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Biological effects of contaminants: the use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors

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Contents

Abstract

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This report describes the methodology for assessing the proportions and different types of embryo aberrations in both sediment-dwelling and nektonic amphipods. Determination of malformed embryos is a sensitive method of detecting the effects of contaminants, such as trace metals and hydrophobic organic contaminants. Furthermore, it is also possible to derive information about non-contaminant environmental effects, e.g. oxygen deficiency and temperature stress, by discriminating between different types of embryo aberrations. Thus, the main advantage of the method is to separate general effects of contaminants from other environmental stressors. It is a general bio-indicator that is sensitive to all kinds of xenobiotics and is applicable for measuring effects of long-term chronic impact of individual chemicals or mixtures of contaminants, as well as acute local effects from point source discharges *in situ*.

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1 Introduction

Biomarkers at the cellular, biochemical, or molecular levels are currently used within monitoring programmes to provide an early warning of future effects on higher levels of organization (Beyer *et al*., 1996; Goksøyr *et al*., 1996; Holth *et al*., 2006; Hylland *et al*., 2006). Species survival depends ultimately on the reproductive success and quality of offspring. Therefore, reproductive variables are sometimes considered more direct markers of significant toxicological effects (Ford *et al*., 2003a). Variables linked to reproduction combine the supposedly higher sensitivity of low organization-level biomarkers with the higher relevance attributed to variables giving more direct information on next-generation and population-level effects (Sundelin, 1983; Tarkpea *et al*., 1999; Cold and Forbes, 2004; Heuvel-Greve *et al*., 2007; Hutchinson, 2007). In contrast to the Vertebrata Echinodermata and Mollusca, the use of embryo aberrations has been underexplored within Crustacea and similarly may provide a valuable biomarker in assessing reproductive effects of contaminants (McKim *et al*., 1985; Laughlin *et al*., 1988; Bay *et al*., 1993).

This report aims to highlight a standardized methodology for assessing embryo aberrations in both sediment and nektonic forms of amphipods, from fresh-water, brackish-water, and marine environments. It describes the general reproductive biology of amphipods, including a standardized description of embryogenesis. The methods for field collecting both sediment-living and nektonic forms of amphipods are outlined, and a series of easily quantifiable embryo aberrations is illustrated. Embryo aberrations observed during field studies are provided for the sediment-dwelling amphipod *Monoporeia affinis* and nektonic gammarid species.

Despite numerous papers reporting reduced fecundity in amphipods inhabiting polluted environments (Borowsky *et al*., 1993, 1997; Zulkosky *et al*., 2002; Ford *et al*., 2003a), most papers have not reported on different types of aberrant development. The main reason for describing the embryonic development of *M. affinis* was to facilitate the use of the embryo variables in field studies of sediment toxicity. This strategy of analysing the frequency of malformed embryos still present in the marsupium of females can also be used for other amphipods in the *in situ* assessment of both marine and fresh-water pollution. The stage descriptions for *M. affinis* embryos and their photographic documentation will facilitate such studies.

1.1 Amphipods in biomonitoring

The general susceptibility of amphipods to pollutants (Swartz *et al*., 1982; Conlan, 1994; Chapman *et al*., 1998) has motivated numerous laboratory and field-based studies focusing on the reproductive effects of contaminants on amphipods (Prato *et al*., 2006, and references therein). Many of these have reported lower fecundity (eggs per female) in amphipods exposed to contaminants (Borowsky *et al*., 1993, 1997; Zulkosky *et al*., 2002; Ford *et al*., 2003a). Twenty years of laboratory ecotoxicological studies of soft-bottom microcosms and studies of field populations collected in contaminated industrial areas have demonstrated toxicant-sensitive variables on the embryonic development of the deposit-feeding Baltic amphipod *Monoporeia affinis* (Sundelin 1983, 1984, 1988, 1989; Sundelin and Eriksson, 1998; Eriksson *et al*., 1996; Eriksson Wiklund *et al*., 2005). When exposed to heavy metals, chlorinated organic compounds, pulp mill effluents, or contaminated sediments in bioassays as well as in field studies, the frequency of malformed embryos has been demonstrated to be significantly higher than control microcosms and reference areas. These effects also arise in low concentrations that do not demonstrably affect the sexual maturation, fertilization rate, fe-

cundity (eggs per female), and rate of embryo development (time to hatching), revealing embryogenesis to be even more sensitive than other variables of the reproduction cycle.

Information about aberrant embryonic development of amphipods in the literature is limited (Langenbeck, 1898; Segerstråle, 1937; Weygoldt, 1958; Rappaport, 1960; Ward, 1985; Steele and Steele, 1986; McCahon and Pascoe, 1988; Scholtz, 1990; Lalitha *et al*., 1991; Ford *et al*., 2003a). However, Bregazzi (1973) reported unfertilized eggs occasionally occurring in the broods of the amphipod *Tryphosella kergueleni* and Sheader and Chia (1970) observed 27% "diseased" broods of the amphipod *Marinogammarus obtusatus* around a sewer pipe.

1.2 General amphipod reproductive biology

Amphipods are found throughout the world in polar, temperate, and tropical regions, occupying both terrestrial and aquatic (fresh-water, brackish-water, and marine) environments, and covering many functional groups with regard to their mode of feeding and habitats (Lincoln, 1979). Amphipods display a diversity of life-history patterns; they may be semelparous or iteroparous and display semi-annual, annual, biannual, or perennial life cycles. The patterns are influenced by latitude, depth, and salinity (Appadoo and Myers, 2004).

Although most amphipod species display considerable sexual dimorphism, the dimorphic characteristics may vary from species to species. For example, many male specimens who display precopular guarding possess enlarged gnathopods compared with females. Male amphipods of species that do not display precopular guarding (e.g. many sediment-dwelling species) often demonstrate an increasing number of antennae segments where the number of chemosensory organs, the aesthetascs, increase during male sexual maturation. Males probably use their aesthetascs to detect female pheromones in the water, facilitating the search for females (Gleeson, 1982; Hallberg *et al*., 1997).

All male specimens are characterized by genital papillae on the ventral surface of the seventh thoracic segment. Female specimens are characterized by the presence of brood plates (oostegites) that hold eggs or developing embryos within the brood pouch (marsupium). Mature females can be differentiated from immature specimens by the presence of setae on the brood plates.

Fecundity (eggs per female) of amphipods varies considerably between different species, e.g. *Gammarus minus* produces 4–9 eggs (Glazier *et al*., 1992), while *Gammarus zaddachi* produces 14–130 eggs (Cheng, 1942). In addition, intraspecific fecundity is related to female size (Cederwall, 1977; Leonardsson *et al*., 1988; Subida *et al*., 2005) and weight (Ford *et al*., 2003a, 2003b; Castro *et al*., 2006; Pockl, 2007). All amphipods demonstrate direct embryo development in the marsupium, which makes them particularly suited to biomonitoring reproduction effects *in situ*. During oogenesis, large quantities of lipids are deposited into the developing oocytes (Herring, 1974; Harrison, 1990, 1997; Wouters *et al*., 2001; Rosa and Nunes, 2003). These lipids, which consist mostly of monounsaturated fatty acids, are utilized and consumed during embryo development (Morais *et al*., 2002; Rosa *et al*., 2003, 2005), potentially leading to increased toxic effects of lipophilic contaminants during embryogenesis. All amphipod species display similar direct embryo development, despite differences in sexual behaviour before mating and during embryogenesis, which differs mainly as a result of ambient temperature (Bregazzi, 1973; Lalitha *et al*., 1991; McCahon and Pas-

coe, 1988). Therefore, this similar development allows for a consistent method of staging embryogenesis among all amphipod species and any resultant aberrations.

Before mating, the female oocytes are separated into easily distinguished eggs (Figure 1A and B). During mating, the male deposits his spermatophores into the marsupium of the female. Eggs from the ovaries are deposited into the brood pouch, where they are fertilized (Figure 1C). A pair of spermatophores is visible in the anterior part of the male body (Figure 1D). Empty sperm sacs and spermatozoa (Figure 1E and F) can be found in brood pouches of females containing recently fertilized eggs, suggesting that males deposit sperm in the marsupium of recently moulted females, whereupon ova are liberated and extruded into the brood pouch where fertilization takes place. Many amphipod species have a precopulatary stage during which the larger males use their larger gnathopods to hold the smaller females until they moult when the marsupium has developed (Steele and Steele, 1986).

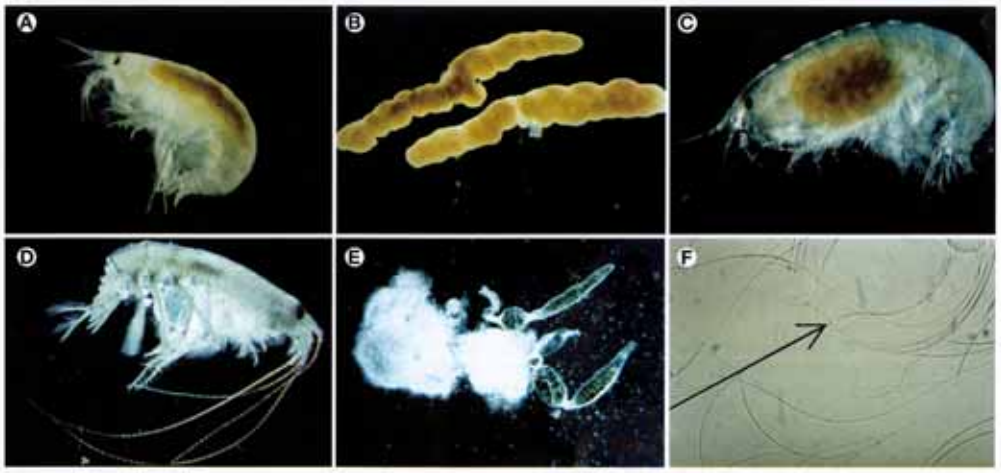

Figure 1. The reproduction cycle of *Monoporeia. affinis*. (A) Unfertilized female with oocytes; (B) Mature female oocytes with unfertilized eggs; (C) Fertilized female with eggs in the marsupium; (D) Sexually mature male with elongated antennae and enlarged complex eyes; (E) Pair of spermatophores with sperm; (F) Sperm–the sperm head and the mobile tail are illustrated with an arrow. Reproduced with permission from Marine Ecology Progress Series, 171: 165–180.

Almost without exception, normal eggs of each brood are the same colour and at the same stage of development. Unfertilized eggs, however, can be differentiated from fertilized eggs. The unfertilized eggs are full of oil droplets and vary in colour from green to bright yellow to orange or brown-red.

Numerous studies have classified embryogenesis in amphipods in distinct quantifiable stages, although the number of stages categorized varies (2–30), depending on the study (Ford *et al*., 2003a; Sheader and Chia, 1970; Sundelin and Eriksson, 1998; Browne *et al*., 2005). For the purpose of the methodology, the nine stages of classification used previously by Sundelin and Eriksson (1998) have been adopted; they are easily applicable to any species. The nine stages are described in Table 1 and illustrated in Figure 2A–O.

The first two cleavage divisions are total (Figure 2A), resulting in four cells, two smaller than the others. The next division results in four small micromeres lying upon four larger macromeres (Figure 2B). The unpigmented and yolk-free micromeres give rise to a ventral germinal disc. The micromeres extend over the egg surface and surround the lipid-rich yolk cells, derived from the macromeres (Figure 2D). Egg size is unchanged until the blastula stage (Stage 4; Figure 2D and E), after which

gastrulation occurs, the outer egg membrane bursts, and the embryo is allowed to increase in size. Somites of the antennulary, mandibulary, and maxillary segments appear, and a ventral groove, the caudal furrow, which separates the developing cephalothorax and abdomen, becomes apparent (Stage 5; Figure 2F), and the characteristic comma-like shape of the embryo develops (Stage 6; Figure 2G–I). The midgut extends backwards, containing large, multinucleate, pigmented yolk cells (Stage 7; Figure 2I–J). Paired, bud-like appendages develop (Stage 8; Figure 2J–K), and the head region with the club-like antenna can be recognized (Figure 2J–L). The head region appears distinctly, the first and second antennae are considerably enlarged, and the pigment of the eyespot is discernible (Figure 2M). Later, the compound eye becomes clearly visible, and the animal is fully formed before hatching (Figure 2N). Peristalsis begins in the midgut, and the limbs and body move. Spasmodic contractions of the body cuticle and movement of the limbs cause rupture of the embryo membrane, and the juvenile (Figure 2O) is liberated. The yolk remains for about 3–4 weeks after hatching, and it is probable that juveniles stay within the marsupium until the yolk is consumed.

Table 1. Description of amphipod eggs in each stage of embryo development. Nine developmental stages were adopted for embryos.

Stage of development	Embryo description
1	The newly fertilized egg during the first three cleavages; the egg membrane and the vitelline membrane, tightly bound to the yolk cells, are visible. Cleavages are initially total but later become superficial. Figure 2A–B.
2	More than 8 cells. There is a synchrony of divisions of the larger macromeres and the smaller micromeres until 64 cells. Figure 2C.
3	The blastula stage. The small micromeres divide more rapidly than the macromeres, resulting in a ventral, non-pigmented, germinal disc, which extends over the surface of the egg, enclosing the yolk cells, and constituting the blastoderm. Figure 2D. Embryonic development until gastrulation takes approximately one month.
4	Gastrulation starts from the posterior region of the germinal disc. Ventrally gastrulation proceeds by the inward proliferation of cells to form the embryonic mesoderm and endoderm. At this stage, the outer egg membrane bursts, and the developing embryo is allowed to increase in size. Later, a narrow groove is formed, which develops into the caudal furrow. Figure 2E–F.
5	The differentiation of appendages and embryo organs. After gastrulation, the embryo is characterized by its comma-like shape, and the dorsal organ is formed. Figure 2G–H.
6	The midgut extends backwards, the cephalothorax is visible, and the bud-like limbs begin to develop. The dorsal organ is at maximum size. Figure 2I–K.
7	Club-like antennae of the cephalothorax develop, appendages are segmented, and the first pigments of the eyespot appear. Figure 2L–M.
8	The dorsal organ has regressed, and the compound eye is fully developed and clearly visible. Peristalsis begins in the midgut, and the limbs and body move. Figure 2N.
9	The newly hatched juvenile. Figure 2O.

Figure 2. Embryonic development of *Monoporeia. affinis*. Nine arbitrary developmental stages of embryogenesis were adopted. (A, B) Stage 1, (C) Stage 2, (D) Stage 3, (E, F) Stage 4, (G, H) Stage 5, (I–K) Stage 6, (L, M) Stage 7, (N) Stage 8, and (O) Stage 9. For information see Table 1 and text above. Reproduced with permission from Marine Ecology Progress Series, 171: 165–180.

2 Sampling methods and laboratory analyses

2.1 Sediment-dwelling amphipods

To obtain a quantitative sample, a grab sampler (e.g. van Veen) should be used to sample amphipods inhabiting sediments but in cases where amphipods display low abundances, a bottom sled could be used to achieve greater sampling efficiency. To collect the sediment-dwelling amphipods, 5- and 1-mm sieves could be used, depending on the size of the amphipods.

For species producing several broods during the reproductive period, the sampling should preferably be carried out in the early mating period when specimens in the population demonstrate more synchronous maturation than in the later part of the reproductive period.

It is also possible to collect sediment and sexually maturing females and males *in situ*, to be incubated in aquaria during the period prior to mating and embryogenesis. The field-collected amphipods should be transported to the laboratory at a temperature matching their habitat, e.g. cold-water species in cold water. Many Baltic glacial relicts are stenotherm cold-water species and are particularly sensitive to temperature stress during oogenesis (Eriksson Wiklund and Sundelin, 2001).

2.2 Nektonic amphipods

Nektonic amphipods can be collected in buckets from fresh-water, estuarine, and marine environments, with collection methods varying depending on the habitat (e.g. subtidal vs. intertidal). Intertidal specimens can be collected by hand from under stones or macroalgae during low tide. Macroalgae can be transported to the laboratory where it can be washed into a sieve using water with appropriate salinity. Subtidal specimens can be collected either by transporting macroalgae to the laboratory, as described above, or in the field by shaking macroalgae vigorously into a large container. Sampling in rivers or shallow waters can also be done by kick-sampling, whereby disturbed sediments can be washed downstream/current and collected within an appropriate net. Many nektonic amphipods are relatively hardy and can survive a considerable time after sampling, provided they are kept in field-collected water with some algae for food/shelter and under cool conditions. For example, *Echinogammarus marinus* can be transported for more than 12 hours in these conditions.

2.3 Laboratory analyses

Usually, gravid females can be distinguished easily because the eggs are clearly visible within the marsupium through the transparent exoskeleton. Fine insect needles and feather forceps facilitate the preparation of eggs from the living female. Larger amphipods with harder exoskeletons can be anaesthetized in carbonated (sea) water and examined under a dissecting stereomicroscope. However, to be able to see the internal organs clearly, the eggs and embryos should be alive, which means that they must be removed from the carbonated water within half an hour using fine forceps and a fine glass pipette. Smaller sediment-dwelling amphipods with softer exoskeletons or newly moulted females can be dissected easily without being anaesthetized. The embryos should be analysed for embryo aberrations under a stereomicroscope with good depth of field. Temperature during embryo preparation must be low for cold-water species in order not to affect the embryos. Preferably, a camera for photographic documentation should be connected to the microscope.

Living females should be examined for fertilization success and fecundity (eggs per female). Eggs and embryos should be analysed for stage of embryo development, different types of malformed embryos (which have been shown not to survive hatching), enlarged embryos with no other visible damages, undeveloped/unfertilized (henceforth called undifferentiated) and dead eggs, and "dead" broods (defined below). Examples of embryo aberrations are provided in Section 4 and in Figure 3A. The protocol for analysing females and embryos is provided in Appendix 1, and Appendix 2 shows examples of different species of amphipod embryos to illustrate the similarity between species (Figures 7–12).

Owing to the correlation between size and fecundity observed in many amphipods, fecundity values should be normalized to weight and/or body length. To avoid risk of subjective assessment when two or more persons are collecting data, approximately 50 broods should be analysed independently by each person, and the results cross-checked for accuracy.

3 Embryo aberrations

There are four main egg/embryo aberrations that have been proven to be easily quantifiable in amphipods (Sundelin and Eriksson, 1998). These include malformed, undifferentiated, single dead eggs and finally "dead broods". The latter term is used when many or all of the brood are in a state of decay and it is too difficult to quantify the number of eggs accurately. Malformed embryos are further subdivided in different categories, and undifferentiated eggs are separated into unfertilized, arrested development before gastrulation, and aberrant cleavage pattern in early developmental stages. Each aberration is illustrated in Figures 3 and 4. Embryos that are slightly malformed are easier to detect in later embryonic stages, while it is easier to detect undifferentiated eggs and aberrant cleavage pattern during early embryogenesis. Because this method is developed mainly to detect effects of contaminants, it is recommended that embryos be analysed in later stages of development (i.e. stages that are most likely to show malformations).

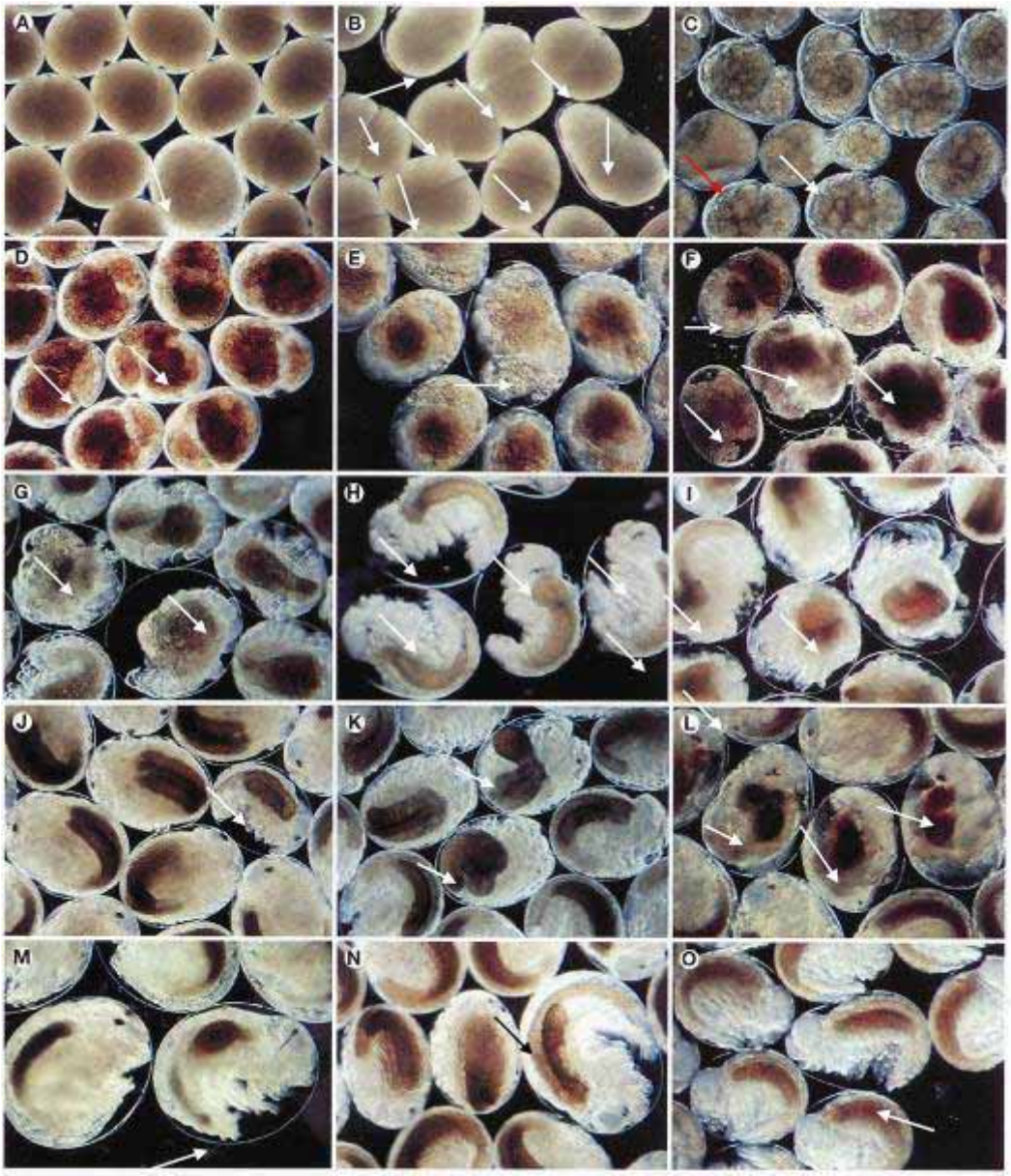

Figure 3. ***Monoporeia. affinis*****. Malformed embryos (A–M) of the amphipod at different developmental stages. Malformed embryos are indicated with white arrows. A single embryo with aberrant cleavage is indicated with a red arrow (C). Non-specific malformations (F, I, J, K, L). Oedema and impaired membranes where lipids had leaked outside the embryo (A, B, C, D, E, G). Malformed eyespot (M). Enlarged embryos without any other visible damages (N, O). Reproduced with permission from Marine Ecology Progress Series, 171: 165–180.**

3.1 Malformed embryos

Embryos are classified as malformed when they manifest various degrees of deformities. The malformations described below are ones that are easily discernible. It should be noted, however, that slightly malformed embryos that are difficult to detect may still occur within a brood, and therefore the percentage of malformations reported should be stipulated as being clearly identified as a minimum level for the population measured. Common deformations are: comma-like compound eyes (Figure 3H and M), shortened, bud-like limbs (Figure 3G and H), shortened, irregular, and club-like midguts (Figure 3I, K, L, and M), and different types of membrane dysfunction. More serious impairments include destroyed membranes where lipids leak out through inner egg membrane and are visible inside the outer membrane (Figure 3D, E, and F).

Minor membrane dysfunctions can result in different shapes of eggs (Figure 3B and C) or disturbed osmotic regulation, causing increased water content of embryos leading to oedemas (Figure 3N and O). Experiments rearing embryos in the laboratory have demonstrated that all malformations, except those described as "enlarged" (with no other visible aberrations), were found to be lethal (Sundelin, unpublished data).

3.2 Undifferentiated eggs

Undifferentiated eggs are either unfertilized or have ceased development prior to gastrulation as a result of, for example, aberrant cleavage (Figure 4H–L).

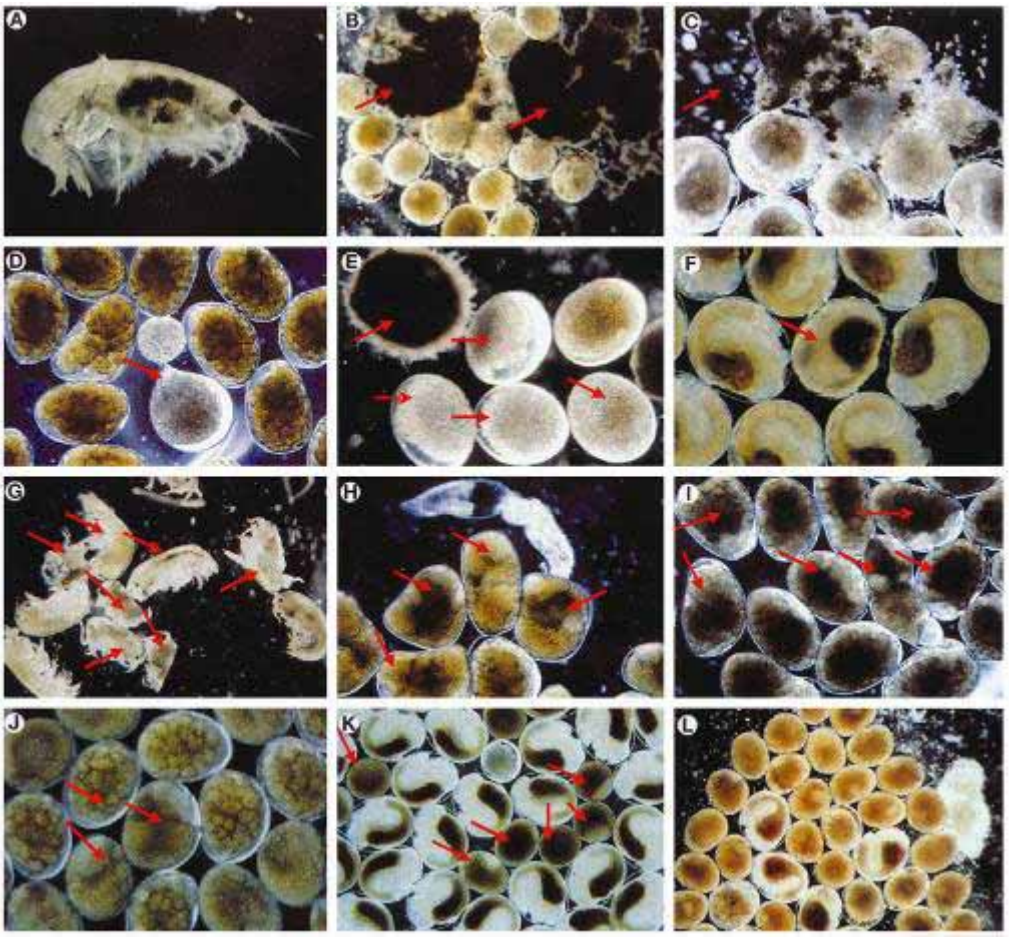

Figure 4. Aberrant embryonic development of the amphipod *Monoporeia affinis*. Aberrations are indicated by red arrows. In Figure L, most eggs are undifferentiated and not indicated by arrows. Female with dead brood (A). Unidentified dead eggs of a brood (B and C). Single dead eggs and juveniles (D–G). Embryos with aberrant cleavages in early embryogenesis (H–J). Undifferentiated embryos, where development ceased before gastrulation when embryos differentiate (K and L). Reproduced with permission from Marine Ecology Progress Series, 171: 165–180.

3.3 Single dead eggs/embryos and dead broods

Single dead eggs (Figure 4D–G) could occur at any stage of embryogenesis and appear as white opaque eggs lacking distinct organs because proteins have denatured. A "dead" brood is a term used when many or all of the brood are in a state of decay (Figure 4A–C). Usually, this has occurred during early embryogenesis, and it is too difficult to quantify accurately the number of original eggs/embryos. The decayed

eggs remain in the marsupium as a fat residue, full of oil droplets and having the same colour as the living eggs.

4 Statistical evaluation

Fecundity data (eggs per female) should be normalized to weight and/or body length. The mean brood size can be compared, using weight and/or length as a covariable in an analysis of covariance (ANCOVA). Data concerning eggs/embryos are categorized into several types of aberrations, and it is recommended to use analysis of variance (ANOVA), general linear models (GLM), or a non-parametrical equivalent as statistical methods. If there are large differences between replicate sizes, we recommend weighting the data, based on the number of individuals in a replicate or sample before testing, using the appropriate method. Data concerning the female are often in the form of binary data, i.e. carrying a dead brood or not, and could be treated that way, using appropriate methods for binominal data (e.g. G-test, Chi-Square, Fishers Exact Test, or logistic regression).

5 Use of amphipod embryogenesis in biomonitoring

A low percentage of amphipod embryos displaying aberrant development can be found in pristine, uncontaminated areas. Therefore, it is important to determine the normal variation in background areas before it is possible to classify a population as affected by contaminants or not. Most biomarkers and bio-indicators are more or less influenced by different types of environmental stressors, such as temperature stress and oxygen deficiency.

The frequency of malformed embryos of the Baltic amphipod *Monoporeia affinis* evidences a comparatively low variation in pristine areas, which means that a minimum of five replicate grab samples, including an average of 15 females or in total about 60 females per station, give sufficient statistical power. Studies in the Baltic and Bothnian seas have demonstrated that a yearly change of 5.7% in malformed embryos will be detected within ten years, with 80% statistical power and a 5% significance level. (Calculations were based on linear regression of the variable over time. The program nQuery was used to determine the correlation in ten years with 80% power.)

About 96% of embryos sampled on reference sites demonstrate a normal development. Results from 12 years (1994–2005) of biomonitoring on five reference sites in the Bothnian Sea, where gravid females of the deposit-feeding amphipod *M. affinis* were sampled during late embryogenesis at the beginning of February, demonstrated a frequency of 2–6% malformed embryos (Figure 5), suggesting that this is the range for normal background levels in this area. Fecundity, measured as eggs per female, varied during this period between 17 and 37 eggs per female at sites in the Bothnian Sea. The fecundity was shown to correlate with carbon content in phytoplankton production during April to May (Eriksson Wiklund *et al.*, 2001).

Malformed embryos are correlated with contaminant exposure, and the percentage of malformed embryos per female was demonstrated to decrease with increasing distance from known sources of industrial pollution (Sundelin and Eriksson, 1998). Also, the percentage of females carrying broods including malformed embryos was significantly higher close to industrial discharges than reference stations (Sundelin and Eriksson, 1998). The malformations arise as a result of exposure to several kinds of contaminants such as trace metals and persistent hydrophobic organic contaminants (Elmgren *et al.*, 1983; Sundelin, 1983, 1984, 1988, 1989; Sundelin and Elmgren, 1991;

Eriksson *et al*., 1996; Sundelin and Eriksson, 1998; Eriksson Wiklund *et al*., 2005), suggesting that the variable is a general bio-indicator of contaminant effects.

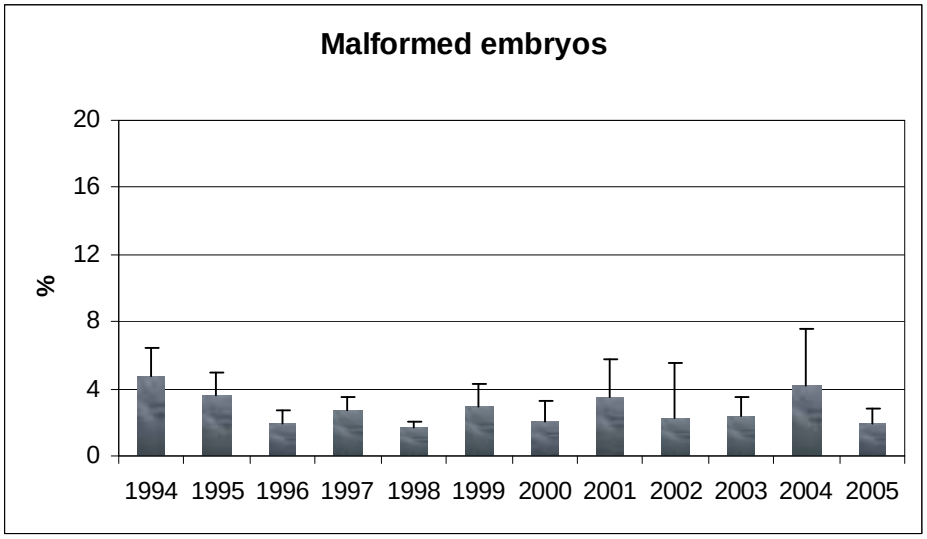

Figure 5. Percentage of malformed embryos of *Monoporeia. affinis* in the Bothnian Sea from 1994 to 2005. Data are presented as ± CI of five replicate grab samples of five sites.

Undifferentiated eggs did not show any correlation with contaminants (Sundelin and Eriksson, 1998; Eriksson Wiklund and Sundelin, 2001), but during laboratory exposure experiments, they were related to increased water temperature before mating and also to oxygen deficiency (Eriksson Wiklund and Sundelin, 2001). However, these relationships have not been demonstrated in the field (Eriksson Wiklund and Sundelin, 2004).

Single dead eggs or dead broods are related to increased temperature prior to mating and oxygen deficiency (Eriksson Wiklund and Sundelin, 2001). In addition, *in situ* females with dead broods are related to oxygen deficiency, as measured by oxygen concentrations in bottom waters (Figure 6), meriting these variables as suitable methods in biomonitoring in the field for effects of oxygen deficiency (Eriksson Wiklund and Sundelin, 2004). Levels of embryo aberrations in *M. affinis* exposed to various types of stressors during laboratory exposure experiments are illustrated in Table 2.

Table 2. Embryo aberrations related to various types of stressors. Results were obtained in laboratory exposure. Superscripts link concentrations of stressors to embryo variables.

Type of stressor	% Malformed embryos	% Dead embryos	% Undifferentiated embryos	% Females with dead broods	References
Cd <0.2[1], 6.3[2], 31[3], 100[4] µg/L	0.15[1], 3.1[2], 5.2[3], 20.5[4]	0[1], 0[2], 0[3], 0[4]	0.5[1], 3.2[2], 1.5[3], 3.7[4]	0[1], 0[2], 0[3], 0[4]	Sundelin, 1983
Pb, <0.1[1], 5[2], 50[3] µg/L	0.15[1], 1.5[2], 5.1[3]	0[1], 0[2], 0[3]	0.5[1], 3.2[2], 3.2[3]	0[1], 0[2], 0[3]	Sundelin, 1984
As <0.5[1], 100[2] µg/L	0.5[1], 20[2]				Sundelin, 1989
Effluent from pulp mills, 0[1], 0.05% BKME[2], 0.5% BKME[3]	3.0[1], 7[2], 18[3]				Sundelin, 1989, 1992
Temperature, normal *in situ*[1], increase of 3.2 C[2]		0.95[1], 14[2]	0.52[1], 5.32[2]	25[1], 68[2]	Eriksson and Sundelin, 2001
Oxygen, 9.7 mg/L[1], 3.2 mg/L[2]				18.5[1], 75[2]	Eriksson and Sundelin, 2001

BKME = Bleached kraft pulp mill effluent

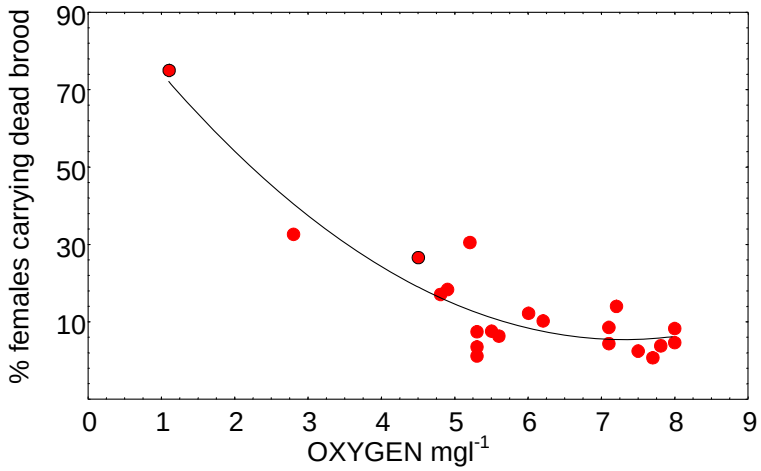

Figure 6. Percentage of females carrying a dead brood in relation to oxygen concentration of the bottom water (2 cm above the sediment surface) in mid-September. Data for dead broods are mean values of 5 replicates per station per year; oxygen concentrations are mean values of 3 replicates per station per year. Reproduced with permission from Marine Ecology Progress Series, 274: 209–214.

6 Additional analysis

Outlined below are some further analyses that can be recorded/undertaken from field-collected specimens.

An assessment of brood loss can be quantified by recording the average number of eggs per female (normalized to weight) in early stages of development and comparing them with those in late stages of development. Ford *et al*. (2003a) used embryo stage assessment to compare both fecundity and brood success (or "relative" fecundity; Ford *et al*., 2003a) in the amphipod *Echinogammarus marinus*, collected from industrially contaminated sites, compared with reference sites on the east coast of Scotland. To assess fecundity (i.e. the initial number of offspring produced), the authors only needed to count eggs/embryos in early developmental stages. Brood success is measured as the ratio of eggs/embryos in the early stages of development (Stages 1–4 in this report) compared with late stages of development (Stages 5–8 in this report). Ford *et al*. (2003a) hypothesized that comparison of the mean number of embryos at early and late stages of development from a population might provide a measure of the loss of embryos from a brood. Brood loss was assumed to occur either via active ejection of non-viable/abnormal eggs or via passive loss through malformed brood plates (oostegites). Results from this study found that *E. marinus* from polluted locations produced fewer eggs, and fewer eggs developed to later stages of development. The authors concluded that previous studies measuring eggs per female might have been underestimating initial fecundity in amphipods by discounting egg/embryo loss during embryonic development. Measurement of brood loss is not an appropriate tool in sediment-dwelling amphipods when compared with nektonic species because eggs/embryos are prevented by larger brood plates and a protective membrane from leaving the marsupium.

Amphipod embryos can also be cultured *ex vivo* and may provide a way of assessing the effects of contaminants on crustacean embryonic development. Lawrence and Poulter (2001) successfully utilized embryonic development in *E. marinus* to assess the effects of copper, pentachlorophenol, and benzo[a]pyrene on embryonic development. Measurable end points can include egg/embryo length, percentage hatching,

time to reach a development stage/hatching, and any specific development abnormality.

In semelparous species, males and females normally synchronize their sexual maturation. The percentage of females and males exhibiting a delayed or interrupted sexual development is a valuable effect variable of different types of stressors. Semelparous amphipods maturing asynchronously can have very dramatic effects on population growth and survivorship. By analysing the status of oocytes in unfertilized females, it is possible to determine whether the females are just delayed in their sexual development or if the development has been interrupted. Owing to various environmental stressors, the whole gonad or single oocytes might be damaged and fertilization is no longer possible.

Because amphipod size correlates to fecundity, measuring female length provides information as to whether low fecundity is the result of smaller size, which is normal, or whether it is an effect of contaminants or other stressors. Furthermore, the frequency of parasites, other visible aberrations, or diseases in females also provides valuable data to aid interpretation. Many microsporidian parasites are known to infest amphipods, some of which have the ability to feminize their hosts. Feminizing microsporidian parasites can result in intersexuality (Kelly *et al*., 2004), causing reduced fecundity (Ford *et al*., 2003b, 2004b), which has been shown to be enhanced in some contaminated areas (Ford *et al*., 2004a, 2006).

7 Required experience of practitioners

Comprehensive experience of amphipod morphology and embryology is not necessary for following this methodology. However, all practitioners require a basic knowledge of crustacean morphology to understand the vocabulary.

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Annex 1. Recommended format for recording data from analysis of embryo aberrations

Nr	Egg/female	Embryo stage	Malformed	Membrane	Enlarged	Undifferentiated	Dead	Degenrated gonad	Dead brood	Parasite	Comments

Annex 2. Embryo aberrations in various amphipod species

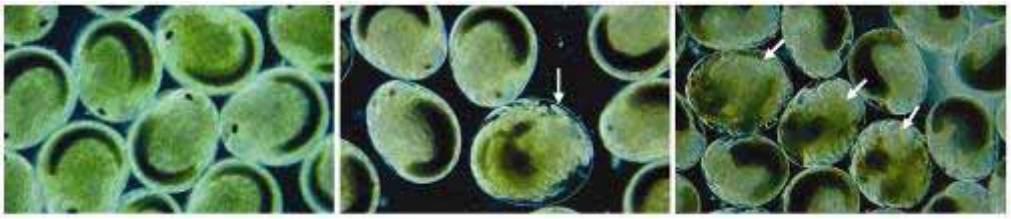

Figure 7. ***Gammarus zaddachi*****. Arrows indicate malformed embryos. The middle panel shows an embryo of Stage 8, where the midgut is broken and yolk-rich cells are leaking into the muscles. The right panel also shows deformities on the midgut of embryos of Stage 7.**

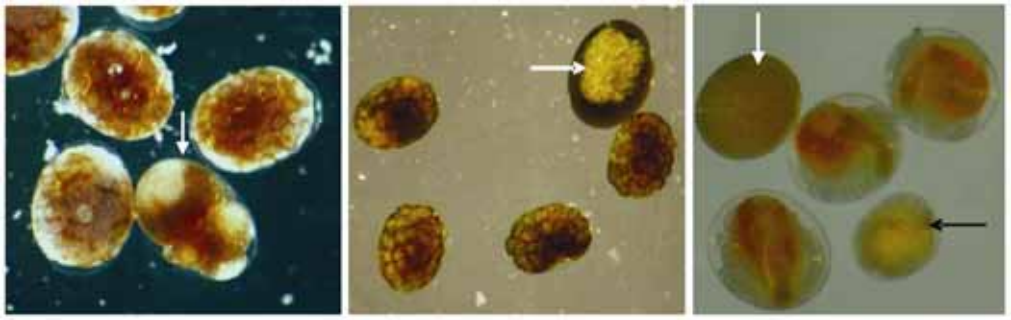

Figure 8. ***Echinogammarus marinus*****. White arrows indicate undifferentiated eggs (left and right panels); crystallization of egg membrane and darkening leading to degeneration (middle panel); black arrow indicates a dead egg starting to degenerate (right panel).**

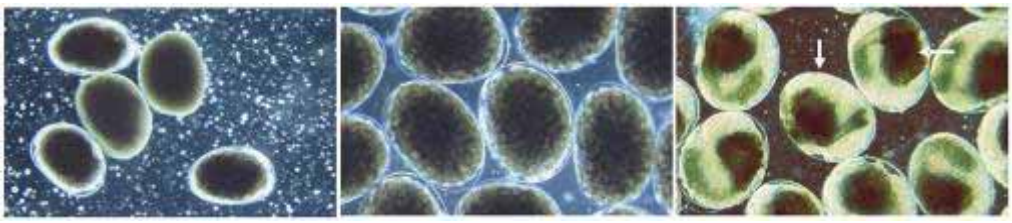

Figure 9. ***Gammarus salinus*****. Arrows indicate malformed embryos, where the embryos are flattened and twisted, leading to an aberrant shape.**

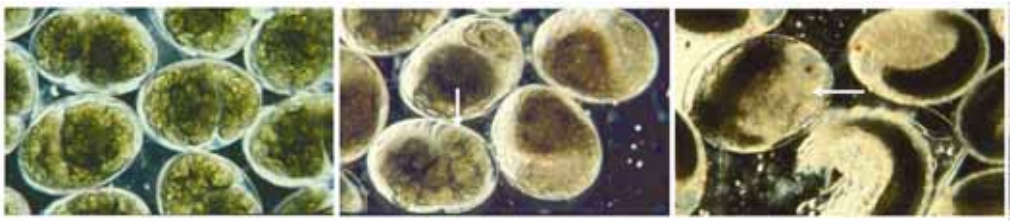

Figure 10. ***Pallasea quadrispinosa*****. The middle panel shows an undifferentiated egg among embryos of Stage 6; the right panel shows a malformed embryo of Stage 8 with membrane dysfunction, where lipids are leaking from both anterior and part of the midgut.**

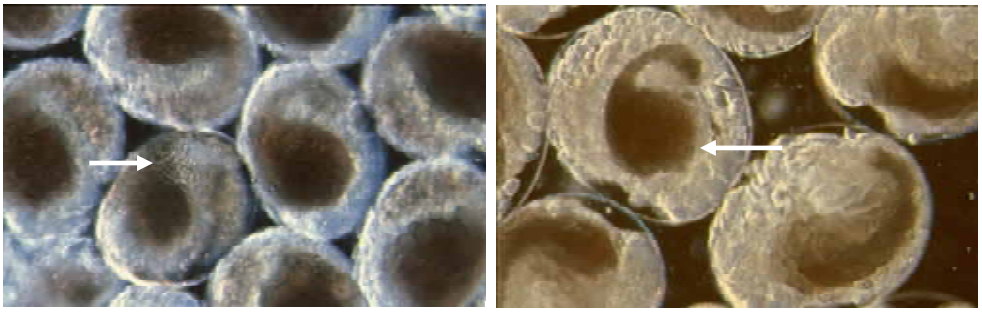

Figure 11. ***Pontoporeia femorata*****. Arrows indicate membrane dysfunction (left panel), where lipids are leaking from the midgut and remain close to the outer egg membrane, and a malformed embryo (right panel), where the diminished midgut is placed in the centre of the animal and appendages are situated around the animal rather than to the ventral side.**

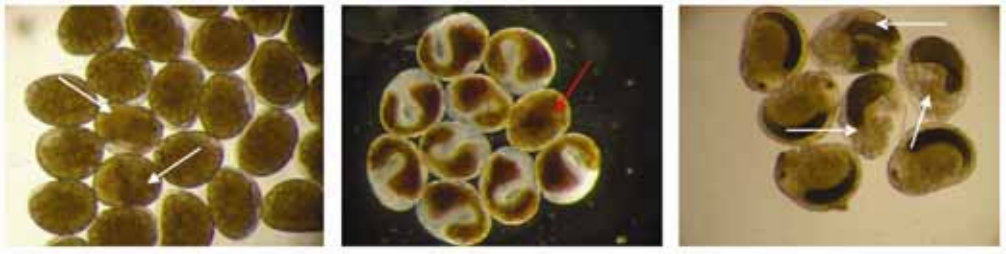

Figure 12. ***Hyalella azteca*****. White arrows indicate malformed eggs (left panel) and malformed midguts and exoskeletons (right panel). The red arrow indicates undifferentiated eggs (middle panel).**

ICES Techniques in Marine Environmental Sciences

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No. 2 Trace metals in seawater: Sampling and storage methods

No. 3 Cadmium in marine sediments: Determination by graphite furnace atomic absorption spectroscopy

No. 4 Lipophilic organic material: An apparatus for extracting solids used for their concentration from seawater

No. 5 Primary production: Guidelines for measurement by ^{14}C incorporation

No. 6 Control procedures: Good laboratory practice and quality assurance

No. 7 Suspended particulate matter: Collection methods for gravimetric and trace metal analysis

No. 8 Soft bottom macrofauna: Collection and treatment of samples

No. 9 Sediments and suspended particulate matter: Total and partial methods of digestion (*videotape available*)

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No. 24 Biological effects of contaminants: Use of imposex in the dogwhelk (*Nucella lapillus*) as a bio-indicator of tributyltin pollution

No. 25 Biological effects of contaminants: Measurement of DNA adducts in fish by ^{32}P-postlabelling

No. 26 Biological effects of contaminants: Quantification of metallothionein (MT) in fish liver tissue

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No. 33 Recruitment studies: Manual on precision and accuracy of tools

No. 34 Biological effects of contaminants: Quantification of d-aminolevulinic acid dehydratase (ALA-D) activity in fish blood

No. 35 Chemical measurements in the Baltic Sea: Guidelines on quality assurance

No. 36 Biological effects of contaminants: Measurement of lysosomal membrane stability

No. 37 Biological effects of contaminants: Use of intersex in the periwinkle (*Littorina littorea*) as a biomarker of tributyltin pollution

No. 38 Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus*

No. 39 Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile

No. 40 Biological effects of contaminants: Measurement of scope for growth in mussels

No. 41 Biological effects of contaminants: the use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors