency, of values for proportions of malformed embryos from impacted stations being higher than and / or close to the threshold values, indicating the effect on amphipod reproduction. Application of the median values would require a larger data set, as many values for embryo aberrations are zero.

Figure 4.2. Proportion of females with more than one malformation per station, all stations (RS: reference station). Red line – threshold value from the HELCOM indicator report (2018). Holbæk Marina plot: mean value for values from both years, deviation lines for maximum and minimum values.



A similar general tendency as with proportions of malformed embryos from impacted stations being higher than values from reference stations is observed in mean values for the proportion of females with more than 1 malformed embryo. This tendency is indicative of the effect on amphipod reproduction at impacted locations. There is generally higher background response for this indicator when compared to the assessment criteria, than for he proportion of malformed embryos.

Fecundity data (eggs per female) were normalized to body length, and the results revealed that the brood size per female was higher in amphipods from reference sites (mean value from all stations= 10.1) than from impacted areas (mean value from all stations= 6.5), indicative of the effects on the *Gammarus spp*. reproduction at the contaminated areas (Sundelin et al., 2008).

4.2 Lethal response in acute-toxicity test on *Corophium volutator*

The results of the sediment bioassays showed that all the tested harbour sediments expressed a low lethal toxicity towards the sediment dwelling amphipod *C. volutator*. All samples showed a mortality level below 20%, even though some of them contained relatively high levels of metals and TBT (see section 4.3).

According to the US EPA (1998), a sediment sample is classified as toxic when it induces an amphipod mortality 20% above the control and the difference is statistically significant. In line with this, ICES (2008b) has also proposed a classification as "elevated" when Corophium mortality is >30% and "high concern" when it is >60%. For instance, the Netherlands has based assessment criteria of toxicity of dredged materials on this kind of Corophium bioassay, using an upper threshold limit for biological effects at 30% mortality (Stronkhorst et al., 2003).

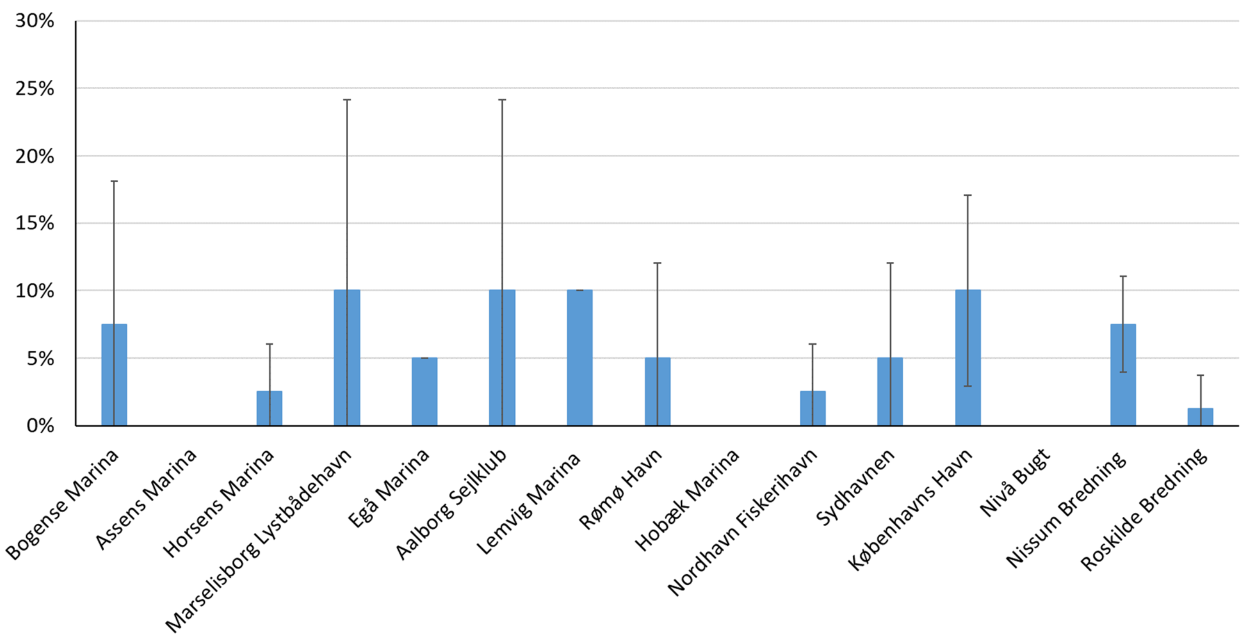


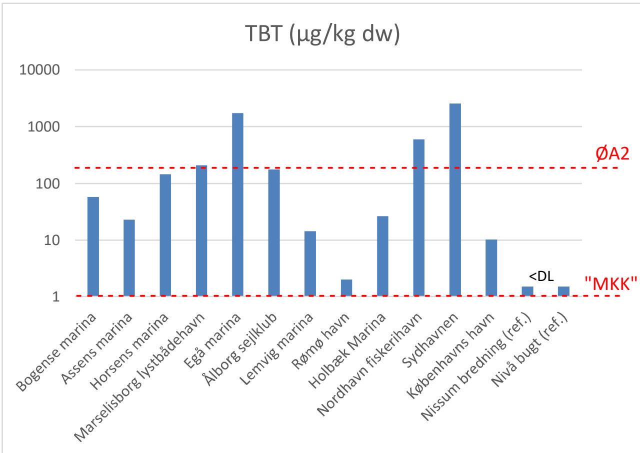
Figure 4.3. Mean values of mortality (%) of *Corophium volutator*. Number of replicates per station was two. Reference stations, number of replicates: Nivå Bugt (n=2), Nissum Bredning, Lemvig (n=2), Roskilde Bredning (n=4).

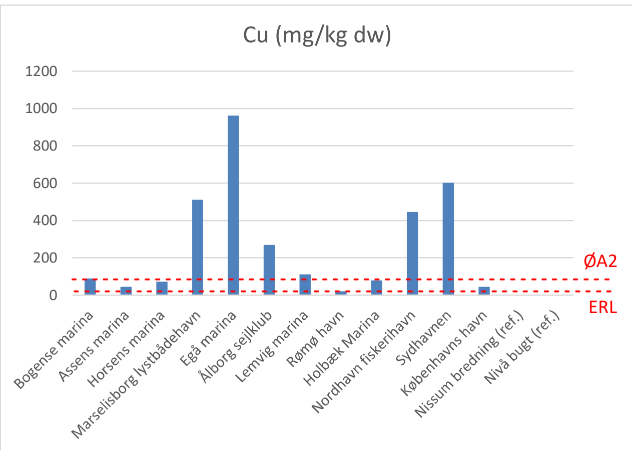
4.3 Contaminant contents in sediments

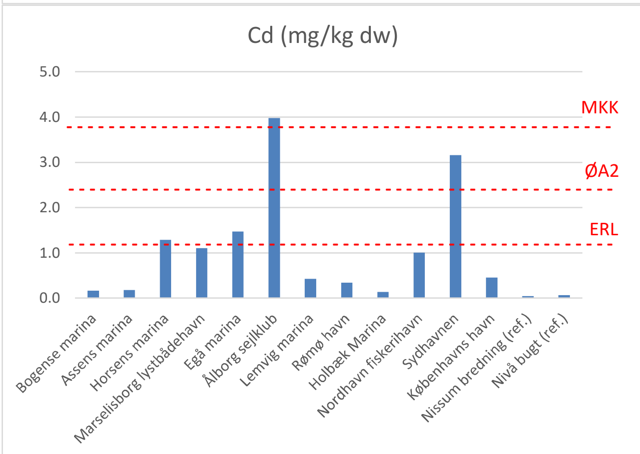
The levels of pollutants in the tested sediment samples showed a high variability in both concentration levels and composition of the analyzed substances of TBT and heavy metals. All the collected harbor sediments were significantly polluted compared to the two reference sites, also when compared to different types of assessment criteria.

In all harbours, TBT levels were significantly above the assessment criteria of 1.6 µg/kg dw, which have been proposed as a national quality standard for TBT, indicating that the TBT levels are posing a risk for causing chronic effects in benthic organisms (Danish EPA, 2021). In four of the harbours, TBT levels were also above the higher action level at 200 µg/kg dw developed for assessing dredged materials.

Also, in several of the harbor samples the levels of heavy metals of e.g. cobber (Cu), cadmium (Cd), mercury (Hg) and lead (Pb) are above the national quality criteria (MKK) for lead and cadmium and national quality standards or the OSPAR recommended ERL-values (Effect range low), indicating that the metal levels also are posing a risk for chronic effects on benthic organisms. In addition, some samples also expressed very high levels, even exceeding the higher action levels for dredged materials.







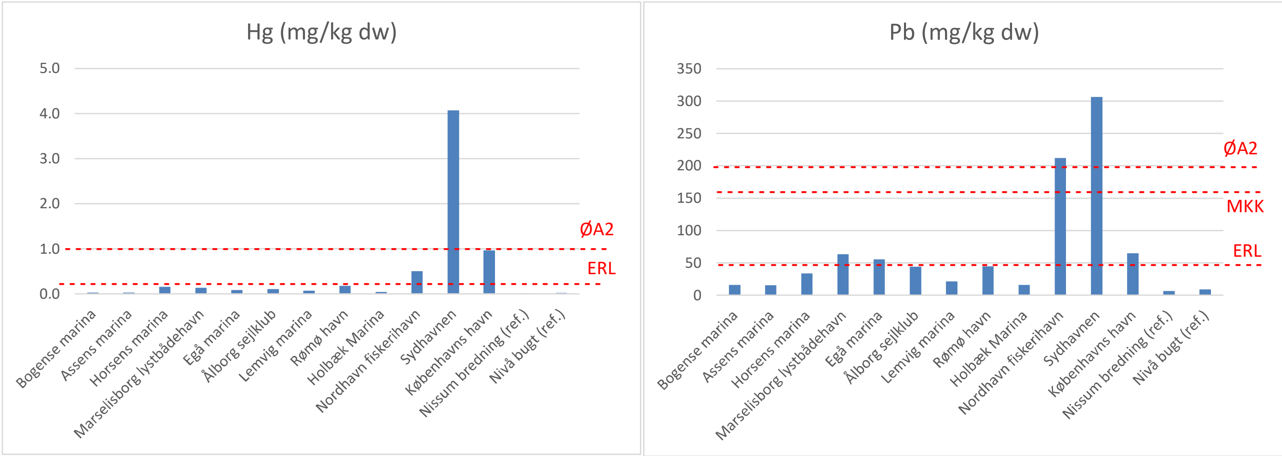


Figure 4.4. Contents of TBT and selected heavy metals in sediments from different harbours/marinas and two less polluted reference sites (ref.), where the sediment samples have also been assessed by sediment bioassay testing using *C. volutator*.

For comparison, different assessment criteria have been shown.
MKK; National quality standards (BEK 1625) developed in line with EQS from the EU water directive. For TBT, it refers to a currently prosed value for MKK. ERL: Effects Range-Low (OSPAR, 2009) is applied by OSPAR for assessing the ecological significance of contaminant concentrations in sediment as proxy for Environmental Assessment Criteria (EAC). ØA2: The higher action level 2 for dredged materials (Danish EPA, 2005), where dredging is not allowed.

Even though the metal and TBT levels in several of the samples can be regarded as significantly polluted, the contaminant levels were not high enough to induce elevated lethal effects in the sediment bioassays with *C. volutator*. This indicates that this type of sediment bioassay is not that sensitive for assessing the environmentally relevant contaminant levels found in this study, even though they were relatively high.

Other studies with the Corophium bioassay have previously shown that even higher pollution levels are needed to induce significant mortality. For instance, for TBT a Dutch study found a LC50-value above 5000 µg/kg dw in sediments that is significantly higher in the sediment tested in this study (Stronkhorst et al., 2009).

5 Conclusions

The results indicate that sub-lethal toxicity response in amphipods *Gammarus spp.* is a sensitive end-point allowing to detect toxic effects of hazardous substances. It is recommended that sub-lethal response in amphipods *Gammarus spp.* is explored as a surveillance indicator in the monitoring of contaminated areas in Danish coastal waters.

5.1 Sub-lethal toxicity response: Reproductive disorders-malformed embryos of amphipods *Gammarus spp.*

The analyses of reproductive effect data, both baseline data (from reference areas) and data from impacted areas (e.g. polluted marinas), indicate following:

- Baseline data: the levels and threshold values for *proportion of malformed embryos* based on baseline values (natural variation from reference stations) are comparable with baseline data presented in the HELCOM supplementary indicator report (2018);

- Data from contaminated areas: the levels and threshold values for proportion of malformed embryos from impacted areas are above baseline values.

  Reproductive effects observed in *Gammarus spp.* can be further explored as potential indicators in line with future development of the HELCOM supplementary indicator. For instance as “surveillance indicators” acting as *early warning systems*, which can be a part of monitoring applied in more impacted areas (HELCOM, 2020).

5.2 Lethal response in acute-toxicity test on *Corophium volutator*

This type of sediment bioassay with *C. volutator* was shown not to be sensitive enough to assess the toxicity, even though the contaminant levels were relatively high, and in several samples were significantly above the relevant quality criteria. Presumably, higher pollution levels in sediments are necessary to induce significant toxicity responses using this sediment bioassay.

6 Suggestions for future studies

In order to strengthen the basis for the use of the sub-lethal toxicity responses observed in *Gammarus spp.*, it is recommended to conduct further baseline studies to determine spatial and temporal variability of the biological response. Baseline studies are recommended that are comprised of repetitive and seasonal field sampling of amphipods at sites with various degrees of pollution, and laboratory studies, comprised of experiments with amphipods exposed to different pollutants (selected according to environmental relevance for DK waters) in order to investigate specificity to pollutants. It is suggested to complement the chemical analysis of sediments with pollutant analysis in bivalves from sites under study. This will allow the investigation of the water-borne, bioavailable fraction of contaminants, which can be bioaccumulated, as *Gammarus spp.* are not benthic amphipods.

Regarding the acute-toxicity test on *Corophium volutator*, since, presumably, the higher pollution levels in sediments are necessary to induce significant toxicity responses using this sediment bioassay, it could be applied at other more relevant sites. The development of more sensitive test-systems including chronic effects, i.e. sub-lethal end-points as reproduction, can be more relevant for environmental monitoring purposes, as these are more sensitive biological responses.

Another biological effect-indicator regularly used in NOVANA, “Reproductive success in eelpout”, has been challenged by low catchment rates of this fish species for the past several years in Danish waters. Future studies employing the reproductive responses to pollution in *Gammarus spp.* can potentially complement or, in some cases, replace this indicator in fish. Hence, the comparison of sympatric populations of amphipods and fish can be an additional focus of a future study.

7 Acknowledgment

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8 References

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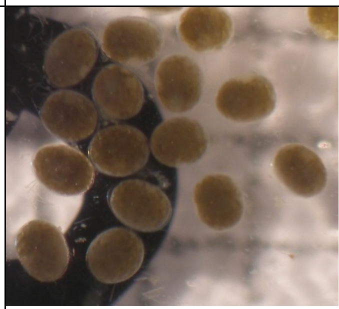
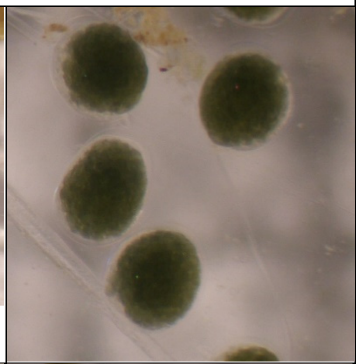
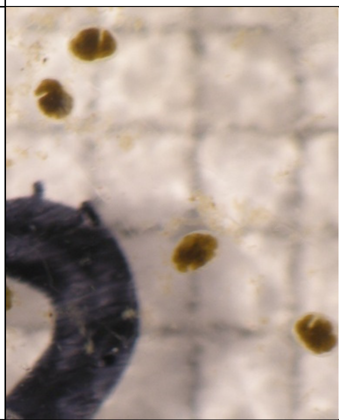
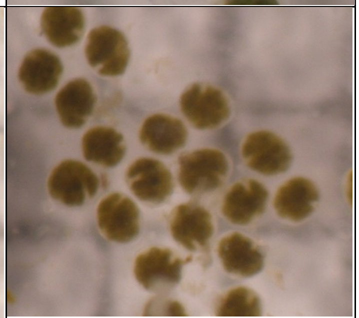
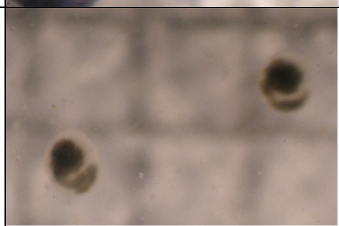
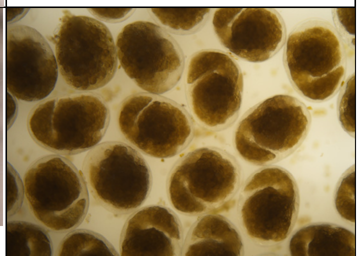
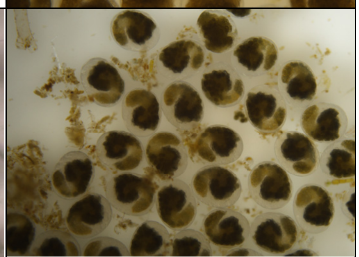
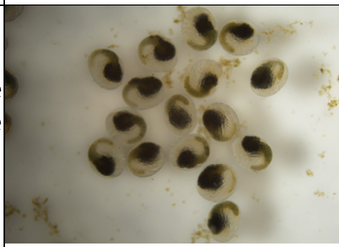
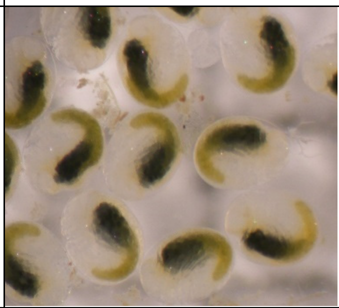
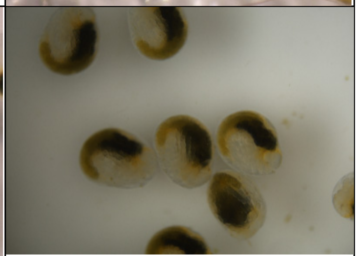
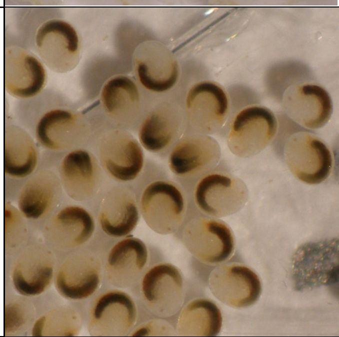
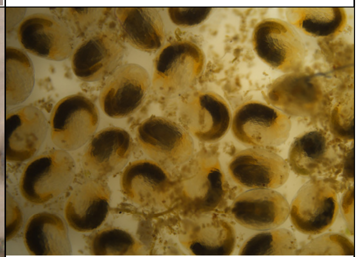
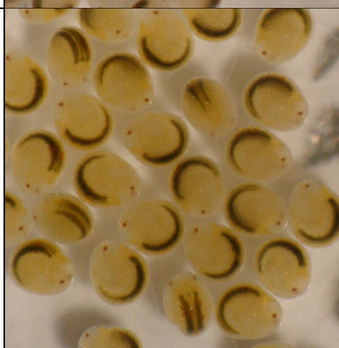
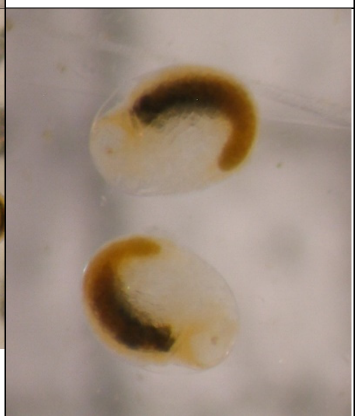
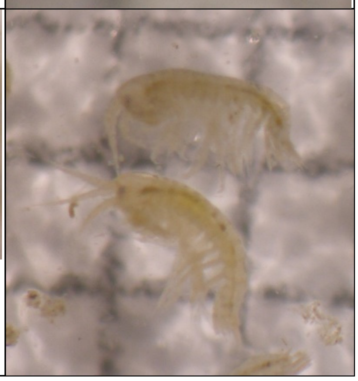
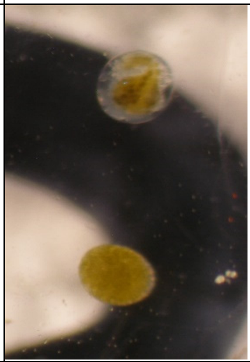
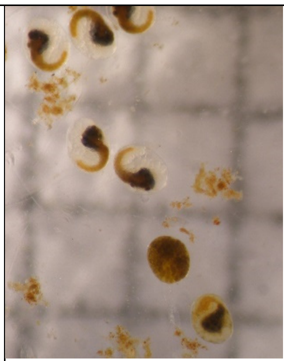
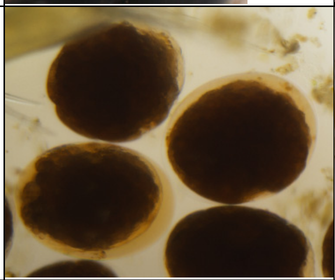
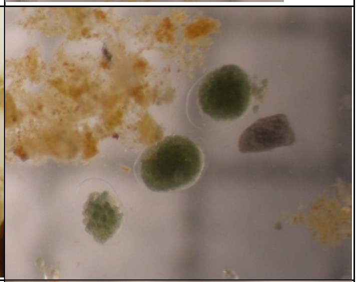
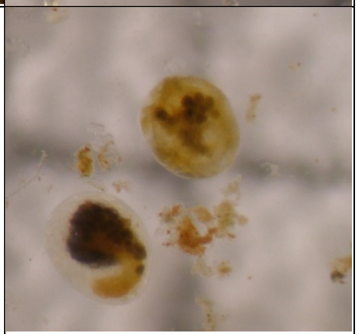
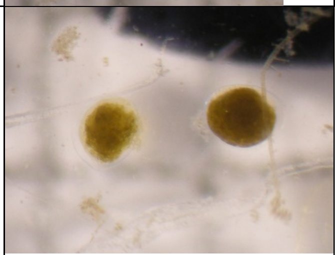
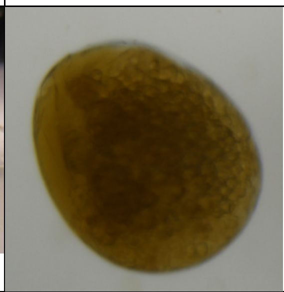
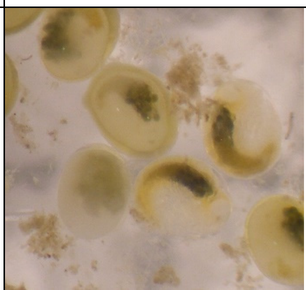
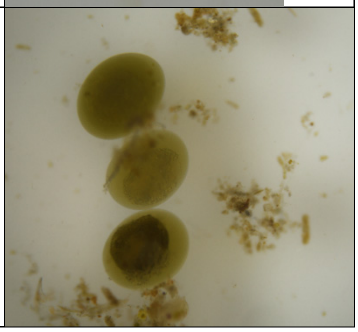
9 Annex

9.1.1 Table 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 off 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.		

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 suggestions 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.		
7	Small copy of adults.	Juveniles, still curved and / or hatching from the egg and / or potentially free swimming.		

9.1.2 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 outermembrane.		
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.		

BIOLOGICAL EFFECT MEASUREMENTS IN *GAMMARUS* SPP. AND *COROPHIUM VOLUTATOR* AS INDICATORS OF TOXIC EFFECTS OF HAZARDOUS SUBSTANCES IN DANISH COASTAL WATERS

The current project aims to investigate the utility of lethal and sub-lethal toxic effect measurements in two species of amphipods *Gammarus* spp (tangloppe) and *Corophium volutator* (slikkrebsen) as indicators for toxic stress of environmentally hazardous substances in Danish coastal waters.
The study should be seen in the context of the monitoring strategy employed under NOVANA and HELCOM for integrated environmental monitoring, where chemical measurements in marine biota and sediment are measured in parallel with biological effects/responses.
The study includes both lethal 10 day acute-toxicity tests and sub-lethal responses in reproduction. *Corophium volutator* was used in lethal tests and *Gammarus* spp. was used to test sub-lethal response study.
The sub-lethal response end-points in reproduction showed the highest sensitivity and are recommended as surveillance indicators for monitoring of spatial and temporal trends in contaminated areas.

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