ABCB1 gene in *Aporrectodea caliginosa*

- A potential ecotoxicological tool

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of the requirements for the Degree of

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by

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Earthworms are exposed to a variety of agrochemicals due to strict export requirements and maintenance of high productivity. *Aporrectodea caliginosa* (grey earthworm) is the most abundant species in New Zealand pastures. In biological systems, the first-line defence mechanism against accumulation of toxicants within cells is mediated by drug efflux transporters of the ATP Binding Cassette (ABC) transporter family. This study describes the discovery of a partial sequence homologous to *ABCB1* gene that encodes the efflux transporter P-glycoprotein (P-gp). The regulation of *ABCB1* gene expression has been associated with exposure to polluted environments in aquatic organisms. The expression of the *ABCB1* gene in annelids is a potential candidate as a molecular biomarker for soil pollution monitoring. The lack of *A. caliginosa ABCB1* genetic information or any other Lumbricidae family member has been a significant drawback.

The novel sequence was identified by using a combined approach of degenerate PCR and sequence retrieval from the draft *Lumbricus rubellus* genome LUMBRIBASE. The novel translated sequence of 134 amino acids (encoding 10.7% of exogenic sequence) has high amino acid sequence identity to P-gp (>78%) in vertebrate and invertebrate species and possesses some of the classical domains of P-gp. A real-time quantitative PCR (RT-qPCR) was developed to measure *ABCB1* gene transcription. In the process of PCR optimisation, another partial gene sequence, homologous to *β-actin* gene was identified in *A. caliginosa* and used as the reference gene for the normalisation of samples. Preliminary analyses in RT-qPCR were performed with earthworms exposed to modulators (rifampicin, cyclosporin A, Ivermectin™) on *ABCB1* gene and P-gp expression used in mammals. Fold-changes in the *ABCB1* transcriptional levels were measured by the comparison between exposed and non-exposed earthworms. The P-gp activity was measured by a biochemical assay using a specific P-gp substrate (fluorescent dye Rhodamine B) and two P-gp inhibitors (verapamil and cyclosporin A). Rifampicin induced the *ABCB1* expression in *A. caliginosa.* However, it did not increase P-gp activity.

This observation suggests the involvement of post-transcriptional mechanisms to produce the functional P-gp. Ivermectin™ also increased *ABCB1* expression in *A. caliginosa*, showing that this drug may have the same effect on *ABCB1* expression observed in studies with nematodes and mammals. In contrast, cyclosporin A did not increase *ABCB1* gene expression, suggesting that P-gp inhibition alone does not lead to overexpression of *ABCB1* to compensate for the inactive P-gps.

The presence of an *ABCB1* gene homologue in *A. caliginosa* and its expression in response to known modulators supported the hypothesis that P-gp is present in *A. caliginosa*. Moreover, the P-gp activity in *A. caliginosa* was demonstrated in the results of the biochemical assay. The results of this research provide baseline data in *A. caliginosa ABCB1* and P-gp expression, and may assist in the advancement of ABC transporter research in earthworms and development of a monitoring system in toxicological risk assessments.

Keywords: *ABCB1* gene, P-glycoprotein, *Aporrectodea caliginosa*, earthworm, biomarker, LUMBRIBASE, Real time quantitative PCR, rifampicin, cyclosporin A, verapamil, Ivermectin™, rhodamine B.

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Chapter 1

Introduction

1.1 P-glycoprotein and *ABCB1* gene

In biological systems, the first line of defence against toxicants is mediated by transmembrane transporter proteins which pump out a variety of endogenous substances and xenobiotics (foreign chemicals) across the cell membranes in an energy-dependent manner (Szakács *et al.*, 2008). The exposure to high levels of anthropogenic pollutants and natural toxins can induce the overexpression of these transporters known as the multixenobiotic resistance (MXR) system (Bard, 2000; Higgins, 1992; Kurelec *et al.*, 2000). The most well characterised transporter in the MXR system is the P-glycoprotein (P-gp) which belongs to the superfamily of ATP-dependent transmembrane transporters, the ATP Binding Cassette (ABC) proteins (Szakács *et al.*, 2008). In mammals, P-gp is expressed and distributed in various organs and tissues and transports a wide variety of substrates (Zaja *et al.*, 2008). P-glycoprotein is encoded by the gene *ABCB1* (Buss & Callaghan, 2008). The expression of either P-gp protein or the *ABCB1* gene in aquatic organisms has been used as a biomarker to detect aquatic pollution (Ame *et al.*, 2009; He *et al.*, 2011; Luedeking & Koehler, 2002).

1.2 Earthworms

Earthworms have been used as bioindicators of soil pollution (Eijsackers, 2004; Reinecke & Reinecke, 2004). The popularity of earthworms as sentinels is because of their natural activities of burrowing and ingestion of large amounts of soil, which continuously expose the earthworm to bioavailable contaminants via both its digestive tract and the outer membrane (Sanchez-Hernadez, 2006). In New Zealand, the most prevalent earthworm found in pastures is *Aporrectodea caliginosa (Fraser et al., 1996)*. However, little is known about the presence and activity of P-gp in these soil organisms. P-glycoprotein has been identified in fungi, bacteria, plants, invertebrates and other mammals (Szakács *et al.*, 2008) and to date, not yet in earthworms.

1.3 Toxicological risk assessment in soil

In New Zealand, there is intensive and extensive usage of agrochemicals in pastures and horticulture to maintain high productivity and to comply with strict export requirements for pest/pathogen-free products (Walker *et al.*, 2004). The impact of agrochemical pollution on soil biota is not well known. P-gp

substrates include several chemicals routinely used in agricultural practice, including insecticides, fungicides, herbicides, antibiotics, alkaloids, immunosuppressants, hormones, and heavy metals (Buss & Callaghan, 2008; Luckenbach & Epel, 2005; Pivčević & Žaja, 2006; Szakács *et al.*, 2008).

P-gp is constitutively expressed in cells, however, it may be induced (or upregulated) on exposure to its substrates (Hennessy & Spiers, 2007). Also, P-gp substrates may increase or decrease *ABCB1* gene transcript levels. The regulation of the *ABCB1* gene in aquatic organisms has been associated with exposure to polluted environments and *ABCB1* expression is now recognised as an appropriate biomarker to determine the hazardous effects of chemicals in contaminated marine habitats (Ivanina & Sokolova, 2008; Luedeking & Koehler, 2004; Zucchi *et al.*, 2010). The response of P-gp in the earthworm's defence against anthropogenic pollutants in soil may be understood by the measurement of *ABCB1* transcriptional levels in *A. caliginosa.* Studies on the *ABCB1* gene expression in earthworms have the potential to be used as an ecotoxicological tool for risk assessments exposed to pollutants including agrochemicals.

1.4 Research aim and objectives

1.4.1 Aim and context

The main purpose of this research was to identify the *ABCB1* gene in *A. caliginosa* and measure the *ABCB1* gene expression and P-gp activity in response to known *ABCB1* inducers and P-gp modulators reported in studies of other species (Cascorbi, 2006; Magnarin *et al.*, 2004; Ménez *et al.*, 2012; Schinkel & Jonker, 2003). The lack of *ABCB1* genetic information from *A. caliginosa* or any other Lumbricidae family member was a significant drawback. The approach in the identification of *ABCB1* in *A. caliginosa* was a combination of degenerate primers and sequence retrieval from the draft *Lumbricus rubellus* genome LUMBRIBASE to be used in PCR. Degenerate primers are a blend of primers with different combinations of nucleotides within its sequence, in an attempt to provide the correct primer sequence that will bind to the specific target sequence (Zheng *et al.*, 2008a) and they are typically designed from multiple sequence alignments from a variety of species when the target sequence in an organism is not known (Dieffenbach *et al.*, 1993). For the measurement of *ABCB1* expression, a quantitative PCR was developed and optimised, and the P-gp activity was measured by a biochemical functional assay.

The identification of the *ABCB1* gene in *A. caliginosa*, and further analysis of *ABCB1* gene expression and P-gp activity across earthworms exposed to different treatments would provide insights into the role of P-gp in detoxification and the variation in its response on exposure to different substrates. It is presumed that such approach could contribute greatly to ecotoxicological assessments of soil in future studies, as

has been the case with studies in aquatic organisms (Luedeking & Koehler, 2004; Shúilleabháin *et al.*, 2005; Smital & Kurelec, 1998b; Timofeyev *et al.*, 2007).

1.4.2 **Objectives**

- To identify the *ABCB1* gene in *A. caliginosa* by amplification of a partial sequence with degenerate PCR primers designed from the conserved areas of published *ABCB1* gene sequences in other species available from public databases such as GenBank (www.ncbi.nlm.nih.gov/), and with primers designed from the draft of *L. rubellus* genome available at LUMBRIBASE (www.nematodes.org/). *Lumbricus rubellus* and *A. caliginosa* are members of the same Lumbricidae family (Chapter 3).

- To use molecular methods such as rapid amplification of complementary DNA (cDNA) sequence ends (RACE) and cloning to identify as much of the *ABCB1* gene (cDNA) sequence as possible (Chapter 3).

- To develop and optimise a reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to measure the *ABCB1* expression in *A. caliginosa* (Chapter 4).

- To expose earthworms to an *ABCB1* inducer (the antibiotic Rifampicin), to a P-gp inhibitor [cyclosporin A (CyA)] and to a widely used agrochemical [Ivermectin^{TM}(IVM)], an anthelmintic drug to control gastrointestinal worms in cattle and sheep)[1] and measure changes in *ABCB1* gene expression levels of each treatment with RT-qPCR (Chapter 4).

- To expose *A. caliginosa* to rifampicin, CyA, IVM, and to another P-gp inhibitor (verapamil), and measure the P-gp activity by a biochemical assay using a specific P-gp substrate, the fluorescent dye Rhodamine B (RB) (Chapter 5).

[1] Drug that expels parasitic worms from the body by either stunning or killing them.

1.4.3 **Hypotheses**

- The *ABCB1* gene has been found in many species, ranging from bacteria and plants to mammals (Hennessy & Spiers, 2007). It is hypothesized that *ABCB1* is present in the earthworm *A. caliginosa*, based on the evidence of P-gp activity in the study of Brown *et al* (2008) with another earthworm species (*Eisenia fetida*).

- The antibiotic rifampicin has been used to induce the *ABCB1* gene expression in mammals (Magnarin *et al.*, 2004; Nannelli *et al.*, 2010). It is hypothesized that exposure of earthworms to rifampicin will: (1) increase the *ABCB1* transcription as measured by the developed RT-qPCR technique in earthworm tissues expressing P-gp, (2) increase the P-gp transport activity in P-gp expressing tissues via outer cell membrane as measured by the RB assay.

- The immunosuppressant CyA has been shown to inhibit the P-gp function in other species (Schinkel & Jonker, 2003). Based on this, it is hypothesized that CyA will: (1) decrease the P-gp transport activity, resulting in accumulation of RB inside cells of P-gp expressing tissues, (2) induce *ABCB1* transcription as a protective mechanism to eliminate RB.

- The anti-parasitic drug IVM has been shown to induce drug resistance in the model nematode *Caenorhabditis elegans* (James & Davey, 2009). This resistance was associated with increased expression of *ABCB1* gene and protein P-gp. It is hypothesized that exposure *of A. caliginosa* to non-lethal concentrations of IVM will increase the *ABCB1* transcription and P-gp transport activity in order to eliminate the drug from the body.

Chapter 2

Literature Review

2.1 Ecotoxicology

Ecotoxicology is a study of the relationship between natural toxins, chemical pollutants, the environment into which they are released and the biota in that environment (Luedeking & Koehler, 2002). In New Zealand, there is intensive and extensive usage of agrochemicals in pastures and horticulture due to both strict export requirements for pest/pathogen-free products and in order to maintain high productivity (Penman & Buick, 1995; Walker *et al.*, 2004). Furthermore, the disposal of sewage sludge in forests and the spray of dairy farm effluents on pastures are common practices in New Zealand and that may have unpredictable consequences on the ecosystem (Müller *et al.*, 2007). The dairy effluent may contain chemicals such as cleaning and disinfection agents used in the milking shed, and veterinary pharmaceuticals excreted by animals. According to the latest New Zealand dairy statistics of 2010-11 (www.dairynz.co.nz, 2011), the growing dairy industry has approximately 11,735 herds with 4.5 million cows. A recent case study has demonstrated that the widespread use of IVM, a common anthelmintic veterinary drug, is of high concern because of its impact on the environmental organisms (Roembke *et al.*, 2010). Agrochemicals often bind to soil organic matter. Therefore, the bioavailability and the impact of such residues on non-target soil organisms such as the earthworm are not fully known.

2.2 Earthworms

The earthworm is a beneficial organism that is widely distributed in soil. Earthworms can comprise as much as 60-80% of total soil biomass under certain conditions (Booth & O'Halloran, 2001). The natural activity of earthworms promotes soil health through soil aeration, drainage, mineralisation and mixing (Edwards & Coulson, 1997). Earthworms play an important role in soil fertility through digestion of organic matter and transformation of this matter into nutrients for plants and soil microorganisms. Earthworm activities of burrowing and feeding, involving ingestion of large amounts of soil, continuously expose the earthworm to contaminants via both, its digestive tract and outer membrane (comprising cuticle, epidermis and muscle) (Sanchez-Hernadez, 2006). These features and the easy sample collection, handling and laboratory maintenance have led to the use of earthworms as sentinel species in many soil pollution studies (Edwards & Coulson, 1992; Reinecke & Reinecke, 2004).

In New Zealand, the most prevalent earthworm found in pastures is the non-native species *A. caliginosa* (Booth *et al*., 2001; Booth *et al*., 2005; Springett, 1992), commonly known as grey earthworm. In the Canterbury plains, *A. caliginosa* comprises approximately 80% of the total earthworm population (Fraser *et al.*, 1996). *A. caliginosa* builds complex lateral burrow systems through all layers of the upper mineral soil and spend most of their life in these burrow systems, where it feed on decayed organic matter and mineral soil, categorised as endogeic (Wallwork, 1983). During wet weather, the adults move to the soil surface, which makes them more susceptible to surface environmental contaminants (Edwards & Coulson, 1997). Moreover, many reports show that *A. caliginosa* is the most susceptible species of earthworm when used in standard toxicological tests versus three other common earthworm species (*E. fetida, Lumbricus terrestris and L. rubellus*) (Edwards & Coulson, 1992; Martin, 1986).

2.3 P-glycoprotein and *ABCB1* gene

Cells have defence mechanisms to protect them against a variety of man-made and natural chemicals. The body responses include, among others, complex systems as the use of biotransformation enzymes for chemical detoxification, and the immunologic responses that produce antibodies against these chemicals (Casarett *et al.*, 2001). The first line of defence against accumulation of toxicants within cells is mediated by transmembrane proteins, which pump out a variety of endogenous substances and xenobiotics across the cellular membranes in an energy-dependent manner (Szakács *et al.*, 2008). It is known that high levels of anthropogenic pollutants and natural toxins induce the overexpression of these proteins which can confer protection against deleterious effects on cells (Bard, 2000). The overexpression is a phenomenon known as MXR in aquatic organisms, similar to multidrug resistance (MDR) in mammals (Smital *et al.*, 2000). The MDR phenotype was initially identified in human tumour cells that were resistant to chemotheurapeutic drugs caused by an overexpression of a P-gp located in the cytoplasmic membrane (Cascorbi, 2006). Recently, P-gp overexpression has also been associated with increased drug resistance in protozoa including *Plasmodium spp* (de Lagerie SB *et al.*, 2008), reducing the efficacy of anti-malarial treatment. P-glycoprotein has been extensively studied in an effort to overcome widespread drug resistance to cancer therapy, and currently it is the most well characterised drug efflux transporter in humans (Dean *et al.*, 2001).

P-glycoprotein belongs to the superfamily of ATP-dependent transmembrane proteins denominated as ABC proteins (Szakács *et al.*, 2008). Most of the ABC proteins are transporters and their genes are both widely dispersed across eukaryotic genomes and conserved between species. In mammals, forty-eight ABC transporters have been identified, and classified on the basis of the phylogenetic analysis into seven distinct gene subfamilies, denominated as ABC A through to subfamily G or formerly known as ABC1,

MDR/TAP, MRP, ALD, OABP, GCN20, and WHITE respectively (Hennessy & Spiers, 2007). P-gp-like proteins are encoded by genes of subfamily B (or MDR/TAP) comprising 11 members (B1 to B11) (Dean *et al.*, 2005). P-gp involved in the transport of xenobiotics is encoded by the gene *ABCB1* (Buss & Callaghan, 2008). P-gp exists in a number of different isoforms which have >70% sequence homology and are encoded by a small family of closely related genes.

P-gp consists of one polypeptide chain of about 1300 amino acids and the molecular mass of the mature protein may vary from 130 to 180 kDa, reflecting differences in glycosylation (Sturm & Segner, 2005). P-gp shows the domain architecture of a prototypical eukaryotic ABC full transporter, composed of two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) (Figure 2.1). A full transporter consists of two homologous halves (each with one TMD and one NBD) arranged in a tandem-like manner whereas half transporter contains only one TMD and one NBD. The ABC sub-family B comprises full transporters (four members, including P-gp) and half transporters (seven members). Full transporter proteins are expressed in the plasma membrane whilst half-transporters are usually found in intracellular membranes of the mitochondria, endoplasmic reticulum and peroxisomes (Rocchi *et al.*, 2000).

Each TMD is formed by 6-11 membrane spanning alpha-helices, which confer substrate specificity and NBD for the ATP activity (Dean *et al.*, 2001). When a substrate binds to P-gp, it activates one NBD to hydrolyse ATP (ADP+P), releasing energy. This energy changes the structure of TMD, resulting in extrusion of substrate out of the cell (Hennessy & Spiers, 2007). Members of ABCB family possess a highly conserved NBD (specifically, ATPase), opposed to TMDs, which do not share great similarity between domains of different ABC transporters (Feldstein *et al.*, 2006). The conservation of NBDs are central to the classification and demarcation of the ABC transporter super-family (Hennessy & Spiers, 2007), which are composed of several distinct sequence motifs with a higher degree of conservation than the rest of domain (Ambudkar *et al.*, 2006). At least eight of such sequences have been described in mammals: A-loop, Walker-A, Gln-loop, X-loop, C-motif (or ABC signature), Walker-B, D-loop and His-loop (Lawson *et al.*, 2008). Several amino acids in these conserved motifs play a role in the ATP binding, coordination with Mg^{2+}, communication between the NBDs and the transport substrate sites and finally, the ATP hydrolysis to release energy to be used in the transport of xenobiotics (Smith *et al.*, 2002).

P-glycoprotein transports a wide variety of substrates, with common properties such as moderate hydrophobicity/amphiphilicity, neutral or positive charge, a basic nitrogen atom and a high molecular weight (Zaja *et al.*, 2008). Some of these substrates include important anti-cancer agents, immunosupressants, anti-viral drugs, steroids, anti-gout agents, pesticides and antibiotics (Ambudkar *et*

al., 1999). Due to its function, P-gp is expressed and distributed in a variety of organs and tissues involved in excretion, metabolism and protection, including lung, liver, kidney, gastrointestinal, adrenal gland, gravid uterus, and brain capillary endothelium (Zhou, 2008). Therefore, the combination of P-gp function and its localisation directly reduce the bioavailability and potential toxicity of xenobiotics to organisms.

2.4 P-glycoprotein in environmental organisms

Many studies have demonstrated the role of P-gp in cellular detoxification in aquatic organisms in polluted waters (Bard, 2000; Kurelec *et al.*, 1996; Smital & Kurelec, 1997, 1998a; Smital *et al.*, 2000). To date, the function of P-gp and other relevant transporters in cellular detoxification and defence against natural and anthropogenic toxicants have been demonstrated in more than 40 aquatic species (Loncar *et al.*, 2010). P-glycoprotein has been also identified in fungi, bacteria, plants, invertebrates and other mammals (Szakács *et al.*, 2008), but not yet in earthworms.

P-glycoprotein has been detected in the excretory and the intestinal cells of the free living soil nematode *C. elegans* (Broeks *et al.*, 1995). Further studies have demonstrated the role of P-gp in defence against toxicants in *C. elegans* and in the parasitic nematode *Haemonchus contortus* (Kerboeuf *et al.*, 2003). P-glycoprotein has been also identified in several aquatic organisms (Loncar *et al.*, 2010), including mussels *(Mytilus spp*) (Luedeking & Koehler, 2002), which are somewhat phylogenetically close related to earthworms (super phylum Lophotrochozoa) (Figure 2.2.) However, despite the susceptibility of earthworms to toxicants, little is known about the presence of *ABCB1* gene and the activity of P-gp transporter in these soil organisms. There is a high probability that P-gp is also present in earthworms because MXR transporters (including P-gp) are highly conserved across species. A previous study utilising a fluorescent dye assay to detect P-gp drug efflux activity provided some evidence for the presence of P-gp in earthworms (Brown *et al.*, 2008).

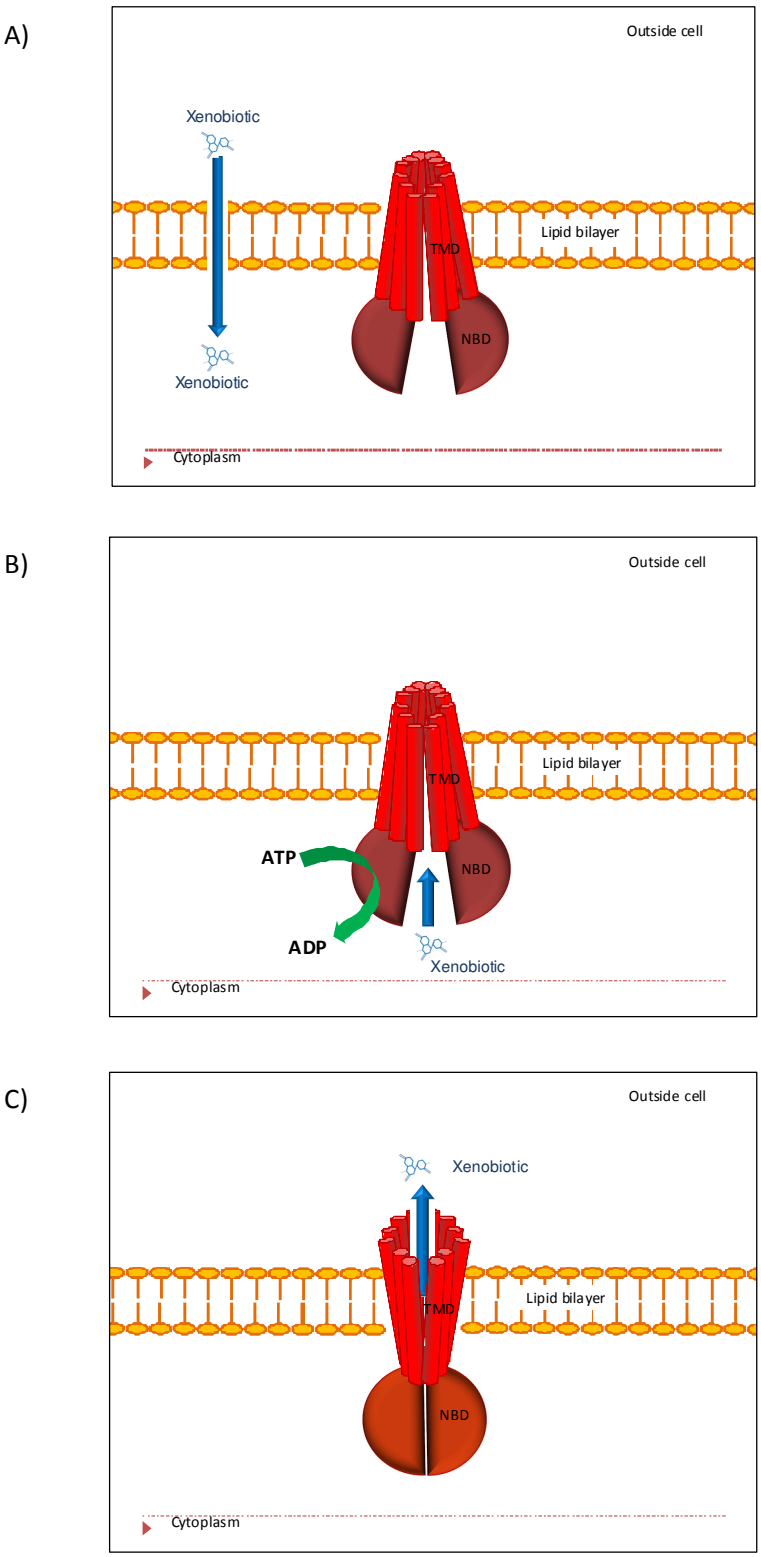

Figure 2-1 Schematic representation of the structure and function of the full transporter P-glycoprotein (P-gp). The transporter is composed of two transmembrane domains (TMDs) and two nucleotide binding domain (NBDs). (A) A xenobitioc crosses the cellular membrane passively. (B) In the cytoplasm, when the xenobiotic binds to P-gp, the NDB is activated to hydrolyse the ATP molecule to release energy. (C) This energy changes the conformation of TMDs and the substrate is expelled out of the cell.

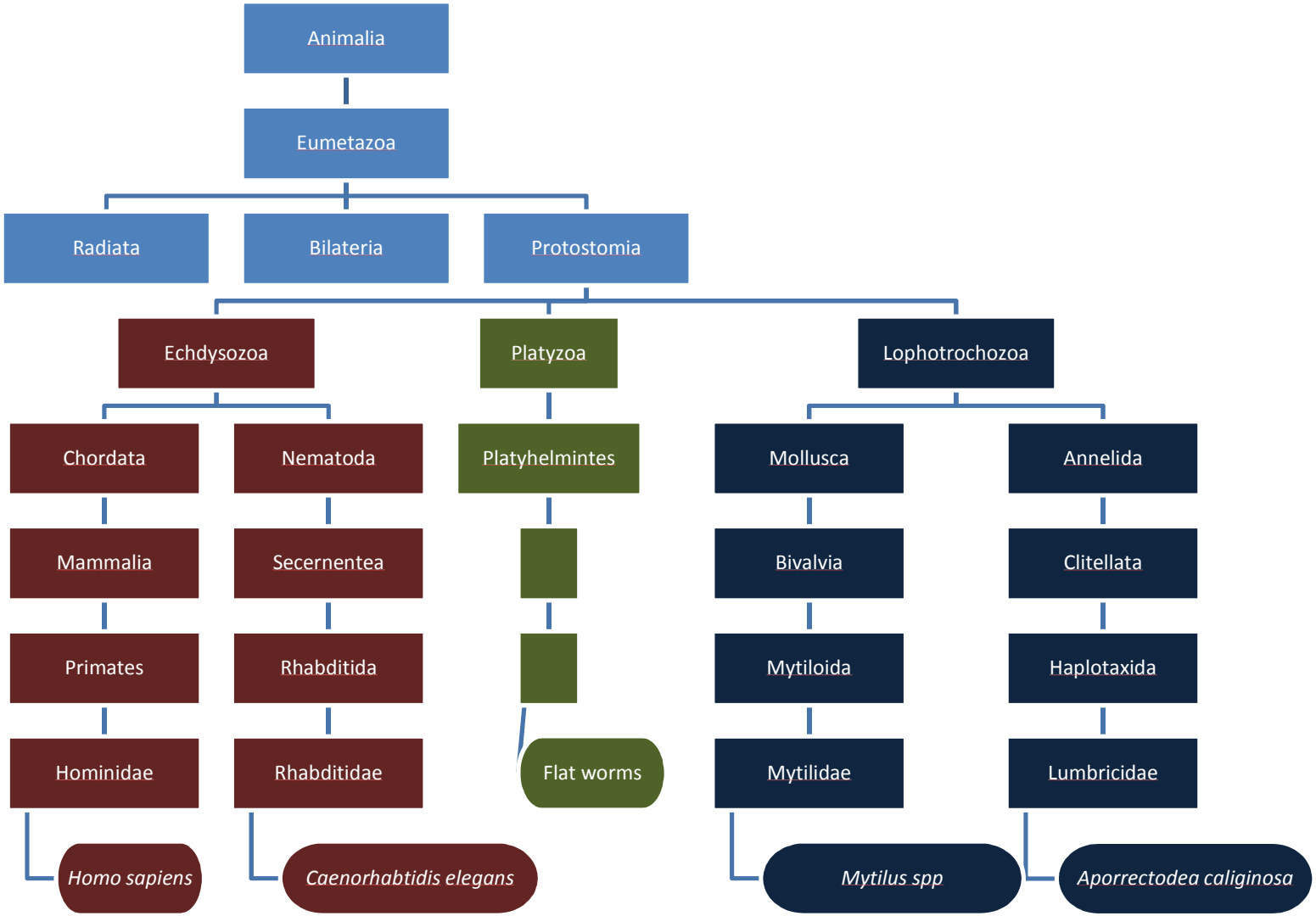

Figure 2-2 Scientific classification: *Aporrectodea caliginosa* (grey earthworm) share the same super phylum (Lophotrochozoa) as *Mytilus spp* (mussels).

2.5 P-glycoprotein modulators

There is a broad range of modulators known to affect expression and function of P-gp in a variety of species (Bard, 2000; Buss & Callaghan, 2008; Lespine *et al.*, 2008; Smital *et al.*, 2004). Experiments with fish and bivalves using environmental toxicants including pesticides, heavy metals, crude oil and pulp mill effluent have demonstrated the induction of P-gp protein expression as detected by immunochemical assays (Bard *et al.*, 2002; Eufemia & Epel, 2000). Moreover, widespread use of IVM, a known P-gp substrate, has been linked to the multidrug resistance phenomenon due to overexpression of P-gp in intestinal worms and nematodes (James & Davey, 2009). With respect to chemicals routinely used in agricultural practice, in addition to the antiparasitic drugs, P-gp substrates include pesticides, fungicides, herbicides, insecticides, antibiotic, alkaloids, immunosuppressant, hormones, and heavy metals (Buss & Callaghan, 2008; Luckenbach & Epel, 2005; Pivčević & Žaja, 2006; Szakács *et al.*, 2008) and the effects of these chemicals on the P-gp regulation in exposed organisms should be further investigated.

Another relevant characteristic of P-gp function is that the transport of substrate may be inhibited by secondary compounds (Luckenbach & Epel, 2005). Chemicals that inhibit or block P-gp function, directly or indirectly, leading to the bioaccumulation of substrates inside the cells and resulting in unexpected toxic effects, are known as chemosensitisers (Smital *et al.*, 2004). The blocking action of chemosensitisers makes the organism more susceptible to the harmful effect of xenobiotics at concentrations well below the safety threshold. Emerging contaminants, such as polycyclic musks (a common fragrance ingredients) found in cosmetics, personal care products, detergents and cleaning agents, have been shown to be strong chemosensitisers which affect the MXR in aquatic organisms (Bard, 2000). A large number of anthropogenic pollutants such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCB), dichlorodiphenyltrichloroethane (DDT) and their metabolites can either induce or inhibit the expression of P-gp in fish, invertebrates and mammals (Sturm & Segner, 2005). One example of this effect was demonstrated in the comparison of PAH-contaminated non-tidal areas with non-polluted areas, where mussels from a contaminated region showed reduced P-gp-related protection in their digestive glands (Einsporn & Koehler, 2008). In addition, many currently used pesticides have been shown to be highly competitive MXR inhibitors (Smital *et al.*, 2004).

Overall, the increase in use and the action of chemosensitisers and P-gp substrates have raised the concerns about their effects on P-gp function and consequences on soil biota. P-glycoprotein substrates and chemosensitisers may increase or decrease *ABCB1* gene transcript levels (Chieli *et al.*, 2010; Diaz de Cerio *et al.*, 2012; Hanke *et al.*, 2010; Rodrigues *et al.*, 2006) and further gene expression studies are necessary to confirm this hypothesis.

2.6 Ecotoxicogenomics

Ecotoxicogenomics is defined as the study of gene and protein expression in environmental organisms that are viewed as important or sentinel species in their responses to environmental toxicant exposures (Snape *et al.*, 2004). The traditional ecotoxicological parameters of earthworms that have been used in risk assessments of contaminated land are classical endpoints such as mortality, reproduction, growth rate, juvenile maturation and hatchability (Booth *et al.*, 2005). Such experiments may produce estimates of xenobiotic effects on populations, but they do not elucidate the mechanism of action. Furthermore, these monitoring parameters are subjected to the variation of organism age, choice of species, and environmental conditions, making the interpretation of results difficult and not accurate. Ecotoxicogenomics studies utilising modern molecular techniques have been shown to be to a large extent more sensitive, stressor-specific, predictive, cost-effective (in the long term) and faster compared to the more traditional earthworm tests mentioned above (Sturzenbaum *et al.*, 2009).

P-glycoprotein is constitutively expressed in cells, but may be modulated by environmental factors such as chemical exposure (Hennessy & Spiers, 2007). It is generally accepted that P-gp expression is increased through upregulation of *ABCB1* mRNA levels as reported in studies with cell lines and human tumours (Chaudhary & Roninson, 1993). The regulation of the *ABCB1* gene in aquatic organisms has been associated with exposure to polluted environments and *ABCB1* expression has been used as a biomarker to determine the level of contamination in marine habitats (Ivanina & Sokolova, 2008; Luedeking & Koehler, 2004; Zucchi *et al.*, 2010). The measurement of *ABCB1* transcriptional levels in *A. caliginosa* may provide information to understand the role of P-gp in earthworm defence mechanism against pollutants in soil. Moreover, the examination of the *ABCB1* gene response could be used as an ecotoxicological tool for risk assessments of soil organisms exposed to agrochemicals.

Recently, transcriptomic profile studies in two standard test earthworms, *L. rubellus and E. fetida,* were created in order to evaluate the response to environmental contaminant exposure (Owen *et al.*, 2008; Pirooznia *et al.*, 2007; Svendsen *et al.*, 2008). Despite a vast collection of genetic data being produced in these studies, there is not yet an annotated reference sequence for the *ABCB1* gene in earthworms. Moreover, whilst the release of the draft genome of *L. rubellus* is imminent, it is not yet available as an annotated sequence (Sturzenbaum *et al.*, 2009). The lack of information of the *ABCB1* gene sequence in earthworms currently presents a challenge to primer design and ultimately the *ABCB1* gene expression analysis.

2.7 Analysis of *ABCB1* gene expression

The analysis of gene expression patterns is an important tool in increasing understanding of the gene and subsequent protein function. A recent experiment whereby constitutive gene expression levels of *ABC* genes were determined in various tissues of rainbow trout (*Oncorhynchus mykiss*, a common aquatic research model organism for ecotoxicological assessments), provided an insight into the spatial distribution of relevant ABC transporters (Fischer *et al.*, 2011).

Today, the gold standard for accurate, sensitive and fast measurement of gene expression is the fluorescence-based reverse transcription quantitative real-time PCR (RT-qPCR) (Derveaux *et al.*, 2010). This approach has been used to measure *ABCB1* expression levels in molluscs and tumours in mammals (Ivanina & Sokolova, 2008; Nakaichi *et al.*, 2007). It is the most sensitive method for the detection and quantification of gene expression levels; in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue samples and for elucidation of minor changes in mRNA expression levels (Bustin, 2004). In this PCR-based process the detection of fluorescence emitted by amplicons enables the visualisation of results during amplification. The quantification is made during the exponential phase of the PCR where the product doubles at every cycle making the number of amplified target directly proportional to the initial amount of target. According to the latest published guidelines for publications of qPCR experiments (Bustin *et al.*, 2009), the quantitative endpoint for expression level calculations is designated as the quantification cycle (C_q). The C_q is defined as the PCR cycle at which the fluorescent signal emitted from amplicon crosses an arbitrarily threshold, above the background fluorescence noise (Bustin, 2004).

Within the range of fluorescent chemistries used in RT-qPCR, SYBR Green I is the most common and cost effective. SYBR Green I is an nonspecific intercalator dye that binds to the minor groove of any double stranded DNA including the specific PCR product, primer-dimers and nonspecific amplicons (Bustin, 2004). However, due to the general affinity of SYBR Green I for all double stranded DNA, RT-qPCR requires the optimisation of some critical quality parameters within the PCR assay to ensure the accuracy and reliability of results (Kubista *et al.*, 2006). Thus, the increase in fluorescence alone is not sufficient for evaluation of replication of target nucleic acid; RT-qPCR using SYBR Green I also requires subsequent melting temperature (Tm) curve analysis to detect formation of nonspecific products and primer-dimers (Ririe *et al.*, 1997).

Data normalisation is required to control for the experimental error introduced during the multi-stage process of isolating, processing RNA and quantification of mRNA transcripts. Technical variations are easily compounded by any variation in the amount of starting material between the samples, sample-to-sample variation, variation in RNA integrity, amplification efficiency differences and cDNA sample loading variation (Kubista *et al.*, 2006). The normalisation of samples is commonly

done by comparing with an internal control gene, referred to as a reference (previously housekeeping) gene, whose expression levels are unaffected by different experimental conditions (Piana *et al.*, 2008). The normalisation of samples is a crucial step to eliminate technical variations during RT-qPCR and the sample C_q values are normalised to a reference gene to account for variation in sample handling and reaction efficiency (Galay-Burgos *et al.*, 2003).

An ideal reference gene is a constitutively expressed gene that does not exhibit changes in its expression between samples from various experimental conditions or time points (Taylor *et al.*, 2010). In the study presented here, the *β–actin* gene, which encodes for a muscle fibre and cell cytoskeletal structural protein, was selected as the reference gene for sample normalisation in RT-qPCR experiments. In gene expression analyses of diverse species, *β-actin* gene was revealed as one of most stable genes together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) when compared to some of the other commonly used reference genes including beta-2-microglobulin (B2M), hypoxanthine phosphoribosyl-transferase I (HPRT1), succinate dehydrogenase complex subunit A (SDHA), and tyrosine 3 monooxygenase/ tryptophane 5-monooxygenase activation protein zeta polypeptide (YWHAZ) (Piana *et al.*, 2008). The *β-actin* gene has previously been shown to be stable in earthworms following metal exposure (Spurgeon *et al.*, 2004) and it has become the prevalent reference gene in gene expression studies in earthworms (Chen *et al.*, 2011; Homa *et al.*, 2010; Liang *et al.*, 2011; Ricketts *et al.*, 2004).

The analysis of data of RT-qPCR is usually calculated by relative quantification where the quantification describes the change in expression of the target gene relative to some control group such as an non-treated control (Livak & Schmittgen, 2001). One of the methods to calculate the relative changes in gene expression is the comparative C_q method also known as the $2^{-\Delta\Delta Cq}$ method (Schmittgen & Livak, 2008). The equation $2^{-\Delta\Delta Cq}$ is used to compare the gene expression in two different samples (a treated group and an non-treated group) and the result demonstrates the fold change in expression of target gene due to treatment.

Quantifying gene expression levels through the measurement of the amount of cellular RNA has become fundamental for many molecular biological studies. However, the main critical step is the integrity of isolated RNA molecule. High quality RNA is DNA-free and must contain undegraded RNA. Degradation of RNA is typically due to cleavage by ribonucleases (RNases) during tissue sampling and isolation. Therefore, precautions must be taken during isolation in order to minimise RNA degradation. Contaminants such as proteins and/or chaotropic agents from the extraction reagents may affect the kinetics of PCR, even when present in small amounts.

2.8 P-gp functional activity assay

A bioassay using the fluorescent dye RB has been successfully used to demonstrate evidence for P-gp activity in aquatic organisms (Kurelec *et al.*, 2000; Smital *et al.*, 2000). Rhodamine B is the model substrate for P-gp (Smital *et al.*, 2003) and the quantification of its fluorescence accumulated in organs, tissues, and cells in the study can be correlated to the presence and function of P-gp. The RB bioassay can lead to a more accurate interpretation of the function and expression of the P-gp response to chemical exposure, since it measures the active protein and not intracellular pools of mRNA. The RB bioassay has been used in many experiments with marine organisms to evaluate the effects of contaminants on the P-gp activity (Shúilleabháin *et al.*, 2005; Smital & Kurelec, 1997; Timofeyev *et al.*, 2007) and in earthworms to demonstrate the P-gp activity (Brown *et al.*, 2008; Hackenberger *et al.*, 2012).

Chapter 3

ABCB1 gene in *Aporrectodea caliginosa*

3.1 Introduction

The *ABCB1* gene encodes the transmembrane protein P-gp which is a member of the large family of ABC efflux transporters responsible for cellular defence against the accumulation of drugs and natural toxins (Szakács et al., 2008). These transporters are ubiquitous in biological systems and are expressed in virtually all living organisms from bacteria to humans (Schrickx & Fink-Gremmels, 2008). None of the ABC gene family has been identified in earthworms. As the ABC genes of sequenced organisms are highly conserved between species (Loncar et al., 2010), the common approach to detect new ABC genes has been through the use of degenerate primers (Baker & Barrett, 1994; Luedeking & Koehler, 2004; Zaja *et al.*, 2008; Zucchi *et al.*, 2010) as described in section 1.4.1. Degenerate primers have been designed by alignment of published ABC gene sequences available from public databases such as GenBank (www.ncbi.nlm.nih.gov/) and, for earthworms, LUMBRIBASE (www.nematodes.org/), an on-going genome project carried out by the Gene Pool Group in Edinburgh to sequence the whole genome of *L. rubellus* (red wiggler earthworm) is available.

The hypothesis that the *ABCB1* gene is present in earthworms is supported by its ubiquitous nature and the detection of *ABCB1* in nematodes such as *C. elegans* (Broeks *et al.*, 1995; Zhao *et al.*, 2007), *H. contortus* and *Onchocerca volvulus* (Kwa *et al.*, 1998). Moreover, the detection of *ABCB1* in mussels (*Mytilus sp*) (Luedeking & Koehler, 2002) , which are phylogenetically more closely related to earthworms than to nematodes (Giribet, 2008) reinforces that earthworms should contain a homologous gene.

The aim of this study was composed of two parts: first, to identify a part of the sequence of *ABCB1* in *A. caliginosa* and second, to extend this sequence to encompass the whole gene. To achieve this, degenerate and specific primers were designed through the identification of conserved areas from *ABCB1* sequence information in different species, and to be used in the amplification by PCR. Once a putative *ABCB1* fragment was obtained, extension of the novel sequence was attempted using two approaches. The first approach used *L. rubellus* contiguous sequences from LUMBRIBASE, with high homology to the *ABCB1* fragment, to design primers to extend the novel sequence information. The second was the use of 3' RACE (Rapid Amplification of cDNA Ends).

3.2 Material and Methods

3.2.1 Detection of *ABCB1* in *A. caliginosa*

3.2.1.1 Design of primers

Degenerate primers

Five degenerate primers were designed from conserved areas identified by multiple alignment of P-gp protein sequences encoded by the *ABCB1* gene from *Caenorhabditis briggsae* (GenBank acession number XM_001672012), C*. elegans* (Pgp-1, NP_502413.1), *Cricetulus ssp* (M59254), *Homo sapiens* (MDR1, EU_854148), *Canis lupus familiaris* (MDR1, NP_001003215.1), *Pan troglodytes* (MDR1, XP_001163417.1) and *Equus caballus* (MDR1, XP_001492073.2) and areas with the low degree of degeneracy were chosen for primer design (Appendix A.1). The most suitable conserved areas for primer design were those that contained amino acids encoded by few triplet codons.

A further three degenerate primers were designed from conserved areas identified by multiple alignment of nucleotide sequences (mRNA sequences) from *Mytilus californianus* (ABCB/Pgp- like, EF_521414), *Mytilus edulis* (P-glycoprotein, AF_159717), *Mimachlamys varia* (mdr1, FM_994159), *O.mykiss* (*ABCB1*, AY_863423), *Rattus norvergicus* (*ABCB1*b, NM_012623), *Homo sapiens* (MDR1, EU_854148), *Gallus gallus* (Pgp, XM_418636) and *Trichoplax adherens* (hypothetical protein, XM_002110919) (Appendix A.2).

Alignments were performed by the software DNAMAN version 4.0 (Lynnon Biosoft, Quebec, Canada). Primers were manually selected based on the following criteria: 18-22 nucleotides in length, avoidance of three or more consecutive bases, G (guanine)-C (cytosine) content between 40-60%, G and/or C at 3' terminal region for stability, avoidance of complementarities between primers, melting temperature (Tm) in the range of 56-62˚C and similar for each pair of primers (Dieffenbach & Dvesksler, 1995; Innis *et al.*, 1990). Melting temperatures were calculated by the formula 4˚C (G+C) + 2˚C (A+T) (Wallace *et al.*, 1979). Primers were synthesised by Invitrogen Life Technologies, New Zealand. All primers were reconstituted and stored following the manufacturer's recommendations.

Primers designed using LUMBRIBASE

The *ABCB1* partial mRNA sequence (446 bp) of *M. edulis* (blue mussel) (AF_159717.1) was used as query to search LUMBRIBASE using the Basic Local Alignment Search Tool (BLAST) in order to find homologous *ABCB1* sequences in *L. rubellus*. Primers were designed manually from the returned *L. rubellus* sequence with highest homology (>50%) to the *ABCB1* mRNA from *M. edulis* and *C. elegans* (Appendix A3). The criteria for design, synthesis, reconstitution and storage of primers followed the same procedures as described in the section of the degenerate primers.

3.2.1.2 Sample collection

Two species of adult lumbricid (Familiy Lumbricidae) earthworms, *A. caliginosa* (Savigny, 1826) and *L. rubellus* (Hoffmeister, 1843) were collected for DNA extraction and used as templates for the detection of *ABCB1* gene. Adult earthworms with well-defined clitellum weighing between 300- 600 mg and measuring approximately 60 to 100 mm in length were collected from an organic apple orchard of Biological Husbandry Unit (organic farm) at Lincoln, using a spade followed by hand-sorting of the collected soil. They were collected during the months of March to November 2010 and species identification was based on the taxonomic features which are described elsewhere (http://www2.uclan.ac.uk).

Other specimens were also collected for the analysis of homology between the *ABCB1* sequences of common earthworms in New Zealand: *Aporrectodea trapezoides* and *Octolasion cyaneum* (both collected from the same area as *A. caliginosa*)*, E. fetida* (kindly donated from Patricia Fraser, AgResearch, Lincoln) *and Allolobophora chlorotica* (collected from an organic dairy farm in Ashburton). *Mytilus edulis* was collected from Dunedin Harbour and its DNA was included as positive control in PCR using degenerate primers, as well the cDNA from *C. elegans* (kindly donated from Susan Stasiuk, AgResearch, Palmerston North).

3.2.1.3 DNA extraction

Earthworms were placed on a glass Petri dish lined with moist filter paper for 48 h to void the soil content in their digestive tract and thus, to minimise DNA contamination from other soil organisms. The earthworms were briefly immersed in a Petri plate filled with sterile distilled water to remove external soil and then placed on absorbent paper to dry. A piece of approximately 10 mm length of the posterior end of worm, without digestive tract contents, was cut with a sterile blade and immediately transferred to a mortar filled with liquid nitrogen, ground to a fine powder and approximately 10 mg of frozen tissue was transferred into a 1.7 ml tube containing 300 µl of cell lysis solution (Puregene™, Gentra Systems, Minneapolis, USA). The DNA extraction followed the manufacturer's instructions for the solid tissue protocol with a few modifications as described below: 1.5 µl of Proteinase K Solution (Fermentas, 900 U/ml, 20.2 mg/ml) was added to the lysate, mixed by inverting 25 times and incubated at 55˚C overnight; followed by the addition of 160 µl of Protein Precipitation Solution to the lysate, and the pellet was hydrated with 20 µl of nuclease–free water (Ultrapure distilled water, Gibco) and incubated overnight at 4˚C.

The purity and quantity of the DNA extract were measured by spectrophotometry (NanoDrop® Technologies Inc., Delaware, USA). To confirm the extracted DNA was of high molecular weight and undegraded, it was run in a non-denaturing 1% agarose (Agarose Molecular Grade Bioline) gel. Two µl of extract was added to 6 µl of nuclease-free water and 3 µl of loading buffer (0.25 %

bromophenol blue, 0.25 % xylene cyanol and 40% sucrose in water) and loaded into each well alongside 5 µl of a 1 kb plus DNA ladder (Invitrogen, Carlsbad, USA). The gel was run at 10 V/cm for 45 min in 1× TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.0). The gel was stained with ethidium bromide (0.5 µg ml^{-1}) for 10 min and rinsed with tap water 3 times. Bands were visualised using a VersaDoc™ model 3000 imaging system (BIO-RAD). Subsequently, all samples were adjusted to a pre-determined concentration (10 ng/µl). DNA extracts were stored at -20˚Cuntil further PCR. The same extraction procedure was used for the frozen tissue of *M. edulis.*

3.2.1.4 Polymerase chain reaction (PCR)

Gradient PCR

A gradient PCR was run initially for each pair of primers in order to determine the optimal annealing temperature to minimise the amplification of nonspecific products. The annealing temperatures were 48.5, 49.1, 52, 55.9, 59.6 and 61.9°C. Each 25 µl of reaction contained 1.25 U of FastStart Taq DNA Polymerase (5 U/ µl Roche Applied Science, Mannheim, Germany), 1 × buffer [500mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20mM $MgCl_2$, pH 8.3], 2 mM of $MgCl_2$, 200 µM of each dNTP (Fermentas Life Sciences, Cat # R0192), 2 µM of the forward primer, 2 µm of the reverse primer, nuclease-free water (Ultrapure distilled water, Gibco) and 20 ng of DNA. A negative control (only water) was included for each PCR. The thermal cycler was set as follows: initial denaturation at 94˚C for 4 min; followed by 40 cycles of denaturation at 94˚C for 45 s, annealing at different temperatures for 45 s and extension at 72˚C for 45 s; and a final elongation step at 72˚C for 7 min. Ten µl of each PCR product was mixed with 3 µl of loading buffer and loaded into a non-denaturing 1.3% agarose gel alongside and 5 µl of a 1 kb plus DNA ladder run at 10 V/cm for 1 h in 1× TAE. Gel was stained and visualised as described in section 3.2.1.3.

PCR optimisation

The amplification with degenerate primers required further optimisation of PCR components to produce single amplicons. The optimisation comprised modifications to the reagent concentrations and cycler parameters of the standard PCR as follows. In the absence of PCR products, the $MgCl_2$ concentration was increased from 2.0 mM to 2.5 mM and the cycle number was increased from 40 to 45. In the event of multiple bands, the template in the reaction mix was decreased from 2 µl to 1 µl and the cycle number decreased from 40 to 35.

Product isolation

In the event that a band of expected size was present among nonspecific products, this band was excised from the agarose gel under ultraviolet (UV) illumination using a sterile scalpel blade. The band was placed in a sterile 1.7 ml tube with 15 µl of nuclease-free water. The band was used either as a template in re-amplification by using 1 µl of melted solution after 95˚C for 2 min or the PCR

product was sequenced directly after purification using the HiYieldTM Gel/PCR DNA extraction kit (Real-Biotech, Taiwan) according to the manufacturer's protocol.

Another approach to produce single amplicons from nonspecific products was the use of nested PCR with primers designed from a region within the amplicon sequence of previous PCR. The master mix and cycler settings were the same as the first PCR and the template for the nested PCR was 1 µl of the first PCR product diluted 1:500 or 1:1000 in water.

3.2.1.5 Sequencing

PCR products were sequenced directly at Lincoln University Sequencing Facility. Sequencing was carried on an ABI Prism 3100-Avant Genetic Analyser installed with a 4 capillary 80 cm array using Performance Optimized Polymer 4 (POP4). Products were sequenced using the original primers on both forward and reverse orientations. Chomatograms were analysed by software Chromas Lite 2.1 (Technelysium PTY Ltd) and areas with ambiguous bases were removed. Trimmed sequences were aligned with DNAMAN version 4.0 software and identities were determined with BLAST using the online nucleotide database, the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For the analysis of the *ABCB1* sequences of different earthworm species, a phylogenetic tree was created using the software MEGA version 4.0 (Tamura *et al.*, 2007).

3.2.2 Extension of novel sequence

Two approaches were used to extend the sequence data of the *ABCB1* novel sequence as follows.

3.2.2.1 Identification of *ABCB1* conserved areas

The first approach was the design of primers located in conserved areas of *ABCB1* homologues identified by alignments between *L. rubellus* contiguous sequences (LUMBRIBASE) and the *ABCB1* sequences of different species. Conserved areas in the downstream regions from novel sequence were used to design new reverse primers to be used in combination with a specific *A. caliginosa ABCB1* forward primer.

The partial mRNA sequence of *M. edulis ABCB1* (AF_159717.1) was used as query in the LUMBRIBASE database. Returned *L. rubellus* contiguous sequences that were overlapping (>65% of similarities) were assembled into one sequence using DNAMAN 4.0 (Appendix A.4). The translated assembled sequence was compared against the *M. edulis ABCB1* protein sequence by the program WISE2 (http://www.ebi.ac.uk/Tools/Wise2/) and homologous areas were identified (Appendix A.5). From these homologous areas, one reverse primer was manually designed using the same criteria on section 3.2.1.1. The new reverse primer was used in combination with forward *ABCB1*-AC primer for *A. caliginosa* template or Lum_*ABCB1*F1 for *L. rubellus*.

Also, the novel *A. caliginosa* sequence was used as query in the LUMBRIBASE database. The returned *L. rubellus* contiguous sequence with highest similarity was then used to query the GenBank protein database (BLASTx) to find other similar sequences. All sequences were aligned and conserved areas were identified (Appendix A.6). Two reverse primers were manually chosen within conserved areas using the same criteria on section 3.2.1.1. Each reverse primer was used in combination with forward *ABCB1*-AC primer to amplify *A. caliginosa* DNA. *Lumbricus rubellus* DNA was included as positive control.

For both strategies, the PCR mixture and cycler parameters were as described in section 3.2.1.4. Any PCR products were directly sequenced and/or cloned and sequenced.

3.2.2.2 Rapid amplification of cDNA ends (3'RACE)

The second approach was the method of rapid amplification of cDNA ends (3'RACE) using the kit 5'/3'RACE kit - 2nd Generation (Roche Applied Science, Germany). The 3'RACE method is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and the 3´ end of the mRNA (Zhang & Frohman, 1998). This method takes advantage of the natural poly (A) tail of mRNA to be a generic priming site for PCR. The specific *A. caliginosa ABCB1* forward primer was used together with generic primer to amplify the unknown flanking regions, allowing the extension of sequence information.

RNA extraction

RNA extraction required general precautions to minimise the contamination by RNAses including wearing gloves during all steps and changing them often, using RNAse-free glassware treated with diethylpyrocarbonate (DEPC at 0.1% in distilled water), commercially supplied RNase and DNase- free plasticware and water, a set of pipettes specific for RNA use and barrier pipette tips.

One randomly chosen earthworm was placed for 48 h on moist filter paper to void digestive tract contents, then briefly washed in a Petri plate filled with sterile distilled water to remove external soil and minimise contamination by RNAses from soil. The earthworm was placed on absorbent paper and a piece of the posterior end (caudal extremity) measuring 10-12 mm in length and weighing 50-60 mg was removed with a sterile scalpel blade. The excised piece was placed into a mortar filled with liquid nitrogen and ground to a fine powder with a pestle and quickly transferred to a 1.7 ml tube. Total RNA was extracted using the Nucleic Acid Isolation kit (E.Z.N.A.® Mollusc RNA isolation Kit, Omega Biotek Inc., Georgia, U.S.A.) according to the short protocol of the manufacturer's instructions with a few modifications. The modifications were the addition of 500 µl of buffer RB/2-mercaptoethanol (at step 2) rather than 350 µl, 500 µl of 70% ethanol gently mixed with a pipette tip (at step 4) rather than vortexing and (before step 5) an additional centrifugation at 10, 000 × g for 15 s at room temperature (RT), then the flow-through liquid discarded and centrifugation repeated

for the remaining mixture. The first elution was with 50 µl of RNAse/DNase –free water, then 2 min incubation at RT prior to centrifugation at maximum speed for 1 min. The elution step was repeated using the first eluate into the same tube. Isolated RNA was placed on ice.

The quantity and quality of the extracted RNA were determined by spectrophotometry and by agarose gel electrophoresis. Ten µl of RNA extract was mixed with 3 µl of loading buffer and loaded into a non-denaturing 1.3% agarose gel alongside 5 µL of the 1 kb plus DNA ladder. The gel was run at 10 V/cm for 1 h in 1 × TAE. The gel was stained and visualised as described in section 3.2.1.3. An aliquot of the RNA extract was separated for subsequent cDNA synthesis and the remaining volume was immediately stored at -80°C.

cDNA Synthesis and amplification

Following the 3'RACE protocol, the mRNA component of the total RNA extract was synthesised into cDNA using reverse transcriptase and an oligo-dT anchor primer that targeted the poly (A) tail region. One µl of RNase inhibitor (RNaseOUT™ Recombinant Ribonuclease Inhibitor; Invitrogen) was added to the cDNA synthesis reaction. After first-strand cDNA synthesis, the specific cDNA was then amplified by PCR using the *ABCB1* forward primer specific to *A. caliginosa* (section 3.3.1) and the supplied anchor primer. The master mix was prepared according to the manufacturer's 3'RACE protocol and the thermal cycle was as follows: initial denaturation 94˚C for 3 min, followed by 40 cycles of 94˚C for 1 min, 60˚C for 30 s, 72˚C for 3 min and one final elongation step at 72˚C for 7 min. After amplification, the PCR product was used in a nested PCR with forward primer AC71rc - 5' GGCGCTGTTATGATTGACGC 3' and reverse anchor primer in order to isolate the target amplicon from the multiple products. The templates were two concentrations of diluted first PCR product in water (1:500 and 1:1000) and an excised band. The nested PCR product was cloned.

3.2.2.3 Cloning

PCR products were cloned into plasmid pGEM®-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instructions. The ligation reaction consisted of 1 µl of PCR product combined with 5 µl of 2 X Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector (50 ng), 1 µl of T4 DNA Ligase (3 Weiss units/ µl) and 2 µl of nuclease-free water. The positive control contained 1 µl of Control Insert DNA instead of PCR product and the negative control used water as the insert. All reactions were mixed by pipetting and incubated overnight at 4˚C.

For transformation, JM109 High-Efficiency Competent cells (Promega, Madison, WI, USA) were used. Two µl of each ligation reaction was transferred to a sterile 1.7 ml tube and kept on ice. The competent cells were allowed to thaw on ice and mixed by gently flicking the tube. Fifty µl of cells were transferred to each ligation reaction and mixed by stirring with a pipette tip. The reactions were then incubated for 30 min on ice, followed by heat-shock treatment at 42˚C for 40 s, and transferred

to ice for 2 min. Two hundred and fifty µl of SOC medium (Appendix C.1) was added to the ligation mixture and placed on the shaker (1,400 rpm, Labnet 211DS) at 37˚C for 1 h. Two volumes, 40 µl and 100 µl, of each transformation culture were plated onto LB/ampicillin plates previously spread with 40 µl of 5-bromo-4-chloro-3-indolyl-b-D-galactosidebromo-chloro-indolyl-galactopyranosisde (X-Gal) (Appendix C.2). Plates were incubated at 37˚C overnight.

Transformed colonies (white colonies) were randomly chosen and amplified by colony PCR using primers complementary to the plasmid region of insertion. Primers were M13 F (5'CTGGCCGTCGTTTTAC 3') and M13 R (5' CAGGAAACAGCTATGAC 3') at a final concentration of 10 µM. The thermal cycler was set as follows: 94˚C for 5 min, then 35 cycles of 94˚C for 30 s, 60˚C for 20 s, 72˚C 1 min and one final extension of 72˚C for 10 min. PCR products of expected size (product size + 257 bp flanking) were sequenced.

3.3 Results

3.3.1 Detection of *ABCB1* gene in *A. caliginosa*

Degenerate primers

Alignment of *ABCB1* amino acid sequences of seven different species identified conserved areas which were used to design five degenerate primers: DEG2487 (forward) 5'GGIGAYAARATIGGIATG 3', DEG6744 (forward) 5' ATIGTIWSICARGARCC 3', DEG6983 (forward) 5' WSIGGIGGICARAARCA 3', DEG2667 (reverse) 5' IACIKYICCIGCYTTIGCRTA 3' and DEG7127 (reverse) 5' GIGCIATIACIATRCAIGT 3' (see appendix A.1 for alignment details). Alignment of the *ABCB1* nucleotide sequences of eight different species was used to design a further three degenerate primers: DEG 1526 (forward) 5' TICAGAGRYTCTAYGAYCC 3', DEG 1804 (reverse) 5' AIICIYTGYTTYTGYCCHCC 3' and DEG 1869 (reverse) 5' GCWGAIGTDGCYTCATCCA 3' (see Appendix A.2 for alignment details). These primers were used in PCR in the combinations listed in table 3.1.

Table 3-1 Sets of degenerate primers used to amplify a partial sequence of *ABCB1* in earthworms.

Set	Pair of primers	Expected amplicon size
A	DEG2487 and DEG2667	180 bp
B	DEG6744 and DEG7127	383 bp
C	DEG6983 and DEG7127	144 bp
D	DEG 1526 and DEG 1804	276 bp
E	DEG 1526 and DEG 1869	343 bp

Using *A. caliginosa* DNA as template none of the primer combinations produced a band with homology to the *ABCB1* gene. Set A amplified a single product of the expected size at an annealing

temperature at 52˚C (Figure 3.1a) which produced ambiguous sequencing results. Set B produced two bands (300 and 650 bp) but these were not the expected 383 bp product (Figure 3.1b). Set C produced one band at the expected size of approximately 144 bp (Figure 3.1c). Following band excision and re-amplification the product was sequenced but produced weak peak signals and high background. Set D produced multiple nonspecific products (Figure 3.1d). Set E produced one distinct band with a greater size than the expected 343 bp (Figure 3.1e) and when sequenced a translated BLAST search showed that it was similar to the protein dynein, axonemal heavy chain 5 from many species.

Caenorhabiditis elegans cDNA and *M. edulis* genomic DNA were used as a positive control. PCR with primer sets D and E produced an amplicon of the expected size in both templates. The single product from set E of *C. elegans* cDNA (Figure 3.b b) was sequenced directly. The product had 98% identity with the sequence of a P-gp related transporter family member in *C. elegans* (GenBank NP_506927.2, results not shown).

Primers designed using LUMBRIBASE

The search in LUMBRIBASE (*L. rubellus* genome) with *ABCB1* partial mRNA of *M. edulis* returned one matching contiguous sequence (contig_344933 of 858 bp) with 54.47% identity and 58.1% coverage, of which the predicted genes were ABC transporters. The alignment between the returned *L. rubellus* contiguous sequence, *C. elegans ABCB1* mRNA (NM_070012.3), and *M. edulis ABCB1* partial mRNA indicated the *ABCB1* homologous regions within the contiguous sequence (see appendix A.3 for alignment details). From the homologous areas, two forward and two reverse primers were designed as following: Lum_*ABCB1*/F1 5'GATACTGAACGGTCTGAACGT3'; Lum_*ABCB1*/F95 5'ATCCAACGCTTCTACGACCC 3'; Lum_*ABCB1*/R255 5'TCCTTCTCTCCCGTATCGAAT3' and Lum_*ABCB1*/R281 5'TCTATCTCAGCCTGGCTCAC3'. The primers were used in sets as illustrated on table 3.2.

Table 3-2 Primer sets to amplify a partial sequence of *ABCB1* gene in *Lumbricus rubellus*.

Set	Pair of primers	Expected amplicon size
F	Lum_*ABCB1*F1 Lum_*ABCB1*R255	255 bp
G	Lum_*ABCB1*F1 Lum_*ABCB1*R281	281 bp
H	Lum_*ABCB1*F95 Lum_*ABCB1*R255	160 bp
I	Lum_*ABCB1*F95 Lum_*ABCB1*R281	186 bp

All sets amplified products of expected size using *L. rubellus* and *A. caliginosa* DNA (Figure 3.2). The sequencing results of *L. rubellus* and *A. caliginosa* (see sequence results on appendixes B.1.1 and B1.2) products in the BLASTx search resulted in matches with high homology to protein sequences of

P-gp in other species. The most relevant matches are on table 3.3 according criteria of highest degree of similarity and lower E parameter values.

Table 3-3 BLASTx search results of novel sequences of *Lumbricus rubellus* (278bp) and *Aporrectodea caliginosa* (208 bp) with highest identity to known protein in other species with query coverage > 94% and E values < 1^{-26}*.

Novel sequence	*Species*	*Protein Name*	*Amino acid identity*
L. rubellus	*Branchiostoma floridae* Florida lancelet	hypothetical protein **(XP_002593897.1)	77%
	Mus musculus house mouse	*ABCB1*(NP_035205.1)	73%
	Strongylocentrotus purpuratus Sea urchin	*ABCB1*a (AFD10328.1)	72%
	Rattus norvegicus Norway rat	*ABCB1*b protein(AAI07561.1)	72%
A. caliginosa	*Branchiostoma floridae* Florida lancelet	hypothetical protein (XP_002593897.1)**	84%
	Trematomus bernacchii Antartic fish	*ABCB1*(ACX30417.1)	81%
	Poeciliopsis lucida clearfin fish	P-glycoprotein(ADQ20481.1)	79%
	Strongylocentrotus purpuratus sea urchin	ATP-binding cassette transporter *ABCB1*a(AFD10328.1)	79%

Expect value (E parameter) represents the number of times this match or a better one would be expected to occur purely by chance in a search of the entire database. Thus, the lower the E value, the greater the similarity between the input sequence and the match.
** contains several recognised ABCB motifs.

The novel fragment of *A. caliginosa* sequence was used to design specific primers: *ABCB1*-AC forward 5' TCGGGTCGAGCGGGTGTG 3' and *ABCB1*-AC reverse 5' GCAAACAGCACTGGTTCCTG 3' with a product of 166 bp. The optimal annealing temperature was set to 60˚C after gradient PCR.

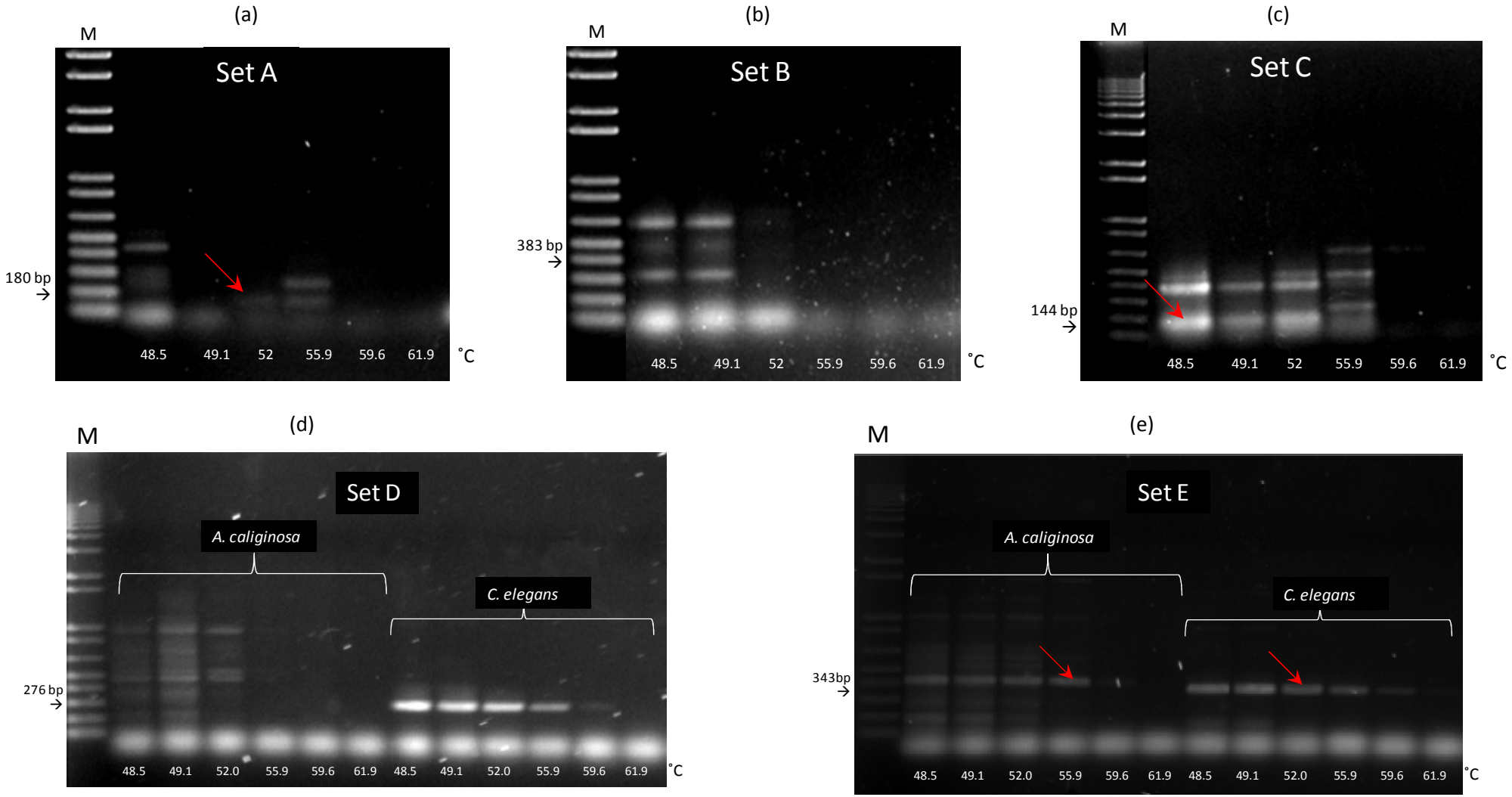

Figure 3-1 Gel electrophoresis of amplicons produced by gradient PCR of *Aporrectodea caliginosa* DNA and *Caenorhabditis elegans* cDNA (with different annealing temperatures denoted at the base of the gels) with degenerate primer sets A (a) , set B (b), set C (c), D (d) and E (e) to amplify a partial sequence of *ABCB1* gene. The black arrow indicates the expected product size and the red arrow indicates product that was sequenced. M is the molecular marker.

Primer set F also amplified products of expected size using DNA from other common earthworms, namely, *A. trapezoides*, *O. cyaneum*, *E. fetida* and *A. chlorotica* (see sequence results on appendix B.1). The sequence of these PCR products also showed high homology to *ABCB1* proteins (data not shown). The neighbour joining tree generated from the novel sequences of six species of pasture earthworms and three homologous sequences of other species (*M. edulis, R. norvergicus* and *C. elegans*) showed that all earthworm sequences clustered together with > 94% similarity (Figure 3.3).

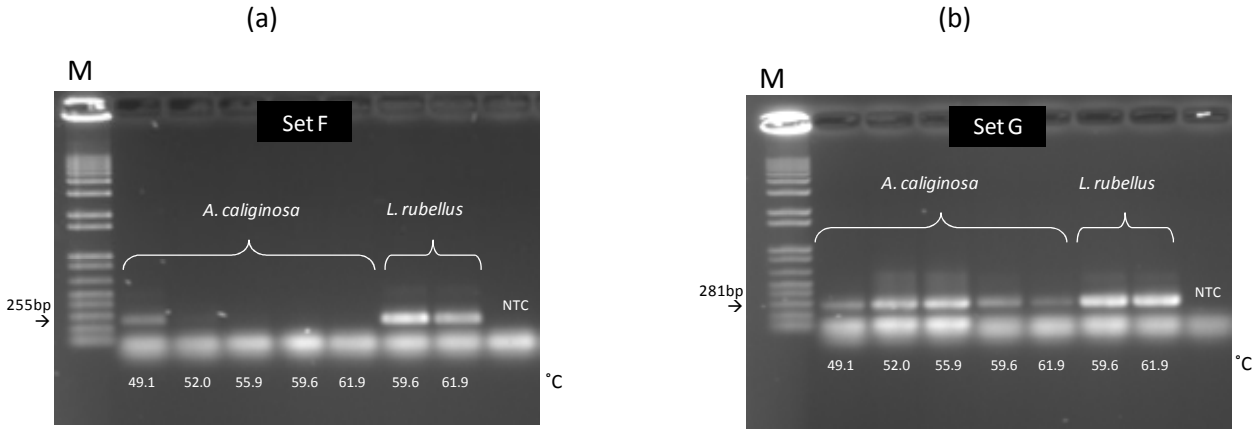

Figure 3-2 Gel electrophoresis of amplicons produced by gradient PCR (increasing annealing temperatures) with specific primer sets F (a) and set G (b) using *Aporrectodea caliginosa* DNA and *Lumbricus rubellus* DNA to amplify partial sequence of *ABCB1* gene. The black arrow indicates the expected product size. M is the molecular marker and NTC is non-template control using water as template**.**

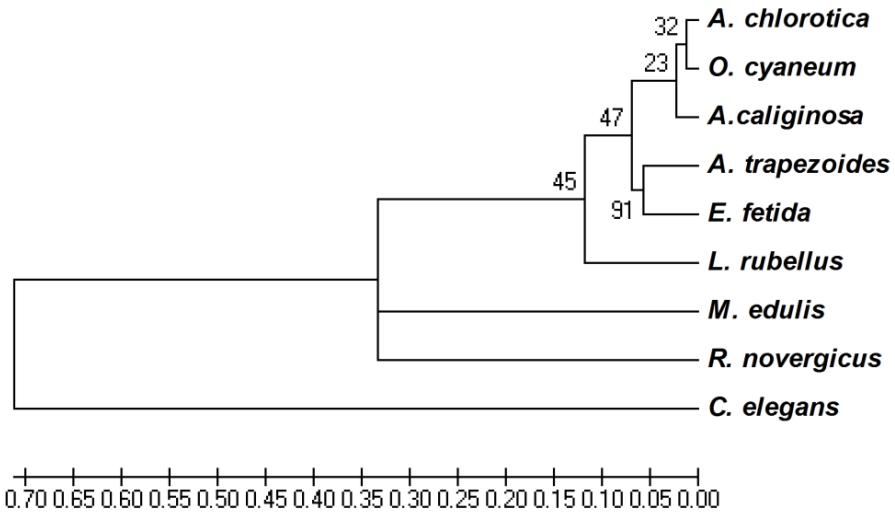

Figure 3-3 Neighbour joining tree of novel sequences homologous to the conserved ATPase domain of *ABCB1* transporter of six species of pasture earthworms (*Alllopobhora. chlorotica, Octolasion cyaneum, Aporrectodea caliginosa, Aporrectodea trapezoides , Eisenia fetida and Lumbricus rubellus* and the *Mytillus edulis ABCB1* (Genbank acession number: AF159717.1), *Rattus norvergicus ABCB1*b (NM_012623.2) and *Caenorhabditis elegans* P-gp1 (NM_070012.3). Bootstrap values are given above the branches based on 1000 bootstrap replicates, using MEGA version 4.0 (Tamura *et al,* 2007).

3.3.2 **Extension of novel sequence**

Identification of *ABCB1* conserved areas to design primers

The first strategy, using *M. edulis ABCB1* to query LUMBRIBASE, resulted in three overlapping *L. rubellus* contiguous sequences (contigs_ 121673, 154463 and 1166) which were assembled into one sequence of 4776 bp (Appendix A.4). Comparison between the *M. edulis ABCB1* protein sequence and the 4776 bp contiguous sequence (Wise2 program) showed that there was a homologous 135 aa sequence with 72.6% identity that was split across two putative exons (Figure 3.3). The two exons within the contiguous sequence were designated here as *L. rubellus* exon 1 (nucleotides 3616-3850 of assembled sequence) and exon 2 (4360-4531). The novel *A. caliginosa ABCB1* sequence was located at *L. rubellus* exon 1, and in order to extend the *A. caliginosa* sequence, a new reverse primer was designed from the *L. rubellus* exon 2 sequence (Lum_exon2: 5'GAGAGCAGACGTMGCTTCATC3') (Figure 3.4).

Amplification of *A. caliginosa* cDNA using the forward *ABCB1*-AC primer (located in putative exon 1 region), and Lum_exon2 produced an amplicon of the expected size (411 bp) (see sequencing results on Appendix B.2.1). Amplification of *A. caliginosa* DNA produced two products with different sizes from the expected size of 920 bp (Figure 3.5). Only the smallest product (between 500 and 650bp) could be reamplified and the resultant sequence was not homologous to *ABCB1* from any species. Amplification with *L. rubellus* DNA using forward Lum_*ABCB1*F1 (based on putative exon1) and Lum_exon2 resulted in an amplicon of the expected size (974 bp) (Appendix B.2.2). The BLAST search of translated sequences of *L. rubellus* DNA and *A. caliginosa* cDNA matched protein sequences of P-gp in other species (Table 3.4).

Multiple Protein Sequence Alignment
Lumbricus rubellus assembled contig sequence, *Caenorhabiditis elegans* P-gp, *Mytilus edulis* P-gp and novel *Aporrectodea caliginosa* P-gp

```
ORIGIN
L. rubellus          ILnGlsLtiakGQTVALVGSSGCGKSTvIqLvqRfYDpLd
M. edulis            ..............................qRfYDpda
C. elegans           ILRGMNLRVNAGQTVALVGSSGCGKSTIISLLLRYYDVLK
A. caliginosa        ................VGSSGCGKSTvIqLiqRfYDpLe

L. rubellus          GcvmIDGtDiRelNikwLRqNigVVSQEPiLFatTIaENI
M. edulis            GqvllDGnnikDlNLnwLRqNigVVSQEPvLFgCTIaENI
C. elegans           GKITIDGVDVRDINLEFLRKNVAVVSQEPALFNCTIEENI
A. caliginosa        GavmIDGtDiRqlNikwLRqhigVVSQEPvLFatTIaENI

L. rubellus          ryGrEGvTdaEiekAaieANAhnFIskLPl<--Intron--
M. edulis            rLGnpnaTitEieqAaKqANAhdFIKsLPq
C. elegans           SLGKEGITREEMVAACKMANAEKFIKTLPN
A. caliginosa        ryGK

L. rubellus          --->kYgTLVGeRGaQLSGGQKQRIAIARALVRdPrILLL
M. edulis                sYNTLVGeRGaQLSGGQKQRvAIARALiRdPrILLL
C. elegans               GYNTLVGDRGTQLSGGQKQRIAIARALVRNPKILLL

L. rubellus          DEATSALDtESEatVQsALDK..................
M. edulis            DEATSALDsESEnIVQeALeK..................
C. elegans           DEATSALDAESEGIVQQALDK..................
```

First exon

Second exon

Figure 3-4 Alignment of amino acid sequences of *Lumbricus rubellus* contiguous sequence, *Mytilus edulis* P-gp, *Caenorhabditis elegans* P-glycoproteins and the translated sequence of the novel *Aporrectodea caliginosa* sequence. Capital letters denote homology between all four sequences, red letters show homology between *Lumbricus rubellus* and *Mytilus. edulis*, green letters between *Lumbricus rubellus* and *Caenorhabditis elegans* and blue are between *Lumbricus rubellus* and *Aporrectodea. caliginosa*. The *Lumbricus rubellus* sequence in a box was chosen to design the reverse Lum_exon2 primer.

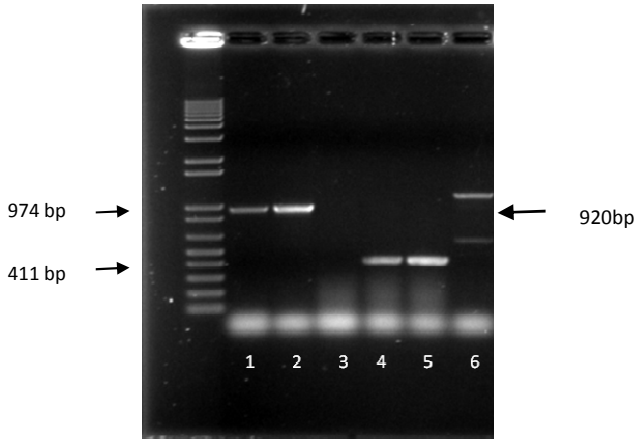

Figure 3-5 Gel electrophoresis of PCR products amplified by primers targeting the *ABCB1* gene. Lanes 1 and 2 are *Lumbricus rubellus* DNA amplified by Lum_*ABCB1*F1 and Lum_exon2 primer with expected product size of 974 bp. Lane 3 is *Lumbricus rubellus* DNA amplified by *ABCB1*-AC and Lum_exon2 with expected product size of 920 bp. Lane 4 and 5 are *Aporrectodea caliginosa* cDNA amplified by *ABCB1*-AC and Lum_exon2 with expected product size of 411 bp and lane 6 is from *Aporrectodea caliginosa* DNA amplified by *ABCB1*-AC and Lum_exon2 with expected product size of 920 bp. Black arrows indicate the expected product size.

Table 3-4 BLAST search results of *Aporrectodea caliginosa* translated sequence (134 amino acids) with highest identity to known proteins in other species with query coverage > 94% and E values < 1^{-58} .

Species	***Protein Name***	***Amino acid identity***
Branchiostoma floridae Florida lancelet	hypothetical protein* (BRAFLDRAFT_131055)	87%
Strongylocentrotus purpuratus *sea urchin*	*ABCB1*a (AFD10328.1)	83%
Danio rerio zebra fish	ABCB4 (NP_00110855)	80%
Heterocephalus glaber naked mole rat	Multidrug resistance protein (EHB12084)	80%
Poeciliopsis lucida freshwater fish	P-glycoprotein (ADQ20481.1)	80%
Crassostrea ariakensis Asian oyster	P-glycoprotein (AET34454.1)	79%
Xiphophorus hellerii swordtail fish	P-glycoprotein (AEV93606.1)	79%
Rattus norvegicus Norway rat	*ABCB1*b (AAI07561.1)	78%

* contains several recognised ABCB motifs.

The alignment of the novel translated *L. rubellus* sequence (974bp) and *A. caliginosa* (411bp) with the protein sequence of *R. norvergicus ABCB1*b (GenBank AAI07561.1) confirmed the presence of two putative exons (see alignment in appendix B.2.3). The exons encoded 110 and 36 amino acids (aa) in *L. rubellus* and 92 and 42 aa in *A. caliginosa*. A 508 bp intron was present in *L. rubellus* and exons had 97.9% and 100% homology respectively.

The typical conserved sub-domains of P-gp (Lawson *et al.*, 2008) were identified in *A. caliginosa* by the alignment with *R. norvergicus ABCB1*, *S. purpuratus ABCB1*a and *D. rerio ABCB4* sequences. The motifs are shown in the figure 3.6.

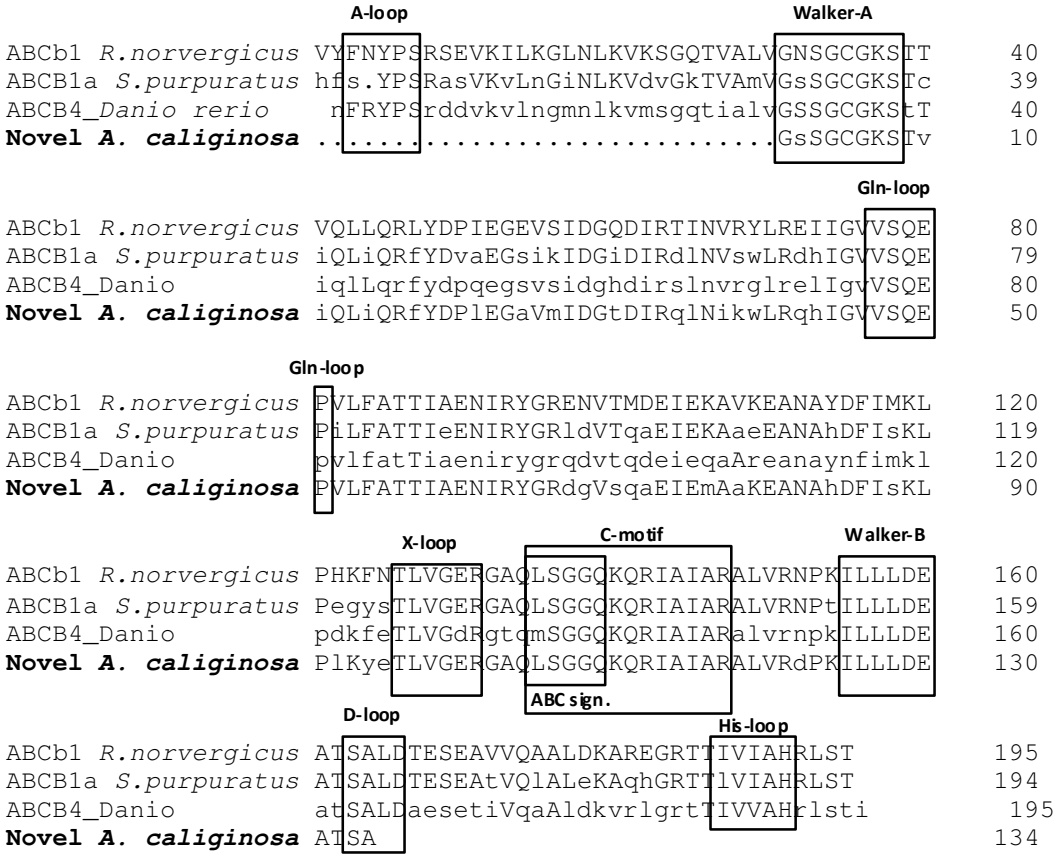

Figure 3-6 Alignment of P-glycoprotein amino acid sequences of *Rattus novergicus* (AAI07561.1*), Strongylocentrotus purpuratus ABCB1*a (AFD10328.1) and *Danio rerio* ABCB4 (NP_00110855) and the translated novel *Aporrectodea caliginosa* sequence. In the boxes are the conserved ABC transporters motifs in P-glycoprotein (Lawson *et al.*, 2008)

The second strategy, using the *A. caliginosa* novel sequence (208 bp), resulted in one contiguous sequence (contig 76678) of 1730 bp with 87% identity to the *A. caliginosa* novel sequence and 84% identity to both *ABCB1* protein sequences, *B. floridae* (XM_002593851.1) and *R. norvergicus* (BC_107560.1). The multiple sequence alignment of *L. rubellus* contiguous sequence, *B. floridae* and *R. norvergicus* identified a conserved area of approximately 180 bp, located at 760 bp downstream from the novel *A. caliginosa* sequence (Appendix A.6). Two reverse primers were designed from the conserved region: BR1 5'GCGATTCTTTGCTTCTGTCC3' and BR2 5'ACCTTGTCGAGAGCCGCC3'. The amplification in *L. rubellus* DNA with *ABCB1*-AC forward and BR2 reverse resulted in a product of approximately 900 bp. BLASTx search resulted in 50% identity to a predicted sequence of similar to ORF2 encoded protein.

3' RACE

The first rapid amplification of cDNA ends using *A. caliginosa* cDNA resulted in a strong product of approximately 1650 bp among multiple products with weaker signals. Following nested PCR, one main 750 bp amplicon was produced which was sequenced. The translated nucleotide sequence showed 92% identity (16% coverage) with methyl-coenzyme M reductase alpha subunit.

3.4 Discussion

3.4.1 Novel sequence

This work describes the discovery of a partial sequence of the *ABCB1* gene in the two most abundant species of earthworms (*A. caliginosa* and *L. rubellus*) found in the pastures of New Zealand, using a combined approach of degenerate PCR and sequence retrieval from the draft *L. rubellus* genome LUMBRIBASE. The novel translated sequence(s) has high amino acid sequence identity (>78%) to P-gp involved in the drug resistance mechanism, encoded by *ABCB1* gene in vertebrate and invertebrate species. According to Hennessy *et al* (2007), homologies at protein level that achieves at least 40% identity compared to known sequences of other species predicts the gene family affiliation. Therefore, for the first time, a new coding sequence of an *ABCB1* gene has been identified from the organisms of Lumbricidae family.

The novel coding sequences from *A. caliginosa*, the species of interest of this study, and the positive control *L. rubellus* shared high similarity. The novel sequences of *A. caliginosa* and *L. rubellus* comprised two putative exons that encompass 10.5% and 12% of the *ABCB1* coding region found in other species respectively (411 and 466 bp out of 3883 bp). The comparison of the lumbricid translated sequences with proteins of other species revealed high homology to P-gp of mammals and aquatic organisms. The highest homology (84%) was with a hypothetical protein sequence from a marine invertebrate (*Branchiostoma floridae*, Florida lancelet), whose genome has been sequenced, but for which not all genes have been annotated. Although this hypothetical protein has not yet been fully characterised, it has the domain architecture of a typical eukaryotic ABC full transporter, composed of two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) (Lawson *et al.*, 2008), including the main ABC signatures within the conserved NBDs of ABC subfamily B (Biemans-Oldehinkel *et al.*, 2006). Since ABC transporters are classified based on the sequences and organisation of their NBDs (Dean *et al.*, 2001), the sequence of the *B. floridae* strongly indicates it is a Pgp-like protein.

The lumbricid novel translated sequences contain the conserved NBD sub-domains (highly conserved sequence motifs), typically found in P-gp sequences, including the classical ABC motifs: Walker-A and Walker-B. Both motifs are conserved among all ABC transporter superfamily members, as well as

other ATP-binding proteins (Leslie *et al.*, 2005). Furthermore, the novel sequences contain the signature sequence "LSGGQ", which is a highly conserved amino acid sequence within another classical sub-domain, the C-motif. This signature distinguishes the novel sequences as an ABC transporter rather than other ATP-binding proteins (Ambudkar *et al.*, 2006; Lawson *et al.*, 2008). In addition, the novel sequences contain sub-domains of subfamily B: the complete motifs Gln-loop and X-loop, and partial sequence of D-loop. The high level of conservation within ABCB signatures was also reported in the multi-alignment of *ABCB1* sequences of *Trematomus bernacchii*, *O. mykiss* and *Homo sapiens* (Zucchi *et al.,*2010). Overall, five complete motifs and one partial within the novel 134 amino acid sequence, were identified from the eight established motifs typically found along the 200 amino acid sequence of conserved sub-domains in P-gp (Feldstein *et al.*, 2006; Luedeking & Koehler, 2002). This information strongly suggests that the *A. caliginosa* and *L. rubellus* novel sequences encode P-gp.

In mammals, there are two classes of P-gp (Sturm & Segner, 2005). P-glycoprotein class 1 is involved in the xenobiotic transport and is encoded by the *ABCB1* gene, and P-gp class 2 is mainly expressed in the liver and transports the bile component phosphatidylcholine, exclusively in mammals. P-glycoprotein 2 is encoded by the *ABCB4* gene and shares high homology to *ABCB1*, including the presence of conserved motifs mentioned above. However, in a phylogenetic analysis comparing ABC proteins of birds and fish to human ABC proteins it was reported that *ABCB4* gene is lacking in birds and fish (Annilo *et al.*, 2006) . This result is supported by the fact that non-mammal species do not contain phosphatidylcholine in their systems, which strongly suggests the *ABCB4* is also absent in invertebrates (Heumann et al., 2012). Moreover, numerous studies in invertebrates demonstrated the presence of *ABCB1*-like P-gps capable of drug transport, but not ABCB4-like P-gps (Heumann et al., 2012). Therefore, the novel sequence, although showing homology to *ABCB4* gene, is more likely to be part of *ABCB1* gene as earthworms do not have the bile component in their system.

Among the *ABCB1* gene family, there are closely related genes encoding for P-gp isoforms involved in the multidrug resistance. Rodents have two P-gp isoforms, Mdr1a (encoded by *ABCB1*a) and Mdr1b (encoded by *ABCB1*b) which share approximately 85% amino acid identity with each other, and share a similar physiological function (Schinkel, 1997). The novel sequences were homologous to a common region of both isoforms of Norway rat (*R. norvergicus*) but, due to its short length; the homology of lumbricid sequences could not be specifically associated to one of the rat isoforms. Until now, there is no report that earthworms have more than one P-gp. However, their presence is hypothesized since multiple P-gp isoforms have been found in the nematode *C. elegans* (Zhao *et al.*, 2007), and in the sea squirt *Ciona intestinalis* (Annilo *et al.*, 2006). *C. intestinalis* has two P-gp-like proteins that are 95% similar, 94% at the nucleotide level of the predicted transcript, and share

approximately 70% similarity at genomic sequence level. The presence of isoforms in lumbricids is unknown and requires further investigation.

In this study, new genetic information related to *ABCB1* gene in other pasture earthworm species (*A. trapezoides, A. chlorotica, E. fetida and O. cyaneum*) was also obtained. Primers based on *L. rubellus* sequence amplified a 208 bp fragment in all six species, resulting in translated sequences comprising one putative exon with high homology to P-gp in other species. In the phylogenetic analysis of the six species, a high degree of conservation within NBD was seen at nucleotide level (> 94% similarity). This outcome is probably because the organisms belong to the same family (Lumbricidae) and was not observed between more phylogenetically distant species used for the design of degenerate primers. This high similarity will be advantageous in future studies characterising the ABC genes of subfamily B in other earthworms using the approach used for *A. caliginosa* and *L. rubellus*.

3.4.2 **Degenerate primers**

The most critical step in designing degenerate primers based on the protein alignment was to find potential sites, preferably clusters of six or seven amino acid sequences that did not contain 6-fold degenerate residues, to avoid high degeneracy level (>512-fold degenerate) (Lang & Orgogozo, 2011). However, these sites were rare in the P-gp alignments and resulted in four primers out of five with degeneracy higher than 1024-fold. The high degeneracy level contributed to the amplification of multiple products in *A. caliginosa* which hindered the identification of target gene by direct sequencing. The primer (DEG2487) was the only one that showed low degeneracy (256-fold), however it was located within the TMDs, and TMDs of different species share little sequence homology (Ambudkar *et al.*, 2006). This may explain the lack of amplification of target gene when using the low degenerate DEG2487. Although degenerate primers with high level of degeneracy have been used successfully in other studies in the search for ABC sub-family B genes (Feldstein *et al.*, 2006; Luedeking & Koehler, 2002), this was not the case here.

A second group of degenerate primers were designed based on the alignment of nucleotide coding sequences of more closely related species, such as mussel and scallops. At that time in this study, there was no annotated *ABCB1* gene in any species of Annelida phylum. The closest species to earthworms, with available *ABCB1* information, were mussel and scallops, which, at least, share the same super-phylum, Lophotrochozoa, with earthworms. Although the mussel and scallops sequences were incomplete, the region was of a nucleotide-binding domain (NBD) with some ABC subfamily B classical features. Degeneracy went down to 72-fold or less in two primers, and they were able to amplify a P-gp-like homologue in *C. elegans* cDNA, which species sequence was not included in the alignment for the design of second set of degenerate primers. For *C. elegans*, the use of cDNA sequence instead of whole genomic sequence contributed to direct sequencing due to minimisation

of nonspecific products, opposed to several nonspecific products observed in the amplification of *A. caliginosa* genomic DNA, which interfered in the direct sequencing. It is likely that the addition of sequences from the species in the same phylum together with the use of cDNA as template may have improved the production of successful degenerate primers. Given the difficulties experienced here with design of degenerate primers using sequences from distantly related species, it is probable that the new lumbricid sequences will be valuable in the design of degenerate primers for other Annelid species.

The success of the degenerate primers may have been improved by incorporating a post-PCR cloning and sequencing step (Allikmets *et al.*, 1998). In this study, although common approaches were used for the optimisation of the degenerate PCR, it is likely that there was considerable amplification of nonspecific products (Lang & Orgogozo, 2011). The success of the use of cloning in the identification of ABC genes fragments, amplified by degenerate primers, has been demonstrated in other studies (Feldstein *et al.*, 2006; Heumann *et al.*, 2012; Löffert *et al.*, 1998; Luedeking & Koehler, 2002; Zaja *et al.*, 2008; Zucchi *et al.*, 2010). The main product of *A. caliginosa* remains to be characterised by cloning techniques.

3.4.3 **Lumbribase**

Access to the draft *L. rubellus* genome, an on-going project to sequence the whole genome was invaluable to this research by providing another option for primer design. Primers were based on a *L. rubellus* sequence homologous to the conserved NBD of *M. edulis ABCB1* containing some of the typical ABC motifs. The close phylogenetic relationship between the two lumbricid species allowed the design of primers without degeneracy. A similar study was done in the isolation of P-gp in the salmon louse (*Lepeophtheirus salmonis*) where the NBDs of the crustacean *Daphnia pulex* were used as queries in the publicly available EST (expression sequence tag) database for sequences encoding sea-louse ABC transporters (Heumann *et al.*, 2012)

LUMBRIBASE information also facilitated the extension of the novel sequence by finding homologous sequences to the downstream conserved motifs. As the draft genome becomes fully assembled and annotated LUMBRIBASE will allow further extension of this gene and expansion to identify ABC transporters and other genes in the Lumbricidae family.

3.4.4 **3’ RACE**

The amplification of the 3’ end of *A. caliginosa* mRNA was the approach to rapidly extend the known gene region. In many studies, the amplification from a defined internal site towards the cDNA ends, either 3’ or 5’, has been the method of choice to obtain the entire open reading frame of transporters (Feldstein *et al.*, 2006; Heumann *et al.*, 2012; Zaja *et al.*, 2008; Zucchi *et al.*, 2010). In

those studies, the products were cloned into a plasmid vector for the isolation of target gene and subsequent identification. It is possible for the products of 3' RACE amplification be inserted into a clone for identification of the target gene sequence. For future studies, the method should be repeated with cloning, and amplification of the 5' end to obtain the whole open reading frame of P-gp in *A. caliginosa*.

3.4.5 **Conclusion**

A new gene has been identified in *A. caliginosa*, the most abundant pasture earthworm of New Zealand, with high homology to the *ABCB1* gene that encodes for the protein P-gp involved in the MXR, a mechanism of defence against accumulation of drugs and natural toxins at cellular level. Although not yet fully characterised, the new sequence encodes classical domain features of an ABC transporter confirming the presence of ABC transporters in earthworms. To date, the presence of P-gp in earthworms has been only evidenced by functional assays using P-gp substrates. Further work is required to obtain the entire sequence of this novel gene, as are studies into the localisation, function, expression pattern, and substrate specificities by methods such as qPCR and immunochemistry assays including Western-blot and *in-situ* hybridization. In addition, the number of P-gp isoforms has yet to be determined in earthworms, as many invertebrates have more than one P-gp (Dean et al., 2006; Sturm et al., 2009; Zhao et al., 2007).

Chapter 4

Development of a reverse transcription-quantitative PCR assay for the analysis of *ABCB1* gene expression in *Aporrectodea caliginosa*

4.1 Introduction

The expression of P-gp is closely regulated, particularly at the level of transcription (Scotto, 2003). The *ABCB1* expression can be induced by a variety of drugs and hormones *in vitro* in mammals (Magnarin *et al.*, 2004). Today, the method of choice for accurate, sensitive and fast measurement of gene expression is the fluorescence-based RT-qPCR (Derveaux *et al.*, 2010). In a number of aquatic toxicological studies, this technique has been used to measure *ABCB1* expression levels in marine organisms in order to understand the role of ABC transporters in the defence against pollutants (Fischer *et al.*, 2011; Ivanina & Sokolova, 2008; Loncar *et al.*, 2010; Luedeking & Koehler, 2004; Zucchi *et al.*, 2010).

The analysis of altered expression of ABC transporter genes in contaminated habitats can serve as biomarkers when environmental factors are correctly taken into account (Luedeking & Koehler, 2004). At present, few transcriptome profiles have been produced in earthworms via microarray technology (Owen *et al.*, 2008; Steinberg *et al.*, 2008; Svendsen *et al.*, 2008), but these did not specifically assess the ABC transporter response. Analysis of *ABCB1* transcript levels in earthworms would contribute to the knowledge of P-gp function in the defence against xenobiotics. Such studies may establish a biomarker for ecotoxicological assessments in soil.

The objective of this work was to develop, validate, and optimise a RT- qPCR assay to measure the *ABCB1* mRNA copies in *A. caliginosa.* Preliminary exposure experiments were done to test the assay and to examine whether *ABCB1* expression in *A. caliginosa* was modulated by chemical exposure. The chemicals were: 1) to the antibiotic rifampicin, a known *ABCB1* inducer at transcriptional level in mammalian cells (Magnarin *et al.*, 2004), 2) to the immunosuppressant CyA, a specific P-gp substrate that inhibits the protein function and has previously been used as a P-gp transport blocking agent in mammals (Saito *et al.*, 2001) and earthworms (Brown *et al.*, 2008) and 3) the widely used anthelmintic IVM, which has been associated with *ABCB1* overexpression in nematodes (James & Davey, 2009).

4.2 Material and Methods

4.2.1 Chemicals

Rifampicin powder (Sigma-Aldrich, R7382) was dissolved in methanol (Scharlau) to obtain a stock solution of 500 µM. Cyclosporin A powder (Fluka, Biochemika, Cat # 30024) was dissolved in 96% ethanol (Scharlau) to obtain a stock solution of 416 µM and stored at 4˚C. Ivermectin™ (commercial concentration = 200 mg/L) was kindly donated by Rob McAnulty (Johnston Memorial Laboratory-Lincoln University). Further dilutions of the three chemicals were made in water.

4.2.2 Pre-treated soil generation

Soil was taken from the same area where earthworms were collected to a depth of approximately 30 cm. Soil was placed in metal trays and oven dried at 30˚C for 7-10 days with daily soil mixing. To eliminate contamination by invertebrates, large pieces of stones and vegetation, dried soil were sieved (2 mm metal mesh) with help of a ceramic pestle to break aggregates of dried soil and push the soil through the sieve.

4.2.3 Pre-treated manure generation

Sheep manure was added to soil as source of organic matter for earthworms. The manure was collected from animals that were not receiving any medication. The fresh dung was air -dried at room temperature and subsequently frozen at –80˚C to diminish contamination by microorganisms (partially sterilised) and to maintain its nutritional value (Lowe & Butt, 2005) or alternatively heated in the microwave at high (1000 W) for 3-5 min (pers. comm. Dr Stephane Boyer, Jan 2010). After thawing, dung was ground with a ceramic mortar and pestle until it was the consistency of a coarse powder and 15 g of dried manure per kg of dried soil was added weekly to the container.

4.2.4 Collection and maintenance of *A. caliginosa*

Adult earthworms of the species *A. caliginosa* were selected for this experiment. Species identification, method and area of collection were described in chapter 3, section 3.2.1.2. Earthworms were transported to the laboratory from the field site inside a bucket covered with soil. They were transferred to a 6 L sealed plastic container (lid with < 5 mm holes for ventilation) containing pre-treated soil. Moisture was adjusted to 30- 35% (v/w) of the dry weight with distilled water. Earthworms were kept in a controlled temperature room (15˚C) with a light programme (16h light and 8h dark) for at least one week for acclimatisation before being exposed to soil treated with chemicals.

4.2.5 Determination of suitable chemical concentrations for *A. caliginosa* bioassays (Range finding study)

Since there is little information available about the effects of the chemicals used in this study on *A. caliginosa*, suitable dosage concentrations to be used in the exposure experiments were determined by preliminary contact tests in filter paper (OECD, 1984) with a range of concentrations. Previous studies in *A. caliginosa* have shown that chemicals are mostly absorbed through the pores of the surface cuticle when exposed to chemicals externally other than via soil (Booth & O'Halloran, 2001).

The chemicals were assessed either in combination with their respective solvents and the solvent alone as a control, in order to identify any negative effects on earthworm physiology that could disrupt the modulatory effect on *ABCB1*. The endpoint of the chemical range was established as the maximal concentration that did not cause any visible physiological change on earthworms.

The tested rifampicin concentrations in *A. caliginosa* were chosen according rifampicin concentrations used to induce the *ABCB1* gene in mammalian cells (Magnarin *et al.*, 2004; Manceau *et al.*, 2010) as follows: 6, 12, 25, 50, 100, 200, 300, 400, 500, 1000, 2000, 2500, 5000 and 10000 µM. Ivermectin^{TM} was tested in four dilutions (20, 2, 1, and 0.2 µg/ml) from the commercial product concentration of 200 µg/ml. Cyclosporin A was not tested for toxicity in this section because a safe and effective concentration that caused P-gp transport blocking, had been already determined in the functional assay in this research (chapter 5) at concentration of 50 µM.

For the contact test of each chemical, a glass Petri dish was lined with filter paper (Whatman Qualitative grade 1 filter paper 63.5 cm^2) and loaded evenly with 1 ml of each target chemical. For each target chemical a control was included which consisted of a Petri dish loaded with 1 ml of the solvent used to make the stock solution. The wetted filter paper was dried by leaving the Petri dish without a lid overnight at room temperature inside a class I laminar flow unit to ensure that solvent would be totally evaporated and protected from light to limit chemical degradation. The following day, the dry filter paper was loaded with 1 ml of non-sterile distilled water to provide moisture for the earthworms. For each treatment, three randomly chosen earthworms were placed onto each filter paper to examine the effects of chemicals in their physiological status or clinical behaviour.

The Petri dishes were transferred into a cardboard box to protect earthworms from light and the box was placed randomly in the temperature controlled room (15˚C). The earthworms were observed for 48 h and any mortality or signs of toxicity were recorded.

4.2.6 **Exposure of chemicals to *A. caliginosa* in soil**

Safe concentrations of chemicals were determined and used in the exposure experiments in soil. Each exposure experiment included one treated group and one non-treated control group (solvent minus chemical). The solutions added to the soil for each experiment were:

- **Rifampicin** – at 10 mM and 5 mM, diluted in 100 mM and 50 mM methanol, respectively. The control was solvent only at the respective concentration.
- **Cyclosporin A** – at 50 μM in 120 mM ethanol. The control was ethanol only at 120 mM
- **Ivermectin™** – at 2.0 μg/ml and 1.0 μg/ml in water. The control was water only.

For all experiments each container contained 40 g of pre-treated dried soil (section 3.2.3) and 15 ml of chemical solution. The mixture was homogenised well with a spatula and then spread onto a metal tray (40 × 30 cm) with layer depth of 5 –8 mm to let the solvent evaporate. The tray was left inside a class I laminar flow unit for 24 h in the dark. The dried mixture was transferred to a mortar and 0.5 g of pre-treated sheep manure (section 3.2.4) was added. Soil clumps were ground with pestle to <1 mm particle size. The mixture was transferred to a 70 ml polypropylene screw top container (Thermofisher Scientific, Albany, New Zealand) with a manually perforated screw top (< 5 mm-hole). Soil was rehydrated with 15 ml of distilled water and mixed with a spatula.

Three adult and active earthworms with healthy appearance were chosen randomly and placed inside each container. The earthworms had previously been placed onto moist filter paper for 48 h to void the soil content in their guts and to stimulate the ingestion of treated soil. Containers were maintained in a temperature controlled room (section 3.2.2) for seven days. Each exposure to rifampicin or CyA was replicated 4 times (4 containers) for both the treated soil (solvent + chemical) and solvent control. The pilot study with IVM had duplicate containers for the treated soil (IVM + water) and non-treated control (water only).

Daily observations were made to check for mortality and, at the end of the trial, clinical alterations such as such as decreased activity, dehydration, impaired and abnormal reflexes, constrictions along body, detachment of osterior end, presence of hemorrhagic spots and death were recorded. Those earthworms were discarded from analysis.

4.2.7 **RNA extraction and DNAse treatment**

After seven days, the earthworms were removed from the experimental containers for extraction of total RNA. RNA was extracted as described in chapter 3 (section 3.2.2.2), but excluding the voiding gut of materials for 48 h on filter paper because the measurement of transcriptional copies had to be

done immediately after exposure of tested chemical. To remove the intestinal contents, the posterior end was gently pressed with forceps to force the soil out from intestinal lumen. The excised portion of earthworm was immediately cut into smaller pieces (~3 mm) and transferred to a RNAse and DNAse- free 1.7 ml tube containing 500 µl of RNA stabilisation reagent (RNAlater, QIAGEN, Cat # 76104). The stabilisation reagent prevented RNA degradation and maintained RNA stability for up to 4 weeks at 2–8°C. For extraction of RNA, the tissue was removed from the stabilisation reagent and RNA extracted as described in chapter 3.

An aliquot of the RNA extract, containing 1 µg of total RNA, was aliquoted into a separate 0.6 ml DNAse/RNAse-free tube for subsequent DNA synthesis and the remaining volume was immediately stored at -80˚C. Genomic DNA contamination was removed using a Deoxyribonuclease I Amplification Grade kit (DNase I, Amp Grade; Invitrogen) as follows. One µg of RNA sample was incubated in 1 × DNase I reaction buffer [200 mM Tris-HCl (pH8.4), 20 mM MgCl2, 500 mM KCl], 1 U of DNase I (1 U/µL) and RNase-free water in a total volume of 10 µL. The reaction solution was incubated at room temperature for 15 min, then 1 µL of 25 mM of EDTA (pH 8.0) was added and the solution heated for 10 min at 65˚C. The DNA-free RNA was then used for cDNA synthesis.

4.2.8 **cDNA synthesis**

The SuperScript ™ III Reverse transcriptase kit (Invitrogen) was used to synthesise cDNA. Into a nuclease-free 0.6 ml tube the following was added: 1 µL of 50 µM oligo $(dT)_{20}$ (Ambion; Texas, USA), 1 µg of DNase-treated RNA sample, 1 µL of 10 mM dNTP (dNTP Mix, 10 mM each , Fermentas Life Sciences) and nuclease-free water to a total volume of 13 µL. The mixture was heated to 65˚C for 5 min and incubated on ice for at least 1 min. Tube contents were collected by brief centrifugation at 5,000 × g and the following reagents were added: 4 µl of 5 × First-Strand Buffer [250 mM Tris HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$], 5 mM of dithiothreitol (DTT), 1 U of RNase inhibitor (RNaseOUT™ Recombinant Ribonuclease Inhibitor; Invitrogen, 40 units/µl), 10 U of SuperScript™ III RT (200 U/µL) to a final volume of 20 µl. The solution was mixed gently with a pipette and incubated at 50˚C for 60 min. The reaction was inactivated by heating at 70˚C for 15 min. The cDNA was stored at -20˚C until use as a template in RT-qPCR.

4.2.9 **Primer design of target and reference genes**

Primers of target gene ABCB1

Primers used for the amplification of target gene (*ABCB1*) were *ABCB1*-AC forward 5' TCGGGTCGAGCGGGTGTG3' and *ABCB1*-AC reverse 5' GCAAACAGCACTGGTTCCTG 3' which produced a product of 166 bp (see section 3.3.2.1 for primer design).

Primers for the reference gene β-actin

The *β-actin* gene was selected as the reference gene for normalisation. *β-actin* sequence information for *A. caliginosa* was not available, so a strategy similar to that used to obtain the *ABCB1* gene (section 3.2.1.1 –primers designed using LUMBRIBASE) was applied here. Firstly, partial sequences of *β-actin* mRNA from species phylogenetically close to the Lumbricidae family were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/): *E. fetida*-accession number DQ286722.1*; Lumbriculus variegatus*- AY157021 and *Taenia taeniaeformis*- AB533224.1 in order to identify conserved regions within this gene. The sequences were used as queries on the LUMBRIBASE database (http://www.nematodes.org/Lumbribase) to find the *L. rubellus β-actin* gene. The returned contiguous sequence from LUMBRIBASE and the three partial mRNA sequences were aligned using DNAMAN 4.0 software (Lynnon Corporation, Quebec, Canada) and homologous areas (>50% of similarity) were identified for primer design (see alignment details in appendix A.7). Primers were designed manually according the criteria described on section 3.2.1.1. The synthesis, reconstitution and storage of primers followed the same procedures as described in the section 3.2.1.1.

The *A. caliginosa β-actin* gene was amplified from RNA using the QIAGEN OneStep RT-PCR Kit. *Lumbricus rubellus* was used as positive control. Each reaction mix contained 1 × RT- PCR buffer [Tris·Cl, KCl, $(NH_4)2SO_4$, 12.5 mM $MgCl_2$, DTT; pH 8.7 (20°C)], 1× Q-solution, 400 µM of each dNTP, 0.6 µM of forward and reverse *β-actin* primers, 1 U of RNase inhibitor (RNaseOUTTM), 1µl of RT-PCR enzyme mix (Omniscript and Sensiscript Reverse Transcriptase and HotStarTaq DNA Polymerase), 200 ng of RNA (DNAse treated) and nuclease-free water to the volume of 25 µl. The thermal cycler was set as follows: reverse transcription at 50˚C for 30 min; then initial PCR activation at 95˚C for 15 min followed by 35 cycles of denaturation at 94˚C for 45 s, annealing at 62˚C for 45 s and extension at 72˚C for 45 s; and a final elongation step at 72˚C for 7 min. The visualisation of products was as described in section 3.2.1.4. The product of *A. caliginosa* was directly sequenced and identity determined by BLASTx results. Primers specific to the*. A. caliginosa β-actin* gene were designed from this sequence. The criteria for design, synthesis, reconstitution and storage of primers followed the same procedures as described in section 3.2.1.1.

The new *β-actin* primers for *A. caliginosa* were tested on *A. caliginosa* genomic DNA with gradient PCR. The DNA template was extracted according to procedures described in section 3.2.1.3. Each 25 µl reaction contained 1× buffer , 2 mM of $MgCl_2$, 200 µM of each dNTP, 2 µM of each primer, 1.25 U of FastStart® Taq DNA Polymerase, 10 ng of template DNA. The thermal cycler was set as follows: initial denaturation at 94˚C for 4 min; followed by 40 cycles of denaturation at 94˚C for 45 s, annealing at 54˚C to 64˚C with 2˚C increments for 45 s and extension at 72˚C for 45 s; and a final elongation step at 72˚C for 7 min. The visualisation of products was according to section 3.2.1.4.

4.2.10 **Optimisation of RT-qPCR**

The RT-qPCR was optimised to improve the amplification kinetics and to limit the formation of any nonspecific products. Amplification kinetics was evaluated by the calculation of amplification efficiency combined with the analysis of the shape of amplification curve (fluorescence value in arithmetic scale versus cycle number). Amplification efficiency was estimated by a standard curve (see details in section 4.2.12) and the optimal RT-qPCR parameters were deemed those that produced amplification efficiency > 80% (Schmittgen & Livak, 2008; Taylor *et al.*, 2010). The expected curve shape was of sigmoidal shape with the steepest exponential phase indicating the highest efficiency of the tested parameters. The formation of nonspecific products was monitored by melting curve (Tm) analysis.

The tested parameters included primer concentrations for both target and reference primers (forward and reverse) which were determined by testing three final concentrations: 0.2 µM, 0.4 µM and 0.6 µM, with the cycling programme and master mix recipe as described in section 4.2.11. Also, two $MgCl_2$ concentrations, 2.0 mM and 2.5 mM, and two dilution rates of the fluorescent intercalating dye SYBR Green I, 1:1,000 and 1:5,000, were tested.

Once the optimal concentration of reagents had been determined, a range of cDNA concentrations were tested to establish the template concentration that would be detectable without inhibiting the reaction. The synthesised cDNA was diluted in water to 10 ng/µl, then further diluted 8-fold to 2 pg/µl. The template concentrations 10, 1.25, 0.156, 0.019 ng/µl, and 2 pg/µl were tested in RT-qPCR according protocol described below (section 4.2.11)

4.2.11 **RT-qPCR protocol**

RT-qPCR was performed on a real-time PCR instrument (ABI PRISM ® 7000 Sequence Detection System-Applied BioSystems, CA, USA) using 96-well reaction plate for real-time (Global Sciences, AYPCR96ABC) and optical adhesive cover for microplate (Raylab, EXTSSRQ100). Each 16 µl of reaction contained the following reagents, except when the process was subject to optimisation, 0.8 U of FastStart Taq polymerase, 1×buffer [500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3], 2 mM of $MgCl_2$,, 200 µM of each dNTPs, 0.4 µM as the final concentration for both *ABCB1* and reference primers (both directions), 1.25×ROX reference dye (Invitrogen, Cat # 12223-012), 0.23 µl of pre- diluted (1:1000 in water) SYBR® Green I (nucleic acid gel stain, 10,000X concentrate in DMSO, Invitrogen Molecular Probes) and 1.25 ng of template. Each template was a sub-sample from a pool of three cDNA samples extracted individually from earthworms from the same experimental container, so to minimise the inherent differences in gene expression levels between individual organisms (Taylor *et al.*, 2010). Each pool was tested in 2–3 technical replicates

to minimise deviations associated with pipetting error, and sample quality or quantity (Taylor *et al.*, 2010). Non-template controls (NTC) in which the cDNA template was substituted with water were run in parallel to ensure all reactions were free of contamination.

The PCR thermal cycler parameters were set to an initial denaturation of 95˚C for 5 min followed by 40 cycles of 15 s at 95˚C (denaturation), 30s at 60˚C (primer annealing) and 30s at 72˚C (extension). The fluorescence signal was measured at 60˚C during the annealing step and results were represented as $C_q$values (Chapter 1). In the RT-qPCR machine utilised, the melting point analysis was automatically carried out by a dissociation stage provided in the manufacturer's software (ABI Prism 700 by DLS Inc., version 1.0) following amplification.

4.2.12 **Data Analysis**

The generated Cq values were transferred to Microsoft Excel and the average of the technical replicates calculated for each pool. The target Cq were normalised by subtracting the endogenous reference gene (*β-actin*) Cq value. Normalisation of target gene expression levels was performed to compensate for intra- and inter-kinetic RT-qPCR variations, otherwise known as sample-to-sample and run-to-run variations (Derveaux *et al.*, 2010).

After normalisation of Cq values, the mean ± standard deviation (S.D.) of experimental replicates were calculated for treated and control groups. Gene expression analysis was performed by the comparative Cq method also referred to as the 2- ΔΔCq method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). The normalised means were applied in the following formula 2− ΔΔCq where ΔΔCq is the difference between normalised Cq mean of treated group and normalised Cq mean of non-treated. The 2− ΔΔCq values represented the fold change in mRNA amounts in treated samples relative to the non-treated control, arbitrarily set at 1. Results greater than 1 indicated increase in gene expression due to treatment (Schmittgen & Livak, 2008). The analysis of variance (P value) between treated and control was done using single factor ANOVA (Microsoft Excel) and a P value < 0.05 was considered statistically significant.

The determination of amplification efficiency for each primer pair was determined by a standard curve which was created by plotting serial 8-fold cDNA dilutions (in triplicate) of a representative sample of cDNA template (1.25, 0.156, 0.019 ng/µl and 2 pg/µl) in the x-axis versus correspondent generated Cq values in the y-axis. The slope of the curve and the coefficient of determination (r^2) were automatically calculated by the manufacturer's software and were used to evaluate the amplification efficiency of each primer pair. The efficiency of RT-qPCR was determined by the formula $E= [10^{(-1/slope)}]-1$, where E is the efficiency value (Bustin *et al.*, 2009).

4.3 Results

4.3.1 Design of reference gene primers

The LUMBRIBASE search resulted in one *L. rubellus* contiguous sequence (contig_194397) comprising 2214 bp with 57.28% identity to the *β-actin* mRNA sequence of *E. fetida* (210 bp), 79.32% with *L. variegatus* (181 bp) and 82.21% *T. taeniaeformis* (675 bp). In the alignment, two conserved areas were identified (see alignment details in the appendix A.7). From these two areas, the following primers were designed: BAC 1219 (forward) 5'CCC AGA TCA TGT TCG AGA CC 3' and BAC 1928 (reverse) 5'CGT ACT CCT GCT TGC TGA TCC 3', which produced a single product of 708 bp in *A. caliginosa* (see novel sequence in appendix B.3). The BLASTx showed that translated product had 99% identity with *L. rubellus β-actin* protein sequence (NCBI P91754.1) encompassing 63.4% of the whole *L. rubellus* sequence (236 aa out of 372 aa). The search also resulted in matches with high homology (95 to 98%) to the protein β-actin in several species of fish and invertebrates. Two primers were designed using this novel *A. caliginosa* 708 bp sequence for use in the RT-q PCR to amplify the reference gene: BAC 697 (forward) 5' CTC GAG AAG AGC TAC GAG C 3' and BAC 876 (reverse) 5' GGC GTA CAG ATC CTT ACG G 3' that produced a single 180 bp product in *A. caliginosa* and *L. rubellus* (Figure 4.1).

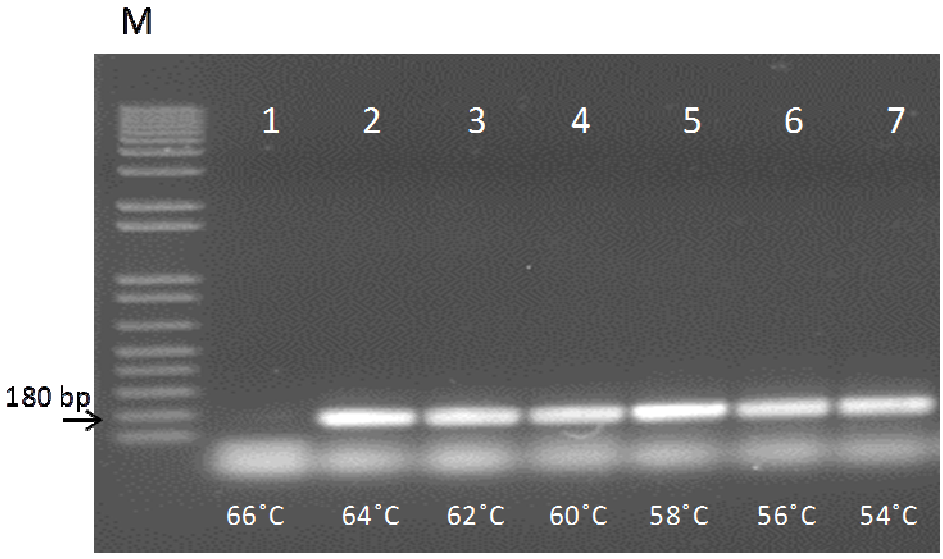

Figure 4-1 Gel electrophoresis of amplicons of *Aporrectodea caliginosa* RNA (with different annealing temperatures denoted at the base of the gels) produced by reverse transcription PCR with species specific ***β-actin*** gene primers. The black arrow indicates the expected product size. M is the molecular marker.

4.3.2 **RT-qPCR performance**

Reagent concentrations for the RT-qPCR protocol were tested to improve the amplification kinetics and to limit the formation of any nonspecific products. The optimal primer concentration for target and reference gene was 0.4 µM which is within the standard range of 0.05–1.0 µM used in general PCR assays (Phillips, 2004). The optimal concentration of $MgCl_2$ was 2 mM (standard range is 1.5–4.0 mM) (http://www.neb.com) and the optimal dilution of SYBR Green I was 1:1,000. The optimal template concentration was 1.25 ng/µl as higher concentrations caused irregular amplification curves (dropping after exponential phase) and lower concentrations resulted in late fluorescence signal (results not shown).

The amplification efficiency of each primer pair was calculated from the standard curve generated in the analysis of samples (Figure 4. 2). Overall, efficiencies of analyses of rifampicin 10 mM, rifampicin 5mM, Cya 50 µM and IVM 2.0 µg/ml treatments had an average of E=0.96 + 0.07 with coefficient of variation (CV) of 8% for target gene, and for reference gene E= to 1.16 + 0.11 with CV of 9.7%, which were within the range (0.8 to 1.2) (Schmittgen & Livak, 2008). Although the theoretical maximum amplification is 1 (or 100%) (Bustin *et al.*, 2009), casual higher values (up to 1.2) were also accepted due to technical variations. A single amplification efficiency for the reference gene in the analysis of CyA experiment (E=1.27) was beyond this range. However, this result was not significantly different from the other results and was included in the normalisation and comparison.

The amplification efficiencies of IVM 1.0 µg/ml experiment were 1.36 (target gene) and 1.44 (reference gene) and were therefore discarded from analysis. The r^2 values were greater than 0.980 for all standard curves which is acceptable for RT-qPCRs (Taylor *et al.*, 2010). The formation of nonspecific products in all experiments was monitored by the analysis of melting curves. The melting points for target and reference gene products were 86.5˚C + 0.63 (S.D.) and 87.5˚C + 0.63 (S.D.), respectively (Figure 4.3).

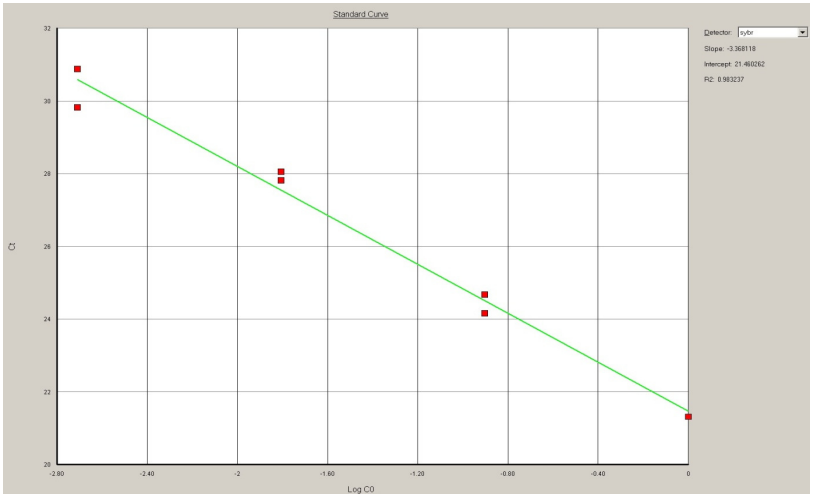

Figure 4-2 Standard curve is generated in the RT-PCR for the calculation of amplification efficiency of reaction. On the x-axis are logarithmic values of known template concentrations and on y-axis are the C_q values. The slope value is applied in the formula $E=[10^{(-1/slope)}]-1$ to calculate amplification efficiency (E). The r^2 value represents how variable the replicates are and how well the data fits the regression line.

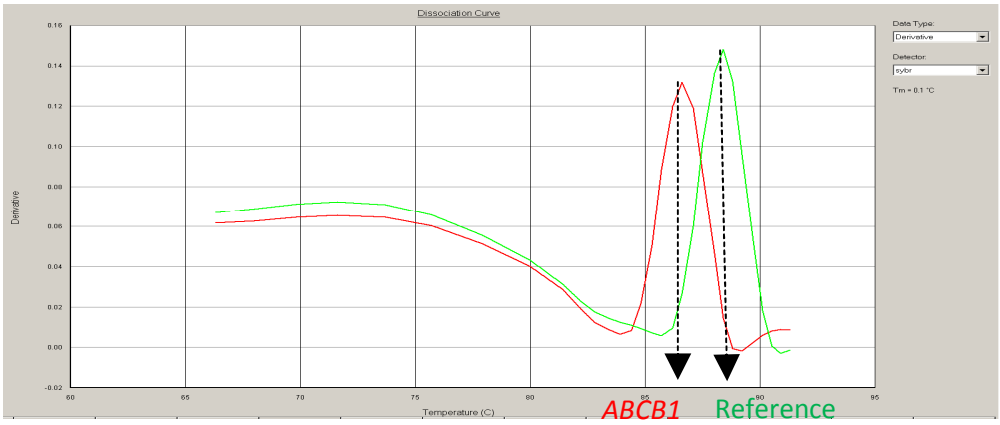

Figure 4-3 Dissociation curve showing the melting points (Tm) from amplicons produced by reverse transcription quantitative PCR using *ABCB1* primers for amplification of target gene (red) and *β-actin* primers as the reference gene (green). The corresponding Tm(s) are 86.5˚C and 87.5°C.

4.3.3 **Toxicity of selected chemicals to *A. caliginosa***

A range finding study was carried out to determine suitable exposure concentrations. The antibiotic rifampicin did not cause any evident toxic effect in *A. caliginosa* even at the highest tested concentration (10 mM). In contrast, exposure to the highest tested IVM concentration (20 µg/ml) resulted in dehydration, abnormal/slow or absent reflex after stimulus and constrictions along body. A concentration of 2 µg/ml on filter paper resulted in some abnormal/slow or absent reflexes after stimulus and a few constrictions along body. Although 2 µg/ml was mildly toxic on filter paper, when

mixed in soil the toxic effects were not evident. Therefore, the 2 µg/ml IVM treatment was included in the RT-qPCR experiments.

4.3.4 *ABCB1* gene expression in *A. caliginosa* following exposure to selected chemicals

Earthworms of the exposure experiments were analysed by RT-qPCR to measure the *ABCB1* mRNA levels of treated and non-treated (control) samples (see data in the appendix D1.1 to D1.4). A significant increase in expression was observed in earthworms exposed to 10 mM rifampicin for 7 days (2.63 fold-change, $P<0.05$) compared to control (Figure 4.4). In contrast, exposure to 5 mM rifampicin did not increase expression (0.58 fold-change). The CyA treatment (1.17-fold) was not significantly different from the control ($P=0.430$).

Exposure to 2.0 µg/ml IVM moderately induced the expression of *ABCB1* by 2.32 and 2.46 in two samples respectively. However, significance could not be calculated due to the lack of adequate replication.

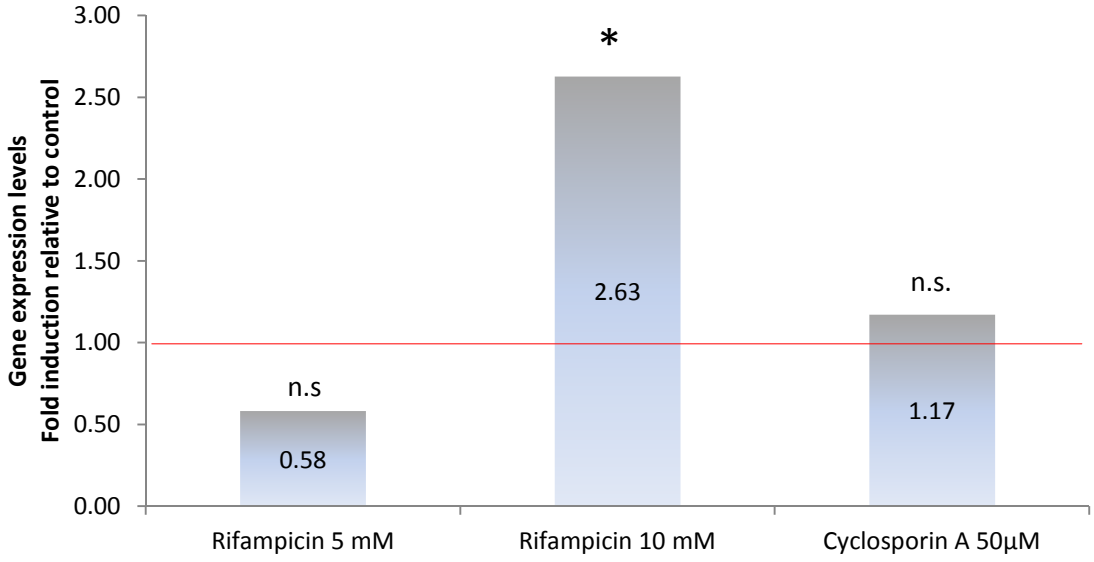

Figure 4-4 Effect of rifampicin and cyclosporin A on *ABCB1 gene* expression in *Aporrectodea caliginosa* (grey earthworm). Expression was determined by comparison with the *β-actin* reference gene. The bars correspond to fold increase of mRNA expression levels of treated samples relative to the corresponding non-treated control (constitutive expression). Significant differences (*) between treated and non-treated groups are denoted by an asterisk (*) ($P\leq 0.05$), n.s denotes not significantly different from the control.

4.4 Discussion

This work describes the development of a RT-qPCR assay to measure the transcriptional mRNA copies of *ABCB1* gene in *A. caliginosa*. Although RT-qPCR using fluorescent probe is a common method to measure gene expression, to date, there are no studies of ABC genes expression in earthworms using this approach, presumably due to the lack of genetic information. As part of the design and optimisation of the RT-qPCR assay, a reference gene (*β-actin)* was identified for the first time in *A. caliginosa*, this will facilitate expression studies for genes in addition to *ABCB1* in this species. This study demonstrated that a mammalian inducer of *ABCB1*, rifampicin, is also suitable as a positive control for examining the expression levels of *ABCB1*.

4.4.1 β-*actin* for reference gene expression

The *β-actin* gene is a well-established reference gene, widely used in gene expression studies in earthworms (Chen *et al.*, 2011; Homa *et al.*, 2010; Liang *et al.*, 2011; Ricketts *et al.*, 2004; Spurgeon *et al.*, 2004; Stürzenbaum *et al.*, 1999; Wang *et al.*, 2011; Zheng *et al.*, 2008b). The *β-actin* gene was demonstrated to be the most stable gene under different experimental conditions (Piana *et al.*, 2008) and it was reported that its expression was constant after exposure to different rifampicin concentrations (Nishimura *et al.*, 2006). However, according to the latest guidelines for real-time PCR experiments (Bustin *et al.*, 2009), the most appropriate and universally applicable method is the normalisation against 3 or more validated reference genes to show strong correlation with total amounts of mRNA present in the samples and to eliminate as much as possible the technical variation (Derveaux *et al.*, 2010). In this study, only one reference gene was used. The approach used here to find the *β-actin* sequence in *A. caliginosa*, could be applied in future earthworm studies to increase the number of reference genes available.

The translated *A. caliginosa* sequence had high homology with protein sequences of *β-actin* in several invertebrates and vertebrates species. The homologies observed in this study confirm that the *β–actin* gene family is conserved across eukaryotic species, and its family members share a high degree of protein sequence homology (Bunnell & Ervasti, 2011).

4.4.2 RT-qPCR optimisation

This RT-qPCR assay was optimised for assurance and quality control issues that influence the accuracy of the results and reliability of the conclusions (Derveaux *et al.*, 2010). The main focus of optimisation was the parameters involving the formation of nonspecific products and amplification kinetics. The primer concentrations were optimised as the excess of primers could cause mis-priming and formation of nonspecific products, and low concentrations could impair efficiency of the reaction (Phillips, 2004). The presence of $MgCl_2$ was essential for the activity of DNA polymerase and either its

excess or deficiency could affect the amplification (Phillips, 2004). The dye SYBR Green I has an important effect on the melting temperature of DNA molecules and its excess could have impaired the denaturation of dsDNA and further amplification (Bustin & Nolan, 2004). Also, the template concentration was determined to avoid inhibition of amplification caused by the excess of template and high concentrations of reverse transcriptase and the reagents of DNAse digestion (Bustin, 2000).

4.4.3 **RT-qPCR performance**

The amplification efficiencies of *ABCB1* and *β-actin* primers were in the acceptable range, between 0.8 and 1.20 and since it is not a strict rule, the outlier result (1.27) was considered as valid. Similar results were reported in the gene expression analysis of ABC efflux transporters in rainbow trout, with an amplification rate range of 0.96 + 0.07 (S.D.) for target gene and 1.03 for the reference gene (Loncar *et al.*, 2010). The poor efficiency observed in the analysis of exposure to IVM 1.0 µg/ml was probably due to human error during preparation of sample mix, as both gene amplifications were affected.

The *ABCB1* primers were designed to bind to the ATPase domain which is conserved across ABC transporters and there was potential for nonspecific amplification of ABC genes. However, the visualisation of a single band with expected size on gel electrophoresis (figure not shown) and analysis of the melting curve confirmed the presence of a single product. Although the product sequence had a high DNA sequence homology to both *ABCB1* and *ABCB4* genes in mammals (Dean *et al.*, 2001; Lawson *et al.*, 2008), it is unlikely that *ABCB4* exists in invertebrates because they do not produce bile. This is supported by the numerous studies on P-gps in invertebrates which have not detected of ABCB4-like P-gps (Annilo *et al.*, 2006; Heumann *et al.*, 2012). Therefore, it is unlikely that there is any nonspecific amplification of other ABC genes in the RT-qPCR.

4.4.4 **Toxicity of chemicals used in this study**

This study has produced new information about the toxic effects of the selected drugs on *A. caliginosa*, either through direct contact on filter paper or through mixing in soil and ingestion of mixture. The antibiotic rifampicin, used in the treatment of the mycobacterial infections actinomycosis and histoplasmosis, did not cause any observable toxic effects on earthworms at the concentrations tested. The low toxicity of rifampicin allowed the addition of solubilised drug to the soil with concentrations 400-500 × higher than concentrations used in experiments with mammalian cells. Magnarin *et al.* (2004) used 25 µM rifampicin in a tubular renal cell line to induce the *ABCB1* gene, which is almost double the standard therapeutic concentration for tuberculosis treatment, equivalent to 12 µM (Kaplan, 2004). In the present study, the use of the highest tested concentration

(10 mM) was to ensure that sufficient amount of ingested rifampicin would reach the intestinal walls of earthworms to stimulate *ABCB1* expression.

Ivermectin^{TM} showed to be more toxic when in direct contact to the earthworm than mixed in soil and ingested. This finding is similar to the study of Svendsen *et al.* (2005) that reported ingestion of IVM at low doses, found in the residues of cattle dung excreted after animal treatment, is innocuous to earthworms and no adverse effects were observed on *L. terrestris* when ingested.

4.4.5 *ABCB1* gene expression due to chemical exposure

The preliminary tests with rifampicin showed *ABCB1* gene upregulation in *A. caliginosa* when exposed to a concentration of 10 mM. There are several studies demonstrating that rifampicin induces *ABCB1* expression in human, mice and other mammalian cells (Buss & Callaghan, 2008; Magnarin *et al.*, 2004; Piana *et al.*, 2008). However, there is no published information on the use of rifampicin as an *ABCB1* inducer in invertebrate species. The induction of expression in *A. caliginosa* (2.63 fold-change) was comparable to the work of Manceau *et al*. (2010), where a human colon cancer cell line (LS174T) was used as positive control for the *ABCB1* gene induction and this resulted in a 4 fold-change after exposure to 20 µM rifampicin for 24 hours. A similar result was also reported in a human hepatocyte cell line treated with 10 µM rifampicin which resulted in a 3 fold increase in *ABCB1* expression (Mills *et al.*, 2004). In mammals, *ABCB1* transcriptional expression has been shown to be regulated by specialized multi-protein complexes that include common basic transcriptional components (transcription factors and promoters) (Scotto, 2003). Rifampicin is a known *ABCB1* transcription factor activator (Ott *et al.*, 2009). The results presented here, suggest that there are likely to be similarities in this induction pathway between mammals and earthworms.

Although the increase in expression between mammal cell lines and *A. caliginosa* was similar, the *A. caliginosa* was exposed to a much higher concentration of rifampicin with a longer exposure period (7 days). This approach was to ensure that an effective drug dosage would reach intestinal walls through ingestion of mixed soil. The drug may have been degraded due to the long exposure, and this outcome may explain the lack of induction in the 5 mM treatment. This may have been exacerbated by soil micro-organisms which are known to metabolise chemicals (Burns, 1991). Long exposure to rifampicin has been shown to be effective in experiments on a pig kidney epithelial cell line, with significant *ABCB1* induction. During that experiment, fresh solutions of rifampicin were added to the culture twice a week during cell sub-culturing, ensuring exposure to the undegraded chemical (Magnarin *et al.*, 2004). For future experiments with earthworms, fresh solutions of rifampicin should be added to the soil, perhaps over a shortened time frame, in order to achieve higher *ABCB1* expression.

Cyclosporin A treatment had a negligible effect on the *ABCB1* expression in *A. caliginosa* indicating that it is not upregulated by P-gp interaction with CyA. This was unexpected as CyA inhibits the P-gp function by blocking the ATPase activity through competitive binding (Smital *et al.*, 2004; Thomas & Coley, 2003), and it is known that that chemicals that interact with P-gp can lead to overexpression of this protein (Romiti *et al.*, 2002). Cyclosporin A produced reversible overexpression of P-gp in the rat (Jetté L, 1996). The 50 µM concentration of CyA was shown to block P-gp function in *A. caliginosa*, as observed in the P-gp functional assay done in this research (chapter 5).Thus, it was hypothesised that the blocking of the P-gp protein would induce *ABCB1* expression in *A. caliginosa*. This result suggests that there is an absence of strict correlation between increase of P-gp expression and induction of mRNA levels, as reported in other studies (Ivanina & Sokolova, 2008; Shirasaka *et al.*, 2006) probably because the overexpression of P-gp has been shown to be controlled partially through post-transcriptional mechanisms, such as mRNA stabilisation (Ménez *et al.*, 2012).

Ivermectin™ was chosen as the candidate agrochemical in this research because of the emerging concern of its effect on the environment and its role in a drug resistance related to P-gp function in free-living and parasitic nematodes (James & Davey, 2009; Kaplan, 2004; Liebig *et al.*, 2010). In this study, two pilot studies were performed to examine the ability of IVM to induce *ABCB1* expression in *A. caliginosa*. The results suggested that IVM can induce *ABCB1* expression in earthworms, as reported in other studies. In a recent study, IVM induced *ABCB1* expression in a murine hepatocyte cell line in both a time- and dose-dependent manner (Ménez *et al.*, 2012).

Elsewhere, researchers have generated resistant populations of the model free living nematode *C. elegans* by selecting survivors from exposure to increasing concentrations of IVM. Gradually, over several generations, a highly resistant population of *C. elegans* was created. In this resistant population a 12 fold-change in *ABCB1* was observed (James & Davey, 2009). This research showed a link of *ABCB1* expression to IVM resistance. To confirm such a response in earthworms would take considerable time, as *A. caliginosa* takes almost four months from larva to adult under optimum conditions. Therefore, IVM is a potential drug to be used as *ABCB1* inducer in future studies with earthworms, possibly without the need to generate a resistant population to obtain the desired effect.

4.4.6 **Final conclusion**

The present study has demonstrated that the developed RT-qPCR assay can measure transcript levels of the *ABCB1* gene in *A. caliginosa*. More reference genes should be included to the assay to strengthen the correlation with total amounts of mRNA present in the samples and to eliminate as much technical variation as possible. The approach used in this research could be used for the identification of other reference genes.

Rifampicin is a suitable *ABCB1* inducer in *A. caliginosa*, with low toxicity and low cost and may be used in the future validation of new reference genes as well as in other studies on *ABCB1* expression in earthworms. The method to expose rifampicin to earthworms should be modified to optimise induction and this may involve assessment of expression levels over time. Furthermore, IVM affects *ABCB1* expression in *A. caliginosa* and since it is a widely used agrochemical its impact on earthworms should be investigated further.

Chapter 5

P-gp protein functional assay

5.1 Introduction

P-gp actively transports a wide variety of structurally and functionally diverse compounds (Hennessy & Spiers, 2007). The common P-gp substrates are natural or anthropogenic compounds with common chemical properties (Kurelec *et al.*, 2000). Due to its function, P-gp is expressed in several important epithelial barriers of tissues involved in secretion and excretion of toxicants (Thiebaut *et al.*, 1987). The number of P-gps in cellular membranes may be increased by constant high exposure to P-gp substrates, conferring the MXR phenotype (Smital & Kurelec, 1997). Several other chemical agents, although not always characterized as P-gp substrates, can inhibit directly or indirectly the function of P-gp and consequently affect the MXR. These chemicals are defined as chemosensitisers because they enhance the effects of natural and anthropogenic toxins, including P-gp substrates (Smital & Kurelec, 1998a). The MXR mechanism has been demonstrated by biochemical, molecular, physiological and toxicological methods in several marine and freshwater organisms in polluted aquatic environments exposed to P-gp substrates (Bard *et al.*, 2002; Kurelec & Pivcevic, 1991; Kurelec *et al.*, 2000; Luedeking & Koehler, 2004; Smital *et al.*, 2004). To date, there were only a few studies about the presence and activity of P-gp in earthworms, exclusively to *Eisenia* genera (Brown *et al.*, 2008; Hackenberger *et al.*, 2012), but there were no records in *A. caliginosa*.

The aim of this study was to demonstrate the presence of P-gp in *A. caliginosa* by establishing a biochemical assay using a common P-gp substrate, the fluorescence dye RB to analyse the P-gp activity when earthworms were exposed to known P-gp modulators (Kurelec *et al.*, 2000; Smital *et al.*, 2003). To achieve this, experiments were conducted in filter paper contact tests (OECD, 1984) with selected chemicals to examine whether P-gp activity in *A. caliginosa* was modulated by chemical exposure. The chemicals were: 1) verapamil, a known chemosensitiser that inhibits the MXR function by blocking the calcium channel (Tsuruo *et al.*, 1981), 2) CyA, another chemosensitiser that inhibits the P-gp function by strongly binding to the ATPase domain and has previously been used as a P-gp transport blocking agent in mammals (Saito *et al.*, 2001) and earthworms (Brown *et al.*, 2008), 3) rifampicin, an antibiotic known to induce P-gp expression at transcriptional level in mammalian cells (Magnarin *et al.*, 2004) et al, 2004), and 4) IVM , the anthelmintic which has been associated with MXR phenotype in nematodes (James & Davey, 2009).

5.2 Material and Methods

5.2.1 Chemicals

Rhodamine B (Sigma-Aldrich, USA, R6626-1006) was diluted in distilled water to 750 mg/L (stock solution) and kept in a dark glass bottle (protected from light) at 4˚C. Verapamil hydrochloride (Sigma-Aldrich, 1 G, V4629) was dissolved to 1 mM in 96% ethanol (Scharlau, Australia) and stored at 4˚C. Propanol-1 was purchased from Scharlau (Australia). Cyclosporin A, rifampicin and IVM (200 µg/ml) were described in section 4.2.1.

5.2.2 Earthworms

Adult specimens of *A. caliginosa* were selected for this experiment. Species identification, method and area of collection are described in chapter 3, section 3.2.1.2. Transportation of earthworms to the laboratory and their maintenance in containers until chemical exposure are described in chapter 4, section 4.2.2. Earthworms were not depurated before the experiment in order to maintain a balanced energy level since the P-gp activity is ATP-dependent (unlike in previous experiments described in chapter 3, where the worms were allowed to empty gut contents before treatment). Only earthworms that appeared healthy and active were selected for experiments. Each earthworm was rinsed briefly in distilled water and placed on moistened loaded filter paper with one earthworm per Petri dish. All dishes were kept in conditions as described on section 4.2.5.

5.2.3 Exposure dishes

The procedures for preparation of exposure dishes (chemical loading, drying and moistening of filter paper) are described on section 4.2.5. A working solution containing 8 mg/l of RB was prepared in water (concentration based on preliminary studies during Summer Scholarship, 2009 at Lincoln University). Briefly, glass Petri dishes lined with filter paper were loaded first with 1 ml of freshly prepared 8 mg/l RB solution, dried, then loaded with 1 ml of each fresh prepared test solutions (stock solution diluted in water) and dried again. The following day, filter papers were moistened with water and earthworms were placed on filter paper containing the RB and test solution (treated groups), and the negative control in RB only.

5.2.4 Experiments

Accumulation assay

Earthworms were exposed to RB (8 mg/l), either alone (negative control group) or in combination with one of selected chemicals (treated group) at varied concentrations (Table 5.1), for a period of 48 h in the dark at 15˚C. Three concentrations of each chemosensitiser (verapamil and CyA) were used. The concentrations of verapamil were based on the unpublished information during a study of the

dairy effluent effect on P-gp function in *A. caliginosa* at Lincoln University (person comm. Dr. Jeff Brown, Jan 2009) and CyA concentrations were based on the study of inhibitors of MXR in freshwater clam (Smital & Kurelec, 1997). Ivermectin™ concentrations were based on the toxicity test results obtained during the measurement of *ABCB1* gene expression due chemical exposure in this research (section 4.3.3), and rifampicin concentrations were based on the studies in *ABCB1* induction in mammal cells (Magnarin *et al.*, 2004; Manceau *et al.*, 2010). Each group consisted of 4-6 worms placed individually in Petri dishes. After the exposure period, earthworms were sacrificed and the RB contained in each individual homogenate was extracted and measured (see method of extraction and measurement in sections 5.2.5 and 5.2.6). The differences of accumulated amount of RB between treated samples and the control was indicative of P-gp activity.

Table 5-1 Treated groups of earthworms (4-6 replicates per group) exposed to different P-gp modulating chemical concentrations combined with fluorescent dye rhodamine B (8 mg/l) in filter paper contact test. Control groups were exposed only to rhodamine B.

Group	*Chemical solution*	*Concentrations*
1	Verapamil	100, 200 and 250 µM
2	Cyclosporin A	25, 50 and 100 µM
3	Ivermectin™	0.2 and 0.02µg/ml
4	Rifampicin	100 µM and 2 mM

Efflux assay

In the efflux assay, one group of 20 earthworms were first exposed to verapamil at concentration of 100 µM (treated group) combined with RB (8mg/l) and another group of 20 earthworms were the negative control (only RB). The chemical exposure procedures were according to the protocol described above in the accumulation assay. The only difference was that after the 48h exposure period, the earthworms were not sacrificed but transferred to a container with clean soil (generation of soil described on section 4.2.3) to let them efflux the dye. The efflux periods were divided in four intervals, starting at 0h (immediately after exposure), 24 h, 48 h, and 72 h and at the end of each interval, five earthworms were sampled from each group (treated and negative control) to measure the RB in their homogenates.

5.2.5 **Dye extraction and fluorescence measurement**

After exposure, each live earthworm was rinsed with distilled water to eliminate material attached to its body including the excreted dye, which would interfere with the accuracy of fluorescence measurement. After rinsing, each earthworm was briefly dried on a paper towel, placed in a small plastic bag with individual identification, and stored in a -80˚C freezer for at least 1 h. Each frozen worm was removed from the bag, cut transversally with a blade at the base of clitellum dividing worm in two pieces: the head part with whole clitellum and the rest of the body containing majority of the intestinal tract. The part with majority of intestinal tract was discarded because it contained extracted dye in its lumen, which would interfere with the fluorescence measurements. The head piece with clitellum was weighed and placed in 12 ml-polypropylene tube with screw top (Labservs LB1280) containing 1.5 ml of 1-propanol. The tube was left overnight in the refrigerator at 4˚C. Samples were homogenised using the Ultra Turrax (IKA-WERK, Janke & Kunkel, # 45621). After each homogenisation, the Ultra Turrax was washed with water and rinsed with 1-propanol after use, to avoid cross contamination between samples. Homogenates were stored for 1-2 h at 4˚C. Samples were centrifuged (Megafuge 1.0R- Heraus) at 4000 rpm for 30 min at 4˚C. A volume of 100 µl of supernatant from each sample was transferred into a well of a 96-well-microplate (Brand Corning, non-treated polystyrene black round bottom wells # CLS3792) in two or three technical replicates. Serial RB dilutions in 1-propanol with known concentrations (from 0.01 mg/l to 0.9 mg/l) were included in triplicates to generate a calibration curve. The fluorescence was measured using a Fluostar BMG with a 544 nm excitation filter and a 580 nm emission filter. Data were transferred to an Excel sheet for calculations of the amount of RB in each sample.

5.2.6 **Data analysis**

The average of fluorescence values of each individual sample was calculated. For every plate, a calibration curve was created by plotting a graph of the standard RB concentrations (x axis) and the respective fluorescence values (y-axis) (one example of a calibration curve is shown in figure 5.1). Linear trendline equation and the R-squared value (R^2) of calibration curve were calculated using the Excel program. The RB concentration of each sample was calculated by extrapolating the fluorescence value in the trendline equation. Sample normalisation was required to correct differences due to experimental variation, which was done by dividing the RB concentration by the weight of respective sample. The normalised ratio was expressed in mg/l g^{-1}. For each treatment, the average of the normalised ratios of experimental replicates and standard deviation (SD) were calculated. The results of treated groups were compared with the respective control group and the statistical significance of the difference between groups (P-value) of each treatment was calculated by ANOVA. For the efflux assay, the normalised ratios of each interval of both groups were plotted in a graph.

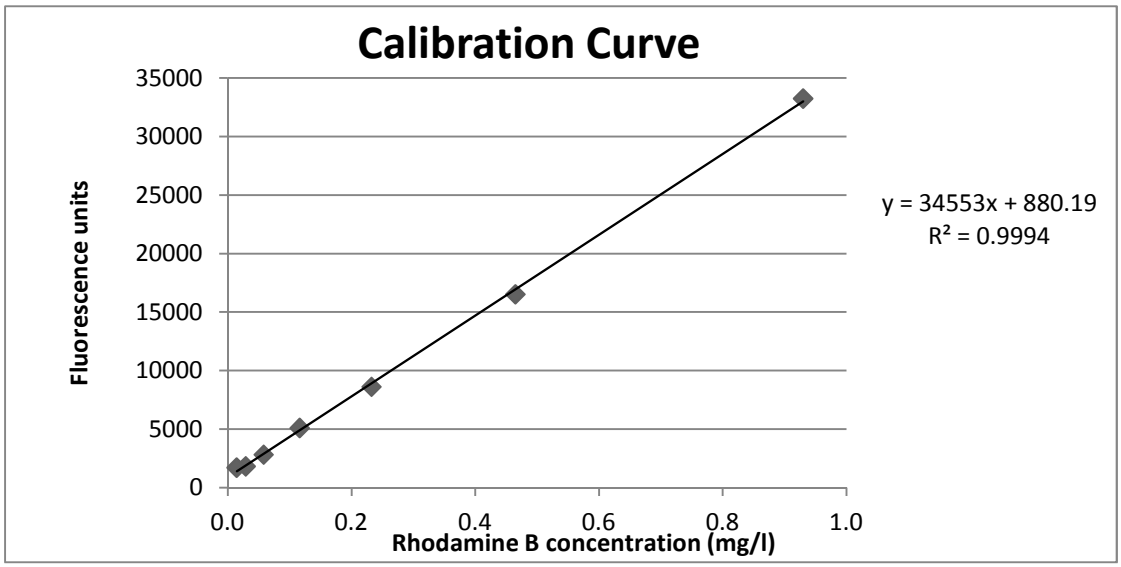

Figure 5-1 Calibration curve of rhodamine B concentrations versus fluorescence values. In the x-axis are the values of known rhodamine B concentrations between 0 and 1 mg/L (0, 0.015, 0.029, 0.059, 0.117, 0.234, 0.469, and 0.937). In the y-axis are the fluorescence units of correspondent measurements by the fluorometer. To calculate the rhodamine B concentration (x value) of samples, the fluorescence values are applied in the y value of the trendline equation (y=34553x+880.19). The R^2 denotes the correlation between x and y axes values.

5.3 Results

5.3.1 **Accumulation assay**

Earthworms exposed to the three concentrations of verapamil for 48 h on filter paper showed higher RB accumulation in their homogenates compared to the negative control (Figure 5.2a). The fluorescence was significantly higher ($P < 0.05$) compared to the negative control showing the inhibitory effect of verapamil on P-gp transport of RB. Earthworms exposed to 25 and 50 µM CyA concentrations also showed significantly higher RB accumulation compared to their negative control (Figure 5.2b), indicating the inhibitory effect on the P-gp function without causing clinical signs of toxicity in earthworms. The 100 µM CyA concentration was toxic to earthworms. It caused dehydration and death and therefore, fluorescence was not measured in those earthworms. Exposure to IVM did not result in significant difference between the treated groups with 0.2 and 0.02µg/ml concentrations and the negative control (Figure 5.2c). Similarly, the rifampicin exposure with 100 µM and 2 mM concentrations did not show a significant difference between the treated and the control (Figure 5.2d). The data of all treatments are shown in the appendix D.2.1 to D.2.4.

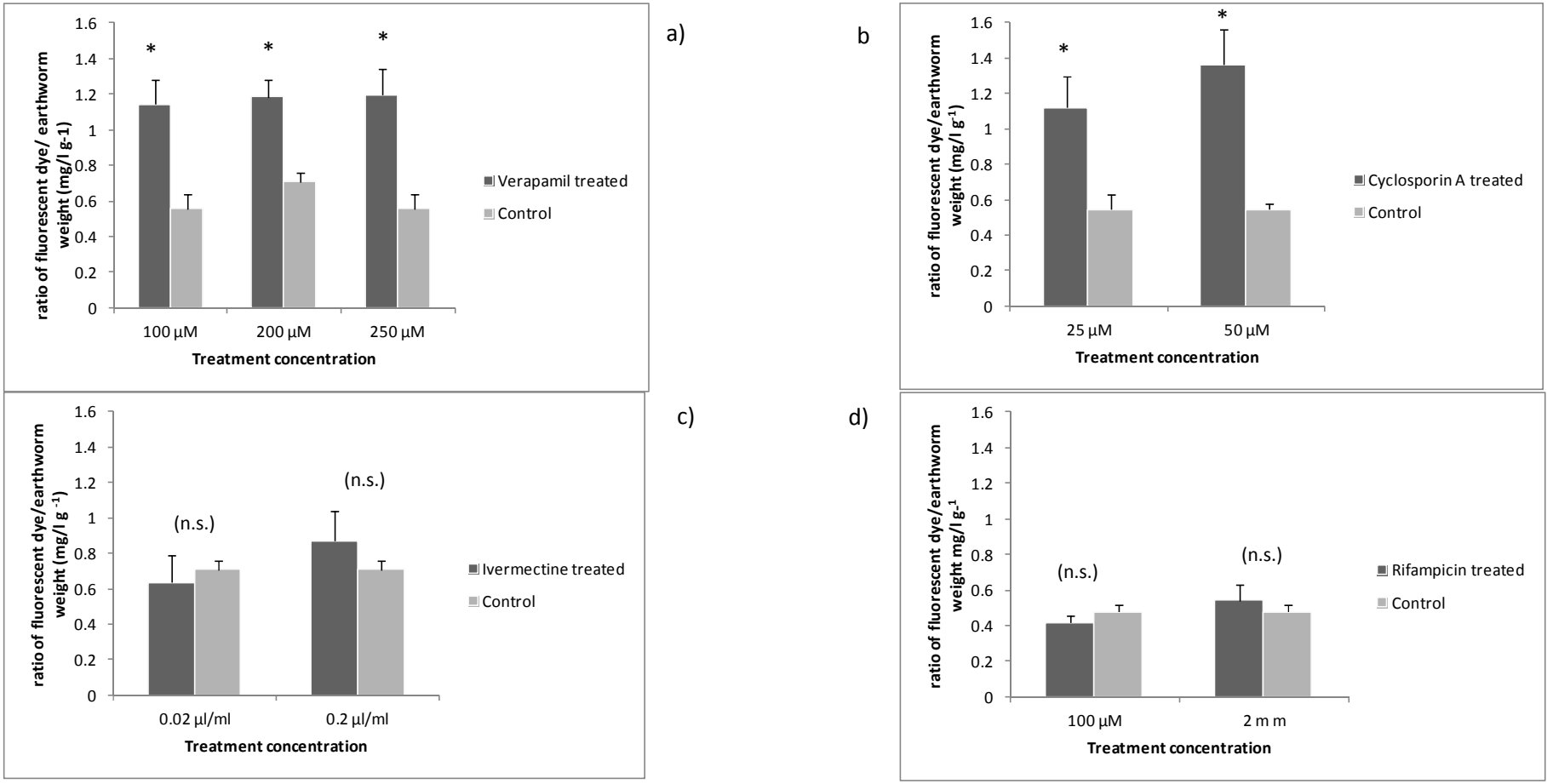

Figure 5-2 Effect of P-gp modulators on the amount of the accumulated fluorescent dye rhodamine B in earthworms *Aporrectodea caliginosa*. a) treated worms were exposed to a mixture of rhodamine B and verapamil and control only to rhodamine B; b) treated worms were exposed to a mixture of rhodamine B and cyclosporin A; c) treated worms were exposed to a mixture of ivermectin and rhodamine B and control only to rhodamine B; d) treated worms were exposed to a mixture of rifampicin and rhodamine B and control only to rhodamine B. Asterisks denotes significant differences ($p<0.05$) between control and treated, indicating the accumulation of rhodamine B in treated worms and (n.s.) denotes non- significant differences, indicating that chemical exposure had no effect in the accumulation of rhodamine B. Data are expressed in amount of accumulated fluorescence dye (mg/l) per gram of anterior piece of earthworm [mean ± standard deviation (errors bars), n= 4 to 6 replicates].

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5.3.2 **Efflux assay**

On exposure to 100 µM verapamil for 48 h, the earthworms showed a higher RB accumulation in the homogenate prior to the efflux period, compared to the negative control. When the treated earthworms were transferred to a clean soil environment, free of verapamil, the earthworms showed the re-establishment of P-gp function as shown by decrease in the fluorescence ratios measured at 3 intervals during efflux (Figure 5.3). After 72 h of efflux, both groups presented almost the same amount of RB detected in their homogenates. The fluorescence measurements and normalised ratios of each interval are shown in the appendix D2.1.

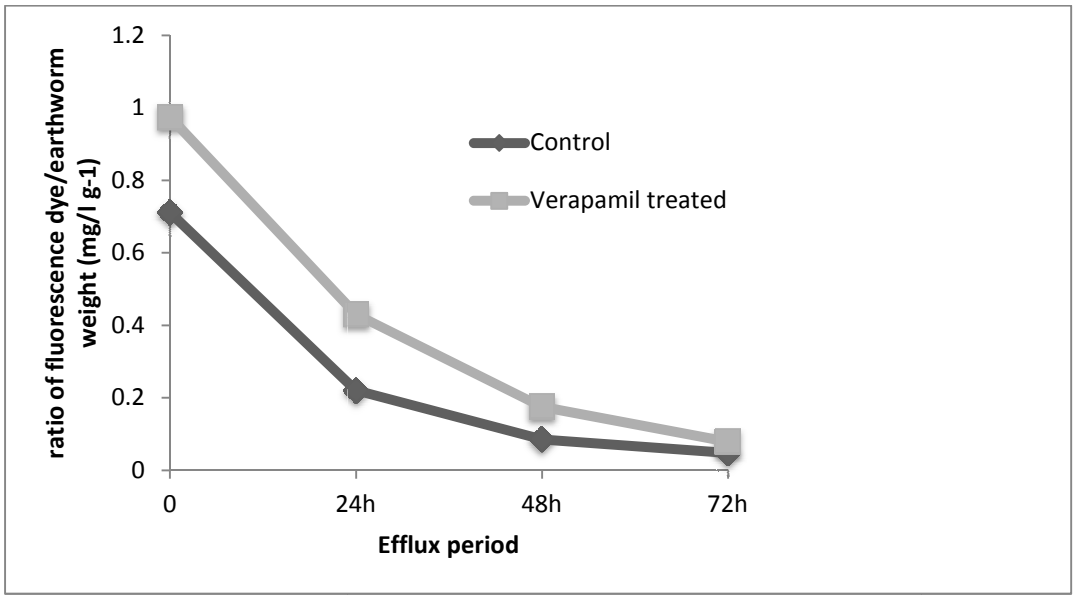

Figure 5-3 Efflux of Rhodamine B dye in earthworms exposed to verapamil 100 µM and the negative control**.**

5.4 Discussion

In this study, the evidence of P-gp presence in *A. caliginosa* was demonstrated by a biochemical assay using a P-gp substrate in combination with MXR inhibitors known to block the P-gp function. The use of MXR inhibitors, verapamil and CyA resulted in significantly higher levels of the P-gp substrate (RB) accumulated in the treated earthworms in comparison to the control earthworms (without MXR inhibitors, only fluorescent dye), indicating their inhibitory effect on P-gp function. The disruption of substrate efflux suggested that P-gp is present in *A. caliginosa*. These results are in agreement with studies using similar assays with RB and verapamil in other earthworm species: *E. fetida* (Brown *et al.*, 2008), and *Eisenia andrei* (Hackenberger *et al.*, 2012). Also, similar findings have been reported in experiments using RB and verapamil in mussels, a species which share a close phylogenetic relationship with earthworms (Kurelec *et al.*, 2000).

Both MXR inhibitors demonstrated to be suitable P-gp blockers in *A. caliginosa*. Verapamil was used in the studies with aquatic species involving marine and freshwater mollusc species to inhibit the function of P-gp and other MXR transporters (Hennessy & Spiers, 2007; Smital *et al.*, 2000). Cyclosporin A has been used in a study conducted in oysters (Ivanina & Sokolova, 2008) where the extruded dye was measured in the water at constant intervals and the results showed a decrease in the rates of RB efflux from oyster gills in the presence of CyA, demonstrating its inhibitory effect on P-gp.

Here, the P-gp activity on *A. caliginosa* was also demonstrated in a modified version of the accumulation assay, the efflux assay. In this study, the efflux assay was conducted to demonstrate the functional activity of P-gp that enables the organism to eliminate the RB dye following exposure to a MXR inhibitor. Their P-gp function was re-established in less than 24 h of efflux, and pre-treated earthworms were able to eliminate RB at a similar rate as the controls. Similar result was reported in a study with marine mussel, where the P-gp response was restored after cessation of the MXR inhibitor exposure (Smital & Kurelec, 1998b). During the efflux period, part of RB extrusion is due to the passive transport of substrate through the cells (diffusion), but the majority is via P-gp transport. It was found experimentally that substrates with low lipid solubility diffuse relatively slowly across membranes compared to chemosensitisers, such as verapamil which traverses lipoprotein bilayers quickly (Bard, 2000). Therefore, the elimination of RB from pre-treated earthworms was mainly from the cessation of verapamil exposure and restoration of P-gp activity.

Earthworms exposed to rifampicin did not present increased transport activity as expected. Rifampicin has been used to induce mRNA expression in mammals (Asghar *et al.*, 2002). In this study, rifampicin induced the mRNA expression after exposure to 10 mM rifampicin solution mixed in soil (Chapter 4) suggesting that an increase of gene expression would result in increase of protein expression and consequently the RB extrusion would be higher than control. One of the possible explanations for the absence of P-gp overexpression in this study is the induction pathway of P-gp. The results are suggestive that the P-gp protein regulation in *A. caliginosa* may be occurring at the post-transcriptional level. The overexpression of *ABCB1* transcripts may have occurred, but the transcripts were not translated immediately and directly to the functional P-gp. Other studies have also noted the absence of strict correlation between mRNA and protein levels of P-gp (Ivanina & Sokolova, 2008; Shirasaka *et al.*, 2006). This meant that the P-gp overexpression did not correspond to the increase of mRNA levels, suggesting that P-gp synthesis occurred at the post-transcriptional level. Moreover, there is evidence from earlier studies that P-gp has to undergo considerable post-translational modification including phosphorylation and glycosylation to become functional (Bard, 2000) and the translocation from intracellular compartments to plasma membrane takes over 7 days (Kota *et al.*, 2010). Therefore, the complexity of post-transcriptional and post-translational steps

involved in the P-gp regulation, the details of which are not known at this time, may explain the lack of increased P-gp activity in earthworms when exposed to rifampicin, a known inducer of mRNA of *ABCB1.*

The exposure to IVM did not increase the P-gp transport activity in *A. caliginosa*. Ivermectin^{TM} has been shown to upregulate the *ABCB1* gene expression and also increase the expression of functional P-gps through post-transcriptional regulation in murine hepatocyte cell line (Ménez *et al.*, 2012). In this study, the concentrations of 2 µg/ml induced mRNA upregulation (Chapter 4), but this concentration was toxic to earthworms when exposed via direct contact on filter paper (Section 4.3.1). Therefore, lower concentrations (0.2 and 0.02 µg/ml) were used in the functional RB dye assay described above. In the study of James & Davey (2009), IVM induced the P-gp overexpression in *C. elegans* after repeated treatments starting with low IVM concentration just below the lethal threshold and increasing gradually. The survivors were selected and allowed to reproduce. The next generation was exposed to an increased concentration and after several generations, a resistant population was created which showed overexpression of P-gp (MXR phenotype). As already mentioned in the previous chapter, the development of a resistant earthworm population to IVM in order to demonstrate the P-gp overexpression, is a long-term project because earthworms take approximately 3-4 months to reach maturity for reproduction under optimal conditions.

In conclusion, the results of accumulation and efflux assays in this study demonstrated the P-gp function in *A. caliginosa*. The extrusion of the specific P-gp substrate (RB) was altered when earthworms were exposed to known MXR inhibitors (CyA and verapamil), but efflux was not significantly changed when exposed to *ABCB1*/P-gp modulators (rifampicin and IVM).

Chapter 6

Final Discussion

6.1 Novel findings

In the present study, new genetic information was generated for *A. caliginosa* (Lumbricidae family), the most abundant pasture earthworm in New Zealand. A partial sequence (10.7% of the exonic sequence) homologous to the *ABCB1* gene was obtained using a combined approach of degenerate PCR and sequence retrieval from the draft *L. rubellus* genome LUMBRIBASE (Chapter 3). The presence of conserved motifs within this *ABCB1* gene fragment indicated that it encodes P-gp, an ATP-dependent transmembrane transporter that is involved in the cellular defence against natural and anthropogenic toxins. Furthermore, partial sequences homologous to the *A. caliginosa ABCB1* gene were identified in other five members of the Lumbricidae family including *L. rubellus* (a common earthworm used in toxicological soil assessments), *A. trapezoides*, *O. cyaneum, E. fetida* and *A. chlorotica*. This work reported for the first time an *ABCB1* gene in *A. caliginosa* or in a member of the Lumbricidae family. The alignment of the six novel lumbricid sequences showed high homology (>94%) at nucleotide level which will facilitate further work to extend these gene fragments in all six species. All translated novel sequences showed high similarities with P-gp sequences of vertebrate and invertebrate species confirming the presence of conserved domains within P-gp.

The new gene sequence from *A. caliginosa* was used to develop a reverse-transcription quantitative PCR (RT-qPCR) assay for measuring *ABCB1* gene expression in this species (Chapter 4). The assay was validated using earthworms treated with the *ABCB1* drug inducer (rifampicin) that had been shown to work in mammals. This RT-qPCR assay was optimised for the numerous critical quality assurance and control that influence the accuracy of the results and reliability of the conclusions (Derveaux *et al.*, 2010). Two key components of this process were (i) the identification of *β-actin* as a reference gene for *A. caliginosa* RT-qPCR assays, and (ii) determining the toxicity of chemicals and their solvents used to induce *P-gp* expression. The assay was also optimised for PCR parameters to improve the amplification kinetics of the reaction and minimise the production of nonspecific products.

Using the optimised RT-qPCR assay, new data was obtained on the effects of selected drugs when exposed to *A. caliginosa.* Rifampicin was demonstrated to be a suitable *ABCB1* inducer for earthworms, with low toxicity and cost. Rifampicin upregulated the *ABCB1* mRNA levels, but it did not increase the amount of functional P-gp measured by the RB assay (Chapter 5), suggesting that there is additional control on the pathway that produces the functional P-gp protein. In many cell lines and human tumours, it is generally accepted that P-gp expression is increased through

upregulation of *ABCB1* gene mRNA levels (Chaudhary & Roninson, 1993), however this may not occur in a straight forward pathway (Hennessy & Spiers, 2007). The overexpression of P-gp has been shown to be controlled partially through post-transcriptional mechanisms, such as mRNA stabilisation (Ménez *et al.*, 2012).

In the functional studies of this research, CyA, a known MXR inhibitor, blocked P-gp function in *A. caliginosa*. Despite blocking protein function, CyA did not increase *ABCB1* gene expression, suggesting that P-gp blocking alone does not lead to overexpression of *ABCB1* to compensate for the inactive P-gps. In contrast, in a preliminary study with IVM, *ABCB1* expression was increased in *A. caliginosa*, suggesting that this specific P-gp substrate has the same effect on *ABCB1* expression seen in other studies (James & Davey, 2009; Ménez *et al.*, 2012). This result was significant as IVM is a widely used agrochemical and there is increasing concern about its effect on the environment. Future research may demonstrate that the measurement of the *ABCB1* gene in earthworms could be used as a biomarker in toxicological risk assessments.

The presence of an *ABCB1* gene homologue in *A. caliginosa* and its expression in response to known inducers supported the hypothesis that P-gp is present in *A. caliginosa*. Although P-gp itself was not isolated or directly measured in this research, the presence of a functional P-gp in *A. caliginosa* was demonstrated indirectly using a biochemical assay with a RB dye as a P-gp substrate and two known MXR inhibitors known to affect the P-gp of mammals and aquatic organisms (verapamil and CyA). The results showed that there was a high level of P-gp substrate accumulation in *A. caliginosa* treated with these inhibitors compared to the non-treated worms indicating that P-gp function was disrupted and therefore, suggesting its presence in *A. caliginosa.* The study contributes to the few that have reported evidence of P-gp in earthworms other than *A. caliginosa* using similar functional assays (Brown *et al.*, 2008; Hackenberger *et al.*, 2012).

6.2 Future work

The partial sequence of the putative *A. caliginosa ABCB1* gene should be extended to obtain the full length genomic sequence. The information obtained from a full-length *A. caliginosa* sequence will further confirm the identity of the gene fragment and clearly differentiate it from closely related genes within the *ABCB1* subfamily. Some organisms, such as rodents, have two members of *ABCB1* (*ABCB1a* and *ABCB1b*) which encode P-gps with similar function (Sturm & Segner, 2005). There is a high probability that earthworms also have more than one P-gp isoform involved in xenobiotic resistance as they do not possess sophisticated protective mechanisms that the mammals have against environmental toxins (Zhao *et al.*, 2007). For example, the free-living nematode *C. elegans* has 15 *ABCB1* gene homologues within a comparatively small genome, but only two are confirmed to be involved in drug transport (Zhao *et al.*, 2004), and the helminth *H. contortus* has 12 P-gp

sequences (Smith & Prichard, 2002). In addition, a full length sequence would provide information on the base sequence and structure of this gene in annelids and may prove useful to facilitate gene discovery in other earthworm species.

The partial *A. caliginosa ABCB1* cDNA could be extended by repeating the 3' RACE method in combination with a cloning step to separate the resultant cDNA sequences and facilitate DNA sequencing. Similarly, 5' RACE would amplify the cDNA towards the 5' end. Primers designed to the cDNA sequence could then be used to amplify the genomic DNA to obtain the complete gene sequence with introns. The complete *ABCB1* sequence information would be useful to design new primers complementary to unique sequences of *ABCB1* gene, preferably from regions encoding the transmembrane domains (TMD), which are less conserved among ABC transporters than the nucleotide domains (NBD). This would improve the RT-qPCR assay by eliminating any possibility of confounding data from nonspecific amplification of other ABC transcripts. Another approach to improve RT-qPCR of the *ABCB1* transcript would be the use of a hybridisation probe instead the DNA intercalator SYBR Green I. Although SYBR Green I has been used in most qPCR studies of *ABCB1* expression in other species (Fischer *et al.*, 2011; Loncar *et al.*, 2010; Ménez *et al.*, 2012), as well as in earthworms (Chen *et al.*, 2011; Ricketts *et al.*, 2004), the use of a probe is a more sensitive and accurate assay. This was observed when a fluorogenic TaqMan probe was used in a 5' nuclease assay in the study of metallothionein gene expression in *L. rubellus,* (Spurgeon *et al.*, 2004).

To further improve the RT-qPCR assay, it is necessary to identify more references genes in *A. caliginosa* for better normalisation of the samples. According to the latest guidelines for qPCR experiments, a quantitative PCR requires at least three or more reference genes to increase accuracy and reliability of gene measurements. The strategy elected for this study to find the novel sequences may be used for the finding of other reference genes. There are a number of common reference genes described elsewhere (Stürzenbaum & Kille, 2001) and this may guide the choice of the additional reference genes.

One of the refinements needed in the process of RT-qPCR analysis of treated earthworms is to improve the chemical exposure methodology. The exposure period to *ABCB1* modulators could be shortened because the *ABCB1* expression is likely to be detected before 7 days. It has been reported that *ABCB1* gene induction was achieved after 72 h in human intestinal epithelial cells exposed to different drug compounds (Haslam *et al.*, 2008) and a rapid induction of *ABCB1* (up to 10-fold) was obtained in human tumours within 10 minutes of exposure to doxorubicin (Abolhoda *et al.*, 1999). More recently, Menez *et al*. (2012) demonstrated that *ABCB1* induction in rodents is transient and the *ABCB1* mRNA levels decline after 72 h of exposure to IVM. As an initial experiment, earthworms could be immersed in an aqueous solution of inducer for 72 h to allow drug absorption and ingestion,

with the advantages that drug is evenly distributed in the medium and the short exposure avoids drug degradation. Another approach to achieve higher induction of *ABCB1* gene expression could be the exposure to a combined P-gp substrate and a P-gp blocker. According to mammalian models, evidence shows that *ABCB1* expression can be induced as part of a general response to cellular stress, such as the accumulation of a P-gp substrate (Sturm & Segner, 2005). In contrast this study, analysed gene expression with samples exposed to Pg- substrate (IVM) and a P-gp blocker (CyA) separately. These new approaches may enhance the assay accuracy and improve the gene expression data obtained from the chemical exposure experiments.

The chemicals used in this study have previously been shown to affect either the *ABCB1* expression or the P-gp function in other organisms. However, there are numerous other chemicals described in the literature that are known to modulate *ABCB1* gene transcription and P-gp function in different organisms (Bard, 2000; Buss & Callaghan, 2008; Lespine *et al.*, 2008; Smital *et al.*, 2004). Testing other chemicals would identify the spectrum of *ABCB1*/P-gp modulators and P-gp substrates for earthworms and they could then be used as reference or control drugs in future experiments. For example, dexamethasone drug induces expression of P-gp in *E. andrei* (Hackenberger *et al.*, 2012), chemotherapeutic agents transiently induce *ABCB1* gene in mammals (Scotto, 2003), and the barbiturate phenobarbitol is a common *ABCB1* inducer in mammals (Manceau *et al.*, 2010). These *ABCB1* inducers may produce higher induction in *A. caliginosa* than observed with rifampicin in this study.

The accurate evaluation of the effect of chemicals on the P-pg and *ABCB1* expression is usually obtained in studies with isolated tissues or pure cell lines because P-gp and *ABCB1* expression is inducible at different levels in several tissues and its regulation can differ between cell types (Sturm & Segner, 2005). It has been reported that rifampicin does not induce the *ABCB1* expression in human lymphocytes and brain cells (Nannelli *et al.*, 2010) as opposed to hepatocytes and enterocytes (Manceau *et al.*, 2010). However, the only established *in vitro* experiment with earthworms used a culture of coelomocytes, but it lasted only 3 h (Quaglino D, 1996). The isolation and culture of cells from specific tissues or organs in earthworms could be attempted but would require considerable skill and specialised equipment.

Alternatively, the localisation of P-gp in earthworm tissues could be performed by immunochemistry. This information would confirm the presence of P-gp in earthworms and would establish which tissues should be targeted for RT-qPCR. Localisation of P-gp in fish, aquatic invertebrates and nematodes has been conducted using antibodies raised against mammalian P-gp (Kerboeuf *et al.*, 2003; Sturm & Segner, 2005; Sturm *et al.*, 2001). Antibodies against the P-gp of any invertebrate species, including earthworms, are not yet available and therefore assays would have to rely on

available mammalian antibodies. The most frequently used monoclonal antibody (mab) C219 recognises an epitope common to all known P-gps. However, not all P-gps are involved in drug resistance. There are other mab that bind specifically to the P-gp product encoded by *ABCB1* gene (Schinkel *et al.*, 1993 ; Taylor *et al.*, 2001) . They include mabs UIC2 and MRK16 (specific for external P-gp epitopes) and JSB-1 and C494 (specific for internal P-gp epitopes), which have been used in mammalian assays (Taylor *et al.*, 2001). However, it is unclear whether they would recognise the P-gp of earthworms. For example, Hemmer *et al.* (1995) used JSB-1 and C494 in *Poecilia reticulata* (guppy fish) with ambiguous results, suggesting that P-gp of fish differ significantly from mammals. In contrast, Riou *et al*.(2005) successfully used mab UIC2 in an indirect immunofluorescence staining assay in the helminth *H. contortus*, showing that P-gp was located in the external layer and cuticle of the worm. To date, there are very few published data about the use of mammalian P-gp mabs in invertebrate species and none for earthworms.

Another approach that would be useful for the localisation of P-gp in earthworms is the detection of *ABCB1* mRNA by *in-situ* hybridisation using frozen or paraffin embedded sections. Smith and Prichard (2002) observed the distribution of *ABCB1* mRNA in transverse cryosections of adult *H. contortus* using a digoxigenin-labelled cDNA encoding the ATP-binding region of *H. contortus* P-gp. The probe sequence had similarity to 12 P-gp sequences previously identified in *H. contortus,* and thus hybridisation gave an overall measurement of the total P-gp mRNA. Similarly, a specific probe could be designed for earthworms from the full length lumbricid *ABCB1* sequence in order to determine the localisation and distribution of P-gp expression. These studies would contribute to the understanding of P-gp function in this species.

With the RT-qPCR optimised and identification of which tissues express P-gp, the next step would be to test field samples to examine whether the *ABCB1* measurement can be used as an indicator to detect the presence of contaminants in soil. Further work is needed to determine the number of earthworms required to minimise the variability inherent in *ABCB1* and P-gp expression due to genetic factors (Hoffmeyer *et al.*, 2000). Identification of additional ABC proteins involved in the MXR would improve the use of *A. caliginosa* as a bio-indicator. The most relevant ABC proteins are the multidrug resistance associated protein (MRP) 1 and 2, encoded by ABCC1 and ABCC2 gene subfamily, and breast cancer resistance protein (BCRP) encoded by ABCG2 which transport a wide spectrum of substances (Leslie *et al.*, 2005; Zaja *et al.*, 2008; Zucchi *et al.*, 2010). Studies in aquatic organisms have shown that exposure to common environmental pollutants modulated the gene expression of these MXR members detected by RT-qPCR, (Diaz de Cerio *et al.*, 2012; Franzellitti *et al.*, 2010; Kingtong *et al.*, 2007; Luedeking & Koehler, 2004).

For a better understanding of the role of P-gp in earthworms, more advanced technology and sophisticated approaches could be used in future studies. To unequivocally establish the role of the P-gp encoded by the *ABCB1* gene, methods such as gene knock-down, direct mutagenesis or transgenic modifications could be attempted. In a transgenic experiment, Zhao *et al*. (2004) demonstrated ABC transporter expression in *C. elegans* using promoter–driven green fluorescent protein (GFP) fusions to address when and where these genes are turned on *in vivo*. For similar experiments to be done in earthworms the challenge of modifying earthworm embryonic cells would have to be overcome. To date, there are no publications of lumbricid embryonic cell manipulation. In the nematode model, *C. elegans* embryonic cells can be obtained from eggs harvested from synchronized gravid adults and then are dissociated using a combination of enzymatic treatment and manual pipetting (Bianchi & Driscoll, 2005). However, it is unknown whether a similar approach would work in earthworms.

Extending genomic sequence of *ABCB1* in *A. caliginosa* to include upstream regulatory regions would improve understanding of *ABCB1* transcriptional regulation in earthworms. In humans, *ABCB1* gene and P-gp expression involves a complex regulatory pattern controlled by several transcription factors (Labialle *et al.*, 2002; Scotto, 2003). The most relevant transcription factor in the multidrug resistance phenomenon is the pregnane X receptor (PXR) (Buss & Callaghan, 2008; Wassmur *et al.*, 2010) that can be activated by a wide array of xenobiotics including numerous pharmaceuticals and other environmental pollutants. When activated, it regulates the expression of P-gp (Wassmur *et al.*, 2010). Rifampicin is a specific PXR agonist (Haslam *et al.*, 2008) and the response of *A. caliginosa ABCB1* gene to rifampicin exposure reported in this study suggests that *A. caliginosa* may have a similar pathway involving transcriptor factors as in mammals for *ABCB1* transcription.

6.3 Final conclusion

The identification of a partial sequence homologous to the *ABCB1* gene and the use of this data to create an RT-qPCR assay for *ABCB1* expression have provided fundamental new knowledge of the *ABCB1* gene in earthworms. In aquatic animals, P-gp overexpression is associated with the MXR and the measurement of *ABCB1* expression has been used to identify resistant organisms and the presence of pollutants. Thus, the results of this research may provide baseline data for similar assays using earthworms. From an ecotoxicological context, information related to *ABCB1* and P-gp in earthworm is important to understand their response to agrochemical use in pastures and in the future, this response may be used as a tool for ecotoxicological research and toxicological risk assessments.

Appendix A- Primer design

A.1 Alignment of P-gp protein sequences for design of degenerate primers

```
C. briggsae          ILRQDIS....WFDTNHSGTLATKLFDNLERVKEGTGDKI     76
C. elegans           ILRQeIS....WFDTNHSGTLATKLFDNLERVKEGTGDKI     76
Cricetulus sp        ffhaimnqeigWFDvhdvGeLnTrLtDdvskinEGiGDKI     80
P.troglodytes        ffhaimrqeigWFDvhdvGeLnTrLtDdvskinEGiGDKI     80
E. caballus          ffhaimqqeigWFDmhdvGeLnTrLtDdvskinEGiGDKI     80
H. sapiens           ffhaimrqeigWFDvhdvGeLnTrLtDdvskinEGiGDKI     80
C. lupus             ffhaimrqeigWFDvhdvGeLnTrLtDdvskinEGiGDKI     80
Consensus                         wfd    g l t l d      eg gdki

C. briggsae          GMAFQYMSQFITGFIVAFTHSWKLTLVMLAVTPIQALCGF    116
C. elegans           GMAFQYlSQFITGFIVAFTHSWqLTLVMLAVTPIQALCGF    116
Cricetulus sp        GMfFQaMatFfgGFIigFTrgWKLTLViLAisPvlgLsag    120
P.troglodytes        GMfFQsMatFfTGFIVgFTrgWKLTLViLAisPvlgLsaa    120
E. caballus          GMfFQsMatFfTGFIVgFTrgWKLTLViLAisPvlgLsag    120
H. sapiens           GMfFQsMatFfTGFIVgFTrgWKLTLViLAisPvlgLsaa    120
C. lupus             GMfFQsiatFfTGFIVgFTrgWKLTLViLAisPvlgLsaa    120
Consensus            gm fq    f  gfi  ft  w ltlv la  p   l

C. briggsae          LIAKSMSTFAIRETVRYAKAGKVVEETISSIRTVVSLNGL    156
C. elegans           aIAKSMSTFAIRETlRYAKAGKVVEETISSIRTVVSLNGL    156
Cricetulus sp        iwAKilSsFtdkElqaYAKAGaVaEEvlaaIRTViafgGq    160
P.troglodytes        vwAKilSsFtdkEllaYAKAGaVaEEvlaaIRTViafgGq    160
E. caballus          iwAKilSsFtdkEllaYAKAGaVaEEvlaaIRTViafgGq    160
H. sapiens           vwAKilSsFtdkEllaYAKAGaVaEEvlaaIRTViafgGq    160
C. lupus             iwAKilSsFtdkEllaYAKAGaVaEEvlaaIRTViafgGq    160
Consensus              ak  s f   e   yakag v ee    irtv    g

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C. briggsae          EVFIDGSEIKTLNPENTRSQIAIVSQEPTLFDCSIAENIV   1034
C. elegans           EiFIDGSEIKTLNPEhTRSQIAIVSQEPTLFDCSIAENIi   1036
Cricetulus sp        tVFlDGkEvnqLNvqwlRahlgIVSQEPiLFDCSIAENIa   1022
P.troglodytes        kVllDGkEIKrLNvqwlRahlgIVSQEPiLFDCSIAENIa   1022
E. caballus          tVllDGtEIKhLNvqwlRahlgIVSQEPiLFDCSIgENIa   1022
H. sapiens           kVllDGkEIKrLNvqwlRahlgIVSQEPiLFDCSIAENIa   1022
C. lupus             sVlIDGkEIKhLNvqwlRahlgIVSQEPiLFDCSIAENIa   1022
Consensus                dg e   ln    r    ivsqep lfdcsi eni

C. briggsae          YGLDPTTVTMSRVEEAAKLANIHNFISELPEGYETRVGDR   1074
C. elegans           YGLDPssVTMaqVEEAArLANIHNFIaELPEGfETRVGDR   1076
Cricetulus sp        YGdnsrvVsqdeiErAAKeANIHqFIesLPdkYnTRVGDk   1062
P.troglodytes        YGdnsrvVsqeeivrAAKeANIHaFIesLPnkYsTRVGDk   1062
E. caballus          YGdnsrvVsqeeivqAAKeANIHpFIetLPdkYnTRVGDk   1062
H. sapiens           YGdnsrvVsqeeivrAAKeANIHaFIesLPnkYsTkVGDk   1062
C. lupus             YGdnsrvVsheeimqAAKeANIHhFIetLPEkYnTRVGDk   1062
Consensus            yg     v       aa  anih fi  lp    t vgd

C. briggsae          GTQLSGGQKQRIAIARALVRNPKILLLDEATSALDTESEK   1114
C. elegans           GTQLSGGQKQRIAIARALVRNPKILLLDEATSALDTESEK   1116
Cricetulus sp        GTQLSGGQKQRIAIARALVRqPhILLLDEATSALDTESEK   1102
P.troglodytes        GTQLSGGQKQRIAIARALVRqPhILLLDEATSALDTESEK   1102
E. caballus          GTQLSGGQKQRIAIARALVRqPqILLLDEATSALDTESEK   1102
H. sapiens           GTQLSGGQKQRIAIARALVRqPhILLLDEATSALDTESEK   1102
C. lupus             GTQLSGGQKQRIAIARALVRqPhILLLDEATSALDTESEK   1102
Consensus            gtqlsggqkqriaiaralvr p illldeatsaldtesek

C. briggsae          IVQEALDRAREGRTCIVIAHRLNTIMNADCIAVVNNGTII   1154
C. elegans           vVQEALDRAREGRTCIVIAHRLNTvMNADCIAVVsNGTII   1156
Cricetulus sp        vVQEALDkAREGRTCIVIAHRLsTIqNADlIvViqNGkvk   1142
P.troglodytes        vVQEALDkAREGRTCIVIAHRLsTIqNADlIvVfqNGrvk   1142
E. caballus          vVQEALDkAREGRTCIVIAHRLsTIqNADlIvVfqNGkvk   1142
H. sapiens           vVQEALDkAREGRTCIVIAHRLsTIqNADlIvVfqNGrvk   1142
C. lupus             vVQEALDkAREGRTCIVIAHRLsTIqNADlIvVfqNGkvk   1142
Consensus             vqeald aregrtciviahrl t  nad i v  ng
```

Note: The letters in red denotes the selected amino acid sequences for the design of degenerate primers.

A.2 Alignment of *ABCB1* nucleotide sequences for the design of degenerate primers

```
M.edulis        ....................................        0
O.mykiss        cagtagtggctgtgggaaaagcaccaccgttcagctgctg   1413
R.norvergicus   caacagtggctgtgggaaaagcacaactgtccagctgctg   1349
H. sapiens      aaacagtggctgtgggaagagcacaacagtccagctgatg   1128
T. adherens     aggtagtggttgcggtaagagcactgtagtacagttaatt   1221
G. gallus       tggcagcggctgtgggaaaagtacaactgttcagctcatc   1413
M.californianus atcgagtggatgtggaaaatctaccatagtcaatctcata   1528
M. varia        ....................................        0
Consensus

M.edulis        CAGAGGTTCTATGACCCAGATGCAGGACAAGTTTTACTTG     40
O.mykiss        CAGcGGTTCTATGAtCCAcAaGatGGAtcAGTgTatgTaG   1453
R.norvergicus   CAGAGGcTCTAcGACCCcataGagGGcgAgGTcagtaTcG   1389
H. sapiens      CAGAGGcTCTATGACCCcacaGagGGgatgGTcagtgTTG   1168
T. adherens     CAaAGaTTCTAcGAtCCAcAgGacGGttgtGTTgaAaTcG   1261
G. gallus       CAGAGaTTtTAcGACCCcaAgGaAGGcacgaTTaccaTTG   1453
M.californianus CAGAGryTCTAyGAyCCAGATGCAGGACAgGTTTTACTaG   1568
M. varia        ........................GACAAGTTTTACTTG     15
Consensus                               g     t     t g

                ....................................

M.edulis        AACACACTAGTTGGAGAGCGTGGAGCTCAGTTATCGGGAG    280
O.mykiss        gAgACtCTAGTTGGAGAcaGaGGAaCcCAGaTgagtGGAG   1693
R.norvergicus   AACACcCTgGTTGGtGAGaGaGGgGCgCAGcTgagtGGgG   1629
H. sapiens      gACACcCTgGTTGGAGAGaGaGGgGCcCAGTTgagtGGtG   1408
T. adherens     gAtACAaTgGTcGGtGAaCGTGGgGCTCAacTtagtGGAG   1501
G. gallus       gAaACtgTgGTTGGAGAaaGaGGgGCaCAGaTgagtGGAG   1693
M.californianus AACACACTAGTTGGAGAGCGaGGAGCTCAGTTATCaGGAG   1808
M. varia        AACACACTAGTTGGAGAGCGTGGAGCTCAGTTATCaGGAG    255
Consensus        a ac  t gt gg ga  g gg  c ca  t    ggdg

M.edulis        GGCAAAAACAACGAGTAGCCATTGCCAGAGCTTTGATCAG    320
O.mykiss        GaCAgAAACAgaGgaTcGCtATcGCacGcGCTcTcgTacG   1733
R.norvergicus   GaCAgAAACAgaGgaTcGCCATTGCCcGgGCccTGgTCcG   1669
H. sapiens      GGCAgAAgCAgaGgaTcGCCATTGCacGtGCccTGgTtcG   1448
T. adherens     GaCAAAAgCAACGtaTtGCtATcGCtcGcGCgTTGgTtAa   1541
G. gallus       GGCAgAAgCAgCGAaTAGCaATTGCCcGtGCTcTGgTtcG   1733
EF521414        GGCAgAAACAAaGAGTAGCCATTGCCAGgGCTTTGATCAG   1848
FM994158        GaCAgAAACAACGAGTAGCCATTGCCAGAGCTTTGATtAG    295
Consensus       grcaraarcarigiit gc at gc  g gc  t  t

M.edulis        AGACCCAAGAATCCTATTGCTGGATGAAGCTACATCTGCA    360
O.mykiss        caACCCcAagATCCTcTTGtTGGAcGAgGCTACcTCaGCc   1773
R.norvergicus   caACCCcAagATCCTtTTGtTGGATGAgGCcACgTCaGCc   1709
H. sapiens      caACCCcAagATCCTccTGCTGGATGAgGCcACgTCaGCc   1488
T. adherens     AaAtCCcAaAATttTAcTatTaGATGAAGCaACtTCaGCA   1581
G. gallus       caAtCCtAaAATtCTtcTGCTtGATGAgGCaACgTCaGCt   1773
EF521414        AGACCCAAGAATCCTATTGCTGGATGAAGCTACATCTGCA   1888
FM994158        AGACCCAAGAATCCTATTGCTGGATGAAGCTACAT         330
Consensus        a cc a  at  t  t  tggatgargchacitcwgc

                ....................................
```

Note: The letters in red denotes the selected nucleotide sequences for the design of degenerate primers.

A.3 Alignment of nucleotide sequences of *ABCB1 C. elegans*, *ABCB1 M. edulis*, and *L. rubellus* contiguous sequence with homology to *ABCB1 M. edulis*.

```
Lumbricus contig    ................GATACTGAACGGTCTGAACGTGG     23
M. edulis           ....................................       0
C. elegans          tcccgcccggatgttccaATtCTacgtGGaaTGAAtcTcc   1320

Lumbricus contig    AGATAAAGCGAGGTCAGACGGTGGCTTTGGTCGGGTCGAG     63
M. edulis           ....................................       0
C. elegans          gtgTAAAtgcAGGTCAaACtGTcGCacTtGTCGGaTCatc   1360


Lumbricus contig    CGGGTGTGGCAAGAGCACAGTCATTCAGCTCATCCAACGC    103
M. edulis           ................................CAgaGg      6
C. elegans          gGGaTGTGGaAAGtcgACgaTtATcagttTgtTgttgCGt   1400

Lumbricus contig    TTCTACGACCCACTAGAGGGCGCTGTGATGATTGACGGAA    143
M. edulis           TTCTAtGACCCAgatGcaGGacaaGTttTacTTGAtGGtA     46
C. elegans          TaCTACGAtgtttTgaAaGGCaagaTtAcGATTGAtGGAg   1440

Lumbricus contig    CGGACATTCGCCAGCTGAACATCAAGTGGTTACGGCAACA    183
M. edulis           acaACATaaaagAttTaAAttTgAAcTGGTTACGaCAgaA     86
C. elegans          tcGACgTTCGggAtaTcAACtTggAaTttTTgaGaaAAaA   1480

Lumbricus contig    TATCGGAGTCGTCAGTCAGGAACCAGTGTTGTTTGCTCTC    223
M. edulis           cATCGGtGTgGTttcTCAGGAACCAGTcTTGTTTGgTtgC    126
C. elegans          TgTgGccGTCGTgtcaCAGGAgCCAGcGTTGTTcaaTtgt   1520

Lumbricus contig    ACGATAGCAGAGAACATTCGATACGGGAGAGAAGGA....    259
M. edulis           ACcATAGCcGAGAACATcCGActaGGaAatccgaatgcaa    166
C. elegans          ACGATcGagGAGAAtATTaGtctCGGaAaAGAAGGcataa   1560

Lumbricus contig    ....................................       259
M. edulis           ccattacagaaatagaacaagcagccaaacaagccaacgc    206
C. elegans          cacgtgaggaaatggttgctgcatgtaagatggccaacgc   1600

Lumbricus contig    ....................................       259
M. edulis           acatgatttcataaaaagtcttcctcagagttacaacaca    246
C. elegans          agaaaagttcatcaaaactctaccaaacggatataatact   1640

Lumbricus contig    ....................................       259
M. edulis           ctagttggagagcgtggagctcagttatcgggagggcaaa    286
C. elegans          cttgtcggagatcgtggaacccagctctccggaggccaga   1680

Lumbricus contig    ....................................       259
M. edulis           aacaacgagtagccattgccagagctttgatcagagaccc    326
C. elegans          aacaacgtatcgctattgctcgtgctctcgtcagaaaccc   1720

Lumbricus contig    ....................................       259
M. edulis           aagaatcctattgctggatgaagctacatctgcattagac    366
C. elegans          gaaaatccttctattggacgaagccacgtcagcactggac   1760

Lumbricus contig    ................GTGAGCCAGGCTGAGATAGAAA    281
M. edulis           tctgagagt..........................        375
C. elegans          gctgaatccgagggaattGTGcaaCAGGCattGgacaAAg   1800
```

Note: The letters in yellow denote the selected nucleotide sequences for the design of *L. rubellus* primers in the forward direction and the green letter denote the primers in reverse direction.

A.4 Assembled sequence of overlapping *L. rubellus* contiguous sequences that were homologous to the partial mRNA sequence of *M. edulis ABCB1*.

LUMBRIBASE contiguous sequences:
121673, 154463, 1166

```
1       CAAGTGCACA TAAAAAATAC AAATGTTAAA GTATGTTTTT CACACCCCGA
51      TTCCAAGTGG ATAATGGTCC AACCCCCTCC CACTTCTTTT TCCACAACTC
101     TTACACTGTA TATCGACAAC GAACAGGAGA AAGAGCAATG CCAATGATCC
151     CTATGTTTTC TATGTATAGA TGACTACAGG CTACTTCAAG TCTTCAACGA
201     ATCCAAATCA ATAATAATTG TTGAAACCAA GCCTTTTCTA AGAACGAGGA
251     CTTCCGACAC ACGGTTAAGT GCATACGATG AATTATAATT ATCAAAGCAG
301     AGTACTGTAT AGGCCAGTAA GCTACTGCAT CTATAATTCA ATCGTGTGGA
351     TTTAAATCGG CGGCCGGCGG GTATATCTTT ATTCGAATCT AAATTAAATC
401     TAAGTTCCCT TCTGATTAAA CTGGATCGAG CAAGTGCGTC GCTTGCCTTT
451     AGTTGGTATA GCCTACTGTT GGTTAGGTTT AATGGCTATG GGAGTGGCTA
501     TAATTATGGG TTGAGAACAG AGTCTCCTGT ATTACGTTCA TGCGGGTTTC
551     AAATCGCAGA ATAAAATTTC AACGCAATGA AATGTCTAAG TGCCCACAGC
601     AGTTAAGTCA AACAGAGACC AGCTTATTTA CCGGCGTAAA AAGAATAAAA
651     AGAGCCAAAG GCTAATCATG AACAAAAAGC CATAGGCTAT AAGCTTAGGC
701     CCATAGTAAT CATGCACTAG TCTATTATGG GTATAGAAGC CACGCATAGC
751     CTACTTCAGC CTCAGGAACC AGTCTTGTTT TATTGCTCCT GGTTAAAATT
801     ATATAATAGT AAAATGTATT TATTACGTCC GTATTTGGGT AGTGCCCTTC
851     ATGCTCTTGC CACAATTAAA GCTCTGAACA AACAGTAATA TTCCGGAAAA
901     AGTTATCACC CGCTAATAAT AGGTAGGCCT ATTGTCCTAT ACTTCAGAGA
951     AACGTCTAAA TATGGCATGC GCCTGAAAGT AGAAATCCCC CTTTGAGCTG
1001    TCGTCTCGAA GACAGCCAAT CATATTCGCT TTTAACTAAA GACTGCTACC
1051    GTGTATGTGT AGACCTAGGC CTACATTGTC TGTAAAACCA GCTGAACTCC
1101    CACTCGAAAG AGTATAAGGA CAAAGGATGA CAGATGACTG AGTTACATTT
1151    GACTCATGAG AGACAGGTGG AATTTAACAT CCCTGTGAAA AAACAGTCCT
1201    TGTATGTGAC ACAGAAATGC AAATCGAACT CAAAATATAT CACTTCAGAT
1251    TTATTTTAAA GAAATCTCAA TATGACCATC CGTCATAGGC CTCAGTGGCG
1301    GATCCAGAAC TGTTCCAAGG GGGGGGGGCA AAATCTTAGA TACATGTGCA
1351    AAATTTTTCG AAATTTGGTT CTTGGTTCCC AAAAAAATCG GTCTTCGGTT
1401    GAGGAAAAAA GGAATTTTCA TCCGCCGAAA AAGTTAAAAG TATATTTTTG
1451    ATAGTAGTTT GACGGTAGGT GTACAGATTT AATTTCTGGA GATCGCCAAT
1501    AAGCAGTTCA TTGCAAAGAT TCAAATGTTT TAACCAATCA ATTTCACGTC
1551    CTTCAATAGT GAAGTTACGG TAGTCCAACA GTTACGGTCA CATTATCACA
1601    GCGCATTAAC CATCATTCCA TGTATGATCA CAATGCACCA CATAGATCAT
1651    TCAGTTTGCA CATGATTTCA TAAAAATTAT ATTGGCGCCA GTTTCTCTAA
1701    AGATAGTTAC TTATCTTTAT ATTTATAGGC CTATCATTGT CTGAAGTAAC
1751    ACACTGGCAT GAATGCACCA TCGGTCCACA GCAGAAGGGT GAAGCGCAGG
1801    CAGAGGCTGG TTATTCCATC GTACTACATC ATGCCTTGTT GGGTTTTCCC
1851    ATCACAGGAG TTGAATGTCA CAGCATTCCA GAAGCATGTT TCTTCATGTG
1901    CATTTTCAGA GTGTAGCTAC GAACAAAACA CTTGAAACAG ATGTCACAGA
1951    CATATGGCTT TTCTCCCGTG TGCATCGCCA TGTGTCGGTT CAACGTGTTG
2001    CTCTTCGCAA ACTGTTTACC GCACTCCATG CAGATAAACC GCTTCTCGCC
2051    AGTGTGTGTC GTCATGTGCG ACATCAGCGT GCTCCGAAGC GCAAATTTCT
2101    TGTCACAAAT GACACAGTCA AACGGTTTCT CGCCAGTGTG GATAAGCATG
2151    TGCTTGTTAA CCTGGCTCTT GTGCGCCAGT TTGCGGTCAC AAATGACACA
2201    GCTGTACGGT GCTTCACCAG AGTGAATACG TTTGTGCGTC TGCAATTGGC
2251    TGTTGTGTGC AAAGCGAACG CCGCATTCGT GGCAGACGAA CGTCTTCACT
2301    CCAGTGTGAG TGACCGAGTG AACCTGAAGG CTGTTTTTGC TCGTAAACTT
2351    CTTATCACAG AGCGAACACT CGTACGGTTT GATGCCCGTG TGCAGCATTC
2401    TGTGGTACTG CAAACCGGCT TTCTGTGCAA AACACCGGTC GCAGATCTCA
2451    CACTTAAATG GCTTTTCACC TGTATGGGTC CGTAGGTGAG ACTTGAGCTG
2501    GCTGGCCAGG ATAAACTGTC GTCCGCAGGC CTTACAATTG AAAGGCTTCT
2551    CCTCTGAGTG TCGAAGCAGG TGGTACTTAA GTGAAAGTGC AACCTTGAAG
2601    CCTTTCCCAC ACAGCTCGCA GGAAAACGGT TTTACGCCTG AATGTGTCCG
2651    TTGGTGCTGC TTAAGCTTGT CACTTCTTGA AAAACACTTT CCACACATAC
2701    TGCATGAATA GGGCTTCTCT CCGGAATGCA CTCGTAAGTG TGCCTTGAGC
2751    TTATCAGAAC GACGAAAGGA CTTTCCACAA TTCGAGCAAA TGTGTTCTTT
```

```
2801   ATTACTACGG CGTTTTCCAT AAAGTCGGCC TTCCTGTTTA GGAGAGTTAT
2851   CTGAAGTCCG TTTTTGAATC TTGACTGCTT TTGACCTCTT TTTAGCAGTG
2901   TTTTTTGCAA CAACATCCGA TGGCTCTTCA ATGTCAGTTG ATTTGTTTTC
2951   AACTTTCACT TCATTGGTTT TGAATTCTGT AACTTCTTCA ACATCAGTCA
3001   TTTCATCATC AACTTCTACT TCATCGGTTT TGAACTCTGT TACTTCTTCA
3051   ACATCAGTCA ATTCATCTTC AACTTTCACT TCATCGGTTT TGAATTCTGT
3101   TACTTCTTCA ATCTCAGTCA ATTCATCTTC AACTTTTACT TCATTGGTTT
3151   TTAATTCTGT TTTTTCATCA ACGCCAGTCA TTTCTTCAAT TCTGACGCTG
3201   CCTTTAGGAC TCTCTTTGAC TCCCATTCCA TCTTCAAAAT CAGTCATTAC
3251   TTCGACTTTG ACTTTGTCAA CGGTTTTTGT AGTGACTTCA GATTGTTTTT
3301   CAGCATTACT CATTTTTGCA GCTCTGACTT TACCCAAAGT TCTTTTGTTG
3351   ACTTGCAGCT TGCATGGGTC TGAAAGAAGA AAAAAATAAA TGAAACTAAA
3401   ATGCTGACAC ATTACTACTC AGACGTGCTC CTGACTATAG CACGGTAAAA
3451   AAAGAAATTT TTGAGTTTAA ATTTTAAAAA TTAATAATTT TTAAAATTAT
3501   TGTGCTGTGT ATTAAACAGA TCTTGAACGG ATTGAGTCTG ACGATAGCGA
3551   AAGGTCAGAC GGTGGCTTTG GTCGGGTCAA GCGGTTGTGG TAAGAGCACG
3601   GTGATTCAAC TCGTCCAGCG ATTCTACGAC CCACTCGATG GTTGCGTGAT
3651   GATCGACGGA ACGGACATTC GTGAGTTGAA CATCAAATGG TTGCGACAGA
3701   ACATTGGCGT CGTCAGCCAA GAACCGATTC TCTTTGCAAC GACGATTGCG
3751   GAGAACATTC GATACGGAAG AGAAGGAGTG ACGGATGCGG AGATCGAGAA
3801   GGCGGCGATA GAGGCGAATG CTCACAACTT CATCAGTAAA CTGCCATTGG
3851   TATGATGATG ATCTGAAATG CTCAATTAAA GACTCTTCAA TATTGATGTT
3901   GTTGTTGTAG ATTAGAGTTG AATGAAAGGT TTAATTAATG AATTGTTTAT
3951   ATACGTGACT AAGGTTGATG TTGCTCTTTT AGCTCTTATA TGTGTCTCTA
4001   AAGGTGTGAG TGCGTGTGTG TGCGCGTGTG TGGTGTGTGT GTGTGTGTGT
4051   GGGTTTTTAT CTGTACGACA TAGTAATGAT TTAGTTTAAG TGCATGGTGT
4101   GGGCTCCAGT CTTTCCTGAT GTAATGACAT TCTGGAAGTC CTTTCTGCAT
4151   AATTGCTCAA GGTTTGATAC AGAGCTCTGC GTTGTAAGGT GCCCATGTAA
4201   TAGCATTGCC ATGTTACGAC ACCATTCAGA CAGTCCTAAT TATTATTGTT
4251   ATTATTATTG TCATCTGTCA CAATGTAATT AAGATCTATG GTTAGGGTTG
4301   TGGTGAACAC TGTTATGCCG TGCAACTGGC TTGCAAGTTG GTTGTTGTCG
4351   TTGTTGTTAG AAATACGGTA CGTTGGTTGG AGAGCGTGGA GCTCAGTTGA
4401   GTGGAGGACA GAAGCAGAGG ATAGCAATTG CACGTGCGTT GGTTCGTGAT
4451   CCACGAATTC TTCTTCTTGA TGAAGCGACG TCTGCTCTCG ACACGGAATC
4501   AGAAGCCACC GTTCAGTCGG CGCTTGACAA GGTAATGTCA TATTATGACC
4551   GTTGTATTCA GTGCTGCATT TGTTAAGCTT AATTCGTTTC AGAATGATAA
4601   ATTATTATTA ATTAATTAAC TAGTAGGTAA GTAGGTTCTT CTTTTGTGTT
4651   GATGTCTAAT AAATGTTTTG AAATAATTTG TTTCAACGTC AGTTGATGCC
4701   AAAGACTTTA ATGGATGACG CACCAAAACC AGTCTAAAAT ATTGATGAGA
4751   ATAATAATAA TATTCTCTTT ATTCAA
```

A.5 Comparison of the translated assembled sequence against the *M. edulis* *ABCB1* protein sequence by the program WISE2

```
M. edulis          1 QRFYDPDAGQVLLDGNNIKDLNLNWLRQNIGVVSQEPVLFGCTIAENIR
                     QRFYDP  G V++DG +I++LN+ WLRQNIGVVSQEP+LF  TIAENIR
                     QRFYDPLDGCVMIDGTDIRELNIKWLRQNIGVVSQEPILFATTIAENIR
L. rubellus     3616 ccttgccggtgaaggagacgtaaattccaagggacgcactgaaaggaac
                     agtaactaggtttagcatgatatagtgaatgttgaactttccctcaatg
                     gacccacttcggccagcttggccaggagctccccaagtctaggtggcta

M. edulis         50 LGNPNATITEIEQAAKQANAHDFIKSLPQ
                      G    T  EIE+AA +ANAH+FI  LP
                     YGREGVTDAEIEKAAIEANAHNFISKLPL
L. rubellus     3763 tgagggagggagaggaggagcataaacctGTATGAT  Intron 1
                     aggagtcacataacctacacaattgatct<0-----[3850 : 4360]
                     caaaaggtggcggggaggttcccctagag

M. edulis         79    SYNTLVGERGAQLSGGQKQRVAIARALIRDPRILLLDEATSALDSE
                         Y TLVGERGAQLSGGQKQR+AIARAL+RDPRILLLDEATSALD+E
                        KYGTLVGERGAQLSGGQKQRIAIARALVRDPRILLLDEATSALDTE
L. rubellus     4358 TAGatgatgggcggctaggcacaagagcgtgcgccacccgggatgcgag
                     -0>aagcttgaggcatgggaaagtctcgcttgacgttttaaccccctaca
                        actggtagtatggtaaggggaatatggtttaattttaggttccga

M. edulis         125 SENIVQEALEK
                     SE  VQ AL+K
                     SEATVQSALDK ....
L. rubellus     4499 tggagctgcga
                     cacctacctaa
                     aacctgggtcg
```

Remaining amino acids from mussel fragment = ...**ARQGRTTLVIAHRL**

L. rubellus P-gp sequence analogous to mussel

```
ORIGIN
1      QRFYDPLDGC VMIDGTDIRE LNIKWLRQNI GVVSQEPILF ATTIAENIRY
51     GREGVTDAEI EKAAIEANAH NFISKLPLKY GTLVGERGAQ LSGGQKQRIA
101    IARALVRDPR ILLLDEATSA LDTESEATVQ SALDK
```

Note: The letters in red show the similarities between sequences and the green letters are the sequence of primer exon2 (reverse direction) to extend the novel ABCB1 sequences of *L. rubellus* and *A. caliginosa.*

A.6 The alignment of *L. rubellus* contiguous sequence (contig_76678), ABCB1 *B. floridae*, ABCB1 *R. norvergicus*, and novel *A. caliginosa*

```
ORIGIN
A.caliginosa        ....................................      0
L.rubellus          tatagcattgaaaagaacacacatttctgcaccgtacaaa     40
B. floridae         ....................................      0
R. novergicus       ....................................      0

                    ---------------------------- (380 bases omitted)

A.caliginosa        ........................GGTCGGTCGAGC     12
L.rubellus          gataaagcgaggtcagacggtggctttgGtcgGGTCGAGC    560
B. floridae         ....................................      0
R. novergicus       ....................................      0

A.caliginosa        GGGTGTGGCAAGAGCACCGTCATTCAGTTGATCCAACGCT     52
L.rubellus          GGGTGTGGtAAaAGCACCGTCATTCAGcTcATCCAACGCT    600
B. floridae         ....................................      0
R. novergicus       ....................................      0

A.caliginosa        TCTACGATCCACTAGAAGGCGCTGTTATGATTGACGGGAC     92
L.rubellus          TCTACGAcCCACTAGAgGGCGCTGTgATGATTGACGGaAC    640
B. floridae         ....................................      0
R. novergicus       ....................................      0

A.caliginosa        AGACATTCGTCAGTTGAATATCAAGTGGTTGCGACAACAC    132
L.rubellus          gGACATTCGTCAGcTGAAcATCAAGTGGTTaCGgCAACAt    680
B. floridae         ....................................      0
R. novergicus       ....................................      0

A.caliginosa        ATCGGAGTCGTCAGCCAGGAACCAGTGCTGTTTGCCACGT    172
L.rubellus          ATCGGAGTCGTCAGtCAGGAACCAGTGtTGTTTGCtctca    720
B. floridae         ....................................      0
R. novergicus       ....................................      0

A.caliginosa        CGATCGCTGATAACATTCG                         191
L.rubellus          CGATaGCaGAgAACATTCGatacgggagagaaggagtgag    760
B. floridae         ....................................      0
R. novergicus       ....................................      0

                    ---------------------------- (760 bases omitted)

L.rubellus          aactgtgaagtatttctcctgttatgtttccggtagaaat   1560
B. floridae         ............................ccacagacat     10
R. novergicus       ..............................cacaaat      7

L.rubellus          acgagactctggttggcgagagaggagcccagttgagtgg   1600
B. floridae         acgagacgctggtcggagagcgaggtgcccagctgtctgg     50
R. novergicus       ttaacaccctggttggtgagagaggggcgcagctgagtgg     47

L.rubellus          tggacagaagcaaagaatcgcaattgccagagctttggtt   1640
B. floridae         cggacagaagcagaggattgcgatcgcccgcgctctggtg     90
```

```
R. novergicus       gggacagaaacagaggatcgccattgcccgggccctggtc     87


L.rubellus          cgcgatccaaagattctgcttcttgatgaggcaacgtctg   1680
B. floridae         cgggacccacggatcctgctattggacgaggccacctctg    130
R. novergicus       cgcaaccccaagatccttttgttggatgaggccacgtcag    127


L.rubellus          cgctcgatacagaatcagaggctacggttcaggcggctct   1720
B. floridae         cgcttgacacagagagcgaggctaccgtacaggctgcact    170
R. novergicus       ccttggacacagaaagcgaagccgtggttcaggccgctct    167

L.rubellus          cgacaaggta                                 1730
B. floridae         ggacaaggca                                  180
R. novergicus       ggataaggctaga                               180
```

Note: The letters in red show the similarities between lumbricid sequences and the green letters denote the conserved area which two reverse primers were designed to extend the novel ABCB1 sequences of *L. rubellus* and *A. caliginosa.*

A.7 Multiple Sequence Alignment for *β-actin* primers

```
L. rubellus          CACCCGGTGCTGCTGACCGAGGCTCCCCTCAATCCCAAGG     40
T. taeniformis       CACCCcGTcCTGCTGACgGAGGCTCCCCTCAATCCCAAGG     40
L. variegatus        CAtCCtGTtCTttTtACtGAGGCaCCttTgAATCCaAAaG     40
E. fetida            ........................................      0

L. rubellus          CCAACCGTGAGAAGATGACCCAGATCATGTTCGAGACCTT     80
T. taeniformis       CCAACaGgGAaAAGATGACCCAGATCATGTTCGAaACCTT     80
L. variegatus        CCAACCGaGAaAAaATGACgCAGATCATGTTCGAGACCTT     80
E. fetida            ........................................      0

L. rubellus          CAACTCCCCAGCCATGTACGTCGCAATCCAGGCCGTCCTC    120
T. taeniformis       CAACTCCCCAGCCATGTACGTCGCcATCCAGGCtGTCCTg    120
L. variegatus        CAAtTCtCCAGCaATGTAtGTtGCAATtCAGGCtGTCCTg    120
E. fetida            ........................................      0

L. rubellus          TCCCTCTATGCCTCCGGTCGTACCACCGGTATCGTCCTCG    160
T. taeniformis       TCCCTgTAcGCtTCCGGTCGTACCACCGGTATCGTgCTCG    160
L. variegatus        TCgCTgTATGCtTCtGGTCGTACaACtGGTATCGTtCTCG    160
E. fetida            ........................................      0

L. rubellus          ACTCTGGCGATGGTGTCACCCACACGGTTCCCATCTACGA    200
T. taeniformis       ACTCcGGCGATGGTGTCACCCACACcGTgCCCATCTACGA    200
L. variegatus        ACTCcGGaGAcGGTGTCACaC                       181
E. fetida            ........................................      0

L. rubellus          GGGTTACGCCCTGCCACATGCCATCCTCCGGCTCGATTTG    240
T. taeniformis       GGGTTACGCCCTGCCcCAcGCCATCCTCCGtCTgGAcTTG    240
E. fetida            ........................................      0

L. rubellus          GCCGGCAGAGATCTCACCGACTACCTGATGAAGATCCTGA    280
T. taeniformis       GCCGGCAGAGATCTCACCGAtTACCTGATGAAGATCCTGA    280
E. fetida            ........................................      0

L. rubellus          CCGAGAGAGGCTACAGCTTCACAACCACGGCCGAGAGTGA    320
T. taeniformis       CCGAGAGAGGCTACAGCTTCACcACCACGGCCGAGcGTGA    320
E. fetida            ........................................      0

L. rubellus          AATTGTTCGTGACATCAAGGAGAAGCTGTGCTACGTTGCA    360
T. taeniformis       AATcGTTCGTGACATCAAGGAGAAGtTGTGCTACGTcGCc    360
E. fetida            ........................................      0

L. rubellus          CTCGACTTCGAGCAGGAGATGGGAACACCGCTTCGCCTCA    400
T. taeniformis       CTCGACTTCGAcCAGGAGATGGGcACtgCcgcctct.TCA    399
E. fetida            ........................................      0

L. rubellus          TCGTCCCTCGAGAAGAGCTACGAGTTGCCCGACGGTCAGG    440
T. taeniformis       TCGTCCCTCGAGAAGAGCTACGAGcTtCCCGACGGTCAGG    439
E. fetida            ........................................      0

L. rubellus          TCATCACCATCGGAAACGAGCGCTTCCGCTGCCCAGAAGC    480
T. taeniformis       TCATCACCATCGGAAACGAGCGCTTCCGtTGCCCAGAgtC    479
E. fetida            ........................................      0
```

```
L. rubellus          CATGTTCCAGCCAAGCTTCCTGGGAATGGAGTCTTGTGGT    520
T. taeniformis       CATGTTCCAGCCAgcCTTCCTGGGtATGGAaTCTgccGGT    519
E. fetida            ....................................        0

L. rubellus          ATCCACGAGACGACCTACAACAGCATCATGAAATGCGATG    560
T. taeniformis       ATCCAtGAGACGACCTtCAACAGCATCATGAAgTGCGATG    559
E. fetida            ....................................        0

L. rubellus          TCGACATTCGTAAAGACCTCTACGCCAACACCGTCCTTTC    600
T. taeniformis       TCGAtATcCGTAAgGAtCTgTACGCCAACACCGTCaTgTC    599
E. fetida            ....................................        0

L. rubellus          GGGAGGCACCACCATGTTCCCAGGCATTGCCGACCGTATG    640
T. taeniformis       cGGAGGCACgACCATGTTCCCAGGtATcGCCGAtCGTATG    639
E. fetida            ....................................        0

L. rubellus          CAGAAGGAGATCACGGCTCTGGCACCAGCCACCATGAAGA    680
T. taeniformis       CAGAAGGAGATCACcagcaTGGCtCCgagCgggATGAAGA    679
E. fetida            ....................................        0

L. rubellus          TCAAGATCATCGCTCCACCTGAGCGCAAGTACTCCGTCTG    720
T. taeniformis       TCAAGATCATtGCTCCACCTGAGCGCAAGTACTCCGTaTG    719
E. fetida            ....................................        0

L. rubellus          GATCGGTGGATCCATCCTGGCGTCCCTGTCCACCTTCCAG    760
T. taeniformis       GATCGGTGGATCCATCCTGGCtTCCCTGTCCACCTTCCAG    759
E. fetida            ......................TcTCCACCTTCCAG      14

L. rubellus          CAGATGTGGATCAGCAAGCAGGAGTACGACGAGTCTGGCC    800
T. taeniformis       CAGATGTGGATCAGCAAGCAGGAGTACGACGAGTCcGGCC    799
E. fetida          CAGATGTGGATCAGCAAGCAGGAGTACGAtGAGTCcGGgC      54

L. rubellus          CATCCATCGTTCACAGCAAATGCTTCTAA               829
T. taeniformis       CATCCATCGTcCACAGgAAgTGCTTCTAA               828
E. fetida          CATCCATCGTcCACAGaAAgTGCTTCTAA                 83
```

Appendix B-Sequencing results

B.1 Novel *ABCB1* sequence in lumbricids

B.1.1 Lumbricus rubellus

```
ORIGIN
1      GATACTGAAC GGTCTGAACG TGGAGATAAA GCGAGGTCAG ACGGTGGCTT
51     TGGTCGGGTC GAGCGGGTGT GGCAAGAGCA CAGTCATTCA GCTCATCCAA
101    CGCTTCTACG ACCCACTAGA GGGCGCTGTG ATGATTGACG GAACGGACAT
151    TCGCCAGCTG AACATCAAGT GGTTACGGCA ACATATCGGA GTCGTCAGTC
201    AGGAACCAGT GTTGTTTGCT CTCACGATAG CAGAGAACAT TCGATACGGG
251    AGAGAAGGAG TGAGCCAGGC TGAGATAGAA ACGGCCG //
```

B.1.2 Aporrectodea caliginosa

```
ORIGIN
1      GGTCGGATCG AGCGGGTGTG GCAAGAGCAC CGTCATTCAG TTGATCCAAC
51     GCTTCTACGA TCCACTAGAA GGCGCTGTTA TGATTGACGG GACAGACATT
101    CGTCAGTTGA ATATCAAGTG GTTGCGACAA CACATCGGAG TCGTCAGCCA
151    GGAACCAGTG CTGTTTGCCA CGACGATCGC TGAGAACATT CGATACGGGA
201    AGAGAAGGA //
```

B.1.3 A. chlorotica

```
ORIGIN
1      ATACTGAACG GTCTGAACGT AGAGATACAG AGAGGTCACG GTAGCAATTG
51     GTCGGGTCGA GCGGGTGTGG CAAGAGCACC GTCATTCAGC TGATCCAACG
101    CTTCTACGAC CCACTAGAGG GCGCCGTTAT GATTGACGGG ACAGACATTC
151    GTCAGTTGAA TATCAAGTGG TTGCGCCAAC ACATCGGAGT CGTCAGCCAG
201    GAACCAGTGC TGTTTGCGAC GACGATCGCT GAGAACATTC GATACGGGAG
251    AGAAGGAA
```

B.1.4 Eisenia fetida

```
ORIGIN
1      TGATACTGAA CGGTCTGAAC GTGGAGATCA AGAGGGGCCA GACGGTGGCC
51     TTGTGGGGTC CGAGCGGGTG TGGCAAAGAG CACGGTCATT CAGTTGATTC
101    AACGCTTCTG CGACCCACTA GAGGGCGCCG TTATGATTGA CGGGACAGAC
151    ATACGTCAGC TGAACATCAA GTGGTTGCGA CAAAACATCG GGGTCGTCAG
201    CCAAGAGCCA GTGTTGTTTG CGACGACCAT CGCTGAGAAT ATTCGATACG
251    GGAGAGAAGG AA//
```

B.1.5 Aporrectodea trapezoides

```
ORIGIN
1      TGGTGGGGTC GAGCGGGTGT GGCAAGAGCA CGGTCATTCA GTTGATCCAA
51     CGCTTCTACG ACCCACTGGA GGGCGCCGTT ATGATTGACG GGACAGACAT
101    ACGTCAGCTG AACATCAAGT GGTTGCGACA AAACATCGGG GTCGTCAGCC
151    AAGAGCCAGT GTTGTTTGCG ACGACCATCG CTGAGAATAT TCGATACGGG
201    AAGAGAAGGA A //
```

B.1.6 Octolasion cyaneum

```
ORIGIN
1       TGGTCGGGTC GAGCGGGTGT GGCAAGAGCA CAGTCATCCA GCTGATCCAA
51      CGCTTCTACG ACCCACTAGA GGGCGCCGTT ATGATTGACG GAACAGACAT
101     TCGTCAGCTT AATATCAAGT GGTTGCGCCA ACACATCGGA GTCGTCAGCC
151     AGGAACCAGT GCTGTTTGCC ACGACGATCG CAGAGAACAT TCGATACGGG
201     AGAGAAGGA //
```

B.2 Extended sequences

B.2.1 Aporrectodea caliginosa

```
ORIGIN
1       TTTGGGTCGA GCGGGTGTGG CAAGAGCACC GTCATTCAGT TGATCCAACG
51      CTTCTACGAC CCACTAGAGG GCGCTGTTAT GATTGACGGG ACAGACATTC
101     GTCAGTTGAA TATCAAGTGG TTGCGACAAC ACATCGGAGT CGTCAGCCAG
151     GAACCAGTGC TGTTTGCCAC GACGATCGCT GAGAATATTC GATACGGCAG
201     AGACGGAGTC AGTCAGGCAG AGATAGAGAT GGCCGCCAAG GAAGCAAACG
251     CACACGACTT CATCAGCAAA CTGCCGCTGA AATACGAGAC TCTGGTTGGC
301     GAGAGAGGAG CCCAGCTGAG CGGTGGACAG AAGCAGAGAA TCGCAATTGC
351     CAGAGCTTTG GTTCGCGATC CAAAGATTCT GCTTCTTGAT GAAGCTACGT
401     CTGCCTCAA//
```

B.2.2 Lumbricus rubellus

```
ORIGIN
1       GATACTGAAC GGTCTGAACG TGGAGATAAA GCGAGGTCAG ACGGAGGCTT
51      TGGTCGGGTC GAGCGGGTGC GGTAAGAGCA CCGTCATTCA GCTCATCCAA
101     CGCTTCTACG ACCCACTAGA GGGCGCTGTG ATGATTGACG GAACGGACAT
151     TCGCCAGCTG AACATCAAGT GGTTACGGCA ACATATCGGA GTTGTCAGTC
201     AGGAACCAGT GTTGTTTGCT CTCACGATAG CAGAGAACAT TCGATACGGA
251     CGAGAAGGAG TGAGTCAGGC AGAGATAGAA ACGGCCGCCA AGGAGGCAAA
301     CGCTCACGAC TTCATCAGCA AACTGCCGCT TGTAGGTGGC GCTAACTGTC
351     CTTTGTGTTC AGATTGCAAT TGTTATTTAT TCACAGTTTC ACAGTTACAC
401     TCCTGCACCA TAAAAAAATC AAAAGAGTGA TTTACTTTGC CTTTGGAAGT
451     GGTACAGTAA CTGAGCGACT AAGGCGTCGC TTTTTGCATA TGCGTCGCGG
501     GTTTAATTCC AGACGTAACA TGTAAAAATA ATTGACATGA GTTTAGATCT
551     TTAATAATCA TGAGCATCTT CTCAAGTCCA GAAAACACTG TTGCTATGAT
601     CGAGATATGC TGCTATGTGC CCAGTGCACG CTGCATCAAG TAAAGGTTAG
651     AATCTGCTGT CAATGTAAAC AAACGTTTAT TGTATGTATT CATATGACCT
701     GATTATCTAA TTCTCAAAAC GCTGCTCTCT ACACGTTTAA AATTAATAGA
751     CTATTTATCT TAGAAACTTA GACCTACAAT GGTCAACAGC TACTCGTTTT
801     AACTGTGAAG TAAGCTTTTT CTCCTGTTAT TTTCTGTTAG AAATACGAGA
851     CTCTGGTTGG CGAGAGAGGA GCGCAGCTTA GTGGTGGACA GAAGCAAAGA
901     ATCGCATTTG CCAGAGCTTT GGTTCGCGAT CCAAAGATTC TGCTTCTTTG
951     ATGAAGCTAC GTCTGCTCTC//
```

B.2.3 Comparison of ABCB1b *Rattus norvergicus* (GenBank AAI07561.1), P-gp homologue *Lumbricus rubellus* and P-gp homologue *Aporrectodea caliginosa*

```
R. norvergicus      SRSEVKILKGLNLKVKSGQTVALVGNSGCGKSTTVQLLQR    440
L. rubellus         ......ILnGLNveiKrGQTeALVGsSGCGKSTviQLiQR     34
A. caliginosa       .....................GsSGCGKSTviQLiQR     16
Consensus                                 g sgcgkst  ql qr

R. norvergicus      LYDPIEGEVSIDGQDIRTINVRYLREIIGVVSQEPVLFAT    480
L. rubellus         fYDPlEGaVmIDGtDIRqlNikwLRqhIGVVSQEPVLFAT     74
A. caliginosa       fYDPlEGaVmIDGtDIRqlNikwLRqhIGVVSQEPVLFAT     56
Consensus            ydp eg v idg dir  n   lr  igvvsqepvlfat

R. norvergicus      TIAENIRYGRENVTMDEIEKAVKEANAYDFIMKLPHKFNT    520
L. rubellus         TIAENIRYGREgVsqaEIEtAaKEANAhDFIsKLPlKyeT    114
A. caliginosa       TIAENIRYGRdgVsqaEIEmAaKEANAhDFIsKLPlKyeT     96
Consensus           tiaenirygr  v   eie a keana dfi klp k  t

R. norvergicus      LVGERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALD    560
L.rubellus          LVGERGAQLSGGQKQRIAIARALVRdPKILLL            146
A. caliginosa       LVGERGAQLSGGQKQRIAIARALVRdPKILLLDEATSA      134
Consensus           lvgergaqlsggqkqriaiaralvr pkilll
```

Note: Amino acids shown in black bold letters belong to an exogenic region and in red to the following exon. Capital letters show the identity between all sequences.

B.3 *β-actin* gene homologue *Apporrectodea caliginosa*

ORIGIN

```
1       TTCGAGACCT TCAACTCCCC GGCCATGTAT GTCGCCATCC AGGCCGTCCT
51      CTCCCTGTAC GCGTCCGGTC GTACCACCGG TATCGTGCTG GACTCCGGCG
101     ATGGTGTCAC CCACACCGTC CCCATCTATG AGGGTTACGC CCTGCCCCAT
151     GCCATCCTTC GTCTCGACTT GGCCGGCAGA GATCTCACCG ATTACCTGAT
201     GAAGATCCTG ACGGAAAGAG GTTACAGTTT CACCACCACG GCCGAGCGTG
251     AAATCGTTCG TGACATCAAG GAGAAGCTGT GCTACGTCGC TCTGGACTTC
301     GACCAGGAGA TGGGAACGGC TGCCTCCTCC TCCTCCCTCG AGAAGAGCTA
351     CGAGCTTCCC GACGGTCAGG TCATCACCAT CGGAAACGAG CGCTTCCGTT
401     GCCCAGAGTC CATGTTCCAG CCAGCCTTCC TGGGTATGGA GTCGGCCGGT
451     ATCCATGAGA CGACCTTCAA CAGCATCATG AAGTGCGATG TCGATATCCG
501     TAAGGATCTG TACGCCAACA CCGTCATGTC CGGAGGCACG ACCATGTTCC
551     CAGGTATCGC CGATCGTATG CAGAAAGAGA TCACGAGCAT GGCTCCAAGC
601     ACGATGAAGA TCAAGATCAT TGCTCCACCT GAGCGCAAAT ACTCCGTATG
651     GATCGGTGGA TCCATCCTGG CCTCCCTGTC CACCTTCCAG CAGATGTGGA
701     TCAGCAAG//
```

Appendix C-Recipes

C.1 SOC Medium

(to make 100ml)

Bacto®-tryptone: 2.0g

Bacto®-yeast extract: 0.5g

1M NaCl : 1ml

1M KCl: 0.25ml

2M Mg^{2+}: 1ml stock, filter-sterilised (as prepared below)

2M glucose: 1ml, filter-sterilised

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring to 100ml with sterile, distilled water. Filter the complete medium through a 0.2µm filter unit. The final pH should be 7.0.

2M Mg^{2+} stock

20.33g MgCl2 • 6H2O

24.65g MgSO4 • 7H2O

Add distilled water to 100ml. Filter sterilise.

C.2 Luria broth/ampicilin/X-Gal plates

Add 12.5 g of Luria broth (L3522-Sigma) and 7.5 g of agar (Davis) into 500 ml of distilled water. After autoclaving, add ampicilin to final concentration of 100 µg/ml. 5-bromo-4-chloro-3-indolyl-b-D-galactosidebromo-chloro-indolyl-galactopyranosisde (X-Gal) (20 mg/mL dissolved in N,N'-dimethyl formamide) was spread onto surface of each plate just before plating the transformation culture.

Appendix D-Data analyses

D.1 Data from RT-qPCR and associated calculations with statistical analysis

D.1.1 Treatment with rifampicin 10 mM

cDNA from treated group earthworms exposed to rifampicin 10mM for 7 days in soil				Normalisation		2e - ΔΔCq
ABCB1 (target)	Cq	Cq	Cq Average	Δ Cq (target-reference)	Average	Fold change
Pool 1	27.14	27.71	27.43	9.78	10.24	2.63
Pool 2	28.18	27.87	28.03	10.77		
Pool 3	28.75	28.19	28.47	10.17		
B-actin (reference)						
Pool 1						
Pool 2	17.66	17.64	17.65			
Pool 3	17.36	17.15	17.26			
	-	18.3	18.30			
cDNA from control (exposed to methanol for 7 days in soil)						
ABCB1 (target)	Cq	Cq	Cq Average	Δ Cq (target-reference)		
Pool 1	30.6	31.3	30.95	12.08	11.63	
Pool 2	29.44	29.85	29.65	11.71		
Pool 3	29.19	29.66	29.43	11.72		
Pool 4	28.48	28.22	28.35	11.02		
B-actin (reference)						
Pool 1	18.85	18.89	18.87			
Pool 2	18	17.87	17.94			
Pool 3	17.69	17.72	17.71			
Pool 4	17.44	17.22	17.33			

Anova: Single FaCqor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*	*Standard deviation*
control	4	46.53	11.6325	0.196358333	0.443123384
treated	3	30.715	10.23833333	0.251008333	0.501007319

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	3.332058333	1	3.332058333	15.26937853	0.011322241
Within Groups	1.091091667	5	0.218218333		
Total	4.42315	6			

D.1.2 Treatment with rifampicin 5 mM

cDNA from treated group (exposed to rifampicin 5mM or 7 days in soil)				Normalisation		2e - ΔΔCq
ABCB1 (target)	Cq	Cq	Cq Average	Δ Cq (target-reference)	Average	Fold change
Pool 1	26.7	27.83	27.27	9.99	9.16	0.59
Pool 2	25.21	25.91	25.56	8.28		
Pool 3	26.57	26.36	26.47	9.32		
Pool 4	26.28	26.55	26.28	9.06		
B-actin (reference)						
Pool 1	16.91	17.65	17.28			
Pool 2	17.35	17.22	17.29			
Pool 3	17.04	17.26	17.15			
Pool 4	17.2	17.25	17.23			
cDNA from control (exposed to methanol for 7 days in soil)						
ABCB1 (target)	Cq	Cq	Cq Average	Δ Cq (target-reference)		
Pool 1	27.67		27.67	8.96	8.39	
Pool 2	26.15	26.03	26.09	8.39		
Pool 3	25.53	26.1	25.82	8.25		
Pool 4	24.9	25.36	25.13	7.99		
B-actin (reference)						
Pool 1	18.72	18.71	18.72			
Pool 2	17.52	17.89	17.71			
Pool 3	17.57		17.57			
Pool 4	17.09	17.2	17.15			

Anova: Single FaCqor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*	Standard deviation
control	4	33.5375	8.384375	0.177184896	0.420933363
treated	4	36.6475	9.161875	0.498505729	0.706049382

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	1.2090125	1	1.2090125	3.578597824	0.107395957
Within Groups	2.027071875	6	0.337845312		
Total	3.236084375	7			

D.1.3 Treatment with cyclosporin A 50 µM

cDNA from treated group (exposed to cyclosporin A 50µM for 7 days in soil)				Normalisation		2e - ΔΔCq
ABCB1 (target)	Cq	Cq	Cq Average	Δ Cq (target-reference)	Average	Fold change
Pool 1	24.16	24.68	24.42	8.61	8.46	1.17
Pool 2	24.34	24.85	24.60	8.40		
Pool 3	23.45	23.67	23.56	7.95		
Pool 4	24.18	25.07	24.63	8.89		
B-actin (reference)						
Pool 1	15.78	15.85	15.82			
Pool 2	16.18	16.22	16.20			
Pool 3	15.56	15.67	15.62			
Pool 4	15.53	15.95	15.74			
cDNA from control (exposed to ethanol for 7 days in soil)						
ABCB1 (target)	Cq	Cq	Cq Average	Δ Cq (target-reference)		
Pool 1	24.94	25.18	25.06	8.60	8.69	
Pool 2	24.5	24.71	24.61	8.65		
Pool 3	23.67	24.06	23.87	8.31		
Pool 4	24.62	25.3	24.96	9.19		
B-actin (reference)						
Pool 1	15.76	17.17	16.47			
Pool 2	15.67	16.24	15.96			
Pool 3	15.36	15.75	15.56			
Pool 4	15.49	16.05	15.77			

Anova: Single FaCqor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*	Standard deviation
control	4	34.745	8.68625	0.134989583	0.367409286
treated	4	33.83	8.4575	0.157025	0.396263801

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.104653125	1	0.104653125	0.716766429	0.429674393
Within Groups	0.87604375	6	0.146007292		
Total	0.980696875	7			

D.1.4 Treatment with Ivermectin™ 2.0 µg/ml

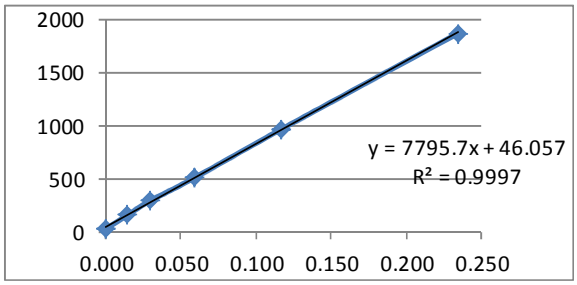
cDNA from treated group exposed to ivermectin diluted to 2.0 µg/ml 7 days in soil)				Normalisation	2e - ΔΔCq
ABCB1 (target)	Cq	Cq	Cq Average	Δ Cq (target-reference)	Fold change
Pool 1	23.39	23.52	23.455	7.89	2.32
Pool 2	24.22	23.32	23.77	7.80	2.46
B-actin (reference)					
Pool 1	15.17	14.82	15.6		
Pool 2	15.97	15.21	15.97		
cDNA from control (exposed to water for 7 days in soil)					
ABCB1 (target)	Ct			**Normalisation**	
Pool 1	24.56	23.66	24.56	9.10	
B-actin (reference)					
Pool 1	15.46	15.04	15.46		

D.2 Data from biochemical assay and associated calculations with statistical analysis

D.2.1 Treatments: Verapamil 200µM, Ivermectin™ 0.02µg/ml, and Ivermectin™ 0.2µg/ml

Sample	weight (mg)	Fluorescence reading		Average FLR	RB conc.mg/l	Normalized rate RB conc/weight $mg/l\ g^{-1}$	Average	STDEV
Neg. Control	144	855	869	862	0.1047	0.727	0.709	0.096
Neg. Control	144	676	694	685	0.0820	0.569		
Neg. Control	102	666	666	666	0.0795	0.780		
Neg. Control	134	831	850	841	0.1019	0.761		
Verapamil 200 µM	132	1010	1030	1020	0.1249	0.946	1.184	0.201
Verapamil 200 µM	128	1455	1481	1468	0.1824	1.425		
Verapamil 200 µM	161	1590	1617	1604	0.1998	1.241		
Verapamil 200 µM	138	1242	1264	1253	0.1548	1.122		
Ivermectin 0.02 mg/l	114	282	290	286	0.0308	0.270	0.635	0.313
Ivermectin 0.02 mg/l	112	929	963	946	0.1154	1.031		
Ivermectin 0.02 mg/l	160	858	893	876	0.1064	0.665		
Ivermectin 0.02 mg/l	218	1013	1026	1020	0.1249	0.573		
Ivermectin 0.2 mg/l	118	1140	1143	1142	0.1405	1.191	0.870	0.330
Ivermectin 0.2 mg/l	139	578	597	588	0.0695	0.500		
Ivermectin 0.2 mg/l	317	1743	1759	1751	0.2187	0.690		
Ivermectin 0.2 mg/l	141	1243	1270	1257	0.1553	1.101		

Std Curve	Fluorescence reading		Average FLR
0.937	6039	5930	5985
0.469	3109	1555	2332
0.234	1895	948	1421
0.117	1012	506	759
0.059	504	252	378
0.029	299	150	224
0.015	157	79	118
0	30	15	23



SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Neg. Control	4	2.836169511	0.709042378	0.00917116
Verapamil 200 µM	4	4.734252026	1.183563007	0.040533141

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.450339655	1	0.450339655	18.12075203	0.005339732	5.987377584
Within Groups	0.149112902	6	0.02485215			

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Neg. Control	4	2.836169511	0.709042378	0.00917116
Ivermectin 0.02 mg/l	4	2.538492006	0.634623001	0.098198868

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.011076487	1	0.011076487	0.206323632	0.665633088	5.987377584
Within Groups	0.322110085	6	0.053685014			

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Neg. Control	4	2.836169511	0.709042378	0.00917116
Ivermectin 0.2 mg/l	4	3.481632162	0.870408041	0.108656565

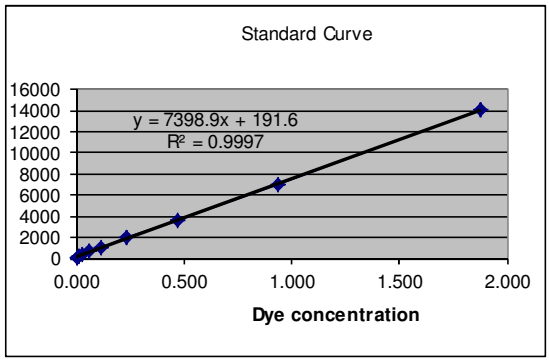
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.052077754	1	0.052077754	0.883964353	0.383410503	5.987377584
Within Groups	0.353483175	6	0.058913862			

D.2.2 Treatments: Verapamil 100 µM, verapamil 250 µM

Sample	weight (mg)	Fluorescence reading			Average FLR	RB conc.mg/l	Normalized rate RB conc/weight mg/l g^{-1}	Average	STDEV
Neg. control	246	1113	998	972	1028	0.113	0.459	0.558	0.207
Neg. control	159	822	708	737	756	0.076	0.479		
Neg. control	100	736	686	698	707	0.070	0.696		
Neg. control	141	568	546	574	563	0.050	0.356		
Neg. control	174	775	744	772	764	0.077	0.444		
Neg. control	141	1051	1329	1045	1142	0.128	0.911		
Verapamil 100 µM	100	1211	1226	1274	1237	0.141	1.413	1.146	0.339
Verapamil 100 µM	140	710	755	766	744	0.075	0.533		
Verapamil 100 µM	112	1014	997	1048	1020	0.112	0.999		
Verapamil 100 µM	154	1547	1641	1624	1604	0.191	1.240		
Verapamil 100 µM	134	1647	1638	1581	1622	0.193	1.443		
Verapamil 100 µM	122	1325	1352	1274	1317	0.152	1.247		
Verapamil 250 µM	221	1636	1732	1800	1723	0.207	0.936	1.198	0.355
Verapamil 250 µM	133	1816	2183	1859	1953	0.238	1.790		
Verapamil 250 µM	156	1683	1580	1604	1622	0.193	1.240		
Verapamil 250 µM	172	1863	2012	1822	1899	0.231	1.342		
Verapamil 250 µM	252	1567	1569	1763	1633	0.195	0.773		
Verapamil 250 µM	127	1112	1253	1336	1234	0.141	1.109		

Std Curve	Fluorescence reading			Average FLR
1.875	15470	13580	13360	14137
0.938	7312	6843	6740	6965
0.469	3726	3697	3497	3640
0.234	2041	1985	2007	2011
0.117	1101	1111	1086	1099
0.059	672	638	677	662
0.029	414	404	432	417
0.015	312	279	285	292
0.000	147	141	129	139



SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	6	3.345	0.5575	0.042699
Verapamil 100 µM	6	6.875	1.145833	0.115183

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.0384083	1	1.038408	13.15429	0.0046364	4.9646027
Within Groups	0.7894063	10	0.078941			
Total	1.8278147	11				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	6	3.345	0.5575	0.042699
verapamil 250	6	7.19	1.198333	0.126031

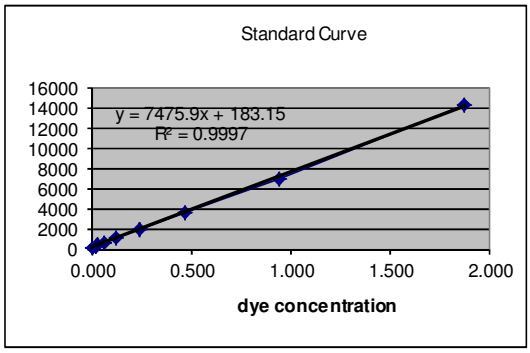
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.2320021	1	1.232002	14.60329	0.0033657	4.9646027
Within Groups	0.8436468	10	0.084365			
Total	2.0756489	11				

D.2.3 Treatments: Cyclosporin A 25 µM and Cyclosporin A 50 µM

Sample	weight (mg)	Fluorescence reading			Average FLR	RB conc.mg/l	Normalized rate RB conc/weight mg/l g^{-1}	Average	STDEV
Neg. control	246	1117	984	967	1023	0.112	0.456	0.546	0.204
Neg. control	159	825	690	706	740	0.075	0.469		
Neg. control	100	722	660	664	682	0.067	0.667		
Neg. control	141	557	533	561	550	0.049	0.348		
Neg. control	174	752	727	764	748	0.076	0.434		
Neg. control	141	1050	1315	1042	1136	0.127	0.904		
Cyclosporin 25 µM	185	1176	1200	1219	1198	0.136	0.734	1.122	0.422
Cyclosporin 25 µM	140	1637	1667	1631	1645	0.196	1.397		
Cyclosporin 25 µM	142	2067	2091	2134	2097	0.256	1.803		
Cyclosporin 25 µM	177	1037	1122	1121	1093	0.122	0.688		
Cyclosporin 25 µM	133	1185	1199	1173	1186	0.134	1.008		
Cyclosporin 25 µM	143	1364	1413	1313	1363	0.158	1.104		
Cyclosporin 50 µM	151	2199	2312	2413	2308	0.284	1.882	1.366	0.473
Cyclosporin 50 µM	153	2218	2655	2300	2391	0.295	1.930		
Cyclosporin 50 µM	142	1448	1390	1409	1416	0.165	1.161		
Cyclosporin 50 µM	163	1001	1063	962	1009	0.110	0.677		
Cyclosporin 50 µM	142	1466	1442	1602	1503	0.177	1.244		
Cyclosporin 50 µM	181	1713	1992	2117	1941	0.235	1.299		

Std Curve	Fluorescence reading			Average FLR
1.875	15669	13701	13469	14280
0.938	7373	6953	6676	7001
0.469	3859	3707	3536	3701
0.234	2039	1984	2021	2015
0.117	1103	1093	1079	1092
0.059	666	631	670	656
0.029	421	390	421	411
0.015	303	275	311	296
0.000	129	123	119	124



SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	6	3.278	0.546333	0.041716
Cyclosporin 25 µM	6	6.734	1.122333	0.178321

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.995328	1	0.995328	9.046901	0.0131669	4.9646027
Within Groups	1.1001867	10	0.110019			
Total	2.0955147	11				

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	6	3.278	0.546333	0.041716
Cyclosporin 50 µM	6	8.193	1.3655	0.224094

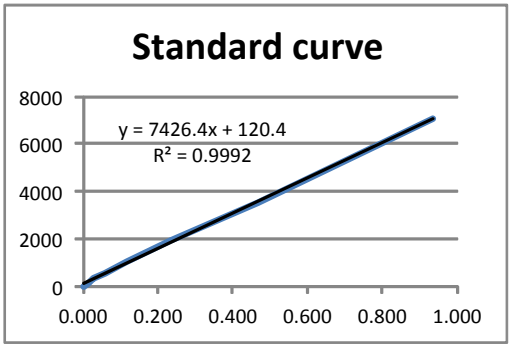
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.0131021	1	2.013102	15.14692	0.0030002	4.9646027
Within Groups	1.3290508	10	0.132905			
Total	3.3421529	11				

D.2.4 Treatments: rifampicin 100 µM and rifampicin 2 mM

Sample	weight (mg)	Fluorescence reading		Average FLR	RB conc.mg/l	Normalized rate RB conc/weight mg/l g^{-1}	Average	STDEV
Control	131	638	662	650	0.071	0.544	0.479	0.089
Control	124	643	653	648	0.071	0.573		
Control	130	462	461	462	0.046	0.353		
Control	160	620	644	632	0.069	0.431		
Control	132	594	613	604	0.065	0.493		
Rifampicin 100 µM	90	409	430	420	0.040	0.448	0.420	0.085
Rifampicin 100 µM	201	623	661	642	0.070	0.349		
Rifampicin 100 µM	183	884	902	893	0.104	0.568		
Rifampicin 100 µM	135	444	460	452	0.045	0.331		
Rifampicin 100 µM	216	747	776	762	0.086	0.400		
Rifampicin 100 µM	70	338	342	340	0.030	0.422		
Rifampicin 2 mM	118	624	655	640	0.070	0.592	0.543	0.221
Rifampicin 2 mM	194	677	703	690	0.077	0.395		
Rifampicin 2 mM	140	679	699	689	0.077	0.547		
Rifampicin 2 mM	140	561	583	572	0.061	0.434		
Rifampicin 2 mM	70	605	624	615	0.067	0.950		
Rifampicin 2 mM	141	467	483	475	0.048	0.339		

Std Curve	Fluorescence reading		Average FLR
0.937	7209	6905	7057
0.469	3459	3669	3564
0.234	1950	2011	1981
0.117	1054	1079	1067
0.059	578	584	581
0.029	314	323	319
0.015	171	172	172
0	33	32	33



SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Rhodmne	5	2.393997285	0.478799457	0.007857987
Rifam 2	6	3.258085064	0.543014177	0.048769528

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.011245992	1	0.011245992	0.367676827	0.559255193
Within Groups	0.275279588	9	0.030586621		
Total	0.28652558	10			

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Rhodmne	5	2.393997285	0.478799457	0.007857987
Rifam 100	6	2.518274109	0.419712351	0.007234128

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	0.009521689	1	0.009521689	1.267631974	0.289337314
Within Groups	0.067602589	9	0.007511399		
Total	0.077124278	10			

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