STAGE- AND SEX-DEPENDENT SENSITIVITY TO WATER SOLUBLE FRACTIONS OF FRESH AND WEATHERED OIL IN THE MARINE COPEPOD *CALANUS FINMARCHICUS*

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Abstract: Acute toxicity differs between species, but also varies within a species. Important intra-specific factors are the exposure duration and properties of the animal such as life stage, sex and physiological status. In this study, the acute toxicity of water-soluble fractions (WSFs) from fresh and artificially weathered oil was followed over time in different life stages of the calanoid copepod *Calanus finmarchicus*, including adult males and females. The life stages differ in size but also in lipid content and physiology. To meaningfully compare the sensitivity of the different stages, we fitted a toxicokinetic-toxicodynamic (TKTD) model from the framework of the General Unified Threshold model of Survival (GUTS) to the mortality patterns over time. The oil WSFs could not be treated as single compounds: the rapid effect at high doses could not be reconciled with the slow effect at low doses. Treating the oil as a mixture of two component blocks could, however, capture these patterns satisfactorily. Even though the early life stages of animals are generally considered to be most vulnerable, the adult males of *C. finmarchicus* turned out to most sensitive, followed by the early copepodites. Naupliar larvae were equally susceptible to oil toxicity as late copepodites and adult females. The relationship between the GUTS model parameters and the physiological traits for the different life stages remains, however, unclear.

Keywords: Copepods *Calanus finmarchicus* acute toxicity GUTS oil toxicity

INTRODUCTION

Acute toxicity plays an important role in assessing the environmental impacts of produced water discharges and accidental oil spills on marine ecosystems. Such assessments are challenged by the fact that oil toxicity differs between species, even closely-related ones [1], but also within a species there may be considerable variation. In most cases, the early life stages of crustaceans are the stages that are found to be most affected by oil exposure [2-4], and this seems to be a general trend for other taxa and compounds as well [5]. Interpreting the sensitivity of different life stages is, however, severely hampered the fact that 'sensitivity' is an ill-defined concept in ecotoxicology. The results from acute toxicity tests are usually summarised as an LC50 (the concentration leading to 50% mortality, relative to the control, after a specified exposure duration). LC50s will decrease over time, and the shape of this time pattern depends on properties of the organism and on the chemical [6]. Therefore, it is impossible to select a single representative exposure duration for the LC50 for all life stages and for all chemicals.

When talking about sensitivity, we first need to distinguish between 'apparent' and 'intrinsic' sensitivity [7]. Apparent sensitivity is the response of an organism to a certain external concentration of a toxicant after a specified exposure time (e.g., a 4-day LC50), whereas intrinsic sensitivity is the relationship between the internal concentration at a target site (e.g., the cell membrane for narcotic compounds) and the physiological process that is affected. Differences in apparent sensitivity may reflect differences in intrinsic sensitivity, but they may just as well be caused by differences in toxicokinetics (TK) or result from the way physiological processes interact to produce the observed effect [7]. The quantification of intrinsic sensitivity requires the use of toxicokinetic-toxicodynamic (TKTD) models. These models use the time course of effects to extract time-independent parameters that have a closer relation to the actual processes leading to the toxic effect. For the endpoint survival, the hazard rate (the instantaneous probability to die) can be pragmatically viewed as such a process [8]. The parameters that govern the relationship between the internal concentration and the hazard rate (in principle) do not depend on time, and therefore provide a straightforward basis for comparing different life stages [9, 10].

Differences in TK are expected between life stages as uptake and elimination rates are influenced by body size [11, 12]. Chemical exchange takes place across a surface area, whereas body concentrations are related to a volume (or weight). Therefore, the rate constants are expected to scale with the surface:volume ratio of the organism, and hence should be inversely proportional to body length [13]. Smaller organisms thus have higher rate constants, which means that they require less time to reach steady-state body residues, and toxic effects will therefore manifest themselves earlier. Gerritsen *et al.* [10] elegantly demonstrated that the difference in apparent sensitivity between adults and young of *Daphnia magna*, exposed to a series of alkylphenols, could be fully explained by a difference in TK between the stages. This finding may, however, not be universally valid, as indicated by the contrasting results obtained for a copepod species exposed to triphenyltin [9].

Intra-specific differences in lipid content may be an additional factor causing differences in apparent sensitivity of the life stages. Boreal and Arctic crustaceans, for example, are subjected to large seasonal variations in food availability. Adaptations to these conditions often include the accumulation of large amounts of lipids when food is available, and periods of low activity or diapause during the winter [14]. Lipid content is obviously related to the bioconcentration factors for hydrophobic chemicals, but a higher lipid content is also expected to lead to a smaller elimination rate constant [11, 12], and has been associated with a decreased sensitivity to oil exposure [15].

In the present study, we examine sensitivity differences between life stages and sexes of the marine copepod *Calanus finmarchicus* (Gunnerus), which is widely distributed in the North Atlantic. Just like other copepods, it goes through several stages during its development. After the egg stage, they develop through six naupliar and five copepodite stages, before closing the life cycle as adults (the reproductive stage). Body size is obviously linked to the stage, but so is the lipid content [16]. Nauplii and early copepodite stages (CI-CII) have a relatively stable low lipid content, which mainly

reflects the membrane lipids. In late copepodite stages (between the CIII and CV), the lipid content increases considerably under abundant food, and may reach more than 50% of the dry weight in CV [14]. These additional lipids are storage lipids (wax esters and triacylglycerols) that are deposited into an oil sac, which constitutes an important storage for gonad maturation, reproduction, and fuel the organism in periods of food scarcity or diapause [14]. To our knowledge, no data exists on the sensitivity of early developmental stages of cold-water marine zooplankton to oil exposure. In the present study, we therefore performed acute toxicity tests using water-soluble fractions (WSFs) of fresh and weathered crude oils on different life stages of *C. finmarchicus*, including adult males and females. The data for survival over time were analysed using a TKTD model from the framework of the General Unified Threshold model of Survival, GUTS [8]. The results are discussed in relation to biologically relevant factors that may explain potential differences, e.g., lipid content and size. The working hypothesis was that stages do not differ in intrinsic sensitivity, but that differences in apparent sensitivity can be explained by differences in toxicokinetics.

MATERIALS AND METHODS

Experimental organisms

C. finmarchicus from the continuous laboratory culture at SINTEF/NTNU Sealab was used for the acute toxicity experiments. The culture is kept at approximately 10°C. Details regarding the culturing have been described previously [17]. The selected stages were nauplii (NIII/NIV), early copepodites (CI/CII), late copepodites (CV), and adults, including both females and males. Photographs of the various stages can be found in the Supplemental Data (Figure S1).

Selection of oil and generation of WSF

Crude oil from the Troll B field reservoir in the northern part of the North Sea was chosen as the representative oil for the study. An aliquot of fresh crude oil was artificially weathered by heating to 200°C [18], and the residue was collected and used for the generation of weathered WSF. The 200°C residue mimics the oil after approximately one day at sea, where the lighter compounds have disappeared due to evaporative losses [19]. WSFs from both fresh and weathered crude oil were prepared according to methodology proposed earlier [20]. 10 L baked glass bottles were filled with filtered (Sterivex, 0.22µm, Millipore, Billerica, MA U.S.) natural sea water leaving a headspace of approximately 25% of the total volume. Oil was then carefully added onto the surface alongside a glass tube until the oil:water ratio in the bottle reached 1:40. To avoid formation of oil droplets, low-energy magnetic stirring was applied for 72 hours before the water phase was tapped from an outlet at the bottom of the bottle and used for the experiments.

Extraction and analyses of water samples

Surrogate internal standards (SIS, o-terphenyl, naphthalene-d8, phenanthrene-d10, chrysene-d12, phenol-d6, 4-methylphenol-d8) were added to the water samples prior to processing, and recovery internal standards (RIS, 5α-androstane, fluorene-d10, and acenaphthene-d10) were added prior to analysis on GC/FID (gas chromatography/flame ionization detection) and GC/MS (gas chromatography/mass spectrometry). For analyses of semi-volatile organic compounds (SVOC) and total petroleum hydrocarbons (TPH), the water samples were spiked with the appropriate surrogate internal standards and serially extracted with dichloromethane (DCM), thereby following a modification of EPA method 3510C [21]. The combined extracts were dried with sodium sulphate and concentrated to approximately 1 mL using a Zymark Turbovap 500 Concentrator. The final extract was spiked with the appropriate recovery internal standards and analyzed on GC/FID and GC/MS.

The samples were analyzed for SVOC (decalins, polycyclic aromatic hydrocarbons and phenols) using GC/MS, for TPH using GC/FID, and for volatile organic compounds (VOC, C5-C9), including BTEX (benzene, toluene, ethylbenzene, and xylenes), by use of Purge and Trap Gas Chromatography Mass Spectrometry (P&T GC/MS). A list of all target components is provided in the Supplemental Data (Table S1). This list includes the recommended components given by Singer *et al.* [20], and is a typical standard list for the target compounds used during post-oil spill damage assessments.

The GC/FID analyses were performed according to a modification of EPA Method 8100 [22]. Resolved and unresolved TPH (C10-C36) was quantified by the method of internal standards using the baseline corrected total area of the chromatogram and the average response factor for the individual C10 to C36 n-alkanes.

The SVOC were quantified using a modified EPA Method 8270D [23]. The mass spectrometer was operated in the selective ion monitoring mode to achieve optimum sensitivity and specificity. The quantification of target compounds was performed by the method of internal standards, using average response factors (RF) for the parent compounds. The PAH and phenol alkyl homologues were quantified using the straight baseline integration of each level of alkylation and the RF for the respective parent PAH compound. The response factors were generated for all targets and surrogates versus fluorene-d10.

The volatiles were analyzed in the water samples. A total of 35 target volatile compounds in the C5 to C10 range were determined by P&T GC/MS using a modification of EPA method 8260C [24]. The samples were spiked with SIS (toluene-d8 and ethylbenzene-d8) and RIS (chlorobenzene-d5). The quantification of individual compounds was performed by using the RFs of the individual compounds relative to the internal standards. All standards and samples were analysed in full scan mode.

For calculations of total hydrocarbon content (THC, C5-C36) in the water samples, concentrations of VOC (C5-C9) and TPH (C10-C36) were summed.

Acute toxicity tests

The WSFs were diluted to a series of seven exposure concentrations with natural sea water, filtered to 1 µm with inline cartridge filters (CUNO, Meriden, CT, USA). To reduce loss of volatile components during exposure, the vessels were filled to the rim before capping. The exposures were performed in darkness in a temperature-controlled room at approximately 10°C for all tests, with monitoring of mortality every 24 hours until termination of the test. The animals were not fed during exposure in any of the tests.

Prior to exposure, nauplii of the desired stage III/IV were identified under a dissecting microscope (Leica M80 with 1x objective, Leica Microsystems, Germany) and captured by a mouth pipette of proper size. Stage identification was done following Marshall and Orr [25]. To avoid dilution of the exposure solution with culture water, the nauplii were transferred to a volume of the exposure solution before moved to the exposure vessel by the mouth pipette. As exposure vessels, 5 ml screw cap clear glass vials were used (model 4-SV, Chromacol Ltd., Hertz, United Kingdom), with four parallel vessels for each exposure concentration, and eight replicate vessels containing filtered sea water as controls. Each replicate consisted of 10-15 animals. To avoid loss of test animals during capping, each individual vessel was observed under a magnifying lamp (Luxo “Wave”, Glamox Luxo Lightning, Norway) while capping. The nauplii were followed for 72 hours. Copepodites I/II were handled as for nauplii, except 12 ml screw cap clear glass vials (model 5183, Agilent Technologies Inc., Santa Clara, CA, USA) were used as exposure vessels to account for the increased biomass. Stage identification was done following Mauchline [26].

For the tests with copepodite V and adults, copepods of the desired stage or sex were sorted and verified under a dissecting microscope (Leica MZ125 with 1x objective, Leica Microsystems, Germany) before being transferred to filtered sea water in polypropylene buckets (5.9 L volume, Emballator, Sweden). Subsequently, the animals for each exposure vessel were concentrated in a sieve (110mm diameter, mesh size 125 µm), partly submerged in filtered sea water, before transfer in a small amount

of sea water to the vessel, topping it up to the rim. As exposure vessels, 500 ml screw cap clear borosilicate glass flasks were used ("Pyrex", Bibby-Sterilin Ltd, United Kingdom). Three replicates were used for each exposure concentration, and six containing filtered sea water as controls. Each replicate consisted of seven animals. The CV and adult copepods were followed for 144 hrs. Identification of stage or sex was done following Mauchline [26]. After exposure, the pH and oxygen saturation were measured in one vessel from the exposure series, and in two from the control series.

Model analysis

The analysis of the toxicity data departs from a particular special case of the GUTS framework: the stochastic-death model with scaled toxicokinetics [8] (an alternative analysis with the individual-tolerance model is provided in the Supplemental Data). We start from the assumption that the *intrinsic* sensitivity at the level of the target site is the same for each life stage or sex, and does not depend on whether the oil is fresh or weathered. The differences in *apparent* sensitivity (e.g., the LC50 or the mortality pattern at a certain exposure) are then solely caused by differences in bioavailability and toxicokinetics. This is equivalent to the assumptions made when comparing the hazard-model parameters of the scaled TK model, within one species, for different chemicals with the same mechanism of action [27]. It makes sense to allow for differences in the elimination rate (k_e) between life stages. However, there may also be differences in the bioconcentration factor and/or differences in bioavailability that lead to differences in the steady-state ratio between the external and internal concentrations. In a scaled TK model, there is no bioconcentration factor, but we can account for relative differences by including a sensitivity factor F_s for each life stage. The scaled internal concentration (C_i^*) is then related to the external concentration (C_w) as:

$$\frac{dC_i^*}{dt} = k_e(F_s C_w - C_i^*) \quad (1)$$

There may also be differences in toxicokinetics and/or bioavailability between fresh and weathered oil, and therefore, separate k_e and F_s parameters are fitted for both oil treatments. F_s is set to 1 for nauplii exposed to fresh oil, so all other values for F_s are relative to this reference situation.

Assuming that oil behaves as a single compound, we can calculate a total hazard rate (h_z) as follows:

$$h_z = k_k \max(0, C_i^* - z) + h_b \quad (2)$$

Where h_b is the background hazard rate, z the threshold for effect, and k_k the killing rate. The total hazard rate h_z can subsequently be integrated over time to calculate the survival probability over time [8]. This model produced a typical misfit: the high concentrations are fitted well, but the slowly-appearing mortality at the lowest doses was not captured. Therefore, we assume that the oil should be treated as a mixture of two compounds; using two scaled TK models (as in Equation 1) for the internal concentrations of both hypothetical components (C_{i1}^* and C_{i2}^*). The same F_s is applied to both components, and also the same exposure concentration C_w is used (the total hydrocarbon content, THC, in each treatment). Using the THC for both hypothetical compounds is valid as long as the fraction of each component in the total remains constant over the test duration. The toxicodynamic parameters (z and k_k) absorb the unknown fraction of each component in the total concentration.

For independent causes of death (i.e., different mechanisms of action), the hazard rates for the two components can be added:

$$h_z = k_{k1} \max(0, C_{i1}^* - z_1) + k_{k2} \max(0, C_{i2}^* - z_2) + h_b \quad (3)$$

The thresholds for effect (z_1 and z_2) and the killing rates (k_{k1} and k_{k2}) can be different for both hypothetical compounds. Alternatively, the two compounds might exhibit the same mechanism of action, which implies that their scaled internal concentrations can be added with a weighing factor:

$$h_z = k_{k1} \max(0, C_{i1}^* + C_{i2}^* W - z_1) + h_b \quad (4)$$

The weight factor W modifies the scaled internal concentrations of compound 2, making it a dilution or concentration of compound 1. If both compounds have the same toxicokinetics (k_{e1} = k_{e2}), the mixture of the two will behave as a single compound again. This additive model, however, could not explain the observed misfit for our data set (see Results and Discussion), and we therefore focus on the independent action model of Equation 3.

The intrinsic sensitivity of a life stage is reflected in the value of the TD parameters (z_1, z_2, k_{k1} and k_{k2}). As we assume the same intrinsic sensitivity for all stages, a single set of TD parameters is used in fitting the model to the survival data. We also keep these parameters the same for both types of oil, assuming that the mechanism of action of both oils will be the same. The same background hazard rate (h_b) is used for all cases, and the same rate constant for the second (slow) TK process (k_{e2}). This latter assumption might not be realistic, but the data provide insufficient detail on the slow toxicity at low doses to warrant further parameters. This gives a total of 25 parameters that need to be estimated from 10 data sets; an average of less than three parameters per data set, which is quite acceptable. The model was implemented in Matlab 2015a, and fitted by maximising the multinomial likelihood [8]. Confidence intervals were generated by profiling the likelihood function. The entire model is schematically drawn in Figure 1.

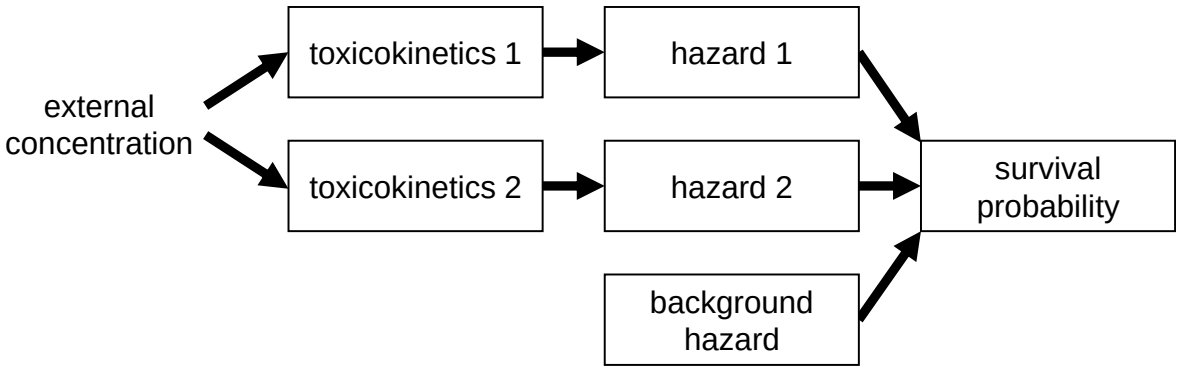

Figure 1. Schematic representation of the model to analyse oil toxicity for *Calanus finmarchicus*, assuming two component blocks with distinct mechanisms. Inter-stage differences are assumed to affect toxicokinetics only.

RESULTS AND DISCUSSION

Water concentrations of oil components

The composition of the generated WSFs, as well as a complete list of components analysed, can be found in the Supplemental Data (Table S1-S2). The total hydrocarbon content (C5-C36) was used as input to the toxicity calculations. The fresh oil WSF was dominated by volatile organic components (VOC). Of the VOC, the mono-aromatic hydrocarbons (MAH), including the BTEX compounds (benzene, toluene, ethyl benzene and o, m, p-xylenes) were dominating, along with cyclohexane, n-hexane and methylcyclohexane. As expected, the concentrations of VOC were lower in the WSF from weathered than fresh crude oil, especially for the aliphatic compounds where only 10% remained after weathering. The C3-C5-alkylated benzenes were more resistant to weathering, demonstrated by the similar concentrations in WSF from fresh and weathered oil. The dominating compound group of the semi-volatile compounds (SVOC) was the naphthalenes (C0-C4) for both fresh and weathered WSF. Some expected decrease in naphthalene content of the WSFs due to weathering was observed, while concentrations of >2-ring PAHs were highly comparable between the two WSFs (Table S2). Previous experiments with weathered Troll WSF have provided similar chemical compositions and concentrations [15].

Model fits

The simultaneous fit to all 10 data sets is presented in Figure 2 (larger graphs with legends, as well as the raw survival data, are provided in the Supplemental Data). The common parameters for all data sets are presented in Table 1, and the parameters that differ between data sets in Figure 3. Overall, the fit for two hypothetical components with different mechanisms of action (Equation 3) provides a good explanation of the data. The fast mechanism 1 is responsible for the deaths at the high doses, and has a rather high threshold for effects (z_1 in Table 1). In contrast, the slow mechanism 2 has a low elimination rate and a low threshold z_2 (the confidence interval includes zero). The nature of these two mechanisms cannot be further elucidated from this data set. However, it is highly likely that they are indeed two separate mechanisms of action; the model for two components with the same mechanism of action (Equation 4) provided a poor fit to the data (fits not shown). Since oil is a mixture of a large number of components, it is conceivable that the two mechanisms reflect two blocks of compounds; within each block, the combined effect can be described as a single compound. Alternatively, it may be that the oil can be treated as a single compound, but the compound itself has two mechanisms of action (for an example, see [28]). Interestingly, Cucci and Epifanio [3] also speculate about two mechanisms of action for oil; one related to a relatively slow accumulation of compounds over time, and one faster, more direct mechanism. As a last possibility, the slower phase of toxicity may have been caused by starvation during the test, making the organisms more sensitive to the oil treatments. In this case, we consider this scenario unlikely; one would expect additional mortality due to starvation to start late and increase more sharply over time at low concentrations [29].

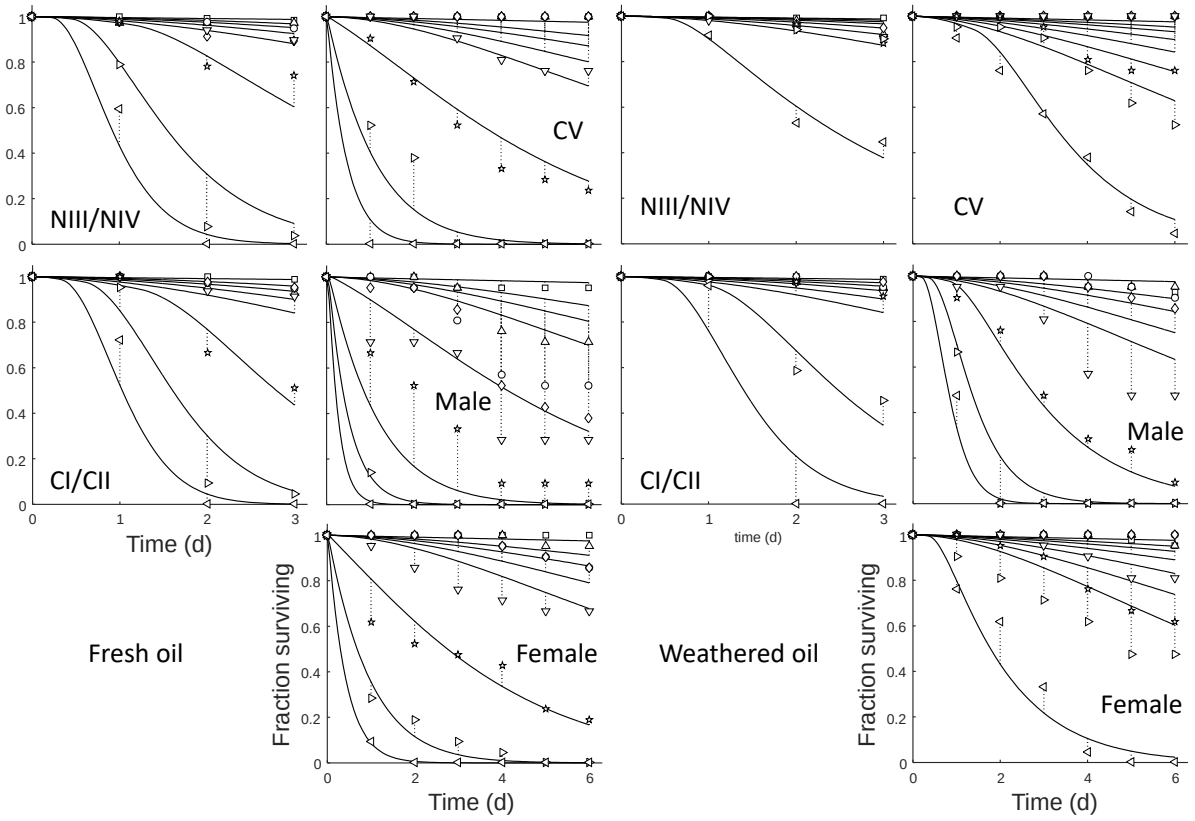

Figure 2. Simultaneous fits to the toxicity data for fresh and weathered oil in different life stages of *Calanus finmarchicus*. Symbols represent different exposure treatments. Note that the time axis is shorter for the nauplii and early copeodites than for the later stages. Parameter estimates are given in Figure 3 and Table 1. Larger figures with legends are presented in the Supplemental Data.

Table 1. Parameter estimates with 95% likelihood-based confidence intervals for the common parameters of all data sets. Parameters that differ between each data set are shown in Figure 3.

Symb.	Parameter	Estimate (95% CI)	Unit
h_b	Background hazard rate	$4.17\ (2.28\text{-}6.79)\cdot10^{-3}$	d^{-1}
z_1	Reference threshold (fast MoA)	2470 (2040-2810)	$\mu g\ L^{-1}$
k_{k1}	Reference killing rate (fast MoA)	$0.395\ (0.316\text{-}0.490)\cdot10^{-3}$	$L\ \mu g^{-1}\ d^{-1}$
k_{e2}	Elimination rate (slow MoA)	0.234 (0.000407-0.546)	d^{-1}
z_2	Threshold (slow MoA)	18.6 (0-131)	$\mu g\ L^{-1}$
k_{k2}	Killing rate (slow MoA)	$0.0773\cdot10^{-3}\ (0.0420\cdot10^{-3}\text{-}2.72)$	$L\ \mu g^{-1}\ d^{-1}$

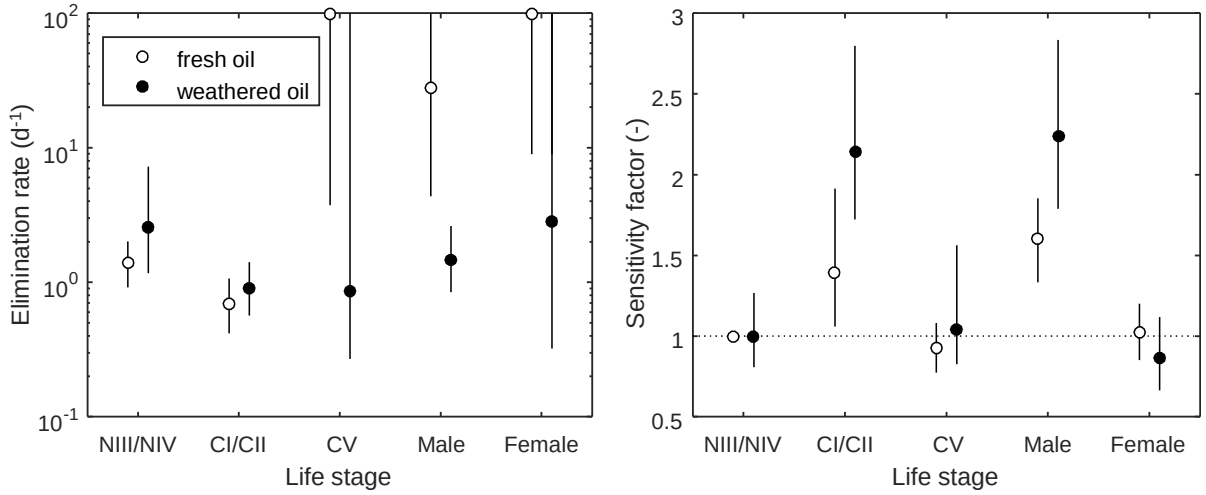

Figure 3. Estimated values, with 95% likelihood-based confidence intervals, for the fitted parameters that are allowed to differ between life stages. Common fitted parameters provided in Table 1. For the elimination rate constants 100 d^{-1} is set as a maximum. The sensitivity factor is relative to NIII/NIV exposed to fresh oil, which is set to 1.

Elimination rates

We expected the elimination rate (k_{e1}) to reflect TK processes, and thus to differ between life stages. As explained in the introduction, large individuals have a smaller surface area:volume ratio than small ones (of the same shape), and hence generally show slower TK (smaller values for k_e). Mean lengths of the individual stages at similar test temperatures are approximately 0.3 and 0.4 mm for NIII and NIV, 0.75 and 1.0 mm for CI and CII, 2.3 mm for CV, 2.6 mm for males, and 2.7 mm for females [16]. We expected the elimination rate to scale inversely proportional to body length, and hence steadily decrease from nauplii to adults by a factor of around eight. Between nauplii and CI/CII, there is indeed a trend for a decrease in k_{e1} with size (Figure 3). For the later stages, k_{e1} cannot be precisely identified from the data, but in any case, there is no indication for the predicted decrease with body size. The large uncertainty in the estimates for k_{e1} for the CV and adult stages may relate to the fact that less animals were used per treatment, compared to the nauplii and early copepodites.

We also expected elimination rates to decrease with increasing lipid content. The lipid content varies during the life cycle of *C. finmarchicus*, and build-up of a lipid sac generally becomes obvious at stage CIII. In the present study, we thus have two life stages without lipid reservoir (NIII/NIV and CI/CII) in addition to later stages with well-developed lipid sacs. The CV stage generally has the highest lipid content of these stages [16, 30], although lipid content was not determined in our experiments. There

is thus no obvious relationship between the lipid content and the observed differences in elimination rate between the stages.

It should be kept in mind that the elimination rate constant in the model (k_{e1}) is estimated from the survival data, and may therefore also be influenced by other processes apart from TK, such as the accumulation of damage and biotransformation [8].

Sensitivity factor

The sensitivity factor F_s shows an interesting pattern over the life stages (Figure 3). Nauplii, CV and females all have a very similar F_s, and there is no difference between fresh and weathered oil. However, CI/CII and males have an elevated sensitivity, especially for the weathered oil (although the confidence intervals for the two oils overlap). It is not straightforward to interpret these differences, but in any case, it implies that these two stages suffer more effect on the death probability at the same external concentration than the other stages. In the way we set up the model, we assumed that differences in BCF would cause these differences in sensitivity. However, we cannot exclude the possibility that the same internal concentration leads to a different degree of toxicity in different life stages (this can work out in the same way in the model). In any case, the differences in F_s cannot be explained by differences in size or in lipid content (Figure 3).

Lipid content has an interesting role to play in TKTD models. Animals with a higher lipid content will have a higher BCF for hydrophobic chemicals. However, if this additional lipid is restricted to storage organs, the body burden in that organ may not contribute to toxicity. The BCF that is representative for toxicity in the copepods may be related to the structural lipid content, and hence may be less dependent on the status of the lipid sac. The lack of a relationship between F_s and lipid content may thus be expected.

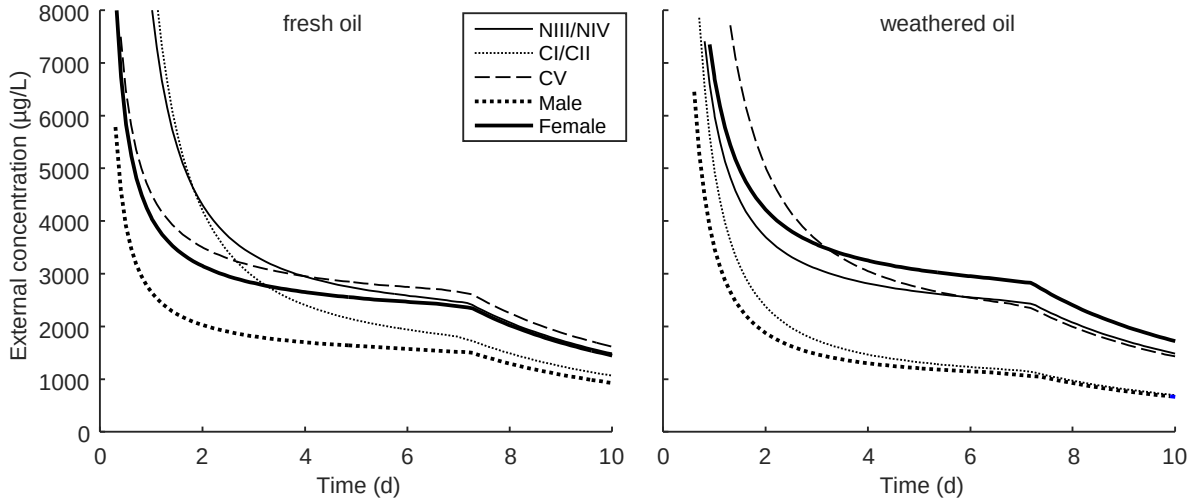

Figure 4. Iso-effect lines for 50% effect over time (LC50), calculated from the parameters in Table 1 and Figure 3.

LC50 versus time

We can use the complete set of model parameters (Table 1, Figure 3) to predict the LC50 over time (Figure 4). It should be noted that the time on the x-axis exceeds the test duration (especially for the first two life stages), so these LC50s are to some extent extrapolations. The rather sharp switch in LC50 versus time around day 7 reflects the point where the second mechanism will begin to dominate the mortality process. The males clearly have the lowest LC50 at each time point. For weathered oil, the early copepodites show a comparable pattern to the males, but less so for short-term exposure to

fresh oil (as a result of a slower elimination rate). For the other life stages, the patterns for the LC50 versus time are rather similar.

Based on the estimated LC50 versus time (Figure 4), *C. finmarchicus* males consistently displayed the highest apparent sensitivity for both WSF treatments compared to the other stages, and were clearly more sensitive than the females. This conclusion is confirmed by the sensitivity factors in Figure 3. Such a difference was not expected, since both sexes from our culture are of similar size, shape and lipid content [30], and other studies also do not indicate a specific sensitivity of male copepods to oil components [31]. We have, however, observed that males of *C. finmarchicus* have much lower RNA content than females (unpublished results), which may result in a lower ability in males to produce biotransformation enzymes like cytochrome P450 and glutathione S-transferase. These enzymes are capable of transforming toxic xenobiotics (including PAHs) into less toxic metabolites, and are considered the first line of cellular defence against PAH toxicity in vertebrates and possibly also in invertebrates [1, 32, 33]. It is therefore conceivable that males either have higher internal concentrations (at the target site), or that similar concentrations produce more effect than in other stages owing to a less effective cellular defence mechanism. Males also spend less time feeding, and have a higher swimming activity than females as they in nature cover large areas in search of females [34]. Irigoien *et al.* [35] estimated that *Calanus* males have a 20% higher respiration rate than the females, whereas the ingestion rate is only 15% of that of the females. The males mainly rely on their lipid storage to sustain them, which may be associated with a higher sensitivity towards toxicants in our study. Furthermore, higher metabolic rates have been implicated in an increased sensitivity towards acute toxicity [36].

Comparison to other studies

Very few studies have systematically compared the sensitivity of individual life stages of marine copepods to oil exposure [4, 31]. Bejarano *et al.* [4] exposed the harpacticoid copepod *Amphiascus tenuiremis* to water accommodated fractions (WAF) of crude oil during a full life cycle. Their results indicated that early stages were more sensitive than later stages, which apparently contradicts our results. The studies are, however, difficult to compare. Bejarano and co-workers followed the animals over a full life cycle (and hence, food was provided), whereas our experiments focused only on survival during specific stages (and no food was provided). In *C. finmarchicus*, and probably also in other copepods, especially the nauplii VI/copepodite I moult is physiologically demanding and may well cause a large fraction of overall ontogenetic mortality. This moult was not part of our experiments, but may have contributed to the apparent higher sensitivity of the early life stages in the work of Bejarano and co-workers.

Lotufo and Fleeger [31] investigated the stage-specific sensitivity in two species of harpacticoid copepod to sediment-associated phenanthrene (a single oil component). For one species (*Schizopera knabeni*), nauplii displayed a higher apparent sensitivity than copepodites, and copepodites were more sensitive than adults. No sensitivity difference between males and females was observed. For the other species (*Nitocra lacustris*), on the other hand, females were distinctly less sensitive than all other stages tested (with no significant differences between the nauplii, copepodites and males). Again, these results are difficult to compare to ours, as the experimental design differs. Lotufo and Fleeger use the 10-day LC50 as their measure of sensitivity, and the animals were fed during the test. Given the short life cycle of both species, this ensures that several moults were included for the non-adult stages within the test duration, including the moult from nauplius to copepodite for the test starting with nauplii.

For another set of PAHs (naphthalene and C2-naphthalene), Saiz *et al.* [37] found that the nauplii of the cyclopoid copepod *Oithona davisae* where roughly a factor of two more sensitive (based on the 1-day LC50) than adults. However, for another copepod species (*Paracartia grani*), no sensitivity differences were observed between nauplii and adults for the same two PAHs [38].

Kulkarni *et al.* [9] used triphenyltin as the toxicant, which is not an oil component, and looked at the response to different life stages of the freshwater cyclopoid copepod *Mesocyclops leuckarti*. In contrast to our study, these authors reported a higher sensitivity for the nauplii. They also used the GUTS model framework to analyse the results, but fitted the toxicity data for each stage separately. Kulkarni and co-workers found that, for the stochastic-death model (as used in our study), body size was a poor predictor for the elimination rate constant, which is consistent with our findings. In fact, the naupliar stages showed the smallest k_e for triphenyltin. The higher sensitivity of the nauplii was related to a lower threshold (z) and a higher killing rate (k_k) compared to the other stages (by a similar factor), which is equivalent to assuming a higher sensitivity factor (F_s) for this stage. No sensitivity differences between males and females were observed.

Some studies with copepods have revealed sex-specific differences in sensitivity for non-oil chemicals. For *Acartia tonsa* exposed to cypermethrin, males were more sensitive to females, but only during the first day of exposure (using the LC50 as sensitivity metric) [39]. In that study, nauplii were considerably more sensitive than males or females. A clearer difference between the sexes was seen for *Microarthridion littorale* exposed to PCBs [40]; the 4-day LC50 for males was two times lower than for females.

In summary, sensitivity differences have been observed for different stages and sexes of copepods, but a general pattern is lacking. In most cases, naupliar stages are found to be more sensitive, contrasting the results from our study. Even though the different studies are difficult to compare due to differences in test design and measure of sensitivity, we should consider the possibility that intra-specific sensitivity variation depends on the species and on the toxicant.

CONCLUSION

Life stages and sexes of *C. finmarchicus* differ in their survival response to fresh and weathered crude oil WSFs. We used a TKTD model based on the GUTS framework to elucidate the nature of these differences in apparent sensitivity. The survival patterns could be captured by representing oil by two abstract components (see Figure 1), at the cost of additional model parameters. The total number of parameters could be kept within acceptable limits by forcing several parameters to be the same in all treatments (see Table 1). A mixture toxicity approach is likely most appropriate for oils (see e.g, [41]), but requires more basic information on the action of the single components.

Given that the mortality pattern is best described by two mechanisms of action, this raises the question what constitutes a 'safe' level of oil in the environment. For exposure up to a week or so, the first (fast) mechanism dominates mortality. The model parameters established in the present study can be used to predict the mortality for the different life stages, given an exposure scenario such as an oil spill. The parameters can also be used to provide estimates of LCx,t for any effect percentage x and exposure duration t (see Figure 4). For longer exposures, however, the second (slow) mechanism begins to dominate mortality. Since the confidence interval for the second threshold (z_2) includes zero, we cannot exclude that even very low oil concentrations will eventually induce mortality after prolonged exposure.

Even though many uncertainties remain, several general conclusions can be drawn. The differences in apparent sensitivity between the groups can be explained by assuming differences in the TK module only; taking different values for k_{e1} and F_s for each group. Contrary to our expectations, these parameters cannot be related to size or lipid content. Furthermore, the nauplii turn out to be no more sensitive than the older, larger and more lipid-rich stages, but the males and the early copepodites are particularly sensitive. The reasons for these findings are unclear, and require further dedicated study. Toxicity testing using *C. finmarchicus* is usually performed with the CV stage and adult females, but the current results suggest that a higher sensitivity of the males and early copepodites should be considered.

TKTD models from the GUTS framework are essential tools to analyse apparent sensitivity differences between life stages and sexes, and to separate differences in intrinsic sensitivity from

other factors, such as differences in toxicokinetics. The mechanistic link between model parameters and physiological traits remains, however, elusive.

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