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REVIEW

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Xenobiotic metabolism and transport in *Caenorhabditis elegans*

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ABSTRACT

Caenorhabditis elegans has emerged as a major model in biomedical and environmental toxicology. Numerous papers on toxicology and pharmacology in *C. elegans* have been published, and this species has now been adopted by investigators in academic toxicology, pharmacology, and drug discovery labs. *C. elegans* has also attracted the interest of governmental regulatory agencies charged with evaluating the safety of chemicals. However, a major, fundamental aspect of toxicological science remains underdeveloped in *C. elegans*: xenobiotic metabolism and transport processes that are critical to understanding toxicokinetics and toxicodynamics, and extrapolation to other species. The aim of this review was to initially briefly describe the history and trajectory of the use of *C. elegans* in toxicological and pharmacological studies. Subsequently, physical barriers to chemical uptake and the role of the worm microbiome in xenobiotic transformation were described. Then a review of what is and is not known regarding the classic Phase I, Phase II, and Phase III processes was performed. In addition, the following were discussed (1) regulation of xenobiotic metabolism; (2) review of published toxicokinetics for specific chemicals; and (3) genetic diversity of these processes in *C. elegans*. Finally, worm xenobiotic transport and metabolism was placed in an evolutionary context; key areas for future research highlighted; and implications for extrapolating *C. elegans* toxicity results to other species discussed.

KEYWORDS
Caenorhabditis elegans; toxicokinetics; pharmacokinetics; xenobiotic metabolism; xenobiotic transport; microbiome; nuclear hormone receptor; genetic diversity; evolutionary toxicology

Introduction

Caenorhabditis elegans has emerged as an important model in biomedical and environmental toxicology. *C. elegans* was first described over 100 years ago by Maupas (1900) and was intermittently studied thereafter until coming to prominence as an exceptionally powerful model organism for developmental biology, neurobiology, and genetics, as a result of pioneering efforts by Sydney Brenner and colleagues in the 1970s (Nigon and Felix 2017). Although there was some early research in toxicologically relevant areas such as DNA damage and repair (Hartman and Herman 1982) and antioxidant defenses (Blum and Fridovich 1983), the first explicit efforts to develop *C. elegans* as a model for toxicological research were carried out by Phil Williams, David Dusenberry, and colleagues beginning in the 1980s (Williams and Dusenbery 1987, 1988, 1990a, 1990b). In the early 1990s, Jonathan Freedman's lab worked on heavy metal response (Freedman et al. 1993; Slice, Freedman, and Rubin 1990), toxicogenomic analysis (Cui et al. 2007), and medium-throughput toxicity testing (Boyd, McBride, and Freedman 2007), and went on to establish a worm toxicology lab at the United States National Toxicology Program (Behl et al. 2015; Boyd et al. 2010, 2015, 2009; Xia et al. 2018). Starting in the mid-1990s, Christian Sternberg's group carried out ecotoxicological studies with aquatic and sediment exposures (Hoss et al. 1997, 1999; Traunspurger et al. 1997), ultimately leading to a number of academic reports (Hagerbaumer et al. 2015; Hoss et al. 2009). Two standardized toxicology testing protocols have been published (International Standard Organization (ISO) 2020, ((ASTM), American Society of Testing and Materials. 2001). Richard Nass established the use of *C. elegans* for chemical-induced neurodegeneration (Nass, Miller, and Blakely 2001, 2002). Further, *C. elegans* has been used to study transgenerational and environmental epigenetics (Kelly 2014;

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Weinhouse et al. 2018). *C. elegans* is now a well-established model for human and ecological toxicology employed by many labs (a non-comprehensive sampling identifies approximately two dozen: (Leung et al. 2008; Boyd et al. 2010; Steinberg, Sturzenbaum, and Menzel 2008; Helmcke, Avila, and Aschner 2010; Meyer and Williams 2014; Tejeda-Benitez and Olivero-Verbel 2016; Hunt 2017; Choi et al. 2014; Honnen 2017; Ferreira and Allard 2015; Allard et al. 2013; Lenz, Pattison, and Ma 2017; Nass, Miller, and Blakely 2001a; Harrington et al. 2010; Cooper and Van Raamsdonk 2018; Liao and Yu 2005; Fitsanakis, Negga, and Hatfield 2014; Menzel et al. 2005; Harlow et al. 2018; Zhao et al. 2013; Brady et al. 2019; Anbalagan et al. 2013; Clavijo et al. 2016; Hoss et al. 2009; Horsman and Miller 2016; Shomer et al. 2019; Zhang et al. 2020; Haegerbaeumer et al. 2018b; Lee et al. 2020; Shen et al. 2019; Harlow et al. 2016; Dietrich et al. 2016; Xiong, Pears, and Woollard 2017)). The number of publications on toxicology and related fields in *C. elegans* has grown rapidly in recent years, with an even more rapid growth in pharmacology-related publications (Figure. 1).

This toxicological research is complemented by extensive investigations of other stress-response pathways by non-toxicologists, including heat shock unfolded protein, DNA damage, antioxidant defense, osmotic stress, hormesis, insulin-responsive pathways, hypoxia, caloric stress, regulation of apoptosis and necrosis, and autophagy (Baugh 2013; Baumeister, Schaffitzel, and Hertweck 2006; Blackwell et al. 2015; Hengartner and Horvitz 1994; Melendez and Levine 2009; Murphy 2006; Nikoletopoulou and Tavernarakis 2014; Prahlad and Morimoto 2009; Rieckher et al. 2018; Ristow and Schmeisser 2011; Rodriguez et al. 2013). Importantly, as reviewed by Hunt (2017) of the U.S. Food and Drug Administration (Center for Food Safety and Applied Nutrition), there is a significant correspondence between *C. elegans* and higher eukaryotes in rank-order acute toxicity of chemicals as noted in multiple studies in several labs.

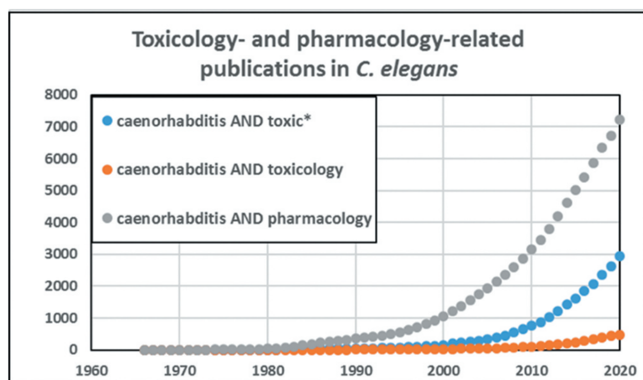

Figure 1. There has been a rapid increase in the use of *C. elegans* for pharmacology and toxicology research.

Explicit discussion of the strengths and limitations of *C. elegans* as a model for toxicological research may be found in several review papers and books (Hunt 2017; Maurer, Luz, and Meyer 2018; Queiros et al. 2019; Wang 2019a, 2019b, 2020; Weinhouse et al. 2018), and thus will not be repeated here. The focus of this review will be on one very important and oft-cited limitation: the absence of detailed understanding of xenobiotic transport and metabolism processes that regulate concentrations of chemicals and metabolites that reach molecular targets. The study of these processes is fundamental to toxicology, pharmacology, and drug discovery. A truism in toxicology is that "the dose makes the poison," and it is not possible to truly understand the toxicity of a chemical without understanding how much of it (or its metabolites) has reached specific molecular sites of action. Similarly, it is not possible to extrapolate effects observed in one species (*e.g.*, worms) to another (*e.g.*, humans) without being able to compare internal toxicant concentrations. In toxicology, a distinction is made between toxico(pharmaco)kinetics (understanding the absorption, distribution, metabolism, and excretion (ADME) processes that regulate xenobiotic transport and transformation processes) and toxico(pharmaco)dynamics (understanding the interactions of xenobiotics with molecular targets including receptors, DNA, and proteins).

Toxicodynamics (TD) appear to be relatively similar between *C. elegans* and higher eukaryotes based on conservation of genes that encode molecular targets such as proteins and signaling pathways, as reviewed in some detail previously (Hunt 2017; Leung et al. 2008; Wang 2019a). Further, the great majority of worm toxicology papers to date studied TD, and these investigations generally support similar mechanisms of action for many chemicals in worms compared to higher eukaryotes. However, genetic, biochemical, and other differences that qualitatively alter potential chemical toxicity exist (reviewed in Maurer, Luz, and Meyer 2018; Queiros

et al. 2019; Weinhouse et al. 2018) and need to be considered. For example, worms (1) do not synthesize heme or cholesterol; (2) lack an adaptive immune system; and (3) lack homologs of some important receptors such as estrogen receptors. Therefore, chemicals that act directly on proteins in these pathways in higher eukaryotes might exert differing or no effects in worms.

Toxicokinetics (TK), in contrast, the focus of this review, is relatively poorly studied in *C. elegans*. Further, based upon the large physiological differences between worms and humans TK are expected to be different in *C. elegans*. For example, the nematode cuticle and eggshell are effective barriers to some chemicals. In addition, ADME processes are likely different in an organism that has a simple gut, but lacks a circulatory system and many discrete organs such as the liver. Significant uncertainty also exists in our understanding of the regulation and catalytic activity of xenobiotic metabolic enzymes and transporters. This review is intended to highlight what is or is not known regarding these processes in *C. elegans*, with the goal of stimulating research to fill in these knowledge gaps and improve the utility of this powerful model organism for toxicology and pharmacology.

Uptake of chemicals by the nematode occurs predominantly either (1) across the cuticle, which has few openings and thus presents an effective barrier against many chemicals and particles, or (2) via ingestion. Description of these barriers is provided in the next section. Emerging evidence suggests that the worm's microbiome exhibits the potential to alter chemicals prior to intracellular uptake, and the following section summarizes our nascent understanding of the role of the worm microbiome in xenobiotic and drug metabolism. The state of knowledge of the biochemical toxicological processes classically referred to as "Phase I, II, and III" (Casarett, Doull, and Klaassen 2008) are present in worms. These processes, which are critically important for organic xenobiotics, but also in some cases important for metals and metalloids, are conceptualized as:

–**Phase I**: enzymatically exposing or adding reactive moieties in parent xenobiotic;
–**Phase II**: conjugating the Phase I-modified (or in some cases, parent) xenobiotic to large, water-soluble molecules, which facilitates excretion;
–**Phase III**: transport of these metabolized compounds out of the cell (some transporters may act to import a parent compound, sometimes described as "Phase 0").

What is known of transcriptional regulation of these processes, including the role of the large number of nuclear hormone receptors present in *C. elegans,* are described below. An important caveat to keep in mind in the context of our discussion of specific genes is that most of what is presented is for the historically heavily-studied N2 Bristol strain. Subsequently a comprehensive catalog from the literature in which actual chemical measurements have been made in worms that can inform us about chemical uptake, transformation, and excretion is provided. The impacts of genetic diversity present across *C. elegans* populations (*C. elegans* is found globally), its use to identify sources of natural variation in toxicological processes, and its role in an evolutionary toxicology context are discussed. Finally, knowledge gaps are summarized and future directions discussed.

Physical barriers and tissue-specific xenobiotic transport and metabolism

A simple cell membrane consists of a lipid bilayer composed primarily of phospholipids, glycolipids, and cholesterol. The phospholipids arrange in such a way that their hydrophobic tails are pointed inwards and their hydrophilic heads are oriented toward the outer and inner membrane surfaces. The membrane barrier is differentially permeable to various xenobiotics based upon their physicochemical properties (movement by transporters is addressed below). Some xenobiotics might passively diffuse across cell membranes; hydrophilic molecules might enter through aqueous pores in the membrane, and hydrophobic molecules might diffuse directly through the lipid domain of the membrane (Benz, Janko, and Läuger 1980). The smaller the molecule, the more rapidly it moves across a membrane, either by aqueous pores or simple diffusion. For large organic molecules, the octanol/water partition coefficient P dictates the rate at which the molecule moves across the membrane, with higher lipophilicity (positive log P) corresponding to higher membrane permeability, except at extreme levels of lipophilicity. In the case

of weak organic acids and bases, ionic compounds move slowly and inefficiently through aqueous pores whereas non-ionized forms diffuse across the lipid membrane. Thus, the rate at which non-ionized organic acids and bases permeate the membrane depends on pK_a/pK_b of the compound and the pH of the surrounding environment (Avdeef 2001; Klaassen 2019; Manallack et al. 2013). However, *C. elegans* is not just a simple cell model system with a single membrane for protection; it is a complex organism with many physical barriers that prevent the passive transport of molecules into its body. In this section, the physical barriers that may impact uptake and release of xenobiotics by *C. elegans* are presented (Figure 2).

The worm's principle physical barrier is its cuticle (Figure 2). The cuticle of the nematode provides structure to maintain body shape, along with protection from its environment (Johnstone 1994; Page 2007; Lints and Hall 2009). The cuticle consists of a complex matrix of collagen and serves as a physical barrier against chemicals and pathogens. The cuticle is well known for protecting the worm from uptake of xenobiotics, and is often viewed as a drawback to using *C. elegans* in high-throughput toxicity assays (Xiong, Pears, and Woollard 2017). Mutants with defective cuticle collagen proteins, of which there are more than 150, were employed to increase uptake in pharmacological screens (Burns et al. 2010; Johnstone 2000; Xiong, Pears, and Woollard 2017). In adult *C. elegans*, the cuticle is roughly 0.5 μm thick and contains 5 distinct zones: surface coat, epicuticle layer, cortical zone, medial zone, and basal zone (Cox, Kusch, and Edgar 1981a; 1981b). The width of the cuticle increases with the age of the worm as the basal zone expands, and in older worms it often wrinkles, potentially due to weakening of the muscles and hypodermis (Herndon et al. 2002). The cuticle might also be damaged from physical interactions with the environment and the process of mating (Woodruff et al. 2014). Each cuticle layer has a distinct composition, suggesting a high degree of specialization between layers (Johnstone 2000). The structure of the cuticle varies at each larval stage along with the composition of each of the layers (Lints and Hall 2009; Page, 2007). *C. elegans* undergoes molting periodically until adulthood. Molting occurs in two stages: lethargus, a period of relative behavioral dormancy and extracellular matrix remodeling in preparation for cuticle shedding, and ecdysis, when the cuticle is undergoing a molt cycle (Lažetić and Fay 2017; Singh and Sulston 1978). At each larval stage, *C. elegans* undergoes ecdysis, in which it sheds the current cuticle and forms a new one. The process of molting enables rapid growth and potentially depuration of any xenobiotic compounds on or within the current cuticle. The existence of varying cuticle structures throughout *C. elegans* development also suggests that critical windows of sensitivity to cuticular xenobiotic uptake

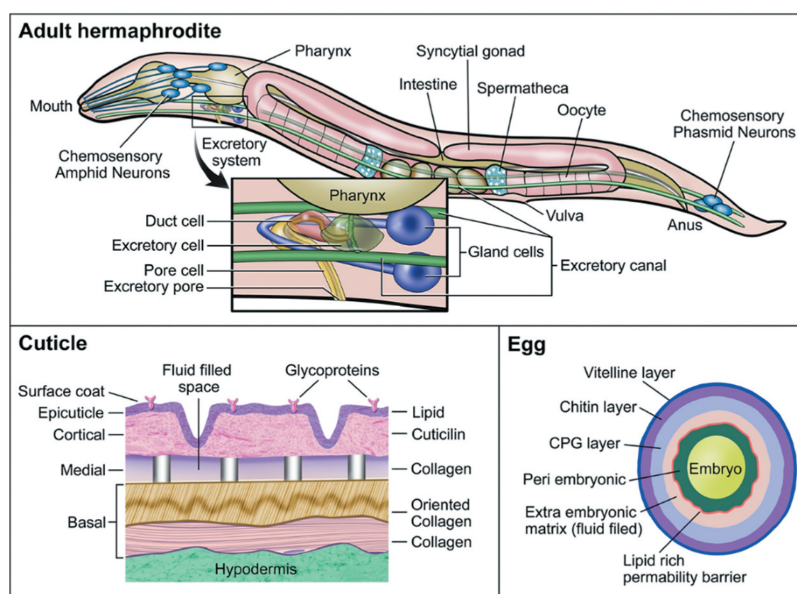

Figure 2. Schematic of physical barriers to xenobiotic diffusion in *C. elegans*.

exist. Dauer larvae, which are highly resistant to many stressors (Erkut et al. 2011; Fielenbach and Antebi 2008), possess thickened basal and epicuticle layers that might offer enhanced protection from their environment (Cassada and Russell 1975; Wolkow and Hall 2011). Early developmental exposures may result in more uptake of xenobiotics because of the difference in protein composition within the cuticle and thinness. Uptake might also be greater in older worms as the cuticle begins to wrinkle and weaken, although little research has been done to address alterations in uptake kinetics throughout the lifespan of *C. elegans*. It is also important to note that many of these physical processes and barriers have been extensively studied in *C. elegans* hermaphrodites but are rarely studied in males. Given the differences in size and morphology of male *C. elegans*, particularly around the tail region, it is possible that the different sexes might have altered uptake kinetics of xenobiotics, but this hypothesis has not apparently been addressed (Emmons 2005; Sulston, Albertson, and Thomson 1980).

Another important barrier is the eggshell, which in *C. elegans* begins to form as soon as the oocyte enters the spermatheca (Figure 2) and is fertilized (Stein and Golden 2015). The eggshell of *C. elegans* is the dominant physical barrier when the nematode is *in utero* and externally (after the egg is laid at approximately 30-cell stage), prior to hatching. The trilaminar outer eggshell is made of an outer vitelline layer, a chitin layer, and chondroitin proteoglycan (CPG) layer (Johnston and Dennis 2012; Olson et al. 2012). Beneath these layers resides the fluid-filled extra-embryonic matrix (EEM), a lipid-rich layer that acts as a permeability barrier to the developing embryo, and an amorphous space referred to as the peri-embryonic layer (Olson et al. 2012). Maternal environment and food supply impact the structure and size of the eggshells produced (Harvey and Orbidans 2011). Production of the egg might serve as a way to excrete xenobiotics, although no apparent research has been done to address this. Similarly, transfer of xenobiotics from mother to egg via other maternally-loaded components, such as vitellogenin is likely to occur. Vitellogenin contains both hydrophobic and hydrophilic domains and is an important vector for the transfer of xenobiotics to eggs in other species (Monteverdi and Di Giulio 2000). After egg laying occurs, the eggshell is exposed to the environment where it serves to protect the developing worm. Molecules that are able penetrate the various layers of the eggshell may be taken up by the developing worm.

C. elegans might also be exposed to xenobiotic compounds through ingestion. *C. elegans* feeds on microbes in the lab, either bacterial lawns or suspension in liquid cultures, through pharyngeal pumping. The pharynx is the neuromuscular pump that connects the mouth to the intestine and contracts and relaxes in order to take in bacteria and expel liquids (Avery and You 2012). The pharynx is lined with a specialized cuticle that helps to form structural elements of the pharynx such as the flaps, sieve, and the grinder, all of which are necessary for initial processing as well as transport of food to the intestine, and protection against diffusion of compounds in the pharynx (Page 2007; Altun and Hall 2009a). The pharyngeal cuticle and the pumping mechanism that expels liquids might serve as a protective barrier from xenobiotic uptake. However, xenobiotics that are bound to or taken up by bacterial food source would still be ingested by the worm. For chemicals that make their way into the intestine, uptake through epithelial cells lining the intestine might occur, as well as transport and trafficking to other locations in the organism. The intestine exhibits high expression of *P*-glycoproteins, involved in trafficking hydrophobic molecules, as well as cytochrome P450s, metallothioneins, and other phase I–III enzymes (McGhee 2013). It is important to note the availability and uptake of xenobiotics in the intestine varies by physicochemical properties and the chemical properties of the gut. As previously noted, membrane permeability to these compounds in the gut is determined by chemical structure, pK_a, and pH of the environment (Casarett, Doull, and Klaassen 2008). Alterations in the *C. elegans* diet and microbiome also lead to changes in intestine pH or metabolites, which might alter the availability of certain compounds (Höss, Schlottmann, and Traunspurger 2011). Other cell types may also play specialized roles; for example, the six coelomocytes are macrophage-like cells that occupy the body cavity, are highly endocytotic, and may, similar to intestinal cells, play a "liver-like" role. Investigators

demonstrated their importance in metal detoxification via endocytosis and metal transport and binding proteins (Maurer et al. 2016; Schwartz et al. 2010; Tang et al. 2020).

C. elegans possesses an excretory system comprising four main cell types. The excretory system includes one pore cell, one duct cell, one canal cell (excretory cell), and a fused pair of gland cells (Altun and Hall 2009b) (Figure 2). The excretory system plays a role in osmoregulation and waste elimination, somewhat similar to the renal system in vertebrates (Sundaram and Buechner 2016). In the adult worm, the excretory canal cell extends from the head to the tail of the animal with the cell body located in the head region. The excretory canal cell is connected to the excretory gland cells, duct cell, and pore cell. The canal cell collects and guides fluids from within the worm toward the cell body for excretion through the duct and pore cells (Nelson, Albert, and Riddle 1983; Nelson and Riddle 1984; Stone, Hall, and Sundaram 2009). The duct cell and pore cell form a channel that opens to the environment, through which fluid might be excreted. Both the duct and pore cells possess a specialized cuticle that provides structure and protects them from the surrounding environment (Lints and Hall 2009; Sundaram and Buechner 2016). Nonetheless, the excretory system might also permit entry as well as exit for xenobiotics.

C. elegans also have a sophisticated chemosensory system that mediates detection and avoidance of noxious conditions. Chemosensation is vital to survival of *C. elegans*, enabling them to find food, mate, avoid harmful environments, and enter or exit the dauer stage (Bargmann 2006). Chemosensory neurons are found in the head and tail regions of the worm. There are 16 pairs of bilaterally symmetric neurons (approximately 10% of the total number of neurons, with neurons comprising approximately 1/3 of all somatic cells) that are involved with chemosensation (Bargmann 2006; Sengupta 2007). These neurons are bipolar, containing a single axon, a single dendrite, and cilia, which are exposed to the surrounding environment through small openings in the cuticle (Sengupta 2007; Ward et al. 1975; Ware et al. 1975). The ability of *C. elegans* to sense and respond to chemical stimuli enables them to avoid potentially toxic environments in the wild and protect themselves from exposures to xenobiotic compounds via chemotaxis. At the same time, the exposure of cilia to the external environment may also be a route of exposure for xenobiotics to infiltrate the neurons (Perkins et al. 1986).

Overall, physical and behavioral barriers play key roles in protecting *C. elegans* from xenobiotic uptake and accumulation, but knowledge of the manner by which most specific xenobiotics and even classes of xenobiotics cross these barriers and alter behavior is lacking.

C. elegans microbiome

The gut microbiome is a complex mixture of microorganisms (Koontz et al. 2019). Changes and alterations to the composition of the gut microbiota are associated with several diseases, including irritable bowel disease, obesity, type 2 diabetes, and neurodegenerative disorders in humans (Scotti et al. 2017). Recently, more research attempted to elucidate the complex interactions between host and the associated gut microbiome. This theme has also increased in toxicology research with many review papers emphasizing the importance and significance of understanding the role of the gut microbiome in toxicology (Claus, Guillou, and Ellero-Simatos 2016; Koontz et al. 2019; Koppel, Maini Rekdal, and Balskus 2017; Mesnage et al. 2018; National Academies of Sciences, Engineering, and Medicine 2018; Pryor et al. 2019).

Humans possess several distinct microbiomes, including the skin, respiratory, reproductive system, and gut (National Academies of Sciences, Engineering, and Medicine 2018). The gut microbiome is the most abundant (National Academies of Sciences, Engineering, and Medicine 2018; Sender, Fuchs, and Milo 2016). When chemicals enter the gut, bidirectional interactions occur: the chemicals may be transformed by microbiota present to be more or less toxic or effective, or chemicals might alter the composition of the microbiome (Koontz et al. 2019; National Academies of Sciences, Engineering, and Medicine 2018). Microbes within the gut primarily use hydrolytic and reductive reactions but are capable of utilizing a wide range of metabolic enzymes including azoreductases, nitroreductases, beta-glucuronidases, sulfatases, and beta-lyases (Koontz et al. 2019; Koppel, Maini Rekdal,

and Balskus 2017) to metabolize or transform environmental pollutants such as some heavy metals, pesticides, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls. Conversely, toxicant exposure might lead to dysbiosis by altering abundance and diversity of the microbiota present (Claus, Guillou, and Ellero-Simatos 2016; Tsiaoussis et al. 2019).

Investigating the human microbiota may be challenging and complex; model organisms provide the opportunity to simplify these studies. *C. elegans* is particularly useful for studying the microbiome because of the ease of creating germ-free progeny through bleaching ability to (1) grow and develop on a monoculture of bacteria, (2) grow worms on bacteria-free media, and (3) use mutant strains (Gerbaba, Green-Harrison, and Buret 2017; Zhang et al 2017). Although, as described above, *C. elegans* has been increasingly employed to study toxicological impacts of xenobiotics, most of these studies were performed without considering what role their gut microbiome might play (Dirksen et al. 2016). Further, most experiments to date were conducted by feeding only with standard strains of bacteria such as the common lab food source, *Escherichia coli* strain OP50. None of the commonly used lab food strains are found in nature; thus, these food sources presumably generate an artificial microbiome, although as discussed below, there is some limited evidence for non-food source bacteria colonizing the worm intestine in the lab.

A few studies examined gut microbiomes in *C. elegans* found in the wild. Zhang *et al.* (2017) presented the first three papers that document non-lab microbiomes in *C. elegans*. The dominant taxa across varying substrates were Proteobacteria (approximately 80%) while the remaining approximately 20% were Bacteriodetes, Firmicutes, and Actinobacteria. These investigators also showed that the composition of the worm gut microbiome was distinct from and to a large extent independent of their environment (Zhang et al. 2017; Berg et al. 2016). The degree to which worm microbiomes vary by geography has yet to be determined. Lee et al. (2020) examined the gut microbiome of N2 Bristol worms grown in lab conditions with OP50 and found that despite the lab culture conditions, these worms also contained a complex microbiome that consisted mainly of Proteobacteria, Firmicutes, and Actinobacteria (*Stenotrophomonas, Bacillus, Microbacterium*). Non-OP50 bacteria might be present because typical worm culture lab techniques are not sterile, such that other bacteria may be found and able to colonize the gut of the worm. However, this study contrasts with other reports that noted that when grown under lab conditions, the N2 strain with OP50 bacteria did not contain microbes other than OP50 within their gut (Dirksen et al. 2016). Clarifying this discrepancy, and the degree to which it depends upon lab culture conditions, is important, because certain bacterial strains influence worm development and reproduction (Dirksen et al. 2020, 2016).

The relationship of *C. elegans* with standard OP50 changes throughout aging. During development, bacteria consumed are destroyed in the worm's grinder, leaving them inactivated in their intestine. However, during middle age, a small number of bacteria consumed pass through the grinder and colonize the intestine. As the worm ages, live bacteria found in the intestine begin to proliferate, eventually contributing to their death (Cabreiro and Gems 2013). Therefore, the microbiome may exert a greater effect on xenobiotic metabolism in older versus younger worms. Presumably, other bacteria might colonize the worm gut at earlier and later ages.

Tools for studying the worm microbiome

With advancing sequencing technology, analyzing the worm's gut microbiome has become more rapid and cost-efficient. However, understanding how differences in diversity and abundance directly affect the worm's health is more challenging. Recently, tools were developed that might help elucidate how microbes impact the health of *C. elegans*.

Dirksen et al. (2020) created CeMbio, a kit of 12 bacterial strains that colonize the gut of worms in one native European environment. Worms may be grown on individual strains or on a strain community. When worms were grown on different individual strains, growth rates changed. Compared to OP50, nine of the strains initiated worms to develop faster, two strains significantly delayed growth, and one strain resulted in similar growth rates. Quantification of bacteria within the gut of worms might be

accomplished by PCR, using strain-specific primers. Thus, this resource facilitates testing how exposures influence single or combined microbial populations, and the manner by which these microbiome changes affect host health.

Another tool, developed by Rutter et al. (2019) permits an *in vivo* analysis of worm-bacterial-chemical interactions by using a bacterial food source engineered to serve as a sensor This sensor is a strain of *E. coli* carrying a plasmid encoding constitutively-expressed mCherry, plus GFP expressed upon exposure to isopropyl β-D-1-thiogalactopyranoside (IPTG). This enables simultaneous visualization of bacteria in the intestine via mCherry, and GFP fluorescence to detect response to IPTG. The ratio of GFP to mCherry therefore provides information both on the bacterial population present, and the response of the bacteria to chemical exposure.

The effect of specific bacterial genes on microbiome-chemical interactions might also be tested via the use of libraries of bacterial mutants. For example for *E. coli* K-12 there are 3985 mutant strains available (Baba et al. 2006); for *E. coli* OP50 (Govindan et al. 2015), approximately 2000; for *Pseudomonas aeruginosa*, approximately 4901 (Jacobs et al. 2003); and for *Comamonas aquatica* DA1877, approximately 5760 (Watson et al. 2014).

Finally, several methods have been described to reduce or eliminate the influence of bacteria on chemical toxicity. These include heat-killing bacteria (Powell and Ausubel 2008); inactivating bacteria with ultraviolet radiation, sometimes using a DNA-damage-deficient strain of bacteria (Meyer et al. 2010b); feeding standardized, lyophilized bacteria lysate (Garcia-Gonzalez et al. 2017); and culturing in axenic media, for which explicit toxicological protocols were provided (Nass and Hamza 2007; Sprando et al. 2009). It is important to note that each of these methods has its own caveats including in many cases reducing caloric intake and/or food quality (axenic media, heat-killed bacteria) or incomplete abrogation of biochemical metabolism (ultraviolet light inactivation).

Gut microbiome and toxicological studies using *C. elegans*

To date, relatively few studies explored how xenobiotics alter the *C. elegans* gut microbiome, or if transformation of xenobiotics occurs within the worm gut. (Garcia-Gonzalez et al. 2017) investigated how microbiota transformed drugs, and how this affected worm health, in order to better understand drug efficacy variability often seen in clinical studies. Other studies examined the manner in which the worm microbiome was aletered after exposure to environmental pollutants.

Cabreiro et al. (2013) using standard lab strain OP50 found that metformin decreased microbial folate production and altered methionine synthesis, which led to an elevation in worm lifespan. However, if worms were grown on metformin-resistant bacteria, or no bacteria, metformin exerted no significant effect on folate production, and reduced lifespan, indicating toxicity. Moreover, worms grown with metformin and OP50 displayed a significant reduction in S-adenosyl methionine levels, which led to diminished in methionine biosynthesis. Since *C. elegans'* main source of methionine is the diet, a decrease in microbial folate and methionine biosynthesis led to a dietary deficiency in methionine. Cabreiro et al. (2013) postulated that deficiency in methionine is the underlying mechanism causing metformin-mediated extended lifespan. Scott et al. (2017) found that different strains of bacteria significantly influenced the minimum inhibitory range of 5 different fluoropyrimidines and that live bacteria were necessary to activate fluoropyrimidines. Supplementation with a vitamin B_6 precursor, pyridoxal enhanced the efficacy of 5-FU in worms (Scott et al. 2017). The addition of pyridoxal to *E. coli* enhances ribonucleotide metabolism of 5-FU thereby increasing the efficacy to the host. Further, data demonstrated that exposure to metformin altered bacterial one-carbon metabolism which is believed to modulate fluoropyrimidines activation. If worms were given both drugs concurrently, activation of 5-FU was inhibited, resulting in a decreased efficacy. These findings show the importance of understanding how the host microbiome, diet, and drug exposure alter drug efficacy and impact host health.

Lee et al. (2020) examined how environmental chemicals alter gut microbiome. OP50-fed worms were exposed to cadmium (Cd) and marked effects on gut microbial composition were noted. Without Cd exposure, Proteobacteria dominated the gut, followed by Firmicutes and Actinobacteria. After

Cd exposure, Firmicutes dominated the microbiota. Firmicutes are postulated to be resistant to Cd, which enables them to proliferate within the gut once the Proteobacteria and Actinobacteria were reduced (Lee et al. 2020). However, when fed a more diverse population that was isolated from an organic farm, Cd exposure resulted in non-significant alterations in taxa by increasing the amount of Actinobacteria present. Lee et al. (2020) demonstrated that worms that possessed a more diverse gut microbiome were more resistant to Cd exposure as evidenced by having a longer lifespan, more progeny, and fewer changes to gut microbial communities compared to worms fed OP50.

Arsenic (As) is biotransformed by microorganisms in the environment where reduction and methylation reactions are particularly important in altering transport and toxicity. In order to investigate how the microbiome might alter As speciation (redox status and methylation) and toxicity, Zhou et al. (2020) used three different types of *E. coli* that either lacked the ability to reduce or methylate arsenic, or might reduce but not methylate As, or might both reduce and methylate As. Zhou et al. (2020) found that bacteria biotransformed As, and that the worm responses (gene expression and reproduction) varied depending upon the strain of bacteria, supporting the importance of the microbiome in mediating As-induced toxicity.

Thus data demonstrated how *C. elegans* might be used as a powerful tool to elucidate the manner in which microbiota alter chemicals to which worms are exposed, as well as how environmental chemicals impact worm microbiota, potentially impacting worm physiology and stress response. It is noteworthy there are caveats associated with differences in *C. elegans* biology that need to be taken into account. For example, the above-mentioned lack of an adaptive immune system *in C. elegans* limits the ability to model mammalian mucosal-microbiota interactions, which when perturbed subsequently might alter xenobiotic metabolism, transport, and gut permeability.

Xenobiotic detoxification in *C. elegans* – Phase I

Phase I metabolic enzymes perform biotransformation of a parent organic compound to introduce or expose functional groups. This typically increases polarity and thus water solubility and reactivity, ultimately permitting conjugation of the compound (Ioannides 2001). Phase I reactions are broadly grouped into three categories: oxidation, reduction, and hydrolysis, with oxidation reactions being the most common and well-studied (Ioannides 2001). In *C. elegans*, all three types of phase I reactions are represented (Lindblom and Dodd 2006), and phase I enzymes are broadly expressed in somatic cells with some cell type specificity for particular isoforms likely driven by exogenous roles such as intestinal expression and endogenous roles such as hormone and/or neurotransmitter synthesis in neurons and other tissues.

Oxidative reactions are mainly carried out by the cytochrome P450 superfamily of monooxygenase enzymes, but also by flavin-containing monooxygenases, alcohol and aldehyde dehydrogenases, monoamine oxidase, and by peroxidases that perform co-oxidation (Ioannides 2001). These oxidation reactions are presumed to have evolved to increase water solubility and thus reduce toxicity from accumulation of chemicals in hydrophobic biological environments and structures. However, many oxidative reactions of benign hydrophobic substrates paradoxically result in formation of a toxic reactive metabolite (also termed 'bioactivation')(Guengerich 2006). A well-known example of this phenomenon is the pollutant benzo[a]pyrene (BaP), which is bioactivated by CYP1A/1B enzymes to a reactive epoxide metabolite that ultimately forms carcinogenic DNA adducts (Shiizaki, Kawanishi, and Yagi 2017).

Reduction reactions are carried out by cellular reductases and have not been well-characterized for their xenobiotic metabolizing activities, even in mammalian systems. In *C. elegans*, several genes have been predicted to exhibit reductase activity but have not been closely studied. Finally, hydrolytic metabolism also occurs by enzymes termed hydrolases. These enzymes cleave their substrates using hydrolysis at functional groups, commonly at esters, amides, and epoxides.

Cytochromes P450 in C. elegans

Cytochrome P450 enzymes carry out the majority of oxidative Phase I reactions and, as illustrated by the example of BaP, are responsible for the majority

of bioactivation reactions (Guengerich 2008; McDonnell and Dang 2013). Cytochromes P450 are monooxygenases that consume one molecule of molecular oxygen and one molecule of NAD(P)H to add one oxygen atom to a substrate to release water and NADP+ as byproducts. Cytochromes P450 are localized in the endoplasmic reticulum, where they associate with the co-enzyme cytochrome P450 reductase (*emb-8* in *C. elegans*) to carry out their reactions. In mammals, P450s also localize to the mitochondria where they associate with adrenodoxin/adrenodoxin reductase (Ahn and Yun. 2010; McDonnell and Dang 2013) (possibly Y73F8A.27 and Y62E10A.6 in *C. elegans*) although the presence of mitochondrial P450s in *C. elegans* is relatively understudied.

The *C. elegans* genome contains genes for at least 86 cytochromes P450 (compared to 60 in humans), and eight of those are predicted to be pseudogenes (Menzel, Bogaert, and Achazi 2001). Three genome-wide RNAi screens collectively identified roles for several of these P450 enzymes (**Table 1**). Perhaps the most well-studied P450 is *daf-9*, an orthologue of human CYP2S1 that is involved in the modification of hormones involved in dauer signaling (Jia, Albert, and Riddle 2002).

Of the 86 cytochrome P450 genes in the *C. elegans* genome, many are induced upon exposure to xenobiotics (**Table 1, 'Inducers'**), suggesting that at least some of the P450 isoforms encoded in the genome are xenobiotic metabolizing enzymes. For the vast majority of these enzymes, the evidence for induction was based upon mRNA levels quantified by microarray, RNA-seq, and/or qPCR or GFP signals in a strain expressing GFP under the control of the CYP promoter. Due to the lack of availability of antibodies for most *C. elegans* proteins, there have not to the authors' knowledge been any studies where P450 induction was quantified on the protein level. This is potentially problematic, because some P450 isoforms are highly regulated post-transcriptionally and mRNA levels often do not correlate well with protein and activity (Chang et al. 2003; Song et al. 1986; Sumida et al. 1999). Further, Abbass et al (2021) recently provided indirect evidence that *C. elegans* might metabolize the commonly studied polycyclic aromatic hydrocarbon benzo[a]pyrene to more-reactive forms, despite confirming previous phenotypic and sequence homology-based evidence (Harris et al. 2020; Leung et al. 2010) that this species lacks the family 1 P450s that canonically produce metabolites that generate bulky DNA adducts. This result highlights the need to study the catalytic activity of *C. elegans* xenobiotic transformation enzymes.

There are a few examples, however, of more direct measurement of P450 metabolism of xenobiotics in the literature (**Table 1, 'Substrates'**). In those cases, the expected metabolite from P450 monooxygenase activity was detected and attributed to a particular isoform. In addition to those examples, P450 metabolites of phenacetin (metabolized by human CYP1A2, abbreviated hCYP1A2), diclofenac (hCYP2C9 substrate), amitriptyline (hCYP2C19/2D6 substrate), clomipramine (hCYP2C19 substrate), dextromethorphan (hCYP2D6/3A4 substrate), and nifedipine (hCYP3A4 substrate) were detected in *C. elegans* but not attributed to a particular isoform (Harlow et al. 2018). Unfortunately, none of those studies compared metabolic activity with and without an inducer, such that the inducibility of *C. elegans* P450 enzyme activity is unclear. In addition, it is interesting that in the case of tolbutamide metabolism, the isoforms identified by RNAi to be metabolizers of tolbutamide were not the closest isoforms identified by sequence homology to the human tolbutamide hydroxylases (CYP2C8/9/19) (Harlow et al. 2018). This observation is an illustrative example of how sequence homology is a poor predictor of substrate specificity for P450 enzymes, and is supported by the observations that although several P450 isoforms have sequence homology to CYP2E1 (**Table 1**), one is not able to detect any CYP2E1-like activity for multiple substrates in *C. elegans* (unpublished observations, JHH and JNM).

Cytochrome P450 enzymes require a heme cofactor (bound to iron) and a coenzyme (cytochrome P450 reductase) to carry out their reactions (McDonnell and Dang 2013). Unlike humans, *C. elegans* cannot synthesize heme, and need to scavenge this component from their diet (bacteria) to incorporate it into newly synthesized cytochrome P450 polypeptides (Sinclair and Hamza 2015). *C. elegans* have a suite of specialized enzymes that deliver environmental heme into the intestine and into other tissues (Chen et al. 2012). Heme

Table 1. *C. elegans* P450 genes with reported human orthologues, phenotypes, substrates, inhibitors, and/or expression patterns.

C. elegans gene	Human orthologue	Knockdown phenotype	Substrates	Inducers (mRNA expression)	Tissue Expression	Reference
daf-9	CYP2S1	Abnormal dauer formation	3-keto-sterols		nervous system, intestine, spermatheca, vulval muscle	(Gill et al. 2004; Mak and Ruvkun 2004; Motola et al. 2006)
cyp-13A1	CYP3A4/TBXAS1	None reported			coelomocyte, gonad	
cyp-13A4	CYP3A4/5/43	Growth delay, Uncoordinated movement			broad somatic expression	(Simmer et al. 2003a; Kamath et al. 2003)
cyp-13A7	CYP3A4/TBXAS1	None reported		tetrabromobisphenol A, rifampcin	intestine	(Liu et al. 2020; Chakrapani, Kumar, and Subramaniam 2008)
cyp-13A8	CYP3A7/51P	Clear body cavity, Sluggish, some Embryonic lethality				(Kamath et al. 2003)
cyp-13A10	CYP3A4/7/43	None reported		tetrabromobisphenol A	neuron, germline, embryos	(Liu et al. 2020)
cyp-13A12	CYP3A4/5/7	None reported		tetrabromobisphenol A, acrylamide	marginal cells	(Liu et al. 2020; Hasegawa et al. 2007)
cyp-14A5	CYP2U1	Short defecation cycle	PCB52		GABAergic neuron, somatic gonad precursor, intestine	(Schafer et al. 2009)
cyp-29A3	CYP4V2	Short defecation cycle	eicosapentaenoic acid	1-methylnicotinamide, chlorpyrifos, 4-bromodiphenyl ether	neurons, intestine, body wall muscle, hypodermis, coelomocytes	(Kulas et al. 2008; Schmeisser et al. 2013; Viñuela et al. 2010)
cyp-31A1 (pseudogene)	N/A	Embryonic lethality				(Simmer et al. 2003b)
cyp-31A2	CYP4V2	Embryonic lethality			hypodermis, germline, neurons	(Simmer et al. 2003a)
cyp-31A5	CYP4V2	Embryonic lethality			germline, AFD neurons	(Simmer et al. 2003a)
cyp-33E2	CYP2J2	Reduced pharyngeal pumping, Extended lifespan, Reduced brood size	Long chain fatty acids, eicosapentaenoic acid		pharynx, intestine, neurons	(Deline et al. 2015; Kulas et al. 2008)
cyp-34A9			tolbutamide			(Harlow et al. 2018)
cyp-35A1	CYP2C18 CYP2D7 CYP2E1	Drug resistant[a]		3-bromopyruvate, β-naphthoflavone, PCB52, lansoprazole, primaquine	intestine	(Gu et al. 2020; Menzel, Bogaert, and Achazi 2001)
cyp-35A2	CYP2C18 CYP2D7 CYP2E1	Drug resistant[a]		3-bromopyruvate, β-naphthoflavone, PCB52, lansoprazole, atrazine	intestine, neurons (PVD/OLL)	(Gu et al. 2020; Menzel, Bogaert, and Achazi 2001; Chakrapani, Kumar, and Subramaniam 2008)
cyp-35A3	CYP2C18 CYP2D7 CYP2E1	Reduced fat storage		chlorpyrifos, β-naphthoflavone, PCB52, lansoprazole, primaquine	intestine, AFD neurons	(Ashrafi et al. 2003; Viñuela et al. 2010; Menzel, Bogaert, and Achazi 2001)
cyp-35A4	CYP2C18 CYP2D7 CYP2E1	Short defecation cycle, Chemical response variant		3-bromopyruvate, β-naphthoflavone, PCB52, lansoprazole, primaquine	muscle, intestine, neurons	(Gu et al. 2020; Menzel, Bogaert, and Achazi 2001)
cyp-35A5	CYP2C18 CYP2D7	Reduced fat storage		3-bromopyruvate (weak), β-naphthoflavone, PCB52, lansoprazole, primaquine, atrazine	intestine	(Ashrafi et al. 2003; Gu et al. 2020; Kulas et al. 2008; Menzel, Bogaert, and Achazi 2001)
cyp-35B1	CYP2C18 CYP2D7 CYP2E1	Reduced fat storage		β-naphthoflavone, ethanol	intestine, hypodermis, dopaminergic neurons	(Ashrafi et al. 2003; Menzel, Bogaert, and Achazi 2001)
cyp-35B2				β-naphthoflavone, lansoprazole, primaquine, ethanol		(Menzel, Bogaert, and Achazi 2001)

(*Continued*)

Table 1. (Continued).

C. elegans gene	Human orthologue	Knockdown phenotype	Substrates	Inducers (mRNA expression)	Tissue Expression	Reference
cyp-35C1	CYP2C18 CYP2D7 CYP2E1	Drug resistant[a], Oxidative stress resistant		3-bromopyruvate, tetrabromobisphenol A, fluoranthene, β-naphthoflavone, PCB52, lansoprazole, primaquine	intestine, neurons (AFD/OLL/PVD)	(Gu et al. 2020; Liu et al. 2020; Menzel, Bogaert, and Achazi 2001; Taubert et al. 2008)
cyp-42A1	CYP4V2	Uncoordinated movement			hypodermis, intestine	(Kamath et al. 2003)

[a]Term "Drug resistant" indicates reducing defects produced by a particular drug treatment (*e.g.* reproduction decline after exposure to xenobiotics)

availability during xenobiotic challenge might limit the xenobiotic response, but this hypothesis has not been explored experimentally. Cytochrome P450 enzymes also require co-expression of cytochrome P450 reductase, also termed P450 oxidoreductase (POR), which is encoded by *emb-8* gene in worms. The *emb-8* protein is expressed in highest levels in intestine, neurons, and pharynx and is also expressed in coelomocytes, hypodermis, and germline (essentially the same tissues where P450 enzymes are expressed). Loss of *emb-8* produces temperature-sensitive embryonic lethality (Miwa et al. 1980), likely due to its role in embryonic polarization (Rappleye et al. 2003).

Finally, longevity associated with reduced mitochondrial function in *C. elegans* requires induction of cytochrome P450 enzymes through Kruppel-like factor 1, suggesting that protection from endobiotic and/or xenobiotic insults is part of the longevity mechanism (Herholz et al. 2019). This hypothesis fits with data from other species illustrating upregulation of xenobiotic detoxification programs in long-lived mutants. An open question is which endo- and xenobiotic compounds underlie this longevity phenotype.

Flavin Monooxygenases

Similar to humans, *C. elegans* possess 5 identified flavin monooxgenase (FMO) genes (*fmo-1 to fmo-5*). FMO enzymes consume oxygen and NADPH to incorporate a single oxygen atom into substrates, usually soft nucleophiles at nitrogen or sulfur atoms. *C. elegans* FMO enzymes are expressed mainly in two patterns: *fmo-1, −2*, and −5 in intestinal cells and excretory gland cells, and *fmo-3* and −4 in the hypodermis (Petalcorin et al. 2005). In addition, expression was reported in duct cells (*fmo-4*), pore cells (*fmo-4*), body-wall muscle (*fmo-3*,), germline (*fmo-4*), hypodermal cells (*fmo-3, −4*), pharyngeal muscle (*fmo-1*), ventral nerve cord (*fmo-4, −5*) and neurons (*fmo-2, −3, −4, −5*).

Similar to cytochromes P450, FMOs contain both endogenous and exogenous substrates and exhibit functions. Nematodes lacking *fmo-1, −4*, and −5 induce neurodevelopmental defects (Gujar, Stricker, and Lundquist 2017). Further, animals deficient in *fmo-4* are extremely sensitive to hypoosmotic stress (Hirani et al. 2016; Petalcorin et al. 2005). *C. elegans fmo-4* is orthologous to human FMO4, and both

contain similar protein structures. However, human FMO4 was not able to rescue hypoosmotic stress sensitivity phenotype in the *fmo-4* deletion strain (Hirani et al. 2016). *C. elegans fmo-2* is upregulated by starvation (Goh et al. 2018) and dietary restriction (Leiser et al. 2015), is involved in dietary restriction-induced lifespan extension, and might extend lifespan when overexpressed (Leiser et al. 2015). In addition, both oxidative stress (Goh et al. 2018) and stress from infection with *Pseudomonas aeruginosa* (PA14) (Dasgupta et al. 2020) or *Staphylococcus aureus* (Wani et al. 2020) induce *fmo-2*, which is required for pathogen resistance (Wani et al. 2020). Further, both *fmo-2* and *fmo-4* are upregulated by hypoxia (Shen et al. 2005).

In terms of catalytic activity, *C. elegans* FMOs (*fmo-1* and −4) are functional for S-oxidation of the prototypical FMO substrate methimazole when expressed recombinantly in insect cells. The overall specific activities (per mg microsomal protein) were approximately 10-fold lower than human enzymes expressed in the same background. However, lack of antibodies for *C. elegans* FMOs precluded estimation of protein expression efficiency in each case (Hirani et al. 2016). To date, this study is the only one that has functionally characterized *C. elegans* FMO activities, and further studies are needed to determine endogenous and xenobiotic substrates of each isoform.

Alcohol and Aldehyde Dehydrogenases

C. elegans possesses two alcohol dehydrogenases, *sodh-1* and *sodh-2*. Both isoforms are widely expressed in the worm including in neurons, muscle, hypodermis, germline, and intestine. In biochemical assays, alcohol dehydrogenase activity appeared as a single band on a polyacrylamide gel. However, the two isoforms are close in sequence and size (349 vs. 351 amino acids for *sodh-1* and *sodh-2*, respectively) and thus may be expected to co-migrate on a gel (Williamson, Long, and Theodoris 1991). *C. elegans* ADH activity is higher for longer-chain alcohols (propanol and butanol) compared to ethanol, and similar to the human enzyme, the worm has a preference for primary over secondary alcohols (Williamson, Long, and Theodoris 1991).

C. elegans contains several aldehyde dehydrogenase genes (*alh-1* through *alh-13)*, most of which have not been functionally characterized and do not have an observable knockdown phenotype (Alaimo et al. 2012). Using gas chromatographic methods to measure internal ethanol accumulation, Alaimo et al. (2012) indicated that *alh-6* and *alh-13* metabolize the ethanol metabolite acetaldehyde to acetic acid.

Monoamine Oxidases

Monoamine neurotransmitters such as dopamine and serotonin are degraded in humans by monoamine oxidases MAO-A and MAO-B. These enzymes also act on xenobiotics that are structurally similar to their natural substrates. *C. elegans* has a single ortholog of these genes, *amx-2* (Schmid et al. 2015), which was demonstrated to metabolize serotonin in the worm (Wang et al. 2017) and presumably would have overlapping specificity with MAO-metabolized xenobiotics identified in mammalian systems.

Hydrolases

Hydrolysis reactions use water to break a chemical bond. Enzymes that perform hydrolysis reactions are referred to as hydrolases. Xenobiotic-metabolizing hydrolases include esterases, amidases, and epoxide hydrolases. Of those, only epoxide hydrolases were studied in detail in *C. elegans*. Although humans express four epoxide hydrolase isoforms including both membrane-bound (microsomal) and soluble (cytosolic) forms, *C. elegans* possesses only two isoforms, *ceeh-1* and *ceeh-2* (Harris et al. 2008). These are most orthologous with the EH3 and EH4 human isoforms, which are the most recently discovered and least well characterized among human isoforms but are postulated to predominantly metabolize lipids. The *C. elegans* enzymes were confirmed to exhibit endobiotic and xenobiotic metabolizing activities, with *ceeh-1* displaying higher activity toward substrates compared to *ceeh-2* (Harris et al. 2008). Further studies are needed to establish the substrate specificity of both isoforms, particularly for xenobiotic substrates.

Phase II in *C. elegans*

Phase II reactions involve conjugation of a substrate with a large, water-soluble group, facilitating excretion. There are 4 major families of phase II enzymes:

UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), and N-acetyltransferases (NATs). Except in rare cases, phase II reactions produce safe and easily excreted metabolites, and therefore this phase of metabolism mainly impacts toxicity by facilitating clearance of the compound. Mutations in phase II enzymes in humans result in various phenotypes, including hyperbilirubinemia from mutations in the UGT1 family and elevated risk of cancers from mutations in various UGT and GST isozymes. In *C. elegans*, Phase II enzymes, similar to phase I, are expressed broadly in somatic cells and enriched in intestine, neurons, and other specialized cells depending upon isoform.

UDP-glucuronosyltransferases (UGTs) are endoplasmic reticulum-localized enzymes that catalyze the conjugation of pollutants, drugs, and endogenous compounds to a sugar group, glucuronic acid. The site of metabolism for UGTs is usually at hydroxyl, carboxyl, or amine functional groups. Individual UGT isoforms are classified into families based upon sequence identity, and substrate specificity for individual UGTs tends to be broad compared to Phase I enzymes (Burchell et al. 1998). Mammals express 22 UGTs from 4 families: UGT1, UGT2, UGT3, and UGT8 (Meech et al. 2019). In contrast, *C. elegans* possess 66 UGT genes (named *ugt-1* through *ugt-66*) which have all been classified into families UGT1, UGT2, UGT3, and UGT8 based upon sequence homology, and most of the *C. elegans* UGT genes are each homologous to multiple human isoforms. Knockouts of individual UGT isoforms did not produce phenotypes other than drug hypersensitivity in some cases (Cui et al. 2007; Fontaine and Choe 2018).

A few studies reported induction of UGT after chemical exposures. Hasegawa et al. (2010) found that allyl isothiocyanate exposure induces expression of *ugt-13* (ortholog of human UGT1A1/2B28/2B7). Di, Zhang, and Lawton (2018) noted that the mycotoxin deoxynivalenol induced *ugt-26* (ortholog of human UGT1A4/1A6/2B10) and *ugt-28* (ortholog of human UGT1A10/1A6/1A8). The insecticide albendazole also stimulated expression of *ugt-16* (ortholog of human UGT3A1/3A2)(Laing et al. 2010), *ugt-22* (ortholog of human UGT1A5/1A6/2B17) and *ugt-63* (ortholog of human UGT1A10/1A5/1A8)(Fontaine and Choe 2018). The latter study found that *ugt-22* upregulation was downstream of the transcription factor *skn-1* and noted that mutation of *ugt-22* markedly protected against albendazole-mediated toxicity. The chemical acrylamide was also reported to induce several UGT isoforms in *C. elegans* (Hasegawa et al. 2007). The chemical inducers fluoranthene and β-naphthoflavone also stimulated several UGT isoforms including *ugt-13* and *ugt-63*, as well as other phase I and II xenobiotic metabolizing enzymes (Taubert et al. 2008). Notably, induction was only detected at the transcriptional level, likely due in part to lack of validated antibodies against *C. elegans* UGT proteins. Further, biochemical activities of UGT enzymes have not been directly measured in *C. elegans* to date.

C. elegans exposed to the insecticide albendazole also produced conjugated albendazole-glucose metabolites, which Laing et al. (2010) suggested may have been formed by UGT enzymes utilizing UDP-glucose as a substrate. The same glucose-conjugated albendazole metabolites were also detected in the helminth *H. contortus* (Cvilink et al. 2008). Further, glycosylated metabolites of the bacterial toxins 1-hydroxyphenazine and indole were also reported in *C. elegans* (Stupp et al. 2013). Taken together, these studies provide support to the postulation that UDP-glucose may be utilized by invertebrate UGT enzymes, although this substrate is uncommon in mammals. A similar concept was previously suggested based upon sequence analysis, that is, some *C. elegans* isoforms that were identified as UGTs may be more correctly termed glycosyltransferases that catalyze the transfer of galactose, glucose, or glucuronic acid (Kapitonov and Yu 1999).

Sulfotransferase enzymes (SULTs) catalyze the conjugation of a sulfonate group from a donor molecule, typically 3-phosphoadenosine-5′-phosphosulfate or PAPS, to a substrate (at a hydroxyl or amino functional group). SULTs might either be membrane-bound in the Golgi apparatus, where they metabolize endogenous molecules, or soluble in the cytosol, where they metabolize xenobiotic and endogenous substrates. The human genome encodes up to 14 SULTs, while the *C. elegans* genome encodes a single cytosolic sulfotransferase enzyme, *ssu-1* (also termed Y113G7A.11 and ceST1)(Hattori et al. 2006). There is no apparent knockdown phenotype, although it is

a suppressor of *unc-1* and *unc-24* phenotypes (thus the naming *ssu*, or suppressor of stomatin mutant uncoordination)(Carroll et al. 2006). Its enzymatic properties were investigated biochemically by recombinant protein expression, and the *C. elegans* SULT enzyme sulfonated prototypical hydroxylated substrates including 4-nitrophenol and 2-naphthol as well as bisphenol A but did not metabolize monoamines or hydroxysteroids. Hattori et al. (2006). created antibodies against the recombinant protein and were able to detect expression in cytosolic fractions of *C. elegans* lysate and induction by the substrate isophenylpropanol. The mRNA is also highly induced in dauer larvae, indicating a potential role for sulfonation in dauer signaling (Hattori et al. 2006).

Glutathione S-transferases (GSTs) conjugate glutathione to a wide variety of endogenous and xenobiotic substrates and are divided into two types: membrane-bound and soluble family members. Membrane-bound GSTs are found in the endoplasmic reticulum and mitochondria and form homo- and hetero-trimers with a single active site, and typically metabolize endogenous leukotrienes and prostaglandins. Human cytosolic GSTs (11 total) are expressed at high levels, are highly polymorphic, and classified into 6 groups: α, μ, ω, π, ς, θ, and ζ. The *C. elegans* genome contains 56 GST genes with most categorized within the ς family, which are the most abundant and conserved within insects. However, there are also GSTs in the *C. elegans* genome classified in the α, π, ζ, and θ subfamilies.

Induction of GSTs by xenobiotics was reported. Wu et al. (2015) noted that BaP exposure resulted in induction of SKN-1 as well as *gst-24* (homolog of human GST ς enzyme hematopoietic prostaglandin D synthase or HPGDS). The toxin indole upregulated *gst-5, gst-6*, and *gst-33* (all orthologous to human HPGDS)(Lee et al. 2017), and the natural compound ursolic acid upregulated *gst-7* (also orthologous to human HPGDS)(Negi et al. 2017). Interestingly, exogenous heme induced expression of *gst-19, gst-7*, and *gst-5*, and these isoforms were proposed to play an endogenous role in heme trafficking (Perally et al. 2008).

Humans have two N-acetyltransferase enzymes that are xenobiotic-metabolizing, NAT1 and NAT2. To date, no N-acetyltransferase genes in *C. elegans* that are homologous to the human xenobiotic-metabolizing NATs have been identified. In addition to the 4 major families of phase II enzymes described here, it is also possible to conjugate xenobiotics to other cellular substrates including amino acids, but these reactions are less studied and less common.

Phase III in *C. elegans*

Phase III of xenobiotic metabolism is the export of xenobiotics and metabolites out of the cell, typically via transporters, processes that are especially critical for those xenobiotics whose physicochemical properties make them unable to pass the cell membrane itself (Kell 2020a; Xu, Li, and Kong 2005). Transporters vary vastly in mechanism, function, substrate, and sequence, and are classified largely along those lines into the families of ATP-binding cassette (ABC) transporters, solute carriers (SLCs), pumps, ion channels, and water channels or aquaporins (Hediger et al. 2013). Mutations in each of these transporter families were associated with altered individual sensitivity to xenobiotic or drug exposures and various diseases (DeGorter et al. 2012).

ATP-binding cassette (ABC) transporters comprise a broad family of transmembrane proteins defined by their two nucleotide binding domains and two transmembrane domains. Serving as both importers and exporters in prokaryotes and predominantly exporters in eukaryotes, ABC transporters bind and hydrolyze ATP to move substrates across the membrane (Rees, Johnson, and Lewinson 2009). ABC transporters have been implicated in drug resistance, including to chemotherapeutic drugs, efflux of many xenobiotics including phase I and especially phase II metabolites, as well as endogenous processes such as lipid and cholesterol transport, pathogen response, and mitochondrial iron homeostasis (DeGorter et al. 2012; Glavinas et al. 2004; Jin et al. 2012; Klaassen 2019; Rees, Johnson, and Lewinson 2009).

With 60 sequences encoding ABC transporters, compared to only 49 in humans, these are the most prevalent family of transporters in *C. elegans* representing 0.3% of the N2 reference gene content (Sheps et al. 2004). Of the 60 identified genes, 15 encode full *P*-glycoproteins (PGPs), 8 encode PGP half-molecules, 8 encode other multidrug resistance

proteins (MRPs), 7 encode members of the abt subfamily, and 1 encodes a cystic fibrosis transporter homolog. The remaining 21 were classified according to HUGO nomenclature into groups D-H but lack a corresponding subfamily name in *C. elegans* and are not discussed in depth. Eight are apparently not expressed, and one may be a pseudogene (Sheps et al. 2004). Though 16 ABC transporter encoding genes are found in tandem duplications, promoter linked fluorescent protein detection revealed differential expression patterns for 15 of the 16 duplicates, indicating they have different roles (Zhao et al. 2004). Notably, these patterns can overlap. Although PGPs are expressed in various cell types, many are expressed more or solely in intestinal cells, highlighting the intestine as a critical detoxification organ (Lincke et al. 1993).

In examining orthology to human ABC transporters, only eight were orthologous (Sheps et al. 2004). With such low conservation, it seems logical that those transporters that are conserved play critical roles in biological functions. Thus, unsurprisingly, two are mitochondrial half transporters that support iron homeostasis, and subfamilies E and F, with the highest conservation between humans, *Saccharomyces cerevisiae*, and *Drosophila melanogaster*, play roles in formation of ribosome associated proteins (subfamily F) and RNase L inhibition (subfamily E) (Sheps et al. 2004).

Knockouts of several of these ABC transporters were examined, and led to increased sensitivity to heavy metals, xenobiotics, and bacterial toxins (Broeks et al. 1996, 1995; Mahajan-Miklos et al. 1999; Peng et al. 2018). Deletion of *pgp-3* enhanced sensitivity to colchicine and chloroquine, and simultaneous knockout of *pgp-1* and *pgp-3* elevated sensitivity to bacterial toxins, Cd, and arsenite (Broeks et al. 1996, 1995; Mahajan-Miklos et al. 1999). *pgp-13* inhibition by RNAi showed that it played a significant role in resistance to imidazolium-based ionic liquids with a suspected similar role for *pgp-14* (Peng et al. 2018). *pgp-12* knockouts are more susceptible to phenazine 14 exposure (Stupp et al. 2013). *pgp-1* and *mrp-1* were both upregulated in ivermectin resistant *C. elegans*, with greater PGP upregulation at higher resistance levels (James and Davey 2009). *pgp-12* and *pgp-13* are upregulated in ivermectin resistance, with *pgp-12* silencing conferring sensitivity (Figueiredo et al. 2018). *mrp-5* is implicated in embryonic vitamin B12 transport from mother to offspring (Na et al. 2018). *pgp-5* plays roles in both heavy metal and bacterial infection resistance (Kurz et al. 2007). *ced-7* promotes the engulfment of apoptotic cells and redistribution of phosphatidylserine (Hamon et al. 2000). *pgp-2* contributes to lysosome formation and lipid storage within the intestine, as well as communication between intestine and AWA neurons.

SLCs are a family of more than 300 membrane-bound, ATP-independent transporter proteins, including vesicular and mitochondrial transporters, passive transporters, coupled transporters, and exchangers (Hediger et al. 2013). Their role in xenobiotic transport is increasingly recognized in humans, with a particular focus in their impact on drug pharmacokinetics (Colas, Man-Un Ung, and Schlessinger 2016; Kell 2020b). SLCs are responsible for the membrane transport of metformin, antineoplastics such as tyrosine-kinase inhibitors, and angiotensin converting enzyme inhibitors such as captopril and quinaprilat, and might also play a major role in xenobiotic transport (DeGorter et al. 2012).

Interestingly, of 17 investigated species across 4 basal eukaryotic branches, *C. elegans* contained the largest number of conserved SLC families compared to *H. sapiens*, with 43 of 46 total present (Höglund et al. 2011). In the same analysis, 31 SLCs were identified in the *C. elegans* genome that could not be classified into known human families, highlighting the lack of understanding of the divergent evolution of these key proteins (Höglund et al. 2011). In total, the *C. elegans* genome encodes 348 SLCs, in comparison to 400 in the human genome (Höglund et al. 2011).

Functionally, SLCs are understudied in humans and even more so in nematodes (César-Razquin et al. 2015; Kell 2020a). It is established that SLC subfamilies 8 and 24 (sodium/calcium exchangers), 14 and 4 (chloride transporters), 17 (vesicular transporters), 1, 5, and 6 (neurotransmitter reuptake) play key roles in neuronal function, but these are not well-characterized functionally in worms. The SLC17 subfamily in particular has 51 members in *C. elegans*, a large expansion compared to the meager nine in humans, but few have been characterized (Hobert 2005). SLC17 member *eat-4* is involved in glutamatergic neurotransmission, and

more recently that *vglu-2* may play a role in collagen trafficking in the cuticle (Serrano-Saiz et al. 2013, 2020). MISC-1, a mitochondrial solute carrier that plays a role in apoptosis (Gallo et al. 2011). Slc17.9, a.k.a., *droe-5*, is upregulated upon dietary restriction (Ludewig, Klapper, and Döring 2014). *hut-1* maintains endoplasmic reticulum homeostasis and is essential for larval development (Dejima et al. 2009). *snf-5* assists in sensing in neurons and transport in intestinal cells of L-aspartate and L-glutamate (Metzler et al. 2013).

The flux of xenobiotics via SLCs and ABCs, in addition to transporters that are less understood or could not be addressed, often serves as the final step of xenobiotic metabolism prior to excretion from the organism. Though this step is increasingly examined, many transporters remain orphan and uncharacterized, providing ample opportunity for future exploration. Similarly as for phase I and II processes, cell-specific expression of genes and isoforms is also in need of better description.

Transcriptional regulation of stress responses in *C. elegans*

Given the paramount role of phase I–III detoxification genes, it is not surprising that their transcriptional regulation is subject to myriad of inputs, not all of which are extensively discussed herein. Specifically, the regulation of such genes by metals and metalloids including Cd, iron, zinc, copper or selenium, metal-containing nanoparticles, exposure to reactive oxygen species (ROS) initiating oxidative stress, mitochondrial dysfunction, genetically or pharmacologically induced longevity, advanced glycation end products such as α-dicarbonyls, and/or various diets or dietary components were described elsewhere and are not covered in detail herein (Blackwell et al. 2015; Chaudhuri et al. 2018; Ferguson and Bridge 2019; Gonzalez-Moragas et al. 2017b; Hoffmann and Partridge 2015; Olsen and Gill 2017; Shore and Ruvkun 2013; Tejeda-Benitez and Olivero-Verbel 2016). The focus of this review is primarily regulation of phase I, II, and III detoxification genes by organic xenobiotic compounds including antihelminthic drugs and compounds used for crop control.

Evolutionary conservation and diversification of transcriptional regulators mediating xenobiotic detoxification

Transcription factors (TFs) and regulatory mechanisms that orchestrate the coordinated induction of a tailor-made detoxification response when an animal encounters a xenobiotic compound are fairly well conserved throughout evolution. Key regulators that control the induction of detoxification genes across species include nuclear hormone receptors (NHRs), the aryl hydrocarbon receptor (AHR; *ahr-1* in *C. elegans*), and the basic leucine zipper (bZIP) protein nuclear factor erythroid 2-related factor 2 (NRF2; *skn-1* in *C. elegans*) (Blackwell et al. 2015; Brinkmann et al. 2019; Gracida and Eckmann 2013; Hoffmann and Partridge 2015; Lindblom and Dodd 2006; Mackowiak and Wang 2016; Tonelli, Chio, and Tuveson 2018). In addition, the GATA-type TF ELT-2 is required to express most *C. elegans* intestinal genes, which includes many phase I, II, and III detoxification genes (McGhee 2013); as such, ELT-2 may play a general, permissive role in the expression of many detoxification gene programs. Recently, Herholz et al. (2019) suggested new roles for additional TFs, such as KLF-1, although these are less well understood, especially in their relevance to defense against acute xenobiotic stress. Below, the current understanding of the roles of these regulators, highlighting emerging mechanistic insights as well as conserved and non-conserved functions is described

C. elegans NHRs are involved in xenobiotic detoxification

Based upon their overall architecture, NHRs are grouped into the NR1-NR6 classes (Nuclear Receptors Nomenclature, Committee 1999; Weikum, Liu, and Ortlund 2018). The NHRs most prominently involved in detoxification gene regulation belong to the NR1J groups in *C. elegans* and *Drosophila melanogaster* and to the NR1I and H classes in mammals. The latter group includes several NHRs with important roles in detoxification such as: the pregnane X receptor (PXR; also known as the steroid and xenobiotic sensing nuclear receptor, SXR, NR1I2); constitutive androstane receptor

(CAR, NR1I3); liver X receptor (LXR, NR1H3), farnesoid X receptor (FXR, NR1H4); and vitamin D receptor (VDR, NR1I1) (Hoffmann and Partridge 2015; Mackowiak and Wang 2016; Oladimeji and Chen 2018). However, other NHRs also regulate detoxification genes in various situations, including the peroxisome proliferator-activated receptors (PPARs) and the Hepatocyte Nuclear Factor 4 (HNF4) type NHRs (Wallace and Redinbo 2013). The latter are especially notable as the *C. elegans* N2 reference genome features a large group of approximately 265 NHRs that appear to have descended and diversified from an HNF4-like ancestor (Taubert, Ward, and Yamamoto 2011). Most of these remain uncharacterized, but recent studies implicated several as putative xenobiotic response regulating NHRs.

Side note on endocrine disruption in *C. elegans*: lack of evidence for conserved receptors at present time

Although vertebrate endocrine-related receptors are not generally thought of as major regulators of xenobiotic transport and metabolism, a digression on the potential for *C. elegans* to be used as a model organism for endocrine disruption is warranted. Endocrine disruptors are molecules that interfere with an organism's intrinsic endocrine systems, which often act by targeting NHRs that regulate endogenous endocrine signals such as estrogen and mammalian NHR estrogen receptor. As such, endocrine disruptors have the potential to disturb the normal physiology and development of an organism. Endocrine disruption is a major concern in environmental toxicology with great relevance for human and wildlife health (Hotchkiss et al. 2008; National Academies of Sciences, Engineering, and Medicine 2017). There are some investigations reporting results of using *C. elegans* to study endocrine disruption, and clearly chemicals that are agonists of vertebrate endocrine receptors exert effects in *C. elegans* (Cao et al. 2020; Chen et al. 2019; Custodia et al. 2001; Fischer et al. 2012; Jeong, Kim, and Choi 2019; Mimoto et al. 2007). However, it needs to be emphasized that conclusions regarding the mechanism by which these effects are mediated be interpreted with great caution. It is far from clear that responses result from presumed receptor agonist or antagonist binding to a worm homolog of the vertebrate receptor. As noted above, sequence comparisons suggest that the NR1I group of classical detoxification NHRs is apparently absent in *C. elegans*. In addition, the families encoding the classical mammalian steroid/thyroid receptors: NR1A thyroid hormone receptors (TRs), NR3A estrogen receptors (ERs), and NR3C 3-ketosteroid receptors including glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and androgen receptor (AR) are also absent (Nuclear Receptors Nomenclature, Committee 1999; Taubert, Ward, and Yamamoto 2011; Weikum, Liu, and Ortlund 2018). Accordingly, to our knowledge, no broad evidence base supports the concept that any *C. elegans* NHR is (in)activated by (ant) agonists of any of these vertebrate NHR, which would render it and the nematode susceptible to endocrine disruption by the presumed pathway, such as by a xenoestrogens acting on a worm "estrogen receptor." Therefore, although it is not impossible that functional homologs of vertebrate endocrine receptors exist, one can argue that the burden of proof is on demonstrating such functional homology, which needs to be tested rigorously. On the other hand, it is entirely possible that *C. elegans* may serve as a useful model for the influence of NHR-activating xenobiotics in invertebrates (Hoss and Weltje 2007). Although evidence for such events is currently also lacking, one cannot rule out that endocrine disruption of DAF-12 driven developmental pathways may occur.

C. elegans NR1J group NHRs

DAF-12

In mammals, the NR1I group NHRs (PXR, CAR, VDR) play central roles in detoxification. The apparent absence of NR1I-type NHRs in the *C. elegans* genome thus suggests that other, perhaps closely related NHRs, might have adopted these roles. The closest *C. elegans* homologs to NR1I class NHRs are the NR1J group NHRs: DAF-12, NHR-8, and NHR-48. Although little is known regarding NHR-48, the other two have been widely studied for their functions in development and physiology.

DAF-12 is perhaps the best understood NHR in *C. elegans*, with important roles in development, aging, and metabolism. To date evidence for a direct link between DAF-12 and the detoxification of organic xenobiotics is lacking. This is

curious because (i) some detoxification genes, including *cyp, gst*, and *ugt* genes, are down-regulated in long-lived *daf-12* mutants; (ii) the CYP450, DAF-9, is a key enzyme in the synthesis of the dafachronic acids (DA), a group of molecules that serve as DAF-12 ligands and modulate its activity; and (iii) reduced DA signaling increases the resistance of *C. elegans* to various types of stress (Fisher and Lithgow 2006; Gerisch et al. 2001; Hoffmann and Partridge 2015; Motola et al. 2006). Possibly, the pleiotropic roles of DAF-12 in aging and development accompanied by lack of experiments directly assessing consequences of its loss on xenobiotic sensitivity and induced gene regulation have obscured a role in systemic detoxification for this NHR.

NHR-8

In contrast to DAF-12, NHR-8 has emerged as one of the most important regulators of xenobiotic detoxification in *C. elegans*, as it controls gene activation in response to xenobiotic exposure and is required for organismal resistance to some compounds. Lindblom, Pierce, and Sluder (2001) first showed that *nhr-8* mutation or RNAi rendered worms sensitive to the toxins colchicine and chloroquine. Menez et al. (2019) demonstrated that *nhr-8* mutants displayed hypersensitivity to the anthelmintic ivermectin, with *nhr-8* silencing in ivermectin-resistant worms enhancing drug efficacy. At the gene regulation level, *nhr-8* mutant worms exhibited reduced expression of several phase I, II, and III detoxification genes, including *pgp* and *cyp* genes known to impact ivermectin tolerance in *C. elegans*, with correspondingly diminished ABC transporter-mediated drug efflux activity (Menez et al. 2019). Importantly, re-expression of the ABC transporter *pgp-6* in *nhr-8* mutant worms elevated tolerance to ivermectin, implicating NHR-8 control of ABC drug efflux transporters as a likely mechanism for drug resistance. Support for this model is provided by Guerrero et al. (https://www.biorxiv.org/content/10.1101/823302v1.full), who noted that induction of three *pgp* phase III detoxification genes by glycosylation inhibitor tunicamycin required *nhr-8*; that *nhr-8* loss-mediated acute tunicamycin sensitivity while NHR-8 overexpression produced tunicamycin resistance; and that chemical inhibition of *pgp* glycoproteins suppressed tunicamycin resistance. Collectively, evidence suggests that NHR-8 is essential to induce many phase I, II, and III detoxification genes in worms exposed to various toxins, perhaps especially phase III drug efflux transporters. Notably, the role of NHR-8 is xenobiotic-specific, as Lindblom, Pierce, and Sluder (2001) found that loss of *nhr-8* rendered worms sensitive to only some, but not all tested xenobiotics. Indeed, NHR-8 likely possesses redundant functions with other transcriptional regulators in regulating xenobiotic detoxification genes, as other studies showed that NHR-8 depletion alone did not broadly abrogate the expression of select phase I and II genes (Chamoli et al. 2014; Jones et al. 2013). Rather, Chamoli et al. (2014) reported that PHA-4/FoxA, NHR-8, and AHR-1 cooperatively induce various *cyp* and *ugt* genes, albeit in a long-lived mutant background, rather than in acute exposure to xenobiotic compounds.

Although clearly important for the response to various xenobiotics, the mechanisms of NHR-8 activation by such compounds is not clear. Chloroquine, colchicine, and several sterol-derived small molecules that bind mammalian LXR failed to activate NHR-8 in a ligand-sensor screen (Magner et al. 2013); thus, evidence that NHR-8 may act analogously to PXR and CAR as a xenobiotic sensor remains lacking. An alternative mechanism of NHR-8 activation was suggested at by Verma et al. (2018), who showed that, in phosphatase *vhp-1* or *flr-4* kinase dead mutants, *nhr-8* loss abrogates the expression of cytoprotective genes. As *vhp-1* is an important negative regulator of mitogen-activated protein kinase (MAPK) activity, NHR-8 may thus require MAPK signaling to activate phase I, II, and III detoxification genes, although it is not clear whether and how this classical stress sensing kinase signaling pathway is activated by xenobiotic exposure.

C. elegans HNF4-like NHRs

NHR-86

Besides the NR1J group NHRs, several HNF4-related NHRs have recently emerged as regulators of detoxification gene programs in *C. elegans*. NHR-86 is required to express phase I detoxification in worms exposed to the immunomodulatory toxin

RPW24, including four *cyp* genes, two *gst* genes, and 14 *ugt* genes. As in the case of NHR-8, however, the requirement for *nhr-86* is not universal, as the highly RPW24-inducible *cyp-35A2, cyp-35A3, cyp-35B1*, and *cyp-35B2* genes remained chemical-responsive in *nhr-86* mutants; whether these genes are instead regulated by other NHRs such as NHR-8 which was not tested (Peterson et al. 2019). It is noteworthy that Peterson et al. (2019) used chromatin immunoprecipitation followed by sequencing to identify direct NHR-86 targets. The 32 genes whose promoters were bound by this TF and that required its activity for RPW24 induced expression included *cyp-35A1* as the most strongly NHR-86 bound gene, as well as three *ugt* genes and *gst-5*. This provides first evidence that detoxification regulatory NHRs might directly bind critical response genes in live worms. Further, NHR-86 binding to several promoters increased upon exposure to RPW24. This suggests that NHR-86, and perhaps other NHRs, might display toxin-induced binding to relevant detoxification promoters as a mechanism to enhance their expression on demand.

NHR-114

Another HNF4-like NHR that is required to express detoxification genes is NHR-114 (Gracida and Eckmann 2013). In this case, the exposure of nematodes to a bacterial diet containing high levels of the amino acid tryptophan induced a broad detoxification program including *cyp, ugt*, and *gst* genes. Notably, some of these genes were *nhr-114* dependent, suggesting that adaptation to a specific bacterial diet involves NHR-114-driven transcriptome rewiring to protect the organism from toxic effects. This scenario highlights that NHR (and TF) dependent rewiring of the detoxification pathways is not exclusive to exposure to "foreign", potentially dangerous, substances, but represents an integrative component of the organism's response to specific diets and the corresponding (endo-) metabolic modulation that their consumption invokes. Although it is not currently known whether NHR-114 binds any endo- or xenobiotic compound, it is tempting to speculate that it, and perhaps other diet-sensing NHRs, might undertake this to adapt xenobiotic and other metabolic pathways.

Other HNF4-like NHRs

Several other HNF4-like NHRs have also emerged as potential regulators of xenobiotic detoxification. Using high-throughput yeast-one-hybrid assays to study TF-promoter interactions, Fuxman Bass et al. (2016) found a significant overrepresentation of phase I, II, and III detoxification genes, including *cyp, gst, ugt*, and *sdr* genes, amongst promoters bound by NHRs. RNAi against 26 NHRs that bound the promoter of at least one detoxification gene in combination with exposure to 16 molecules that are toxic for *C. elegans*, including some xenobiotics, revealed new roles in xenobiotic resistance for 9 NHRs (NHR-42, −66, −72, −102, −109, −142, −178, −216, and −273). This unbiased study strongly supports the notion that xenobiotic resistance is an important role of HNF4-like NHRs in *C. elegans*.

Targeted investigations of individual NHRs also support such roles for NHRs. Jones et al. (2013) showed that, despite its role in the response to several toxins, *nhr-8* is not universally essential for xenobiotic detoxification (nor were *skn-1, ahr-1, hif-1*, and *mdt-15*). Studying chloroquine, dazomet, imidacloprid, and thiabendazole, and performing candidate RNAi screens of 387 TFs, Jones et al. (2013) found 12 genes required for activation of some of 12 phase I, II, and III detoxification genes in response to toxin exposure. All 12 genes were NHRs. Most prominently, *nhr-176* depletion enhanced susceptibility to the anthelminthic thiabendazole. A follow-up study demonstrated that NHR-176 directly binds thiabendazole and is required for thiabendazole induced *cyp-35* gene transcription (Jones, Flemming, and Urwin 2015). In contrast, *nhr-176* is dispensable for resistance to 5-hydroxythiabendazole. Thus, Jones, Flemming, and Urwin (2015) proposed that the xenobiotic response of *C. elegans* is rather specialized and might involve combinatorial actions of multiple regulators.

Several other NHRs also regulate *cyp* genes. However, these P450 enzymes may not be phase I detoxification genes, but rather participate in lipid synthesis and modification, or act dually in both functions. These functions include the aforementioned regulation of DAF-9 by DAF-12, a feedback loop wherein DAF-9 contributes to the synthesis of

the DAF-12 ligands; in this context, DAF-9 is not known to play roles in detoxification. Similarly, the HIF-1 regulated P450, *cyp-36A1*, is required for the downstream activity of NHR-46 and might contribute NHR-46 ligand synthesis (Pender and Horvitz 2018). CYP-36A1 is related to the CYP2 family, which function in both detoxification of xenobiotics and metabolism of endogenous molecules (Nebert, Wikvall, and Miller 2013), and as such might also perform detoxification reactions. Similarly, although initially characterized as a regulator of lipid metabolism, NHR-49 also regulates *cyp* and other detoxification genes in long-lived mutants and when worms experience oxidative stress (Chamoli et al. 2014; Goh et al. 2018; Hu et al. 2018). Whether or not NHR-49 is directly involved in xenobiotic compound sensing and adaptation is not clear.

In sum, phase I, II, and II detoxification genes appear to be common targets for *C. elegans* NHRs, especially NHR-8 and a growing number of HNF4-related NHRs. These TFs likely act in a combinatorial fashion to induce detoxification gene programs and consequently organismal resistance and tolerance. Underlying mechanisms may involve combinatorial promoter binding, heterodimer formation, or sequential action to evoke a customized response to a toxin(s) an individual worm may encounter. The extraordinarily large number of NHRs in the *C. elegans* genome, combined with similar, albeit smaller expansion of the *cyp* and *ugt* families, suggests that individual NHRs, or NHR dimer pairs, might help *C. elegans* fine tune its detoxification system in the face of ever changing environmental xenobiotic diversity.

Mechanistic considerations of xenobiotic sensing by *C. elegans* NHRs

The mammalian NHRs that orchestrate the response to xenobiotics are activated via direct and indirect mechanisms. That is, their activity is increased both by their ability to directly bind xenobiotic compounds that then act as ligand agonists, and also through additional independent mechanisms not involving direct physical contact between the molecule in question and an NHR. Such indirect signaling involving kinases and phosphatases alters NHR activity by inducing nuclear translocation, protein stabilization, and/or interaction with transcriptional coregulators (reviewed in (Mackowiak and Wang 2016)).

Unlike mammalian PXR and CAR, *C. elegans* NHRs have not been studied in as much detail for putative molecular mechanisms underlying their regulation of detoxification gene programs. However, collectively, the studies described above hint that direct and indirect mechanisms might contribute to xenobiotic sensing and signaling by *C. elegans* NHRs. Specifically, evidence exists suggesting that (i) NHRs can bind some xenobiotic compounds; (ii) their chromatin occupancy in the regulatory regions near detoxification genes increases when xenobiotics are sensed; and (iii) they activate the expression of said genes, thus promoting xenobiotic resistance and tolerance. Together, these studies agree with a model of direct, ligand-like activation of *C. elegans* NHRs by xenobiotics. Indirect signaling, possibly by the MAPK pathway, could also be required for NHR activation.

MDT-15 – a co-regulator shared between NHRs that control detoxification gene programs

To exert effects on gene regulation, NHRs (and other TFs) need to interact with co-regulators, which help activate and repress gene expression as necessary. An important co-regulator in eukaryotes is the Mediator complex, which is composed of 25–30 subunits, some of which serve as direct docking sites for TFs (Grants, Goh, and Taubert 2015). Notably, one such subunit, MDT-15, interacts physically with several of the above noted TFs, including NHR-8, NHR-49, NHR-86, and SKN-1 (see below) (Arda et al. 2010; Reece-Hoyes et al. 2013; Taubert et al. 2006). Accordingly, *mdt-15* is required to induce numerous phase I, II, and III detoxification genes in worms exposed to xenobiotic molecules, and its mutation or deletion results in sensitivity to xenobiotics such as fluoranthene and RPW24 (Pukkila-Worley et al. 2014; Taubert et al. 2008). A model whereby MDT-15 binds NHRs that have been activated in direct or indirect fashion by the presence of a xenobiotic molecule and co-activates the expression of pertinent detoxification genes is thus plausible. As co-regulators might be rate-limiting for expression of inducible gene programs, it would be interesting to further investigate the role of MDT-15 in NHR driven detoxification responses.

AHR-1 is unlikely to perform major xenobiotic detoxification functions in *C. elegans*

In addition to NHRs, mammalian *cyp* and *ugt* genes are regulated by the aryl hydrocarbon receptor (AHR) in response to xenobiotics, especially aromatic (aryl) hydrocarbons including polycyclic aromatic hydrocarbons, dioxins, and polychlorinated biphenyls for which AHR is an important sensor (Bock 2014; Nebert et al. 2004). The *C. elegans* genome also encodes an AHR gene, *ahr-1*, as well as a gene encoding its obligate partner, the AHR nuclear translocator (ARNT) *aha-1* (Powell-Coffman, Bradfield, and Wood 1998). However, whereas mammalian AHR directly binds several aromatic xenobiotic compounds, *C. elegans ahr-1* does not bind the classical AHR ligand, dioxin (Powell-Coffman, Bradfield, and Wood 1998). Further, most functional investigations on *ahr-1* to date suggested that it is predominantly involved in development, especially of the nervous system (Huang, Powell-Coffman, and Jin 2004; Zhang et al. 2013). Similarly, transcriptome analyses in two *ahr-1* mutants revealed a gene program largely pertinent to neuronal function and development (Aarnio et al. 2014), although some genes identified as *ahr-1* dependent did feature regulatory elements annotated as xenobiotic responsive elements (XREs). Recently Brinkmann et al. (2019) suggested that *ahr-1* may modulate life span in *C. elegans*, perhaps by modulating the effects of metabolites from commensal bacteria. In any case, the central role of mammalian AHR in the defense against certain aromatic hydrocarbons does not appear to be recapitulated by *C. elegans ahr-1*, and thus might arise later in evolution.

SKN-1

SKN-1 is a TF that belongs to the basic leucine zipper (bZIP) family of TFs that regulates stress response pathways across species (Blackwell et al. 2015). In *C. elegans*, SKN-1 is especially important for oxidative stress and starvation adaptation and is induced by complex regulatory pathways involving MAPK and insulin signaling as well as negative regulation by the WDR-23 protein, which appears to promote SKN-1 for degradation, an inhibitory response that is alleviated by oxidative stress (Blackwell et al. 2015). In addition to its role as an antioxidant regulator, SKN-1 also plays important roles in xenobiotic detoxification. Specifically, *skn-1* loss sensitizes worms to the common benzimidazole albendazole, and *skn-1* gain-of-function mutations increase tolerance to this drug. *skn-1* regulated genes include albendazole induced *cyp*, *gst*, and *ugt* genes, of which *ugt-22* loss also enhances albendazole efficacy (Fontaine and Choe 2018). Similarly, induction of phase I and II detoxification genes by acrylamide also requires *skn-1*, at least in part, and unbiased genetic screens for genes involved in stimulation of *gst* genes by acrylamide identified known components of the SKN-1 pathway, including the negative regulator *wdr-23*, and the metabolic enzyme *alh-6* (Fukushige et al. 2017; Hasegawa and Miwa 2010). The Skp1 homologs *skr-1/2*, components of Skp-Cullin-F box ubiquitin ligase (SCF) complexes that regulate SKN-1, are also essential for the SKN-1 detoxification response to acrylamide (Wu et al. 2016). The regulation of SKN-1 during xenobiotic stress is thus apparently similar as that seen in oxidative stress, involving derepression from WDR-23 and SKR-1/2 mediated degradation. How xenobiotic molecules act to relieve WDR-23 action on SKN-1 is not known at this time, but the effects of *skr-1/2* are notably independent of classical stress activated MAPK signaling, implicating alternative pathways. Indeed, SKN-1 activity is highly regulated, and studies on its activity in oxidative stress conditions revealed numerous novel regulators (Crook-McMahon et al. 2014). It would be interesting to test whether any of these factors are necessary for elevated SKN-1 activity in response to xenobiotic exposure.

Conclusions: transcriptional regulation of xenobiotic responsive genes

In sum, the transcriptional response of *C. elegans* to xenobiotic molecules is both similar and distinct from that found in other animals. Although NHRs and SKN-1 clearly play central roles in toxin resistance and the underlying gene regulation, these NHRs are distinct, belonging to NR1J and HNF4-like groups rather than the mammalian NR1I group, which appears to be absent from the *C. elegans* genome. Similarly, the role of AHR appears to be marginally conserved with regard to

xenobiotic metabolism. Future investigations need to focus on identifying mechanisms pertaining to how xenobiotics activate *C. elegans* xenobiotic responsive TFs and what specificity or partnerships exist between different TFs to enable tailor-made detoxification and resistance in this worm. Attention also needs to be paid to cell-specific expression to inform our understanding of which cells contribute to xenobiotic metabolism and transport; the existence of publically-available datasets on cell-specific gene expression should facilitate this effort.

Empirical toxicokinetics (TK)

Toxicokinetics(TK) encompass uptake, distribution, metabolism, and excretion of xenobiotics. Understanding the rates at which these processes occur is a critical part of toxicological assessments, because these dictate the actual doses at internal sites of molecular interaction that initiate toxicity. Comparison of internal doses across lifestages, tissues, and species is critical to intraspecies extrapolations (e.g., why does toxicity occur in one cell type and not another?) as well as interspecies extrapolations (e.g., is the toxic effect observed relevant in another species?). Empirical studies of the kinetics of uptake, metabolism, and excretion are an important first step. For example, the absorption rate might be estimated by incubating the animals for different time periods and subsequently measuring the internal doses. Ultimately, TK models are developed, as has been done in many other species, including humans, rats, mice, and zebrafish. To date, a full TK model has not been developed for *C. elegans*, but some aspects of TK were determined and reported. A variety of methods were used to empirically measure xenobiotic uptake (and in some cases clearance) in *C. elegans*. The concept of internal dose used here is as defined by the U.S. EPA Exposure Factor Handbook (2011; see https://www.epa.gov/expobox/about-exposure-factors-handbook).

Burns et al. (2010) systematically investigated the uptake of drugs by *C. elegans* by surveying accumulation of 387 compounds. Unfortunately, Burns et al. (2010) needed to set a high detection limit (19 μM, or roughly half of the external dose of 40 μM), which limited their ability to determine average uptake across the chemical space. However, under these conditions, only 6.7% of the compounds were detected above the detection limit in worm lysates after 6 hr incubation. Burns et al. (2010) went on to construct a structure-activity relationship to predict which physicochemical properties would enable significant accumulation of a compound within *C. elegans*. Extension of this analysis to additional chemicals and classes of chemicals such as (1) both inorganic and organic, as well as molecules with a wide range of K_{ow} and pK_a values would be beneficial. Knowledge of additional lifestages and exposure times would also be of great value to the field.

Unfortunately, despite the thousands of toxicology- and pharmacology-related publications in *C. elegans*, the number that report internal doses appears to be quite limited. In **Supplemental Table 1**, results from publications are summarized that were identified that report analysis of xenobiotic uptake in *C. elegans* (Luz et al. 2016, 2017; Au et al. 2009; Yang et al. 2014; Maurer et al. 2015; Crone et al. 2015; Wyatt et al. 2016b; Helmcke et al. 2009; Allard and Colaiacovo. 2010; Chen et al. 2016; Zheng et al. 2013; Roh, Lee, and Kwon 2016; and others). While it may be challenging to draw broad conclusions from these studies because of variability in experimental design such as liquid versus agar plate culture, lifestage, or exposure time course, there are a few general patterns and several important limitations to the current state of the literature can be identified.

Some combination of poor absorption through the cuticle, and perhaps behavioral changes that reduce oral intake, appear to comprise a fairly effective barrier to uptake of many chemicals. For example, Luz et al. (2017) found that uptake of arsenite in 8-day adults was approximately 1% in 24 hr (1 μM internal dose, 100 μM exposure concentration). However, this is not always the case: cisplatin uptake in 2 hr in L4s was higher, approximately 10–15% (Crone et al. 2015), and a 48 hr developmental exposure to methylmercury, which biomagnifies in aquatic food webs, was present internally at >100-fold higher concentration compared to the exposure medium (Wyatt et al. 2016b). In general, it is probably not surprising that uptake varies significantly by chemical, which underscores the importance of such measurements in future

research. It would also be helpful to distinguish, as much as possible, absorption through the gut from cuticular uptake.

Perhaps the more critical question for human health risk assessment is not how the internal dose in *C. elegans* is compared to the exposure concentration in the medium, but how the measured internal dose in *C. elegans* is compared to exposure concentrations in other lab models (e.g., cell culture), or internal doses in the human population. Data to date show that for many chemicals, internal doses are reasonable compared to other systems. For example, the internal dose of cisplatin with doses between 150–350 μM result in internal dose less than 1 μM (Crone et al. 2015), and patients undergoing cisplatin infusion have peak plasma levels of around 3–5 μM (Hanada et al. 2001; Rajkumar et al. 2016). Internal doses in *C. elegans* approximating human blood levels were also been reported for ethanol (Alaimo et al. 2012). However, it needs to be emphasized that the literature to date is quite limited. Important areas for future research beyond analysis of additional chemicals include detailed time courses to establish not just internal doses but actual kinetics of uptake and excretion.

A second major limitation even when uptake has been measured is uncertainty regarding target tissue concentration within the worm, and cell-and tissue-specific TK. For example, analytical chemistry techniques are typically quantitative but do not usually provide fine-scale information regarding distribution (*i.e.*, inside the worm versus on the cuticle, and which cells and subcellular organelles have the highest concentrations). Conversely, microscopic or X-ray spectroscopy methods provide more spatial distribution information, but are rarely quantitative. Interestingly, these methods revealed striking spatial variation in concentration, often with higher concentrations in the gut than elsewhere (Brinkhaus et al. 2014; Gonzalez-Moragas et al. 2017a). On the other hand, Jackson et al. (2005), using synchrotron X-ray spectroscopy, found large differences in the intra-organismal distribution of copper (which was fairly evenly distributed) and lead (which located mostly to the head region). Jackson et al. (2005) were also able to demonstrate that the uptake of lead was almost certainly entirely via ingestion, not across the cuticle. Further, the physicochemical properties of the compound determine how quickly it is taken up into the worm, particularly to peripheral tissues beyond the gut, and how quickly it might be lost after removing the compound. For example, chlorpyrifos reaches peak internal doses and remains stable after approximately one hr exposure, and when the pesticide is removed it is quickly cleared with more than half disappearing within one hr and the remaining chlorpyrifos clearing by 20 hr (Roh, Lee, and Kwon 2016). In contrast, ethanol was reported to diffuse readily through the cuticle and might be lost even during wash steps, making accurate estimates of internal doses and TK difficult (Mitchell et al. 2007).

Finally, for chemicals that are taken up poorly, even if internal doses comparable to other systems can be achieved with high exposure concentrations, there are practical issues with the low uptake of some chemicals by *C. elegans*. Higher exposure concentrations require using more chemical, which may be costly, and often is limited by the solubility of the drug or toxicant, even with the use of carriers such as DMSO. A solution that was proposed is to use cuticle-defective mutants such as *bus-5*, which were demonstrated to display increased permeability to drugs. However, this presents additional challenges including needing this mutation in the background of all other genetic/transgenic strains used, reduced fitness resulting from the compromised cuticle, and possible compensatory changes in gene expression in response to the mutation that may have unintended consequences in the study (Xiong, Pears, and Woollard 2017). As a further aid to experimental design, **Tables 2 and Tables 3** provide factors that affect uptake of chemicals and nanoparticles in *C. elegans*, including both aspects of the worm's biology (life-stage and genetics) and the worm's environment (type of medium, and presence of food or other organic matter).

Genetic diversity: *C. elegans* as a model for natural population variation in xenobiotic response

Different individuals respond to xenobiotics in diverse ways. The same exposure might lead to extreme toxicity or to mild effects depending

Table 2. Factors impacting toxicokinetics in *C. elegans*.

Factor	Process(es) Likely Impacted	References
Worm Age	Uptake (due to cuticle thickness), Metabolism (age-related changes in gene expression), Sensitivity to toxicity	(Helmcke et al. 2009)
Worm strain	Uptake (cuticle integrity, transporter expression), Distribution (transporter expression), Metabolism (gene expression), Excretion (transporter expression), Sensitivity to toxicity	(Crone et al. 2015; Davies et al. 2003)
Delivery Method or Medium	Apparent External Dose, Uptake (pharyngeal pumping rate, cuticle diffusion, co-solvent delivery)	(Zheng et al. 2013; Alaimo et al. 2012; Burns et al. 2010)
Presence or Absence of Bacteria	Apparent External Dose, Uptake (delivery of compound by adsorption/internalization by bacteria, pharyngeal pumping rate)	(Yang, Lin, and Liao 2017; Spann, Goedkoop, and Traunspurger 2015; Höss, Schlottmann, and Traunspurger 2011; Offermann, Matthäi, and Ahlf 2009)
Bacterial Density	Apparent External Dose, Uptake (delivery of compound by adsorption/internalization by bacteria, pharyngeal pumping rate)	(Offermann, Matthäi, and Ahlf 2009; Höss, Schlottmann, and Traunspurger 2011)
Presence of Organic Matter	Apparent External Dose, Uptake (co-solvent delivery)	(Hoss et al. 2009; Haitzer et al. 1999)
Washing Worms Before Extraction	Apparent Internal Concentration	(Mitchell et al. 2007)
Co-Exposures	Uptake (competition for transport, co-solvent delivery), Metabolism (competition for metabolic enzymes), Sensitivity to toxicity	(Wyatt et al. 2016a)

upon the genetics of the individual. For example, toxicants that pose high risks to biological systems are ubiquitous in the environment, yet individuals vary greatly in the degree to which these exposures initiate diseases. Knowledge of specific xenobiotic responses is limited to cases with a high known exposure source, which is not typical of a daily exposure scenario. A major missing link between individual responses and exposures comes from a limited understanding of the role of genetic variation in responses to xenobiotics. Toxicity assessment techniques are lacking that properly account for genetic variation in toxicological studies because sample sizes are too limited to accurately reflect true human genetic diversity. Similarly, there is also an absence of methods to correlate genotypes to disease phenotypes in human populations that thoroughly account for heterogeneous environmental exposures because this knowledge is essentially impossible to capture in an informative manner. Using a model system, one can address both weaknesses in xenobiotic assessments ethically and robustly. However, a gap exists in the translation of xenobiotic toxicity mechanisms in most model organisms because most assessments of xenobiotic responses use only one genetic background. This situation is akin to making xenobiotic-induced disease risk assessments from a single subject. Therefore, in order to define population-level risk factors, one requires a discovery platform that determines whether xenobiotic response pathways vary across genetically diverse individuals, and *C. elegans* is ideally suited for this purpose.

The *C. elegans* species has genetic diversity similar to humans and genetically distinct individuals are found worldwide (Andersen et al. 2012; Lee et al. 2020). Recent samples collected from nature have levels of genetic diversity from before the domestication of the lab strain N2 (Cook et al. 2017; Crombie et al. 2019; Sterken et al. 2015). Using these strains, one can begin to characterize the spectrum of xenobiotic responses present in the *C. elegans* species to make broader conclusions regarding how xenobiotics affect diverse individuals. Quantitative genetics studies correlations between phenotypic differences (*e.g.* xenobiotic responses) and genotypic differences (*e.g.* genetic backgrounds) to discover the genes that might underlie population-wide differences. *C. elegans*

Table 3. Factors impacting nanoparticle toxicokinetics in *C. elegans*.

Factor	Process(es) Likely Impacted	References
Presence of Food	Apparent External Dose, Uptake (delivery of compound by adsorption/internalization by bacteria, pharyngeal pumping rate)	(Yang et al. 2014; 2017; Collin et al. 2014)
Environmental Transformation	Uptake, Distribution	(Starnes et al. 2015)
Presence of Organic Matter	Apparent External Dose, Uptake (co-solvent delivery)	(Yang et al. 2014; Collin et al. 2014)
Particle Charge and Coating	Uptake, Sensitivity to toxicity	(Ahn et al. 2014; Gonzalez-Moragas et al. 2017b, Meyer et al. 2010a)
Particle Size	Uptake, Sensitivity to toxicity	(Ahn et al. 2014; Gonzalez-Moragas et al. 2017b; Meyer et al. 2010a; Roh, Lee, and Kwon 2016)

offers a unique opportunity to leverage natural genetic variation to identify novel genetic sources of variability in xenobiotic metabolism and transport. Diverse *C. elegans* populations encounter many xenobiotics in their natural rotting substrates, soil, and groundwater ecosystems. For this reason, subpopulations of *C. elegans* have likely adapted to either periodic or constant toxicant exposure and retained advantageous genetic differences that produced increased survival rates in these conditions. Alleles that have arisen naturally might be easily translated to human populations because several xenobiotic response pathways are conserved between *C. elegans* and humans (Ardelli 2013; Harlow et al. 2016; Jones et al. 2013; Shaye and Greenwald 2011). Powerful insights into the genetic basis of xenobiotic responses n may therefore be derived from *C. elegans* as a sentinel of toxic exposure.

Only the beginning of understanding mechanisms of xenobiotic resistance across natural *C. elegans* populations is known. Although drugs are inarguably essential, chemotherapeutic medications may be classified as xenobiotic compounds with known molecular targets and conserved TK properties (Zdraljevic and Andersen 2017). A relatively rare allele of the *scb-1* gene induces bleomycin susceptibility in a large recombinant *C. elegans* population (Brady et al. 2019). This allele also confers resistance to several other chemotherapeutic drugs, particularly drugs that produce DNA double-stranded breaks (Evans and Andersen 2020; Evans et al. 2018). The results are less straightforward with heavy metals and pesticides, two common types of agricultural and industrial xenobiotics. Differential responses to these compounds might be explained by genetic differences among individuals. In particular, three regions of the genome were linked to variation in susceptibility to the pesticides paraquat, chlorpyrifos, and chlorothalonil, as well as the heavy metals silver and copper. However, when one of these regions was isolated through a series of genetic crosses, it alone could only explain variation in one specific trait in response to silver (Evans and Andersen 2020; Evans et al. 2018). Therefore, resistance to heavy metals and pesticides is a complex trait governed by a set of genes with varying functions. As is the case with the majority of complex traits, individual resistance loci typically exert relatively small effects in isolation and are problematic to characterize. Further, these loci might participate in either antagonistic or synergistic interactions, making individual genetic effects even more difficult to detect and interpret. Nevertheless, some individual genes have outsized influences on xenobiotic resistance, even though the entire set of genes is difficult to infer.

One unique example of the power of *C. elegans* for translational TK insights is the discovery of a novel pathway to As resistance among natural populations. Genome-wide association (GWA) mapping involves measuring a phenotype of interest among a collection of genetically diverse individuals. The unique advantage of GWA compared to lab crosses is that it leverages past relatedness to detect resistance alleles with potentially low representation in traditional lab strains. In *C. elegans*, the molecular genetic toolkit is broad and tractable so that causal relationships between genetic variants and phenotypic variation may be established. GWA mapping, followed by a series of metabolomic experiments in edited *C. elegans* strains and human cell lines, confirmed that one cysteine-to-serine missense variant within the *dbt-1* gene enhanced resistance to As by modulating branched chain fatty acid biosynthesis (Zdraljevic et al. 2019). Arsenic sensitivity mediated by differential metabolism of branched-chain amino

acids is a novel observation that is consistent with other features of As-mediated toxicity in human populations. Most critically, these results demonstrate the unique opportunity for the toxicology community to leverage natural genetic variation in *C. elegans* to identify novel mechanisms of differential toxicodynamics in the human population and learn how individuals differ in xenobiotic metabolism.

Natural *C. elegans* populations likely encounter many xenobiotics for which there are poor assessments of human health risk. Nematodes need to find a way to survive in environments contaminated by industrial byproducts such as Cd and Pb. For this reason, *C. elegans* is sometimes considered a useful environmental indicator (Custodia et al. 2001: Hoss et al. 2009) and, in turn, lab assessments have identified important metal resistance genes (Broeks et al. 1996: Swain et al. 2004), including phytochelatins (Vatamaniuk et al. 2001), which play a key role in *C. elegans* but are not expressed in higher eukaryotes. Genetically diverse wild isolates can be sampled from many environments and screened against the battery of toxicants for which detailed human assessments are scant. *C. elegans* isolates can be collected from polluted sites and their xenobiotic responses compared to more traditional lab strains, offering a unique way to take advantage of natural populations. High-throughput phenotyping platforms (Andersen et al. 2015) enabled precise and rapid phenotypic characterization of thousands of individuals. Highly scalable xenobiotic screening efforts enable us to continue to explore *C. elegans* natural variation and expand the scope of large-scale quantitative genetics analyses (Cook et al. 2017). This endeavor might undoubtedly reveal a rich set of genomic regions linked to variation in xenobiotic metabolic and transport capacity (Evans et al. 2018), and provide new opportunities to discover drug-initiated cellular targets for xenobiotic-induced disease treatment.

Evolutionary toxicology: applications of inter-species variation to understanding stressor responses

Intraspecific differences in toxicant responses across a single species might serve as a powerful tool to discover new mechanisms of toxicity and toxicity mitigation. This technique can identify natural variation in genes involved in toxin responses, but some genes might not be conserved with other species because *C. elegans* likely has experienced evolutionary selection by chemical exposure differently. By broadening the number of species where natural history differences in chemical responses are measured, it is possible to increase the likelihood the discovered genes and toxin responses mechanisms might be connected to other species, including humans. The underlying assumption of this hypothesis is that different species have experienced similar evolutionary pressures by chemical exposure, such that mechanisms that were used to adapt are similar. In other words, evolutionary trajectories in different species all lead to the same mechanisms of toxicity mitigation. This hypothesis is likely not universally true, but when it is, the statistical likelihood that genome-wide association studies identify conserved toxin response loci is higher. Thus, some researchers used comparison of toxicological responses of *C. elegans* to those of other nematode and invertebrate species to infer expected ecotoxicological responses in other invertebrates (Boyd and Williams 2003; Haegerbaeumer et al. 2018a, 2019; Peredney and Williams 2000). Similarly, others investigators previously advocated for using evolutionary genetics to inform human health risk assessment (Brady et al. 2017; Hahn 2019; Leung et al. 2017).

Nematodes are incredibly ecologically diverse, either living freely in soils, freshwater, or seawater or as a parasites in plants or animals (De Ley 2006). The chemical exposure of nematodes is as diverse as their natural habitats. It includes organic compounds and minerals in soils (Ekschmitt and Korthals 2006), pesticides used in agriculture (Rich, Dunn, and Noling 2004), as well as defensive compounds secreted by bacteria, fungi, plants, and animals (de Veer, Kemp, and Meeusen 2007; Williamson and Kumar 2006). Some nematodes have evolved to survive desiccation and high salinity in extreme arid environments such as deserts (Treonis and Wall 2005). The panoply of abiotic and biotic stresses has likely contributed to the high abundance and diversity of many of the xenobiotic metabolism and transport-related gene classes discussed above. Even within a single species like *C. elegans*, the diversity of xenobiotic response genes is far larger than the suite of genes found in

the lab-adapted strain N2 (https://www.biorxiv.org/content/10.1101/2020.07.23.218420v1.abstract). The power of *C. elegans* GWA mapping approaches might be expanded to related self-fertilizing species like *Caenorhabditis briggsae* and *Caenorhabditis tropicalis*, when reference genomes are completed and species-wide natural variation has been characterized. Comparative genetics and genomics across these three species will discover conserved *Caenorhabditis* toxin responses along with species-specific toxin responses. The conserved responses are more likely to be conserved with humans. To further supplement the comparative genetics and genomics approach, natural variation in toxin responses across nematode species beyond *Caenorhabditis* or even other invertebrate species (*e.g., Drosophila melanogaster*) need to be studied. The "worm community" has begun in-depth investigations of the biology of multiple related *Caenorhabditis* species (Elsworth, Wasmuth, and Blaxter 2011; Fitch 2005; Martin et al. 2015). However, the total number of nematode species is estimated at approximately one million (Kiontke and Fitch 2013), and only approximately 30,000 species have been described. A limited number of nematode species important in agriculture, biology, and medicine were also examined and provide a basis for comparison, including *Ancylostoma duodenale* (hookworm), *Brugia malayi* (Lymphatic filarial nematode), *Meloidogyne incognita* (root-knot nematode), and *Steinernema carpocapsae* (entomopathogenic nematode). As xenobiotic response loci are discovered in additional species, comparative approaches might increase in power and enable connections of discovered genes, pathways, and mechanisms to humans.

Finally, although this review focuses on *C. elegans* as a toxicology model for human health, *C. elegans* and other nematodes may also serve broader ecological health goals. Nematodes, or communities of nematode species, may serve as useful bioindicator species in ecotoxicology. The species diversity of nematode communities is one measure of ecological integrity, and is sensitive to environmental changes (Franco et al. 2019). Morphological identification of nematode species may be time-consuming and requires expertise. However, recent studies demonstrated that metabarcoding samples of nematode communities is similarly efficient to morphological species identification, and may be implemented rapidly (De Ley et al. 2005; Schenk et al. 2020). Further, given relevance for both human health and ecotoxicology, and the ability to be studied both in the field and in the lab, experiments with *C. elegans* has strong potential to help bridge ecotoxicological and human health research. Research with nematodes might be used in frameworks that were developed for integrating data from different species from molecular (Mattingly et al. 2006), biochemical (Harborne 1988), and pathological (Snyder et al. 2010) to ecological (Monosson 2012) levels, approaches that more recently were incorporated into the EcoHealth and OneHealth concepts (Brooks et al. 2020; Haschek et al. 2019; Perez and Pierce WiseSr. 2018).

Knowledge gaps, opportunities, and future directions

It is hoped that this review will serve to orient *C. elegans* researchers who are not toxicologists to these important pharmacological and toxicological parameters, and to orient toxicologists and pharmacologists who are new to *C. elegans* to what is known and not known regarding relevant aspects of *C. elegans* biology. This review is somewhat narrowly focused on biology relevant to TK, largely excluding some pathways that are nonetheless relevant, including non-enzymatic xenobiotic defenses (metallothioneins, phytochelatins, glutathione, antioxidant molecules), and antioxidant enzyme systems.

It is also hoped that this review will stimulate additional research into some of the less well-understood aspects of TK in this species. Because of the long history of genomic and genetic research in this species, a remarkable understanding of genes involved in TK-related processes exists. Unfortunately, our understanding of the function (*e.g.*, substrate specificity, enzymology, etc.) of the gene products is less complete, and it is recommended more research be done in this area. There is also a limited understanding of which specific cell types are responsible for carrying out many xenobiotic transport and metabolism functions (i.e., which cells act as hepatocytes or renal cells). Similarly, although a handful of careful analyses of internal doses of chemicals in worm exposure studies

were carried out, many more are needed. Work to date permits a tentative conclusion that internal doses of *C. elegans* are often comparable to exposure concentrations in (1) cell culture studies, (2) vertebrate model organisms, and (3) human tissues in exposed individuals. However, the database of chemicals for which internal doses and exposure concentrations have been quantified in *C. elegans* exposure studies is still far too small to make extrapolations with confidence, or to model likely uptake kinetics based upon chemical characteristics and first principles. Exposure studies need to routinely test internal doses. Finally, there is a remarkable opportunity to tie research in these traditional toxicological areas into more general aspects of the stress response (thermal stress, osmotic stress, hyperoxic and hypoxic stress, or caloric stress). Stress responses, broadly defined, are poorly integrated in any species; i.e., how do multiple different stress-response pathways integrate in the context of combined stressors, chemical and otherwise? Such exposures are the reality for individuals and species that one attempts to protect with toxicological studies, and are therefore critical to study. The opportunity to do combination studies and examine the role of multiple stress response pathways is great in *C. elegans*, in which the non-xenobiotic stress response pathways are exceptionally well-studied and tractable.

Better toxicological knowledge will be critical in permitting rigorous use of *C. elegans* for toxicological research that might be used to protect human and environmental health. This is true for in-depth, mechanistic toxicological research, such as on the role of specific enzymes in mediating toxicity of specific chemicals (Harris et al. 2020) and the ability to extrapolate worm studies to other species. It will also permit adoption of this powerful genetic model to "functional toxicology" studies (Gaytan and Vulpe 2014). Finally, it will be crucial to the adoption of the *C. elegans* model for New Approach Methodologies that seek to reduce time, cost, and vertebrate animal use in chemical safety regulation and risk assessment. The Frank R. Lautenberg Chemical Safety for the 21st Century Act (2016) mandates the U.S. Environmental Protection Agency (U.S. EPA) to explore the use of non-vertebrate models for toxicity testing (U.S. Congress 2016). In 2019, the U.S. EPA Administrator Andrew Wheeler signed a directive to reduce funding for animal research 30% by 2025 and eliminate it by 2035 (U.S. Environmental Protection Agency Environmental Protection 2019). *C. elegans* has attracted interest of a number of regulatory agencies and governmental labs (Boyd et al. 2016; Gong et al. 2018; Hunt et al. 2018). Yet, at the time of this publication, only two standardized testing documents were developed (International Standard Organization (ISO) 2020, (ASTM), American Society of Testing and Materials. 2001), and none by the U.S. EPA exist. Closing these knowledge gaps and developing standardized protocols will support the use of *C. elegans* model to address key knowledge gaps in risk assessment, such as developmental neurotoxicity (Hunt et al. 2018), neurodegeneration (Sammi, Agim, and Cannon 2018), and mixture effects (Wittkowski et al. 2019).

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