

Effects of petrogenic pollutants on North Atlantic and Arctic *Calanus* copepods: From molecular mechanisms to population impacts

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ABSTRACT

Oil and gas industries in the Northern Atlantic Ocean have gradually moved closer to the Arctic areas, a process expected to be further facilitated by sea ice withdrawal caused by global warming. Copepods of the genus *Calanus* hold a key position in these cold-water food webs, providing an important energetic link between primary production and higher trophic levels. Due to their ecological importance, there is a concern about how accidental oil spills and produced water discharges may impact cold-water copepods. In this review, we summarize the current knowledge of the toxicity of petroleum on North Atlantic and Arctic *Calanus* copepods. We also review how recent development of high-quality transcriptomes from RNA-sequencing of copepods have identified genes regulating key biological processes, like molting, diapause and reproduction in *Calanus* copepods, to suggest linkages between exposure, molecular mechanisms and effects on higher levels of biological organization. We found that the available ecotoxicity threshold data for these copepods provide valuable information about their sensitivity to acute petrogenic exposures; however, there is still insufficient knowledge regarding underlying mechanisms of toxicity and the potential for long-term implications of relevance for copepod ecology and phenology. Copepod transcriptomics has expanded our understanding of how key biological processes are regulated in cold-water copepods. These advances can improve our understanding of how pollutants affect biological processes, and thus provide the basis for new knowledge frameworks spanning the effect continuum from molecular initiating events to adverse effects of regulatory relevance. Such efforts, guided by concepts such as adverse outcome pathways (AOPs), enable standardized and transparent characterization and evaluation of knowledge and identifies research gaps and priorities. This review suggests enhancing mechanistic understanding of exposure-effect relationships to better understand and link biomarker responses to adverse effects to improve risk assessments assessing ecological effects of pollutant mixtures, like crude oil, in Arctic areas.

1. Introduction

Oil pollution in marine ecosystems is of major environmental concern (Barron et al., 2020). Oil and gas exploration and production

has taken place in the North Sea since the 1960s and in the Norwegian Sea since the 1980s. The Norwegian continental shelf areas where most oil and gas extraction has taken place until now, are highly biologically productive areas, e.g., providing spawning grounds for commercially

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important fish species, like the Atlantic cod (*Gadus morhua*) (González-Irusta and Wright, 2016). Petroleum production activity has shifted northwards towards Arctic areas of the Northern Atlantic and Barents Seas, a move facilitated by declining sea ice caused by rising surface temperatures. Ship traffic in the Arctic has already increased by 60 % in the last decade (Champine et al., 2019). Expanded industrial and maritime activities increase the risk of accidental spills of petrogenic pollutants (crude oils, fuel oils and produced water) from production platforms and maritime traffic to the Arctic marine environment. Although the petroleum industry has implemented a zero-discharge policy for certain northern areas (Knol, 2011), meaning that discharges of produced water will be limited, accidental discharges may still occur e.g. from leakages during reinjection and from the reservoirs, affecting potentially sensitive cold-water ecosystems.

The fate of spilled oil in the sea may differ based on the type of crude oil, location of spill, type of spill (surface or sub-surface), weather conditions, use of chemical dispersants etc. Hence, estimating the environmental impacts and damage of oil spills is a highly complex process, which requires detailed information about oil type, weather condition, oceanography, and local biota. Environmental models are often used as tools to predict oil spill trajectories, evaluate oil spill response and mitigation actions, and estimate risk and impact on the marine environment (French-McCay, 2004; Nepstad et al., 2021; Reed et al., 1999; Reed and Rye, 2011). Exposure of marine organisms to oil may be difficult to predict and can have impacts at different levels of biological organization, from molecules to populations. Effects of oil exposure within organisms are often assessed by using biomarkers to provide benchmarks for biological exposure and effects (Depledge et al., 1993; Depledge and Fossi, 1994). However, biomarkers often fail to predict population-level effects to estimate ecologically protective exposure thresholds (Forbes et al., 2006). Reduction in population size or changes in population structure of marine species due to oil pollution are ecologically relevant and unfortunate scenarios, but the risk for such events to occur is difficult to predict and hard to evaluate even in post-spill assessments. Organisms may have complex life histories, and for many marine species, life history traits are not well characterized, and the underlying mechanisms of toxicity are often unknown. Efforts to provide causal mechanistic relationships between the initial (early) molecular responses and intermediate physiological changes (effects) that ultimately lead to adverse effects (outcomes) are thus urgently needed. The adverse outcome pathway (AOP), originally conceived by the US EPA as a conceptual knowledge framework to organize, evaluate and portray mechanistic relationships within hazard assessment, is proposed to facilitate more efficient ecological risk assessment of pollutants (Ankley et al., 2010). Through use of existing biological and toxicological data, the AOP approach aims to translate mechanistic data into adverse impacts meaningful for ecological risk assessment (e.g., reduced survival, development, reproduction, and subsequently reduced population size). AOP structured construction utilizes available data to describe causal linkages between molecular initiating events (MIE) and key events (KE) representative of key and critical ecotoxicological effects across different levels of biological organization that ultimately lead up to adverse health impact. As such, the AOPs may facilitate evaluation of the causal linkage between biomarker responses (e.g. the MIE and KEs) and adverse outcomes in more mechanistically-informed ecological risk assessment (Ankley et al., 2010; Villeneuve et al., 2014a, 2014b).

Planktonic copepods of the genus *Calanus* are keystone species in Arctic and boreal marine ecosystems, being the most important prey for many fishes, especially at the earlier life stages. The *Calanus* assemblage in the north Atlantic and Arctic marine waters is dominated by three species: *C. finmarchicus*, *C. glacialis* and *C. hyperboreus*. These *Calanus* species share some common traits such as a complex life cycle, feeding dependency on the spring diatom bloom, accumulation of energy as lipids, diel vertical migrations (DVM) to avoid predators and a diapause stage for overwintering at depth during periods of low availability of

food. These three species can constitute around 80 % of the mesozooplankton biomass in subarctic waters of the Barents Sea (Aarflot et al., 2018). While there is biogeographical overlap, their centers of distribution and depth preferences differ: *Calanus finmarchicus* is a North Atlantic oceanic species that can be found from the Gulf of Maine to the North Sea and subarctic waters, *Calanus hyperboreus* is the main grazer in the Arctic Ocean and can be found in deep water, and *Calanus glacialis* inhabits shallow waters in the continental shelf and the fjords of northern latitudes and can be the dominant species in arctic water masses (Aarflot et al., 2018; Conover, 1988; Daase et al., 2008). Another *Calanus* species, *C. helgolandicus*, can also be found on the continental shelf of the North Sea but its distribution is more southern, reaching the Mediterranean (Bonnet et al., 2005). Given the abundance and key function of *Calanus* copepods in high latitude marine food webs of the north Atlantic and Arctic marine waters, understanding the impacts of oil on these species is essential to assess the potential impacts of petrogenic pollution in Arctic and north Atlantic marine ecosystems.

Among the *Calanus* congeners, *Calanus finmarchicus* has served as a model species in exposure studies of petroleum products and crude oils. Being pelagic, *Calanus* copepods may be exposed to dissolved components and particulate oil droplets (oil dispersions) as spilled oil continuously undergoes weathering in the marine environment. *Calanus* may also be subjected to chemicals used for oil spill responses, like dispersants and herders, or to other chemicals used for oil production operations released with produced water discharges. Despite the ecological importance and pivotal role of *Calanus* species in high-latitude food webs, the molecular mechanisms underlying their complex life history (ontogenetic development, reproduction, molting, and diapause) and physiology (digestion, neural processes and membrane physiology) have remained poorly characterized. Several studies have, however, investigated the impacts of petroleum exposure on *Calanus*, providing mechanistic insights into the toxicity of these complex mixtures of chemicals. This review provides a comprehensive overview of literature published on ecotoxicological effects of petroleum on *Calanus* and attempts to link molecular mechanisms to effects of petroleum on higher levels of biological organization. We discuss how different life history strategies are regulated and how they may be affected by petrogenic oil exposure. We then examine how petrogenic exposure may impact *Calanus* populations and their ecology, and finally, how the current knowledge may be used to expand existing AOPs and facilitate development of new AOPs relevant for crustaceans in general and copepods in particular.

2. Exposure pathways for petrogenic compounds

Crude oil consists of thousands of individual components (Booth et al., 2007), and from a toxicological perspective, polycyclic aromatic hydrocarbons (PAHs) comprise the most studied component class. Most studies of oil ecotoxicity to copepods have focused on identification and quantification of PAHs in water and copepod biomass during exposure. Uptake mechanisms for oil components include, as for most aquatic species, passive uptake through diffusion, bioaccumulation through filtration and subsequent ingestion of contaminated food or oil droplets, and adhesion of oil droplets to surfaces (Fig. 1). Passive uptake of dissolved oil components through body surfaces is driven by non-equilibrium conditions between the water and organism. Some studies on *Calanus* copepods have included data of PAH body burden following laboratory exposure experiments (Almeda et al., 2021; Frost et al., 1998; Hansen et al., 2017, 2018, 2015; Jager et al., 2017; Jensen et al., 2012).

Lighter and less hydrophobic PAHs, like naphthalenes, are taken up and eliminated faster than heavier and more hydrophobic PAHs, like benzo(a)pyrene (Frost et al., 1998; Jensen et al., 2012; Øverjordet et al., 2018). Accumulation and sequestration of heavy PAHs in lipids cause lower elimination rates, as evidenced by observations in crude oil-exposed *C. hyperboreus* where lipid-rich copepods displayed much lower elimination rates than lipid-poor ones (Øverjordet et al., 2018).

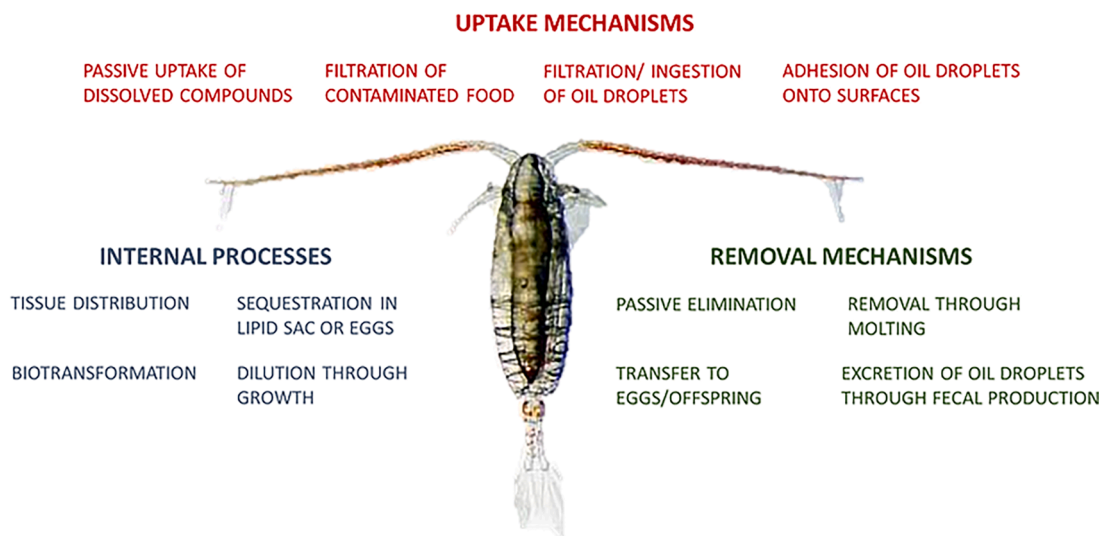


Fig. 1. Mechanisms of uptake, removal, and internal mobilization of oil components in *Calanus* copepods.

PAH body residue dynamics are therefore best explained by representing copepods with two toxicokinetic compartments (structural biomass and lipid storage) (Jager et al., 2017). Assuming that the structural biomass includes key sites for short-term and acute toxicity (Jager et al., 2017), PAH accumulation in lipid structures may prevent acute toxicity (Hansen et al., 2013, 2011), but introduces the risk of delayed toxicity through remobilization of lipid-accumulated oil compounds during gonad maturation and diapause (Toxværd et al., 2019). PAH accumulation in lipid stores of females also introduces the risk of maternal transfer of oil compounds to eggs (Hansen et al., 2017) and transfer to higher trophic levels feeding on lipid-rich stages (Agersted et al., 2018). Maternal transfer of PAHs from ovulating female *C. finmarchicus* to their eggs is associated with a temporal shift in egg hatch timing and differential gene expression in nauplii between treatments (Hansen et al., 2017).

Oil spills and produced water discharges generate micron-sized oil droplets small enough to be entrained in the water column and bioavailable for filter-feeding organisms. Laboratory studies have shown that when *C. finmarchicus* is exposed to crude oil dispersions, they filter oil droplets (Hansen et al., 2018, 2015; Nordtug et al., 2015; Olsen et al., 2013). Exposures to either oil dispersions (containing dissolved fraction and oil droplets) or water soluble fractions (WSF) from filtered dispersions (dissolved fraction without droplets) showed that PAH body burden and mortality were higher for oil dispersions (Hansen et al., 2018). The filtration rates for oil droplets in copepods decreased with exposure concentration, suggesting reduced filtering activity in the presence of high dispersion concentrations (5.6 mg oil L⁻¹) (Nordtug et al., 2015). Modeling studies have shown that, due to their high abundance and filtration rates, *C. finmarchicus* can have a significant impact on the mass balance and fate of oil spills (Nepstad et al., 2015). However, few studies have reported concentrations of oil compounds in field-sampled copepods (e.g., Hansen et al., 2020).

3. Standard toxicity testing and sensitivity to acute oil exposure

Acute toxicity testing is widely used for regulatory purposes, and prior to the establishment of the continuous laboratory culture of *C. finmarchicus* at SeaLab in Trondheim, Norway (Hansen et al., 2007), wild-caught animals were used for this purpose. The culture enabled a continuous supply of animals at relevant developmental stages with a predictable feeding history for regulatory toxicity testing. A standardized protocol for acute toxicity testing was adopted based on the International Organization for Standardization (ISO) guideline ISO

14669:1999 (Water quality — Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea)) mainly used for *Acartia tonsa* (ISO, 1999). The main modifications from the ISO guideline were prolongation of the exposure time to 96 h, adjustment of the temperature to suit *C. finmarchicus* (10 °C) and the use of larger vessel volumes (500 mL) to accommodate the larger body size. Using this protocol, acute toxicity testing has been conducted with copepodite stage 5 (C5) or adult animals for different types of crude oils (Hansen et al., 2022, 2011; Jager et al., 2016), fuel oils (Faksness et al., 2015; Hansen et al., 2013), produced water (Hansen et al., 2017b), PAHs (Hansen et al., 2008b; Jager et al., 2017; Olsen et al., 2013), production chemicals (Farkas et al., 2020), drilling mud (Farkas et al., 2017) and chemicals used for oil response mitigation actions (Hansen et al., 2022, 2014). An early life stage test was also developed for *C. finmarchicus* nauplii based on the ISO guideline 16,778:2015: Water quality — Calanoid copepod early-life stage test with *Acartia tonsa* (ISO, 2015), where development from the naupliar stages (N3) to the first copepodite stage (C1) is assessed. This test has also been used for standard chronic toxicity testing of production chemicals (Farkas et al., 2020) and crude oils (Hansen et al., 2022; Jager et al., 2016).

The critical body residue (CBR) for mortality is the internal concentration of a given narcotic chemical associated with 50 % mortality, and CBRs reported is usually within the range of 2 – 8 mmol kg⁻¹ (Escher and Hermens, 2002). In a study of *C. finmarchicus* (CV and adults) exposed to the PAH dimethylnaphthalene, CBR for mortality was estimated to 7.9 mmol kg⁻¹, suggesting that *C. finmarchicus* is relatively tolerant to dimethylnaphthalene exposure (Jager et al., 2017). The sensitivity of *C. finmarchicus* to dispersed oil exposure appears to be dependent on their lipid content as acute exposure caused higher mortality in copepods with low lipid content compared to lipid-rich copepods (Hansen et al., 2016). This may be explained by slow kinetics in lipid-rich copepods, as explained above. Sequestration of contaminants in storage lipids may postpone the onset of toxic effects, so that individuals with the highest lipid content survive for longer during constant exposure. A similar trend was shown for *C. hyperboreus*, where lipid-rich copepodites at stage C5 were less sensitive than lipid-poor copepods (C3 stage) (Øverjordet et al., 2018). Jager et al. (2016) reported, however, no clear differences in sensitivity to oil exposure between C5 (lipid-rich), females (somewhat less lipid-rich) and nauplii (no lipids). In that study early copepodites (C2/C3) and adult males, were significantly more sensitive than the other stages.

A species sensitivity distribution (SSD) generated for acute toxicity data of the dispersant Corexit 9500A (Hansen et al., 2014), showed that

both *A. tonsa* (48h-LC₅₀: 6.5 mg L⁻¹) and *C. glacialis* (96 h-LC₅₀: 11.8 mg L⁻¹) were more sensitive than *C. finmarchicus* (96 h-LC₅₀: 20.6 mg L⁻¹). *C. finmarchicus* displayed higher sensitivity to crude and fuel oil exposures compared to *C. glacialis* (Hansen et al., 2013, 2011). *C. glacialis* was the least sensitive species in a comparative study on acute toxicity of produced water where 10 different aquatic species were tested (Camus et al., 2015). This study, as well as a similar study on methylnaphthalene (de Hoop et al., 2011), concluded that even though acute toxicity thresholds for Arctic and temperate species were comparable, chronic toxicity data for Arctic species are largely absent. Thus, responses at different levels of biological organization should also be studied to reveal potential differences in sensitivities between Arctic and boreal organisms and ecosystems. This is a clear call for a better understanding of the underlying mechanisms for sub-lethal impacts of petrogenic oil exposure in cold-water species, but unfortunately so far relatively little has been done to mechanistically link petrogenic exposure to sub-lethal effects. In the following, we describe the current knowledge of different biological processes in *Calanus* copepods from molecular to population levels, providing guidance for future research.

4. Transcriptomics in *Calanus* species

Despite their global importance, there is only one published whole-genome assembly for any calanoid copepod, the recently released *Eurytemora affinis* genome assembly (Choi et al., 2021). Within the genus *Calanus*, publicly available genomic data are limited to five mitochondrial genomes (*C. glacialis*, *C. finmarchicus*, *C. sinicus*, *C. hyperboreus* and *C. similimus*) (National Center for Biotechnology Information (NCBI) genome database, accessed September 2022). Genome size and complexity have been a significant barrier for whole-genome assemblies of any *Calanus*. The haploid genome sizes of *Calanus* species is estimated to be ca. 6.5 Giga-base-pairs (Gbp) for *C. finmarchicus* and *C. pacificus*, ca. 8.5 Gbp for *C. sinicus*, ca. 11 Gbp in *C. helgolandicus* and *C. marshallae*, and 12.5 Gbp in *C. glacialis* and *C. hyperboreus* (McLaren et al., 1988). More broadly, *Calanus* genome is greater compared with the *Eurytemora* spp. whose genome assembly has been recently released and estimated to be 0.7761 Gbp (Choi et al., 2021). Furthermore, for reference, the *C. finmarchicus* genome is similar in size to the human genome (ca. 6.3 Gbp) (Piovesan et al., 2019) and much larger than the *Drosophila melanogaster* genome (ca. 180 Mbp) (Adams et al., 2000). Moreover, copepod genomes have low guanine and cytosine (GC) content and a complex genome architecture with the presence of many repetitive sequences (Bron et al., 2011; Grishanin, 2014; Tarrant et al., 2019) making them a challenge to assemble.

As an alternative to genome sequencing, *C. finmarchicus* has been targeted for sequencing of messenger RNA. By 2009, a large dataset of expressed sequence tags (ESTs, >10,000) became available for *C. finmarchicus* (Christie et al., 2008; Lenz et al., 2012) followed by multiple shotgun transcriptome assemblies (Hartline et al., 2023; Lenz et al., 2014; Lizano et al., 2022; Tarrant et al., 2014; Yadetie et al., 2022). These resources have been used to investigate gene diversity, physiology and functional ecology in *C. finmarchicus*. Genes that regulate key developmental processes such as diapause, oogenesis, lipid metabolism and detoxification pathways have been identified and have contributed to our understanding of copepod environmental adaptations, including responses to starvation and toxicants (Christie et al., 2013c; Lenz et al., 2014; Tarrant et al., 2016, 2014).

The four transcriptomes for *C. finmarchicus* were generated from individuals belonging to different developmental stages (pre-adults C5, females, and mixed stages) and from different populations including the Gulf of Maine (PRJNA236528; (Lenz et al., 2014)), Greenland (Disko Bay) (PRJNA236983; (Smolina et al., 2015)), northern Barents Sea (PRJEB51404; (Yadetie et al., 2022)) and Trondheim Fjord (PRJNA231164; (Tarrant et al., 2014)). Transcriptomic databases for other species include *C. glacialis* (4 resources available), *C. marshallae* (5), *C. helgolandicus* (2), *C. hyperboreus* (2), *C. pacificus* (1) and

C. propinquus (1). The resource generated from Gulf of Maine individuals (Lenz et al., 2014) has been used in gene discovery papers (Christie et al., 2014a, 2014b, 2013c, 2013a, 2013b; Porter et al., 2017; Roncalli et al., 2021b) and as a reference for gene expression studies (Häfker et al., 2018; Lenz et al., 2021; Payton et al., 2022, 2020; Roncalli et al., 2016a).

The “gene discovery” studies have focused on gene families, whose expression or function may be impacted by exposure to petrogenic oil substances. The Gulf of Maine transcriptome has been searched for the presence and diversity of genes associated with detoxification (Roncalli et al., 2021b, 2015), neurochemical signaling systems, including gas transmitters (Christie et al., 2016, 2014a, 2013c), toxin resistance (voltage-gated sodium channel) (Roncalli et al., 2017), photoreception (Porter et al., 2017) and chemical communication (chemosensory related genes) (Roncalli et al., 2022) have been characterized.

The molecular underpinnings of sensory systems and neural function are of particular interest since their function could be impacted by oil pollution. Porter et al. (2017) reported the presence in *C. finmarchicus* of the conserved two groups of opsins (middle-wavelength-sensitive opsins and pteropsins) and of two additional opsins that were not common to all copepods, the peropsins and rhodopsin 7 (Rh7) opsins. These two additional opsins were found only in another calanoid *Eurytemora affinis*, and in two cyclopoids (*Paracyclops nana* and *Lernaea cyprinacea*) out of 12 copepod species (Porter et al., 2017).

The first report of multiple voltage-gated sodium channels (Nav₁) genes in a protostome was based on the analysis of transcripts found in the *C. finmarchicus* shotgun assembly (Lenz et al., 2014). Further analyses revealed the presence of a mutation in one splice variant that confers resistance to saxitoxin (Roncalli et al., 2017). However, the expression of this gene was unresponsive to saxitoxin-producing dinoflagellate *Alexandrium fundyense* challenge in *C. finmarchicus* adult females. Instead, the mechanism of resistance relied on the regulation of digestive enzymes, possibly to complement channel resistance by limiting toxin assimilation via the digestive process (Roncalli et al., 2016a).

A recent study reported the presence of 5-histidylcysteine sulfoxide synthase (OvoA), a gene involved in ovothiol biosynthesis. OvoA is found in almost all metazoans, however, it was thought to be absent in arthropods (Roncalli et al., 2021b). Ovothiol is a powerful antioxidant acting in marine organisms as a defense against oxidative stress during development and in response to environmental cues. *In silico* mining of the *C. finmarchicus* transcriptome revealed the presence of a transcript encoding full-length OvoA that is up-regulated in females exposed to a toxic diet and during the copepodite stage, a developmental stage that includes significant morphological changes (Roncalli et al., 2021b).

Comparisons across transcriptomes could provide insights into genetic differences between *C. finmarchicus* populations, and across *Calanus* congeners. A recent phylotranscriptomic analysis of seven *Calanus* species used single copy orthologs to resolve phylogenetic relationships. Briefly, maximum-likelihood tree resulted in three well-supported clades with a group including *C. sinicus*, *C. helgolandicus* + *C. pacificus*, a second group with *C. finmarchicus* and *C. glacialis* + *C. marshallae* and a third monophyletic group with *C. hyperboreus* (Lizano et al., 2022). The publicly available transcriptomes are an important resource for future studies.

5. Molecular defensome

Like other animals, copepods are constantly exposed to physical, chemical, and biological challenges that impact their homeostatic state. To deal with these stressors, metazoans have evolved a complex “defensome”, a collection of gene families and pathways that help them to counteract homeostatic disruptions. The chemical defensome protects the organism from environmental chemicals and includes genes that encode defensive proteins used to sense, transform, and eliminate the toxicants (Goldstone, 2008; Goldstone et al., 2006). Due to the diversity

of the chemical stressors, which can include microbial products, natural occurring organic and inorganic compounds, and anthropogenically derived compounds, the chemical defense includes classes of proteins that can activate either a specific or a non-specific defense response. Sensing the environment, the first step of the defense, occurs via chemical sensors, such as nuclear receptors (NR) and PAS (Per-ARNT-SIM) proteins; these genes sense the stress and regulate the expression of the other genes downstream. After sensing a potential threat, the first line of cellular defense against amphipathic or slightly lipophilic compounds involves activation of adenosine triphosphate (ATP)-binding cassette (ABC) or multidrug efflux transporters including p-glycoproteins and multidrug resistance proteins (MRP) (Goldstone, 2008; Goldstone et al., 2006; Leslie et al., 2001). The roles of these transporters are to alter the entry of the toxicant into the cell to impair its movement across membranes or to actively excrete them. Once a toxicant succeeds in entering the cytoplasm, the organism depends on biotransformation, which is generally based on two phases of detoxification. In phase I, chemicals are oxidated into more hydrophilic metabolites by oxidative enzymes including cytochromes P450 (CYP), flavoprotein monooxygenases (FMO) and aldehyde dehydrogenases (ALDH). In phase II, detoxification enzymes either reduce, conjugate or hydrolyze the toxicants. Some of the enzymes involved in this phase are glutathione-S-transferases (GSTs), sulfotransferases (SULTs), UDP-glucuronosyl transferases (UGTs), and aldo-keto reductases (AKRs) (Goldstone et al., 2006). Then, antioxidant enzymes such as superoxide dismutases (SODs), catalases (CATs) and peroxidases (e.g. glutathione peroxidase, thioredoxins) are activated to reestablish cellular homeostasis via the inactivation and the elimination of endogenous signaling molecules (e.g., steroids, reactive oxygen species [ROS], lipid peroxides). Lastly, the transformed or conjugated toxicants are eliminated or excreted by the efflux transporters (e.g. ABC proteins) (Leslie et al., 2001).

In addition to the chemical detoxification responses described above, the defense includes genes that are activated in response to specific stressors, such as metallothioneins, and genes that are activated by damage to cellular components, like heat shock proteins (HSP). Metallothioneins (MT) are active in response to excess metal stress but may also play roles in micronutrient homeostasis and in apoptosis (reviewed in Vařák and Hasler (2000)). Metal detoxification is also performed by other metal-binding proteins, such as Cu- and Fe-binding proteins. HSPs are molecular chaperones involved in cellular damage repair and they react to a wide variety of stressors, including exposure to heat stress, heavy metals, or free radicals (Feder and Hofmann, 1999). This family is highly diversified and includes members of the HSP 100, 90, 70, 40, and small α -crystalline HSPs (HSP20s). The HSP70 class contains the highest number of genes which have been used as biomarkers of stress in many metazoans.

The chemical defense is broadly conserved across metazoans, as suggested by the published genome analyses for the sea urchin *Strongylocentrotus purpuratus* (Goldstone et al., 2006), the sea anemone *Nematostella vectensis* (Goldstone, 2008) and the tunicate *Oikopleura dioica* (Yadete et al., 2012). While the first two studies investigated the entire collection of defense-relevant genes, the *O. dioica* analysis focused only on the defense genes activated in response to specific xenobiotics (carcinogenic polycyclic aromatic hydrocarbon benzo[a]pyrene [BaP] and Clofibrate [Clo]). To date there are no similar analyses available for copepods which is partially due to the lack of genomic resources for processing. However, in a recent study, an evolutionary analysis of three detoxification-related gene families (ABCs, CYPs and GSTs) has been reported for the calanoid *E. affinis* (Choi et al., 2021). Using the new *E. affinis* genome, Choi and coauthors (Choi et al., 2021) identified the genes belonging to the three detoxification classes and performed an orthologous relationship analysis with the cyclopoid *P. nana*, and the two harpacticoids *Tigriopus japonicus* and *T. kingsejongensis*. The reported differences as number of defensive-genes among the different copepods, highlight the importance to have more

genomic studies to uncover lineage-specific adaptive strategies in copepods. Nevertheless, considering the good quality and the completeness of the Gulf of Maine transcriptome, we surveyed this *C. finmarchicus* molecular resource for defensive genes that included efflux transporters (e.g., ABC, phase I (CYP450, FMO, ALDH), phase II (GST, SULT, UGT, AKR) and antioxidant (SOD) enzymes; this subset of defense gene classes was chosen to enable comparisons with previous studies from *S. purpuratus*, *N. vectensis* and *E. affinis* (Choi et al., 2021; Goldstone, 2008; Goldstone et al., 2006). *C. finmarchicus* transcripts encoding proteins were identified by name in the annotated transcriptome, which had been generated through blast-based comparisons against the SwissProt database (Roncalli et al., 2016a). As shown in Fig. 2, the *C. finmarchicus* transcriptome includes representatives of all classes. The distribution of genes is similar to the one found for *E. affinis*, although the number of transcripts in *C. finmarchicus* is always greater; this is not surprisingly due to the differences in the resource mined for the identification (genome vs. transcriptome). With the exception of the ABC, GST and AKR classes, the number of copepod defensive genes (and transcripts) is lower compared with the ones identified in *S. purpuratus* and *N. vectensis* (Fig. 2). Exception was SOD class which was over-represented in *C. finmarchicus* compared to all other organisms.

We summarize the current state of knowledge of key components of the molecular defense (cytochrome P450 enzymes, glutathione-S-transferases, antioxidant enzymes, and heat shock proteins), and what is known regarding their potential role in responses to petrogenic pollutants in *Calanus*.

5.1. Cytochrome P450 enzymes

Cytochromes P450 (CYPs) form a large, evolutionarily conserved, highly diversified and duplicated family of enzymes. In vertebrates, a well-known primary mechanism of PAH toxicity occurs through binding of PAHs to the aryl hydrocarbon receptor (AhR), leading to increased CYP1A expression and generation of oxygenated metabolites (e.g., epoxides and dihydrodiols) that may be carcinogenic or otherwise toxic (Baird et al., 2005; Nebert and Dalton, 2006). While this specific AhR-CYP1A signal transduction pathway is unique to vertebrates (Hahn et al., 2017), copepods and other invertebrates possess large numbers of CYPs, many of which change expression in response to contaminant exposure and are likely involved in phase I detoxification of petrogenic pollutants.

Patterns of CYP expression following exposure of *Calanus* species to petrogenic pollutants have been investigated in a few studies. In *C. finmarchicus*, expression of a CYP (originally annotated as CYP1a using blast-based homology) was induced after exposure to naphthalene (Hansen et al., 2008b). A second study found that responses to phenanthrene (Phe) and benzo[a]pyrene (BaP) varied among species (Yadete et al., 2022). With Phe exposure, CYP expression was induced in both *C. finmarchicus* (CYP6A13/CYP6A21 and CYP2I3) and *C. hyperboreus* (CYP2C23, CYP6K1, CYP18A1, CYP12A5 and CYP3A16), but not in *C. glacialis*. In contrast, CYP expression (CYP2J3 and CYP18E1) was induced in both *C. glacialis* and *C. hyperboreus* after BaP exposure, but not in *C. finmarchicus*. The regulation of CYP330A1 gene expression to pollutants in *C. finmarchicus* is variable; down-regulated after naphthalene exposure (Hansen et al., 2008b), no response following exposure to the WAF of a naphthenic crude oil (Soloperto et al., 2022), a response dependent on lipid-sac fullness following exposure to the WSF of a weathered crude oil (Hansen et al., 2009).

In addition to their role in the metabolism of xenobiotics, CYPs are involved in the synthesis and metabolism of lipid-signaling molecules, including ecdysteroids, retinoids, and fatty acids. Thus, they are involved in the regulation of development, reproduction, and other physiological processes. In *C. finmarchicus*, the role of specific CYPs in development or reproduction is suggested by higher expression of CYP330A1 and CYP305A1 in stage C5 individuals compared with adults and by the higher expression of CYP201a1 in post-reproductive females

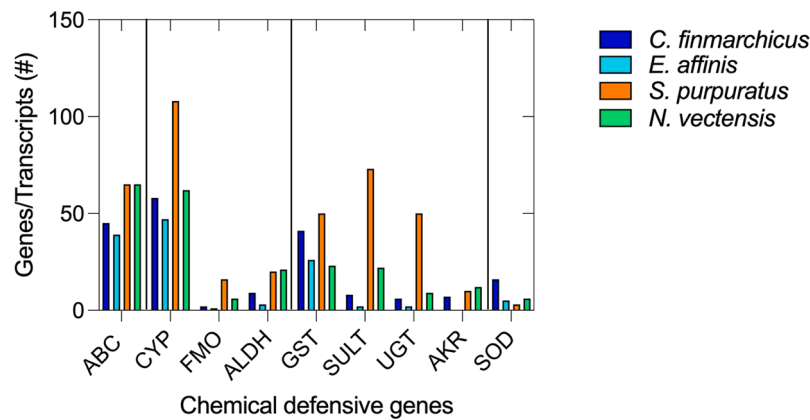


Fig. 2. *Calanus finmarchicus* chemical defensome. The number of *C. finmarchicus* transcripts encoding proteins involved in different phases of the chemical defense was compared with the genes previously reported for *Strongylocentrotus purpuratus* (Goldstone et al., 2006), *Nematostella vectensis* (Goldstone, 2008) and *Eurytemora affinis* (Choi et al., 2021). Chemical defensive genes have been divided accordingly to their role (lines): 1) ABC: ATP-binding cassette transporters (first line and/or phase III); CYP: cytochrome P450; FMO: flavoprotein monooxygenase; ALDH: aldehyde dehydrogenase (phase I detoxification); 3) GST: glutathione S-transferase; SULT: sulfotransferase; UGT: UDP-glucuronosyl transferase; AKR: aldo-keto reductase (phase II detoxification); 4) SOD: superoxide dismutase (antioxidant). The number of SOD includes Cu/Zn SOD (11), Mn SOD (4) and Fe SOD (1) transcripts.

and males compared with stage C5 and reproducing females (Hansen et al., 2008a). The positive relationship between CYP330A1 expression and lipid sac size in C5 could indicate an involvement in lipid metabolism (Hansen et al., 2008a). Additional studies will be required to better understand and characterize the *Calanus* CYP gene family and the diverse functions of these genes.

5.2. Glutathione S-transferase

Glutathione S-transferases (GSTs) comprise a large family of detoxification enzymes found in prokaryotes and eukaryotes. GSTs catalyze the conjugation of endogenous and exogenous electrophilic compounds, including products of phase I metabolism, to the reduced form of glutathione (GSH) to facilitate elimination of toxins from the body. This diverse gene family is divided into cytosolic, microsomal and mitochondrial classes (reviewed in Lauritano et al. (2021)). Detoxification is carried out by cytosolic GSTs, but cytosolic GSTs also play important roles in other cellular functions. A total of 39 GST transcripts have been identified in *C. finmarchicus*, with the largest portion belonging to the cytosolic class (Roncalli et al., 2015). Relative expression for several cytosolic GSTs changes across development, with low levels of expression in embryos, and higher expression in late-stage copepodites and adult females. In contrast, the expression of the mitochondrial (Kappa) and microsomal GSTs is stable across developmental stages (Roncalli et al., 2015).

Upregulation of individual GSTs has been reported in *C. finmarchicus* exposed to crude oil (Hansen et al., 2011, 2009), fuel oils (Hansen et al., 2013), produced water (Hansen et al., 2017b), naphthalene (Hansen et al., 2008b) and mercury (Øverjordet et al., 2014; Tollefsen et al., 2017), suggesting a role in the detoxification of these compounds. Moreover, exposure to water-accommodated fractions (WAFs) of crude oils also induced an increase in the GST enzymatic activity in *C. finmarchicus* consistent with a response to oxidative stress. The authors suggested that GST enzymes help the organism in handling lipid peroxidation (Soloperto et al., 2022). The expression of GST genes was also upregulated in *C. glacialis* exposed to the WAF of crude oils (Hansen et al., 2011) and in both *C. glacialis* and *C. hyperboreus* exposed to phenanthrene (Yadeti et al., 2022).

5.3. Oxidative stress and antioxidant enzymes

Oxidative stress is induced by disturbances in the normal redox state of cells, which occurs when there is an imbalance between the presence

of reactive oxygen species (ROS) and the ability of cellular antioxidants to defend against them. Oxidative stress, through the production of peroxides and free radicals (e.g., H₂O₂ - hydrogen peroxide, O₂⁻ - superoxide radical, ●OH - hydroxyl radical), can induce damage to macromolecules, including proteins, lipids, and DNA. Further, because some ROS compounds act as cellular messengers, excess production of these compounds can disrupt normal mechanisms of cellular signaling. Antioxidant defense systems are based on the activity of enzymes eliminating ROS (e.g., SOD, CAT, GPX), internal lipid peroxidation products, toxic secondary radical oxidation products, and non-enzymatic antioxidants. Oxidative stress can be induced by environmental conditions, both natural (e.g., hypoxia, UV radiation, and bioactive compounds) and anthropogenic (e.g., chemical contaminants). ROS production can also be induced by cellular aging or under diapause conditions. Consistent with a role in diapause, in *Neocalanus flemingeri*, high expression of antioxidant enzymes, including peroxiredoxins, a SOD, a GST-sigma and aldehyde dehydrogenases, was reported in diapausing females compared with post-diapause females (Roncalli et al., 2021a, 2018). Due to the non-specificity of the antioxidant responses and the considerable variability of various biological and environmental factors, the use of antioxidant enzymes as biomarkers of environmental stressors in copepods is not straightforward (Glippa et al., 2018). In *C. finmarchicus*, exposure to crude oil did not alter the gene expression or enzyme activity of SOD and CAT, or the expression of the glutathione synthetase gene or total glutathione concentration (Soloperto et al., 2022).

5.4. Heat shock proteins

Heat shock proteins (HSPs) are a superfamily of proteins that act as molecular chaperones. Their diverse functions include inducible cellular responses to heat and other proteotoxic stressors (Chen et al., 2018; Driedonks et al., 2015; Feder and Hofmann, 1999). Large cytosolic heat shock proteins, particularly Hsp70, can be strongly induced by stressors. Thus, HSP70 gene expression is often used as a biomarker of stress exposure in copepods (reviewed by Tarrant et al. (2019)). Aruda et al. (2011) identified multiple HSP70 transcripts in *C. finmarchicus*, which seemed to originate from different genes. In contrast to the robust induction of HSP70 by thermal stress (Voznesensky et al., 2004), HSP gene expression does not appear to be a robust biomarker of exposure to oil pollution in *C. finmarchicus* as exemplified by lack of modulation of HSP70 and HSP90 expression after naphthalene exposure (Hansen et al., 2008b). Similarly, HSP70, HSP90, and ubiquitin expression were not affected by crude oil exposure (Soloperto et al., 2022).

HSPs play important physiological and developmental roles in facilitating protein folding and degradation. During diapause, increased expression of specific HSPs has been described in insects and brine shrimp and attributed to protection of proteins from degradation (King and MacRae, 2015; MacRae, 2016; Malitan et al., 2019). In *C. finmarchicus*, expression of HSP22 is upregulated during diapause (Aruda et al., 2011) while this signal is absent in *N. flemingeri* diapausing females, where there was no significant regulation of any HSPs (Roncalli et al., 2021a, 2018). Given the large number of HSPs present within *C. finmarchicus*, a transcriptomic approach may be useful in future studies to better understand the diversity of genes and differentiate between genes that are associated with endogenous processes and those that respond to environmental stress.

6. Ontogenetic development, growth and molting

Eggs of northern high-latitude *Calanus* copepods are released during the spring bloom, developing to the last copepodite stage (C5) during summer, and overwintering in a dormant state (diapause) prior to maturation and reproduction; however, this phenology varies regionally (Baumgartner and Tarrant, 2017; Fiksen, 2000). *C. finmarchicus* eggs hatch within 48 h and progress through 12 developmental stages during their life cycle, including 6 larval (naupliar) stages, 5 juvenile (copepodite) stages, and an adult stage (males and females) (Fig. 3). The first two naupliar stages are non-feeding, whereas the third naupliar stage (N3) requires food for continued development (Cook et al., 2007) and is prolonged relative to all other naupliar stages (Campbell et al., 2001). Growth rates are negative during the non-feeding stages and are highest during the late naupliar and early copepodite stages. Growth rates decrease during the transition from the 6th naupliar (N6) to the first copepodite stage (C1) and in the later copepodite stages (Campbell et al., 2001). Development time from N3 to C6 ranges from 30 to >80 days under experimental conditions. It varies with temperatures and with food availability (Campbell et al., 2001).

Copepods, like all arthropods, must shed their exoskeleton and

produce a new exoskeleton to grow. For copepodites, progression within a molt stage can be assessed by examining the attachment of the epidermis to the exoskeleton at the site of tooth formation (Miller et al., 1990; Tarrant et al., 2014). Although limited information exists about the mechanisms that regulate molting in *C. finmarchicus* or other copepods, molting in other arthropods (i.e., insects and decapod crustaceans) is regulated by a suite of hormonal signals (Miyakawa et al., 2018; Nakagawa and Henrich, 2009; Song et al., 2017), among which ecdysteroids serve as molt-stimulating hormones (Chang and Mykles, 2011). Ecdysteroids primarily act by binding to the ecdysone receptor (EcR), a nuclear receptor that forms heterodimers with the ultraspiracle protein (Usp) and modulates transcription of target genes (Henrich et al., 1990; Nakagawa and Henrich, 2009; Yao et al., 1992). In *C. finmarchicus*, ecdysone levels are higher in C5 copepodites than in adults (which do not molt) (Hansen et al., 2008a). Within the C5 copepodite stage of *C. pacificus*, ecdysone levels were low during diapause (consistent with a developmental delay), and highest in the early premolt portion of the stage (Johnson, 2003). Additional insight into molt regulation has come from studies characterizing the expression of genes associated with molting in *C. finmarchicus* or with known roles in regulating molting in other arthropods. A laboratory-based study of *C. finmarchicus* C5 copepodites revealed that EcR expression was highest during early premolt (Tarrant et al., 2014). A field-based study found that during diapause, EcR expression was negatively correlated with the size of the C5 lipid stores (Tarrant et al., 2008). This suggested that EcR expression increased as the copepods consumed their lipid stores during diapause and prepared for the terminal molt.

Little is known still about the effects of petrogenic pollutants on the different developmental stages or molting processes of *Calanus*; however, a few studies suggest a higher sensitivity of early developmental stages. Exposure of females and eggs to water-soluble fractions of crude oil ($10.4 \mu\text{g PAHs L}^{-1}$) reduced *C. glacialis* hatching success 4-fold (Jensen and Carroll, 2010). Naupliar development of *C. glacialis* and *C. finmarchicus* was delayed when exposed to pyrene, and even stopped at high concentrations (up $20 \mu\text{g L}^{-1}$), with feeding naupliar stages more

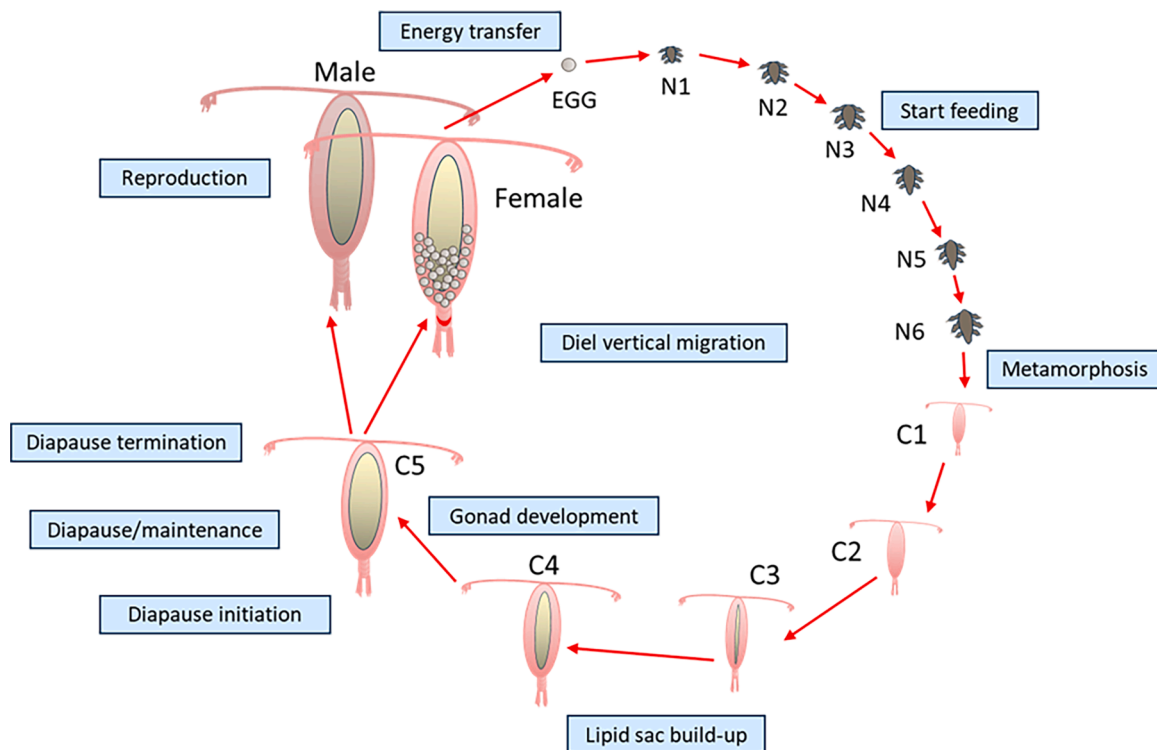


Fig. 3. Overview of the life cycle of *Calanus* copepods from egg, through six naupliar stages (N1-N6), five copepodite stages (C1-C5) to adulthood. Note that entry into diapause is facultative for some *Calanus* species, including *C. finmarchicus*.

sensitive to pyrene than non-feeding naupliar stages (Grenvald et al., 2013). In contrast, egg hatching of *C. finmarchicus* and *C. glacialis* was not affected by 5–6 days exposure to pyrene (up to $20 \mu\text{g L}^{-1}$) (Grenvald et al., 2013; Hjorth and Nielsen, 2011; Jensen et al., 2008).

Several studies have demonstrated that exposure of *Calanus* adults to petrogenic pollutants can impact survival and development of the next generation. Exposure of adult *C. finmarchicus* to dispersed oil and filtered crude oil dispersions caused delayed hatching of eggs, and in offspring (nauplii) affected expression of genes with roles in xenobiotic metabolism, antioxidant defenses and inflammatory responses (Hansen et al., 2017). Differences were observed between dispersions with and without oil droplets, emphasizing the importance of uptake and transfer of heavier oil components from droplets to copepods and their offspring. These results suggested transgenerational activation of stress responses; however, the relatively small number of differentially expressed genes suggested a minor long-term effect on offspring following maternal exposure (Hansen et al., 2017). In a mesoscale experiment, physiological condition of *C. glacialis* females, quantified by prosome length, dry weight, carbon and nitrogen content, fecal pellet production and egg production, was unaffected by exposure to differently treated oil in ice. However, higher proportions of deformations were observed in nauplii of mothers exposed to chemically dispersed (Finasol® OSR 52 dispersant-treated) oil than in unexposed controls (Toxværd et al., 2018a).

7. Energetics

7.1. Feeding and defecation

All *Calanus* species generate feeding currents with their mouth appendages to concentrate and select food particles (Huntley et al., 1983; Koehl and Strickler, 1981; Leiknes et al., 2014). Planktonic copepods can chemically distinguish among prey depending on their toxicity or nutritional value (Huntley et al., 1983; Kjørboe et al., 1985). For example, copepods can discriminate between particles of the same size but differing in nutritional value, as shown by higher ingestion rates of algae when provided algae and plastic beads of similar size (Huntley et al., 1983; Xu et al., 2022) as well as higher ingestion rates of faster-growing versus slower-growing cells (Cowles et al., 1988). Clearance rates of copepods typically increase during development and with increasing body size (Meyer et al., 2002) and decrease with increasing food concentration (Frost, 1972).

Oil droplets have the potential to be ingested by *Calanus*, and ingestion of droplets can affect their feeding rates and the consequent nutritional condition. Complete feeding suppression has been observed in *C. hyperboreus* when exposed to water contaminated from oil seeps in Baffin Bay (Gilfillan et al., 1986) and when exposed to crude oil droplets and plastic microspheres in the laboratory (Almeda et al., 2021). Acute oil spills and produced-water discharges cause exposure to micron-sized dispersed oil droplets that overlap in size with the normal prey. Ingestion of oil droplets by copepods, including *Calanus* species, has been observed in the field after oil spills (Conover, 1971) and in the laboratory (Almeda et al., 2014; Hansen et al., 2017, 2015, 2012, 2009; Nordtug et al., 2015). Some copepod species exhibit a size threshold for particle discrimination (approx. 5–10 μm for copepods of 1 mm), but oil droplets smaller than the size-threshold can be passively ingested with the feeding current. Little is known about the size-threshold for discrimination of oil droplets in *Calanus* copepods. Also, when copepods are exposed to high concentrations of particles (e.g. microplastics), copepods make mistakes and ingest some undesirable particles (Xu et al., 2022).

Exposure to oil dispersions reduces clearance rates (Hansen et al., 2017a, 2012; Nordtug et al., 2015) and fecal pellet production in *Calanus* (Almeda et al., 2021; Toxværd et al., 2018a). A 4-day exposure of *C. finmarchicus* to oil dispersions ranging from 0.25 mg L^{-1} to 5.6 mg L^{-1} with a constant supply of algae showed that algal uptake was reduced at

higher oil concentrations, which consequently reduced the accumulation factor of oil in a concentration-dependent manner (Nordtug et al., 2015). Exposure to dissolved PAHs decreased both the feeding rates and fecal pellet production of *Calanus* copepods (Jensen et al., 2008). Reduced grazing after exposure to petrogenic compounds can be related to narcosis caused by cell membrane destabilization due to the interference of non-polar hydrocarbons with lipidic membranes. Other related modes of toxicity like osmoregulatory failure and neurotoxicity can affect activity of copepods exposed to oil (Hansen et al., 2017). Exposure to oil causes a decrease in homarine (N-methyl picolinic acid), a compound that influences osmoregulation in crustaceans (Gasteiger et al., 1960), likely due to the disruption of membranes. It has also been suggested that oil dispersions cause a neurotoxic effect in *Calanus* due to a reduction in the concentration of the neurotransmitter acetylcholine (Hansen et al., 2017). The dissolved oil components are expected to be the main driver for toxicity; however, exposure to oil droplets also triggers a starvation-type response, manifested as a reduction in metabolite concentrations (homarine, acetylcholine, creatine and lactate) in *C. finmarchicus* (Hansen et al., 2017a).

7.2. Lipid storage and metabolism

Upon grazing, *Calanus* converts low-energy carbohydrates and proteins from phytoplankton into high-energy lipids (Falk-Petersen et al., 2009), contained in a discrete lipid sac during the late copepodite stages (reviewed by Lee et al., 2006). In *C. finmarchicus* C5 copepodites, the oil sac extends along the body cavity and can comprise up to 50 % of the body volume, and storage lipids may comprise over 50 % of dry mass (Jonasdottir, 1999; Miller et al., 2000). These energy-dense lipids fuel copepod maintenance during diapause and help to support post-diapause development and oogenesis (Fiksen et al., 2004). The high lipid content of *Calanus* provides a major source of energy for fish, as well as sea birds and large marine mammals, encapsulating the key role of *Calanus* species for energy transfer in sub-Arctic and Arctic marine food webs (Falk-Petersen et al., 2009; Tande and Slagstad, 1985).

Wax esters are the primary storage lipid in *C. finmarchicus* copepodites and adults (Miller et al., 1998), and these are concentrated in the lipid sac. Triglycerides may be obtained from the diet and indicate recent feeding; they are, together with phospholipids, incorporated into eggs and sequestered in droplets throughout the body (Miller et al., 1998; Ohman and Runge, 1994). In addition to serving as a major energetic reserve, it has been suggested that lipid storage plays a key role in triggering *Calanus* diapause through variations in lipid-derived hormone levels, determining the overwintering depth in relation to the convective mixed layer, and mediating adaptations to hydrological conditions (Irigoin, 2004).

A few studies have addressed effects of pollutant exposure on energetic metabolism in *Calanus*. Exposure may affect overall metabolic rates; for example, respiration rates of *C. glacialis* increased when exposed to crude oil (Almeda et al., 2023). In addition, exposure to petroleum-based pollutants can create oxidative stress that leads to lipid peroxidation. Under laboratory conditions, lipid peroxidation can be detected through measurements of malondialdehyde, which is produced during oxidation of polyunsaturated fatty acids (Draper and Hadley, 1990). Exposure of *C. finmarchicus* adult females to WAF from a petrogenic crude oil for 24–72 h resulted in elevated malondialdehyde concentrations, suggesting lipid peroxidation as an important mechanism of oil-induced toxicity (Soloperto et al., 2022). Exposure of *Calanus* to petroleum-based pollutants can also impact the catabolism of lipid stores. In one experiment, a mixture of *C. finmarchicus* and *C. glacialis* C5 copepodites were exposed to WAF of a petrogenic crude oil. The exposed copepodites exhibited slower consumption of lipids in the oil sac and downregulation of genes in the β -oxidation pathway (Skottene et al., 2019). While the ecological consequences of these metabolic shifts have not yet been demonstrated, changes in lipid consumption during diapause may affect the timing of diapause emergence, with the

potential for cascading trophic effects within the ecosystem.

8. Neurology, environmental perception and behavior

Behavioral responses to environmental stimuli are rapid, and can be used as a sensitive and early warning tool (Peterson et al., 2017). Hyperactivity and hypoactivity are two common responses to oil contaminants and dispersants reported in several calanoid species (Cailleaud et al., 2011; Cohen et al., 2014; Michalec et al., 2013; Seuront, 2011; Thor et al., 2021). However, the underlying mechanisms are not always clear, since behavior depends on both multisensory input and proper locomotor function (Legradi et al., 2018). The physiological basis of hypoactivity could be due to a direct narcotic effect through interaction with cellular membranes or membrane proteins, where the downstream toxic effects involve interference with either neuronal or muscle function. Alternatively, hyperactivity may be an adaptive response to minimize exposure to a toxicant by attempting to move away from the source. In addition, exposure to oil pollution leads to changes in photic behavior (Miljeteig, 2013). The oil itself, or water-soluble compounds within, may be damaging to sensory systems, such as chemoreceptors. Copepod males, including *C. finmarchicus*, possess large chemosensory setae on their antennae that are used in mate detection and tracking (Boxshall et al., 1997; Doall et al., 1998; Katona, 1973; Ohtsuka and Huys, 2001; Strickler, 1998; Yen et al., 2011). Thus, successful mating depends on the male's ability to detect and follow pheromone trails released by females (Tsuda and Miller, 1998). While the exact mechanism is unknown, Seuront (2011) reported lower mating success in the copepod *Temora longicornis* exposed to hydrocarbons (water-soluble fraction of diesel oil), even at the lowest experimental treatment (0.01 %) (Seuront, 2011). A similar effect may occur in *Calanus*, although it has not been studied experimentally.

The mechanisms underlying neurotoxicity has been investigated in other aquatic model organisms, such as zebrafish (Legradi et al., 2018). Chemical pollutants impede synapse function by interfering with neurotransmitters, hormones, and/or receptors (Legradi et al., 2018). While such studies have rarely included copepods, many of these genes have been described for *C. finmarchicus* (Christie et al., 2014b, 2014a, 2013c; Porter et al., 2017). Furthermore, morphological studies have produced maps of the nervous system, including sensory structures (Elofsson, 2006; Hartline and Christie, 2010; Mercier et al., 2013; Weatherby et al., 2000; Weatherby and Lenz, 2000) providing the background for future studies on the neurotoxic effects of oil pollution on *Calanus*.

9. Copepod migration

9.1. Diel vertical migration

To balance the risk of predation with feeding and growth, many zooplankton, including *Calanus* copepods, perform diel vertical migration (DVM). DVM typically involves feeding in upper water layers during the night and seeking protection from predators in deeper waters during daytime (Dale and Kaartvedt, 2000). In fact, due to the widespread DVM occurrence among zooplankton, it represents one of the largest synchronized animal migrations in terms of biomass (Hays et al., 2003) and has a huge impact on oceanic biogeochemical cycling (Behrenfeld et al., 2019; Steinberg et al., 2001; Steinberg and Landry, 2017). For *C. finmarchicus*, the daily migration range may vary from a few meters to several hundred meters. Depth distribution can be stage-dependent, as early copepodites (C1-C3) are primarily found in upper water layers whereas late copepodites (C5) and adults are found in deeper waters (Kwasniewski et al., 2003).

DVM is strongly cued by environmental light levels, but the behavior and associated physiological changes are also modulated by an underlying circadian clock. The *C. finmarchicus* circadian clock consists of "clock genes" that form the core regulatory component of the circadian machinery (Christie et al., 2013a; Häfker et al., 2018; Hüppe et al.,

2020), and these genes interact via their proteins through an intricate network of feedback loops resulting in daily rhythms (Häfker et al., 2018). While clocks are entrained to environmental cues, particularly daily light/dark cycles (Cohen et al., 2009), circadian cycles can persist to varying extents in the absence of external cues (Häfker et al., 2018). Observations of DVM in Arctic zooplankton during the polar night does not correspond to any externally detectable changes in light (Berge et al., 2012), and internal circadian rhythms may be important in maintaining this behavior.

Consistent with DVM behavior, *C. finmarchicus* C5 copepodites and adult females display negative phototaxis. Interestingly, the opposite has been observed for adult males, which also displayed higher photosensitivity (Miljeteig et al., 2014). While the effects of petrogenic pollutants on migration behavior in *Calanus* copepods are unclear, it should be noted that exposure of late copepodite (C5) *C. finmarchicus* to oil ($309 \pm 32 \mu\text{g L}^{-1}$ total hydrocarbon, $20.37 \pm 0.51 \mu\text{g L}^{-1}$ total PAH) for up to 96 h significantly increased positive phototactic behavior, suggesting that the depth distribution of this species could be altered following an oil spill.

9.2. Diapause and overwintering

The abundances of *Calanus* in surface waters of the Northern Atlantic and Arctic Oceans peak in the spring and early summer, in synchrony with the phytoplankton bloom. In mid-summer, lipid-rich C5 copepodites stage migrate to deep waters and enter a period of low metabolism and arrested development (Hind et al., 2000; Hirche, 1996; Marshall and Orr, 1972). This ontogenetic migration represents a large transfer of biomass to surface waters and to depth (Jónasdóttir et al., 2015). The timing and duration of diapause varies among species and regions. For example, the *C. finmarchicus* population in the Norwegian Sea completes a single life cycle per year (Tande and Hopkins, 1981), while at lower altitudes there are multiple generations per year (Miller et al., 1998). What triggers the diapause program, remains unknown (Baumgartner and Tarrant, 2017; Irigoien, 2004), although many factors have been suggested, including temperature, food restrictions, photoperiod (Miller et al., 1991) and the presence of predators (Kvile et al., 2021). Lipid content has also been suggested as the diapause trigger (Irigoien, 2004), since overwintering individuals have higher lipid levels than individuals remaining in surface waters (Jonasdottir, 1999; Miller et al., 2000).

Thousands of genes are differentially expressed between stage C5 individuals that are in pre-diapause and directly developing ones (Lenz et al., 2021; Tarrant et al., 2014). Marker genes that were robustly upregulated in the pre-diapause individuals were associated with oogenesis, RNA metabolism and fatty acid biosynthesis (Lenz et al., 2021). Furthermore, during diapause, expression of genes associated with increased stress tolerance (ferritin and HSP22) is high (Aruda et al., 2011; Skottene et al., 2019; Tarrant et al., 2008). As animals progress through diapause, lipid catabolism increases, with elevated expression of master regulators of lipid metabolism (SREBP, TAp63, HFN4 and NHR-E75). Approaching termination of diapause, the β -oxidation pathway is downregulated, reflecting lower energetic metabolism as the C5s prepare to molt into adults (Skottene et al., 2019).

It is unknown whether or how pollutants alter diapause initiation, but a few studies have investigated the impact of exposure on *Calanus* during diapause. Exposure of *C. finmarchicus* to a WSF of crude oil for 5 days during diapause caused a temporary pause in lipid catabolism, as indicated by slower utilization of lipid stores and downregulation of several genes in the β -oxidation pathway (Skottene et al., 2019). Because lipid content and composition may be an important trigger for termination of diapause, the authors suggested that exposure during diapause or diapause termination may offset timing of diapause termination and subsequent migration to the surface. A similar effect was observed in *C. glacialis* exposed to pyrene ($60 \mu\text{g L}^{-1}$ initial concentration) during diapause. Over a 3-month period there was increased mortality, and the mobilization of lipid reserves from the oil sac was reduced, suggesting

that pyrene intensified the metabolic depression during diapause (Toxværd et al., 2018b). After diapause, copepods were fed (in clean sea water) but the effects of pyrene exposure persisted in the form of decreased rates of lipid accumulation, fecal pellet production, and lower egg production. A comparable study on *C. hyperboreus* exposed to pyrene during diapause found no differences in survival and egg production between controls and pyrene-exposed copepods. However, exposed copepods exhibited delayed effects; lipid sacs were replenished more slowly than controls after emerging from diapause, and their eggs had lower hatching rates, (Toxværd et al., 2019).

10. Reproduction

Gonad development in *C. finmarchicus* is initiated at the late C4 stage, and by late C5, the sexes can be differentiated visually (Hirche, 1996), and is associated with changes in gene expression during the C5 stage (Tarrant et al., 2014). However, in diapausing C5 individuals, gonad maturation is delayed and begins during the “activation phase” towards the end of diapause (Hirche, 1996). In these individuals, gonad maturation depends on the catabolism of wax esters that are stored in the lipid sac (Hernández Ruiz et al., 2021; Sargent and Falk-Petersen, 1988). During copulation, males attach a spermatophore to the female gonopore, and spermatozoa are subsequently transferred to spermathecal sacs. There they are stored until eggs are mature and fertilized at spawning (Hirche, 1996; Marshall and Orr, 1972). Spawning eggs, with a diameter of 145 µm, contain 15 % lipids on a dry mass basis and are slightly negatively buoyant (1.074 g ml⁻¹) (Hirche, 1996).

The three dominant *Calanus* species in the northern high-latitude marine ecosystems, *C. finmarchicus*, *C. hyperboreus*, and *C. glacialis*, share many traits, as explained in the previous sections, but they differ in their reproductive strategy (Falk-Petersen et al., 2009; Sainmont et al., 2014). Directly developing *C. finmarchicus* are income breeders, using concurrent food intake to produce eggs. Diapausing *C. finmarchicus* fuel early oogenesis using stored lipids, but egg maturation requires new resources (Niehoff, 2000; Niehoff et al., 2002; Plourde and Runge, 1993). *C. glacialis* can start reproduction in the absence of food using only stored lipids (Hirche and Kattner, 1993), but reproduction is closely related to the food availability, including the abundance of ice algae and the timing of phytoplankton blooms (Conover, 1988; Hirche, 1989; Niehoff et al., 2002; Søreide et al., 2010). The larger *C. hyperboreus* is a pure capital breeder, spawning in very deep waters during the winter using stored lipids accumulated during the previous spring bloom (Conover, 1967; Hirche and Niehoff, 1996). These differences in reproductive modes are likely to have implications for the extent to which prior and current exposure of female copepods to contaminants affects the number and condition of their offspring.

Relationships between lipid metabolism, reproduction and toxicity in *Calanus* are complex and may be affected by the developmental program (direct development vs. diapause). The susceptibility to oil exposure may be modulated by female condition, as lipid-rich females appear to experience lower mortality than lipid-poor ones (Hansen et al., 2016). Significant decreases in feeding and reproductive rates of *C. finmarchicus* and *C. glacialis* females were found in the laboratory when exposed to pyrene at high concentrations (100 nM), but not at the lower ones (≤ 10 nM). The copepods acquired pyrene through ingestion, and its effect on reproduction was cumulative (Jensen et al., 2008). Exposure of *C. finmarchicus* females to mechanically dispersed crude oil caused a concentration-dependent decrease in fecundity and an overall reduction in reproductive output within the first five days after exposure (Olsen et al., 2013). Larger adverse impacts were observed in females exposed to chemically dispersed oil compared with mechanically dispersed oil (Hansen et al., 2015). In both cases, a decrease in production of eggs and hence, nauplii, was observed after a 96-hour exposure to medium (1 mg oil L⁻¹) and high (5 mg oil L⁻¹) doses. However, after about two weeks of recovery in clean sea water, a compensatory effect was observed in females exposed to high doses of

mechanically, but not chemically dispersed oil, where egg and naupliar production returned to levels comparable to controls (Hansen et al., 2015; Olsen et al., 2013).

Transcriptomic approaches may be used in the future to gain a mechanistic understanding of how petrogenic pollutants affect *Calanus* reproduction. These approaches have been used to study the effects of natural algal toxins (oxylipins and saxitoxins) on the reproduction of *C. finmarchicus* and *C. helgolandicus*. Organismal level effects include decreased egg production, reduced egg hatching rates, and abnormal naupliar development (Carotenuto et al., 2002; Ianora et al., 2004, 2003, 1995; Romano et al., 2003; Roncalli et al., 2016b). Two studies that used transcriptomic approaches to explore these effects found generalized stress responses that involved many biological processes but could not be linked to specific reproductive impacts (Carotenuto et al., 2014; Roncalli et al., 2016a). Few studies have investigated gene expression patterns within the context of specific developmental or reproductive processes, though some progress has been made in this area. In direct developing *C. finmarchicus* C5 copepodites, a suite of genes involved in oogenesis change their expression as the copepod approaches final molt (Lenz et al., 2021; Tarrant et al., 2014). In another study, gene expression patterns were related to specific phases of oogenesis from diapause to end-of-life in *N. flemingeri* (Roncalli et al., 2021b, 2018). These studies and similar approaches could contribute to a better understanding of how genes shown to be responsive in ecotoxicological studies might be linked with reproductive impacts.

11. Population impacts

The potential population impacts of petrogenic pollutants on northern high-latitude *Calanus* are not well understood (Broch et al., 2020). Effects on a population level are determined by multiple factors including where the exposure takes place (both geographically and within the water column), when it takes place, and how the population is structured at the time of exposure (Broch et al., 2020).

Based on mortality observed from acute exposure experiments using C5 copepodites or adults (e.g. Hansen et al., 2018), impacts on *Calanus* populations are only expected at high concentrations of oil. However, potential impacts of oil on early developmental stages (nauplii and early copepodites) of *Calanus* are unclear, as these stages seem to be more sensitive to oil pollution than adults (Jager et al., 2016) and potentially affected by maternal transfer of pollutants (Hansen et al., 2017). Field data are more limited (Hansen et al., 2020), but feeding suppression has been observed in wild populations of the Arctic copepod *C. hyperboreus* at environmentally relevant concentrations of oil (Gilfillan et al., 1986).

Model simulation studies indicate that in a food-limited environment, increased mortality of *Calanus* due to petroleum exposure may be partially compensated for by increased food availability to unaffected animals (Broch et al., 2013). This is assuming the animals are in a feeding state and not e.g., in diapause. Major ocean current systems may provide protection from localized oil spills, since they can be a barrier against oil exposure of *Calanus* populations across large areas (Broch et al., 2020).

The long-term impacts of acute and chronic exposure to petrogenic pollutants need to be considered to better evaluate potential effects of oil pollution on the population dynamics of *Calanus*. For instance, survival during diapause is critical for the population dynamics of *Calanus* species, and particularly relevant in the Arctic, where mortality of *Calanus* can be up to 94 % during diapause in deep waters (Daase et al., 2014). The percentage survival necessary to sustain a viable population is not known, however. Both surface and deep water oil spills may have sub-lethal effects on *Calanus* populations prior to and during diapause (Broch et al., 2020). In both cases, and regardless of the base mortality, direct exposure to petroleum hydrocarbons can negatively affect the dormant stages, for example, by reducing lipid mobilization as mentioned above with a potential for delayed surface migration (Skotene et al., 2019; Toxværd et al., 2019). Delayed surface migration may

lead to a mismatch in alignment with the spring phytoplankton bloom, which could lead to food shortages, and reduced egg production, decreased survival of offspring, and subsequent impacts on *Calanus* biomass and population structure. Given the complex life cycle and ontogeny of *Calanus* species, toxicity may not only manifest immediately after acute exposure, but also later in the life cycle. If an oil spill occurs during the spring bloom, when *Calanus* feed and store energy as lipids, the feeding rates will be reduced, lipid stores will take longer to build, and toxic petroleum hydrocarbons will be accumulated in the lipid sacs with the potential to cause delayed effects.

Many open questions remain regarding how *Calanus* populations could be affected by oil exposure in a future ocean climate that is generally warmer and with lower pH than at present. On an individual level, oil exposure in combination with increased temperatures can increase mortality compared to oil-exposure alone in naupliar *Calanus* stages (Grenvald et al., 2013). Pyrene exposure led to reduced egg production across a wide temperature range in *C. finmarchicus*, but less so in *C. glacialis* (Hjorth and Nielsen, 2011). Lowered pH may also significantly increase the mortality of some stages and ecotypes of *C. glacialis* (Thor et al., 2018), but little is known about how this interacts with oil exposure. Again, the timing and demographic structure of the *Calanus* population may play a role. With further projected increases in sea temperatures, and reduction in ice cover, there is evidence of range shifts, with *C. finmarchicus* surviving in regions previously more suitable for *C. glacialis* (Ellingsen et al., 2008; Tarling et al., 2022). Population level effects of oil pollution may have effects far outside of the directly oil-impacted region. For example, there are indications that *Calanus* from the North Norwegian shelf can be advected as far as the Northern Svalbard Shelf region (Wassmann et al., 2019). Thus, the Arctic population may be affected by animals exposed in temperate regions.

12. Linking mechanistic information to regulatory-relevant adverse effects

Efforts in broad-content generation technologies such as transcriptomics and other “omics” technologies have expanded the knowledge about initial molecular responses and adverse effects of several chemicals and their mixtures in *Calanus*. Several potential toxicity responses have been proposed based on the responses of biomarkers, but efforts to link them to adverse effects have been undertaken only to a limited degree. Adverse Outcome Pathways (AOPs) have emerged as an efficient approach to provide causal linkage between molecular (biomarker) responses and effects considered regulatory-relevant, as manifested at higher levels of biological organization (e.g., at the individual, population and community). The AOPs organize, evaluate and portray the chemical perturbations from one or more chemicals on biological targets at the molecular, biochemical, phenotypical,

individual and/or the population level (i.e. the MIE, KE and AO) and the causality between these events (i.e. Key Event Relationship, KERs (Fig. 4).

The AOPs themselves are organized in a dedicated data repository, the AOPwiki (www.aopwiki.org), which currently hosts close to 500 AOPs, more than 2000 events (MIEs and KEs) and about 700 prototypical chemical and non-chemical stressors (July 2023). The construction of AOPs is collaborative in nature and often based on scientific crowd-sourcing activities of data generation, data review and evaluation, where the AOPs undergo maturation, expansion and peer-review after initial submission to the OECD AOP development program (Chauhan et al., 2022). Although several AOPs have been developed for arthropods and/or crustaceans (www.aopwiki.org), the number of toxicity mechanisms and associated adverse effects described for non-vertebrate organisms still remains low. However, as AOPs are modular, chemical agnostic, and evolve through collaborative efforts to expand the knowledge domain to new areas of applicability, the wealth of data from *Calanus* would be a highly valuable resource for developing, consolidating and/or expanding AOPs and associated AOP events relevant for oil-related chemicals. This applies in particular to oil-related chemicals such as PAHs (e.g. dibenzo[a,h]anthracene, benzo(a)pyrene, benzo(k)fluoranthene, phenanthrene), phenols (e.g. 4-nonylphenol and 4-propoxyphenol) and heavy metals (e.g. cadmium, lead, copper, iron, nickel, zinc, arsenic), that have been proposed as prototypical stressors in one or more AOPs (e.g. AOP# 12, 131, 257, 296, 310, 397, 499, 413, 499). As per July 2023, no AOPs are specifically developed for *Calanus* or other marine copepods, although crustaceans are part of the taxonomic applicability domain of several chemical-relevant AOPs (e.g. AOP# 4, 16, 113, 160, 161, 201, 286, 287, 290, 291, 336, 337, 341, 358, 359, 360, 361), whereof some biological (biomarker) responses and effects have been the subject of the current review. This particularly applies to several toxicity pathways associated with disruption of molting, neurotoxicity, mitochondrial toxicity and generation of reactive oxygen species (ROS) that potentially may affect survival, development and reproduction. Table 1 provides examples of AOPs potentially relevant for *Calanus* sp. Although poorly developed in the AOP community, behavioral traits (e.g., diapause and anti-predatory behavior) may introduce new and novel knowledge to expand AOPs towards assessing the role of and adaptability to environmental cues and pollutant exposures.

13. Concluding remarks, future research needs and recommendations

Recent toxicity research has used a multiplicity of measurements to quantify responses to oil pollution at several levels of biological organization, such as relative gene expression, changes in lipid storage, stage-specific survival, feeding rates and reproductive output. These

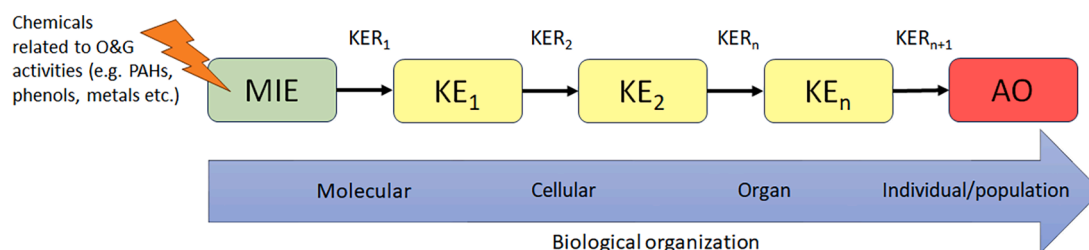


Fig. 4. Schematic representation of an Adverse Outcome Pathway (AOP) modified from Vinken (2013). The AOPs is a structured organization of empirical data that portray the causal relationships between the initial molecular initiating event (MIE) and an adverse outcome (AO) of regulatory relevance, connected with a series of intermediate key events (KE) that represent the most critical steps of a toxicity pathway spanning multiple levels of biological organization (e.g., molecular, biochemical, phenotypical and population). The individual events are interconnected by specific key event relationships (KERs) that describe the causal relationship between two adjacent events and where the biological (toxicological) plausibility, essentiality of the events, empirical evidence and quantitative understanding is critically evaluated using defined criteria as part of a weight of evidence (WOE) assessment based on modified Bradford-hill criteria (Hill, 1965). Detailed description of the AOP, AOP components and WOE assessments can be found elsewhere (Becker et al., 2015; Villeneuve et al., 2014a, 2014b).

Table 1

Examples of molecular initiating events (MIEs), key events (KEs), adverse outcomes (AOs) and AOPs relevant for toxicity observed in *Calanus* sp. (this review). Data is retrieved from the AOPwiki (www.aopwiki.org) and assigned to different levels of biological organization.

Mechanism of toxicity	AOP id	AOP title	Event id	Event name	Event type	Biological organization
Molting disruption	4	Ecdysone receptor agonism leading to incomplete ecdysis associated mortality	103	Increase, Ecdysone receptor agonism	MIE	Molecular
			1264	Increase, Nuclear receptor E75b gene expression	KE	Molecular
			1265	Increase, Fushi tarazu factor-1 gene expression	KE	Molecular
			988	Decrease, Circulating ecdysis triggering hormone	KE	Tissue
			1266	Decrease, Circulating crustacean cardioactive peptide	KE	Tissue
			1267	Decrease, Ecdysis motoneuron bursts	KE	Tissue
			1268	Decrease, Excitatory postsynaptic potential	KE	Tissue
			993	Decrease, Abdominal muscle contraction	KE	Tissue
	358	Chitinase inhibition leading to mortality	990	Increase, Incomplete ecdysis	KE	Individual
			350	Increase, Mortality	AO	Individual
			1806	Inhibition, Chitinase	MIE	Molecular
			996	Decrease, Digestion of old cuticle	KE	Organ
			1524	Increase, Premature molting	KE	Individual
			350	Increase, Mortality	AO	Individual
	359	Chitobiase inhibition leading to mortality	1807	Inhibition, Chitobiase	MIE	Molecular
			996	Decrease, Digestion of old cuticle	KE	Organ
			1524	Increase, Premature molting	KE	Individual
	360	Chitin synthase 1 inhibition leading to mortality	350	Increase, Mortality	AO	Individual
			1522	Inhibition, Chitin synthase 1	MIE	Molecular
			1523	Decrease, Cuticular chitin content	KE	Tissue
Neurotoxicity	16	Acetylcholinesterase inhibition leading to acute mortality	1524	Increase, Premature molting	KE	Individual
			350	Increase, Mortality	AO	Individual
			12	Acetylcholinesterase (AChE) Inhibition	MIE	Cellular
			10	Acetylcholine accumulation in synapses	KE	Cellular
			39	Increased Cholinergic Signaling	KE	Organ
			445	Respiratory distress/arrest	KE	Organ
			1703	Dysregulation of heart rate and vascular tone	KE	Organ
			351	Increased Mortality	AO	Population
			360	Decrease, Population growth rate	AO	Population
			160	Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality	762	Activation, ionotropic GABA Receptor chloride channel
	1012	Increased, Inhibitory postsynaptic potential			KE	Cellular
	1014	Induction, Somatic muscle paralysis			KE	Tissue
	761	Increased, Chloride conductance			KE	Cellular
	1015	Increased, Neuronal synaptic inhibition			KE	Cellular
	1016	Inhibition, Feeding			KE	Individual
	351	Increased Mortality			AO	Population
	1018	Activation, Glutamate-gated chloride channels			MIE	Molecular
	1015	Increased, Neuronal synaptic inhibition			KE	Cellular
	1012	Increased, Inhibitory postsynaptic potential			KE	Cellular
	161	Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality	1016	Inhibition, Feeding	KE	Individual
761			Increased, Chloride conductance	KE	Cellular	
1019			Induction, pharyngeal muscle paralysis	KE	Tissue	
351			Increased Mortality	AO	Population	
1446			Decrease, Coupling of oxidative phosphorylation	MIE	Molecular	
1771			Decrease, Adenosine triphosphate pool	KE	Cellular	
1821			Decrease, Cell proliferation	KE	Cellular	
1521			Decrease, Growth	AO	Individual	
263	Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation	1446	Decrease, Coupling of oxidative phosphorylation	MIE	Molecular	
		1771	Decrease, Adenosine triphosphate pool	KE	Cellular	
		1821	Decrease, Cell proliferation	KE	Cellular	
		1521	Decrease, Growth	AO	Individual	
		1446	Decrease, Coupling of oxidative phosphorylation	MIE	Molecular	
264	Uncoupling of oxidative phosphorylation leading to growth inhibition via ATP depletion associated cell death	1771	Decrease, Adenosine triphosphate pool	KE	Cellular	
		55	Cell injury/death	KE	Cellular	

(continued on next page)

Table 1 (continued)

Mechanism of toxicity	AOP id	AOP title	Event id	Event name	Event type	Biological organization
	265	Uncoupling of oxidative phosphorylation leading to growth inhibition via increased cytosolic calcium	1521	Decrease, Growth	AO	Individual
			1446	Decrease, Coupling of oxidative phosphorylation	MIE	Molecular
			2064	Increase, Cytosolic calcium	KE	Cellular
			55	Cell injury/death	KE	Cellular
			1521	Decrease, Growth	AO	Individual
	266	Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased Na-K ATPase activity	1446	Decrease, Coupling of oxidative phosphorylation	MIE	Molecular
			1771	Decrease, Adenosine triphosphate pool	KE	Cellular
			1562	Decreased Na/K ATPase activity	KE	Cellular
			1527	Increase, Cell membrane depolarization	KE	Cellular
			55	Cell injury/death	KE	Cellular
			1521	Decrease, Growth	AO	Individual
			1879	Bulky DNA adducts, increase	MIE	Molecular
DNA damage	397	Bulky DNA adducts leading to mutations	155	Inadequate DNA repair	KE	Cellular
			185	Increase, Mutations	AO	Molecular

results suggest that it may be possible to use relative gene expression as an efficient method to profile an organism's cellular and organ responses to pollutants in general. The application of transcriptomics and other broad-content molecular tools such as proteomics and metabolomics will continue to advance our understanding of how crude oil affects cellular responses and organism function. However, establishing robust links across biological processes will require a more in-depth analysis of existing molecular resources and additional comparative experiments. The goal would be to use omics patterns to predict responses at higher levels of organization and provide causal relationships between those early molecular effects and adverse effects occurring at apical levels of biological organization that are relevant for risk assessment. For example, low expression of genes for digestive enzymes is indicative of depressed or halted feeding, which in turn may be detrimental to successful growth, development and reproduction in populations of *Calanus*. Although principally similar for all omics technologies, advances in transcriptomics suggest that this enabling technology would offer the largest short-term benefits to such efforts and pave the road towards holistic approaches involving multi-omics initiatives. Comparisons across gene expression studies could for instance be conducted through continued annotation of a master reference transcriptome generated from existing resources (e.g., the Gulf of Maine *C. finmarchicus* transcriptome described earlier). Such a master reference file could be used to cross-reference differentially expressed genes identified in RNA-Seq and RT-qPCR studies not only within a species, but also in studies of homologous genes in other *Calanus* species. While expression of individual biomarkers can be useful in some cases, identifying transcriptome-wide sets of responsive genes can be a powerful alternative for identifying cellular and organ responses that are indicators of organism fitness. To date, *Calanus* gene expression studies have all been conducted using homogenized whole organisms, which may obscure organ-level impacts. In the future, single-cell sequencing approaches may be used to gain a more detailed mechanistic understanding of toxicity to single chemicals and complex mixtures of these such as those represented by exposure to crude oil, WAFs and produced water.

Adverse outcomes are usually quantified at the organismal and population levels. While it is relatively easy to predict the adverse outcomes when toxicants are lethal, sub-lethal effects are more complex. Our current knowledge about the effects of petroleum pollution from a point source indicates that lethal effects may be limited to the immediate surrounding area, but a much larger fraction of the population will experience sub-lethal effects. These effects could lead to decreased organismal fitness, which in turn could lead to local population declines. Current studies have started to address fitness by measuring reproduction and offspring quality and viability. However, more studies are needed to consider how timing of a pollution event might affect

individual fitness and how this might impact interannual recruitment. Immigration from other non-affected regions could be a mitigating factor, however, more populations studies are needed to quantify this.

Successful linkages between molecular responses and adverse outcomes would require a systematic, standardized, and transparent approach for organizing and evaluating the causal relationships between different toxicological responses and adverse effects observed. The AOP has emerged as such a suitable knowledge framework and has undergone substantial evolution over the past decade towards a more mechanistically-informed hazard characterization for chemical and non-chemical stressors (Chauhan et al., 2022). Although being biased towards human toxicity pathways, which in many cases are not relevant for copepods such as *Calanus*, increasing efforts are undertaken to develop new AOPs and to expand the applicability domain of existing AOPs to also non-human species such as crustaceans (Schmid et al., 2021; Song et al., 2020b, 2020a, 2017; Tollefsen et al., 2022; Toyota et al., 2022). As the principles of AOPs would be equally applicable to all organisms, toxicity mechanisms and stressors, they would be particularly useful for characterizing the knowledge domain, but also to identify knowledge gaps and prioritize future research efforts. This also applies to the advancement of AOP networks (Tollefsen et al., 2022) and quantitative AOPs (Cao et al., 2023; Moe et al., 2021; Song et al., 2023, 2020b), and integrated testing and assessment, IATA (Tollefsen et al., 2014), that represent key hallmarks in the advancement towards mechanistically-informed risk assessment (Perkins et al., 2019). Petrogenic pollution involves a plethora of chemicals that in turn could have direct effects on multiple molecules and through multiple toxicity pathways, which may represent a challenge for clearly identifying one or a few particularly relevant AOPs. Efforts to decipher the effects of different stressors at different levels of biological organization using AOPs have been proposed and to some degree demonstrated for simple mixtures of stressors (Song et al., 2018; Xie et al., 2022) and would provide initial suggestions for how to delineate single stressor effects from those of multiple stressors.

CRedit authorship contribution statement

Bjørn Henrik Hansen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Ann M. Tarrant:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Petra H. Lenz:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Vittoria Roncalli:** Conceptualization, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Rodrigo Almeda:** Writing – original draft, Writing – review & editing. **Ole Jacob Broch:** Writing – original draft,

Writing – review & editing. **Dag Altin:** Writing – original draft, Writing – review & editing. **Knut Erik Tollefsen:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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