To observe the effects of environmentally relevant concentrations of CBD and CBD-related compounds using the invertebrate model *Lumbriculus variegatus*.

Ben Williams

Submitted to Swansea University in fulfilment of the requirements for the Degree of Toxicology and Pharmacology, MSc by Research

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Declaration and statements

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed Date 29/09/2025

This work is the result of my own independent study/investigation, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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SWIRL collaboration

SWIRL, the laboratory in which this research was conducted, works in a collaborative environment with both postgraduates and undergraduates collecting and analysing data. The author's role has been to plan all the experiments and ensure that where data is collected by another participant, the methodology and data collection has been completed ethically, accurately and in a safe manner. Although data may have been collected collaboratively, data analysis and presentation has been completed by the author. Where data generation has used methods in which there may be inter-individual differences in the scoring, such as for stereotypical movement, it has been the authors responsibility to ensure that when collecting the data, there has been supervision to ensure we are following the same scoring and during data analysis, to check that there have been enough repeats completed to identify any outliers. Along with my supervisory team, the authors role has also been to manage the laboratory in ensuring that any reagents used are stored safely and where needed, are handled with the correct procedure.

Acknowledgements

I was unsure whether I would be able to start this masters, but I (at the time of writing) did it (viva pending)...

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I dedicate this to you.

I may have a reading processing disorder and confuse my English, but I made it through this journey through my commitment to overpromise and underdeliver.

Final word of wisdom?
CBD BAD.

Abstract

Pharmaceutical contamination of the aquatic environment is increasing, with CBD emerging as of one particular concern due to its medicinal potential and rising use in over-the-counter and therapeutic products. It is the major non-psychoactive component of cannabis, one of the most widely used recreational drugs globally. CBD has since been detected in the environment raising questions regarding its ecotoxicological profile and impact on wildlife. The lipophilic property of CBD and tendency to partition to the sediment necessitates an urgent need to elucidate its effects on aquatic communities.

This study used the aquatic annelid *Lumbriculus variegatus* as an invertebrate model to investigate the toxicity of CBD and its associated compounds on behavioural, physiological and biochemical responses of *L. variegatus*.

The lipophilicity of CBD, 7-OH-CBD, abn-CBD and O-1918 were estimated *in silico* and toxicity was assessed through an *in vivo* toxicity assay. Compounds were tested for their effects on stereotypical and unstimulated movements of *L. variegatus*. The physiological effects of CBD and 7-OH-CBD on *L. variegatus* were further investigated through regeneration, respiration and pulse rate assays. The biochemical response of CBD and 7-OH-CBD were then investigated through quantification of cholinesterase activity and the total energy budget (proteins, carbohydrates and lipids) of *L. variegatus*. CBD exhibited the greatest toxicological impact with significant effects on stereotypical movement, unstimulated movement, regeneration, respiration, pulse rate and energy metabolism. 7-OH-CBD exerted significant effects on stereotypical movement, unstimulated movement and respiration. Both abn-CBD and O-1918 exhibited significant effects in stereotypical movements and unstimulated movements.

Considering its increasing global use and environmental presence, CBD poses the greatest risk for aquatic biological systems of the investigated compounds with potential risk across trophic levels through bioaccumulation and biomagnification. Further comprehensive research into the chronic exposure, multi-generational impact and biomagnification potential of CBD is essential for future environmental risk assessments.

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Publications and presentations

- The science of weed: Understanding the science behind cannabinoids.

  Aidan Seeley, **Benjamin S. Williams**, Georgeena Jomy.

  15th February 2024

  The University of the Third Age

- To observe the effects of environmentally relevant concentrations of CBD and CBD-related compounds using the invertebrate model *Lumbriculus variegatus*.

  Benjamin Williams.

  21st May 2024

  Faculty of Medicine, Health and Life Science Postgraduate Conference

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  10th December 2024

  Pharmacology 2024

- Investigating the effects of cannabidiol (CBD) and 7-hydroxy-cannabidiol (7-OH-CBD) on the regeneration of *Lumbriculus variegatus*.

  Georgeena Jomy, **Benjamin S. Williams**, Megan Flanagan, Grace S. Hawkes, James McRobbie-Aston, Nia A. Davies, Lisa J. Wallace, Aidan Seeley.

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  Pharmacology 2024

- Investigating the *in vivo* effects of the synthetic cannabinoid, O-1918, using *Lumbriculus variegatus*.

  Megan Flanagan, Grace S. Hawkes, James McRobbie-Aston, **Benjamin S. Williams**, Georgeena Jomy, Nia A. Davies, Lisa J. Wallace, Aidan Seeley.

  10th December 2024

  Pharmacology 2024

- The behavioural, physiological, and biochemical responses of *Lumbriculus variegatus* exposed to cannabidiol and its metabolites.

  Benjamin S. Williams, Georgeena Jomy, Julanta J. Carriere, Megan Flanagan, Grace Stella Hawkes, James McRobbie-Aston, Nia A. Davies, Claire L. Price, Melisa J. Wallace, Aidan Seeley.

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- Ecopharmacovigilence.

  Benjamin S. Williams.

  13th June 2025

  Women's Institute

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Abbreviations

Table 1: Common abbreviations used throughout.

7-OH-CBD	7-hydroxycannabidiol
Abn-CBD	Abnormal cannabidiol
CBD	Cannabidiol
DBV	Dorsal blood vessel
DMSO	Dimethylsulfoxide
ddH_2O	Distilled water
ECS	Endocannabinoid system
IVTA	*In vivo* toxicity assay
PIE	Pharmaceuticals in the environment

1. Introduction

1.1. Pharmaceuticals in the environment

1.1.1. Detection of pharmaceuticals in the environment

While efforts in the pharmaceutical industry towards 'greener' practices have been made, such as reducing waste or using more environmentally friendly solvents, the pharmaceuticals produced have posed their own stress on the environment (Kümmerer, 2010). The exposure of pharmaceuticals in the environment (PIE) can have untold implications on the health of ecosystems with effects reaching humans. The ecotoxicological impact of PIEs have been studied, however, due to the limited number of PIEs that have been studied and the different analytical methods used for detection of these PIEs, it can be difficult to truly quantify the adverse extent globally (Wilkinson *et al*., 2022).

In the literature, it is common to use a molecule's Log K_{ow} to provide inference in how it will interact with the environment. Log K_{ow} is the logarithmic value of the octanol/water partition coefficient and describes the concentration ratio between two immiscible solvents, octanol and water (Figure 1.1A), and can be used to infer hydrophobicity or lipophilicity and the ability of a chemical to bioaccumulate in organisms or the environment (Cumming & Rücker, 2017; Lambert *et al*., 2022; Thomann, 1989). It is accepted that Log K_{ow} values of >3 are lipophilic and >5 have the potential to bioaccumulate (Arnot & Gobas, 2003; Mackay & Fraser, 2000; Mastroianni *et al*., 2013). Bioaccumulation is defined as the uptake and enrichment of an exogenous compound from the environment over time (Beek *et al*., 2000; Ratte, 1999), often used when discussing harmful environmental pollutants. Conversely, biomagnification is defined as the transfer of an exogenous compound through trophic levels which may result in increasing bioconcentration (Beek *et al*., 2000). Bioaccumulation and biomagnification are visually described in Figure 1.1B.

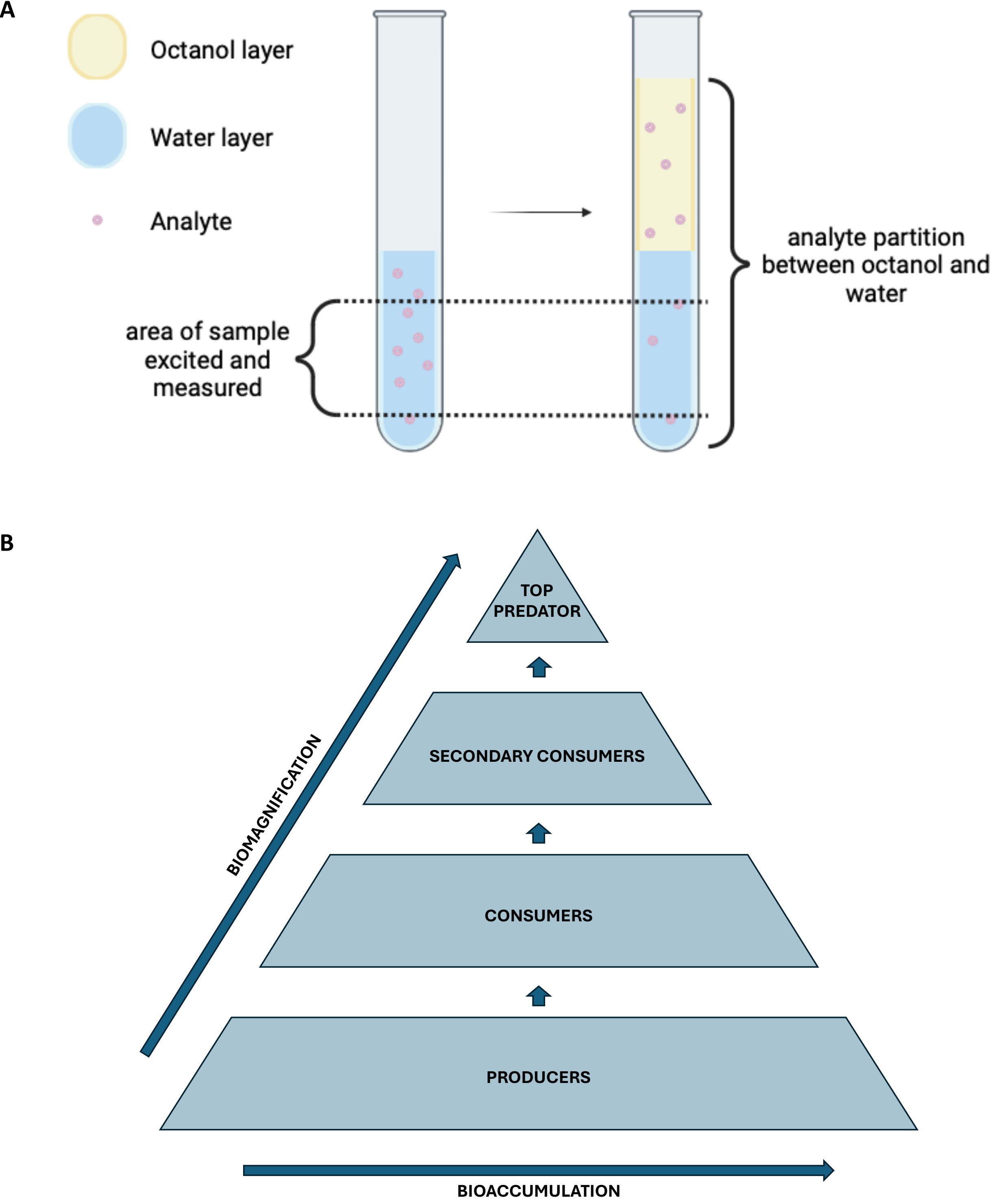


Figure 1.1: Depictions of Log K_{ow}, bioaccumulation and biomagnification. (A) Determination method of a chemicals Log K_{ow}. Image adapted from (Cumming & Rücker, 2017). Created with BioRender. (B) Bioaccumulation and biomagnification simplified model. Bioaccumulation refers to the uptake and increase in bioconcentration of exogenous toxicants in a species over time. Biomagnification refers to the potential stepwise increase in bioconcentration of an exogenous toxicant through higher trophic levels. Image adapted from InsightsIAS, (n.d).

1.1.2. Pharmaceuticals detected in the environment

Due to the efforts and success of modern medicine, we have seen global increases in life expectancy with an ageing population (Boudoulas *et al.,* 2017). Through the achievements of pharmaceuticals, the elderly who face multimorbidity, the suffering of two or more chronic conditions, can manage their health through polypharmacy and multiple pharmaceutical prescriptions (Masnoon *et al.*, 2017). However, this presents the unfortunate case of exacerbating PIES by potentially increasing the environmental contaminants from unused, improperly discarded drugs and unmetabolised drugs excreted through human waste (Kümmerer, 2010). To date, numerous prescribed pharmaceuticals of varying classes have been detected in the environment with adverse and consequential effects (Table 1.1).

Table 2.1: Pharmaceuticals previously observed in the environment and their associated adverse effects on specific species. This is not an exhaustive list of pharmaceuticals detectable in the environment.

Toxicant	Class	Species	Adverse effect	Source
Ethynylestradiol	Oestrogen modulator	*Danio rerio*	Endocrine disruption	Nash *et al.*, 2004
Diclofenac	NSAID[1]	*Gyps bengalensis*	Renal failure	Oaks *et al.*, 2004
Fluoxitene	SSRI[2]	*Elliptio complanate*	Nonviable offspring	Bringolf *et al.*, 2010
		Bufo arabicus	Impaired predator avoidance	Barry, 2014
Carbamazepine	Antiepileptic	*Venerupis* spp.	Reduced health Oxidative stress	Almeida *et al.*, 2014
Propranolol	Beta-blocker	*Phaeodactylum tricornutum*	Photosynethic impairment Growth inhibition Oxidative stress Increased lipid metabolism	Duarte *et al.*, 2020

1 Nonsteroidal anti-inflammatory drug

2 Selective seretonin reuptake inhibitor

1.2. Cannabinoids, *Cannabis,* and cannabis

The term cannabis has evolved to encompass colloquial and scientific classifications, which reflects its diverse applications recreationally and medicinally. *Cannabis* refers to the genus within the dioecious Cannabaceae family of which there are two commonly referred plants: *Cannabis sativa* and *Cannabis indica*, however, there is uncertainty and debate on whether *C. indica* is a subspecies of *C. Sativa* (Acosta *et al*., 2022; McPartland, 2017). Cannabis is also used to describe the illicit products of various *Cannabis* strains each with differing ratios of cannabinoids including psychoactive compounds, primarily Δ-9-tetrahydrocannabinol (THC), and the major non-psychoactive cannabinoid, cannabidiol (CBD) (Austin *et al*., 2024; Murray *et al*., 2016; Pandopulos *et al*., 2020). *Cannabis* that has been cultivated for use outside of recreational and medicinal use have been referred to as hemp and have had uses as a source of stem fibre, seeds and seed oil (Small, 2015). In the United States of America, legislation goes further to classify hemp pharmacologically as *Cannabis* crop containing <0.3% (w/w) THC whereas cannabis/marijuana are crops containing >0.3% (w/w) THC as described in the Agricultural Improvement Act 2018 (Acosta *et al*., 2022). Cannabis is now recognised as one of the most commonly used psychoactive substances globally (Johnson & Colby, 2023). Furthermore, human use of cannabis has been proposed by palaeobotanists as early as 10,000 years ago and there is much in the way of evidence of its health benefits to humans, but data is often anecdotal or contradictory (Chesney *et al*., 2020; Okazaki *et al*., 2011). Medicinal and academic interest in *Cannabis* spp. is experiencing a renaissance concurrently with cannabinoid compounds approved for clinical use including nabiximols and Epidiolex (MacCallum & Russo, 2018) with cannabinoid compounds proposed to be neuroprotective, antiepileptic, anxiolytic, antipsychotic, anti-inflammatory, analgesic and possessing anticancer properties (Peng *et al*., 2022).

1.3. Cannabinoids and the endocannabinoid system

Cannabis has a complex phytochemical profile with >100 phytocannabinoids including the major psychoactive and non-psychoactive compounds, THC and CBD, respectively (Austin *et al*., 2024; Johnson & Colby, 2023; Pandopulos *et al*., 2020). These phytocannabinoids are primarily produced in the glandular trichomes found on the female flowers, although can be

found in different structures of the plant (Figure 1.2A) (Jastrząb *et al*., 2022; Livingston *et al*., 2019). CBD and THC share a common precursor, with both being biosynethsised from cannabigerolic acid (CBGA) (Figure 1.2B). CBGA is converted to cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA) by enzymatic action of CBDA synthase and THCA synthase, respectively. These compounds are further decarboxylated via combustion or extraction to produce CBD and THC (Livingston *et al*., 2019; Ribeiro *et al*., 2024).

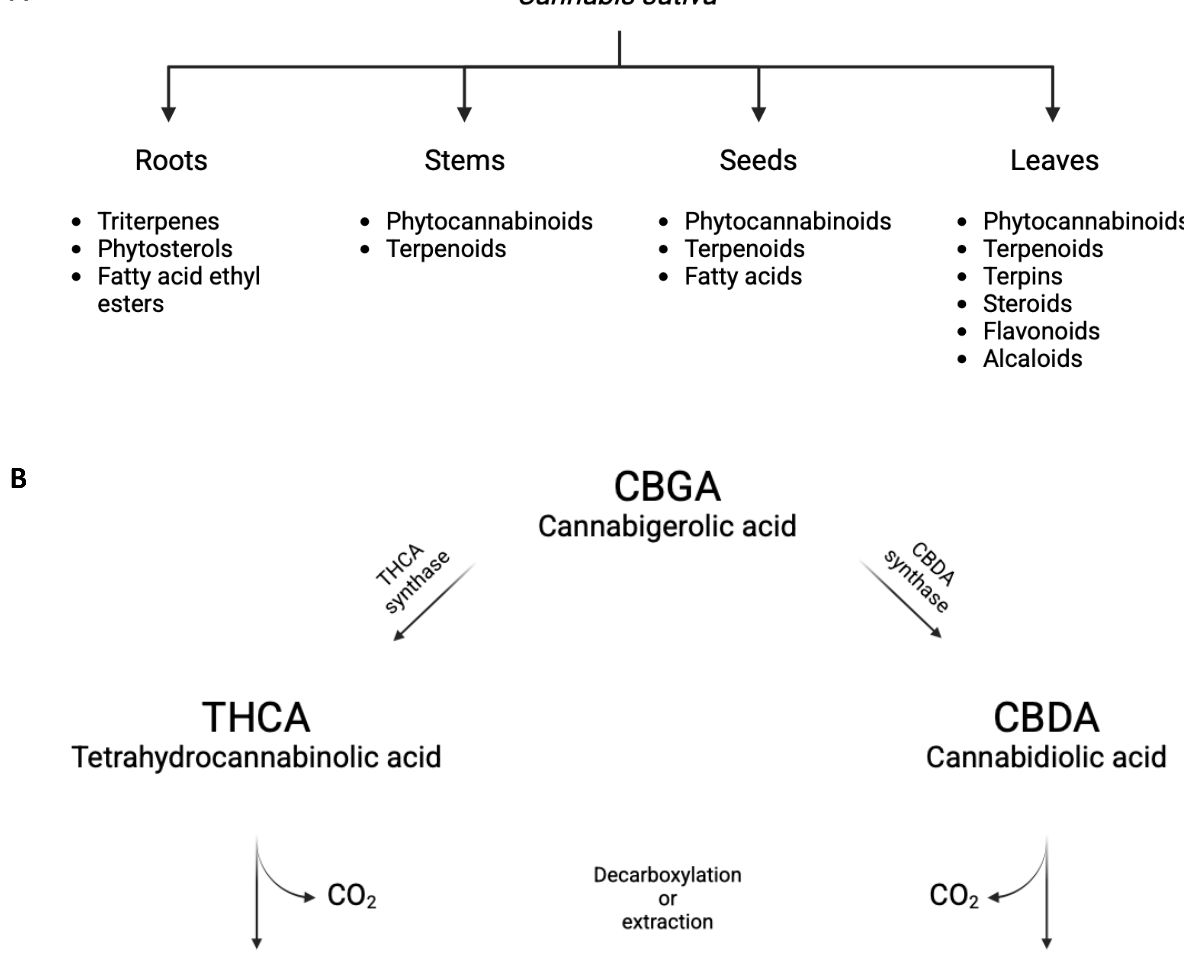


Figure 1.2: Chemical composition of *Cannabis sativa* and the biosynthesis of THC and CBD. (A) Composition of compounds of the different structures of the plant *C. sativa*. Image adapted from (Jastrząb *et al.*, 2022). (B) Biosynthesis pathways of THC and CBD from the parental compound CBGA. Image adapted from (Coogan, 2019)

Despite the potentially beneficial properties of CBD therapy as reviewed by Huestis *et al*., (2019), the illicit status of cannabis due to the psychoactive activity of THC has inhibited cannabis and cannabinoid research due to legal and regulatory constraints (Cooper *et al*., 2021). In the United Kingdom, cannabis is currently classified as Class B under the Misuse of Drugs Act 1971 and Schedule One under the Misuse of Drugs Regulations 2001. This dictates that cannabis holds a moderate risk of harm while offering no therapeutic potential (Monaghan *et al*., 2021). However, Sativex, the cannabis-derived oromucosal spray of 27 mg

THC and 25 mg CBD (Russo *et al*., 2007), is listed as Schedule Four being recognised for its therapeutical applications in treating muscular sclerosis-related spasticity (Collin *et al*., 2010; Monaghan et al., 2021). In the United States of America, cannabis is listed as a Schedule I item by the United States Controlled Substances Act, deeming it to have a high potential for abuse with no currently accepted use in medicine (Cooper *et al*., 2021). While it holds illegal status federally, cannabis has been legalised in 35 states and the District of Columbia for medicinal use and 15 of these sates with the District of Columbia legalising the recreational adult-use of cannabis (Cooper *et al.,* 2021). This mismatch between state and federal laws creates challenges with the regulatory landscape inhibiting the advancement of cannabis research in the United States of America.

While phytocannabinoids have been studied primarily in isolation, *Cannabis* extracts may produce enhanced effects when administered in mixture. The entourage effect was first defined by Ben-Shabat *et al.* (1998), later refined by Mechoulam & Ben-Shabat (1999), it details the potential of phytocannabinoids to work synergistically together to potentiate the therapeutic potential of cannabis over that of using phytocannabinoid isolates such as CBD or THC. A study by Gallily *et al*. (2015) observed that extract of *C. sativa* clone 202, which when analysed by gas chromatography and mass spectrophotometry (GC/MS) contained 17.9% CBD and 1.1% THC, produced a positive correlation between increasing dose and increasing effects of anti-inflammatory and anti-nociceptive in Sabra mice. In comparison to the bell-shaped curve produced by pure CBD treatment, this suggested that whole plant treatment allowed for the synergy of other extracts with CBD to potentiate the desired effects.

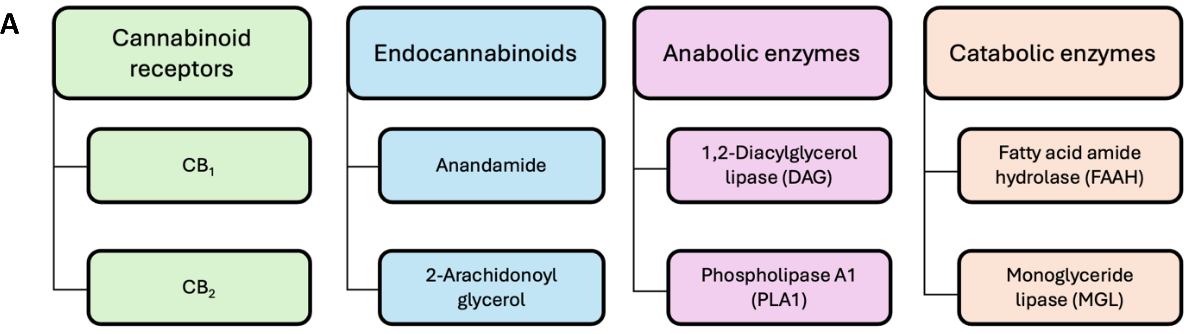
A meta-analysis by Pamplona *et al*. (2018) regarding the use of CBD therapy in refractory epilepsy, where seizures are not controlled by standard medications, collated data from 11 studies with a total of 670 patients. There was a statistical significance between the two groups regarding patient improvement with a 71% increase in the CBD-enriched cannabis group compared to the 46% pure CBD group. However, only 37% of the CBD-enriched group and 42% of the pure CBD group met the clinical threshold of ≥50% seizure frequency, with no statistical significance between the two groups. It should be noted that there was a 76.3% reduction in the average dose administered between the enriched group and pure group

(25.3 mg/kg/day and 6.0 mg/kg/day, respectively) with statistically significant reduction in mild and severe adverse effects in the CBD-enriched group. It was concluded by Pamplona *et al*. (2018) that this reduction in adverse events was perhaps due to the lower average dose, and therefore, the entourage effect of the synergistic behaviour of CBD and other cannabis extracts.

1.3.1. The endocannabinoid system

Cannabinoids exert their effects through the endocannabinoid system (ECS), either directly or indirectly in the case of THC and CBD, respectively. The ECS is a phylogenetically ancient and highly conserved cell-signalling system in vertebrates (Mosca *et al.,* 2021). Within humans, the ECS has neuromodulatory activity (Clarke *et al*., 2021; Elphick, 2012; Levichev *et al*., 2023; H.-C. Lu & Mackie, 2021) and is comprised of two types of cannabinoid receptors; Cannabinoid Receptor 1 (CB_1) and Cannabinoid Receptor 2 (CB_2) (Figure 1.3A), which belong to the superfamily of G-coupled protein receptors and are activated by the endocannabinoids such as anandamide and 2-arachidonoyl glycerol (2-AG), and enzymes responsible for the synthesis and breakdown of endocannabinoids (Bie *et al*., 2018; Lu & Mackie, 2021) (Figure 1.3A). Furthermore, CB_1 and CB_2 can also be targeted by exogenous cannabinoids, such as CBD and THC (Lu & Mackie, 2021). CB_1 receptors are reported to be class A G-protein-coupled receptors that exhibit a 97-99% similar amino acid sequence identity across mammalian species, further indicating the phylogenetic conservation of function for CB_1 receptors (Katona & Freund, 2012). CB_1 receptors are primarily found within terminals of central and peripheral neurons while CB_2 receptors are primarily found within the immune system (Figure 1.3B) (Pertwee *et al*., 2010). CB_1 and CB_2 share common ancestry, and it has been deduced that it is unlikely that any chance of a third, phylogenetically related cannabinoid receptor will occur (Pertwee *et al*., 2010). However, GPR18 and GPR55 are reported to be potentially putative endocannabinoid receptors, being observed to recognise cannabinoid ligands (Ramírez-Orozco *et al*., 2019). Furthermore, GPR119 although being more closely rated to CB_1 and CB_2 phylogenetically, no cannabinoid has been observed to interact with it with significant potency (Ramírez-Orozco *et al*., 2019). 1,3-dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methyle thenyl)-2-cyclohexen-1-yl]benzene (O-1918), a synthetic cannabinoid and analog of CBD, acts as a silent antagonist for putative 4-[(1*R*,6*R*)-3-Methyl-6-(1-

methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol (abn-CBD) receptors (Pertwee, 2020), one of which being the aforementioned GPR18 (Caldwell *et al*., 2013).



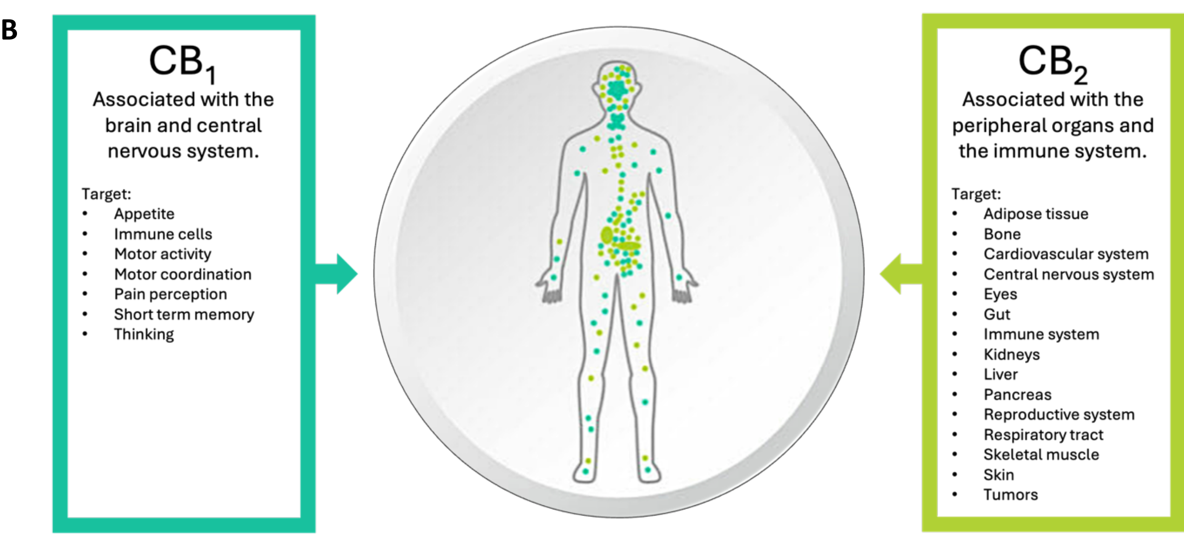


Figure 1.3: The endocannabinoid system (ECS). (A) Components of the ECS. Image adapted from (Bie *et al*., 2018). (B) Endocannabinoid receptors. CB_1 and CB_2 present in the human body and associated targets. Image adapted from (Lopez, 2023).

While cannabinoids have been studied using invertebrate models, they do not possess an ECS akin to that in mammals. However, species such as *Caenorhabditis elegans* have been reported to possess orthologous and homologous signalling pathways that respond to

cannabinoids, making them appropriate models to investigate cannabinoids (Estrada-Valencia *et al*., 2023; Levichev *et al*., 2023). Mosca *et al*. (2021) reviewed 15 species and genes associated with the ECS (Figure 1.4) which demonstrated the increased conservation of the ECS in vertebrates, namely CNR1 and CNR2 which codes for the cannabinoid receptors CB_1 and CB_2, respectively (Sophocleous *et al*., 2017); TRPV1 which is a receptor activated by endocannabinoids such as anandamide (Muller *et al*., 2020); NAPE-PDL which is an enzyme that can act to biosynthesise anandamide (Mock *et al*., 2020); and FAAH, an enzyme that can act to catabolise endocannabinoids (Dainese *et al*., 2020). A study investigating the lifelong exposure of CBD to *C. elegans* reported that there is a lack of long-term toxicity with physiologically relevant concentrations of CBD derived from human clinical trials (Land *et al*., 2021), however, while there is evidence to the contrary that CBD can exhibit adverse effects on *C. elegans* it is at doses that exceed those recommended for human consumption (Camacho *et al*., 2024).

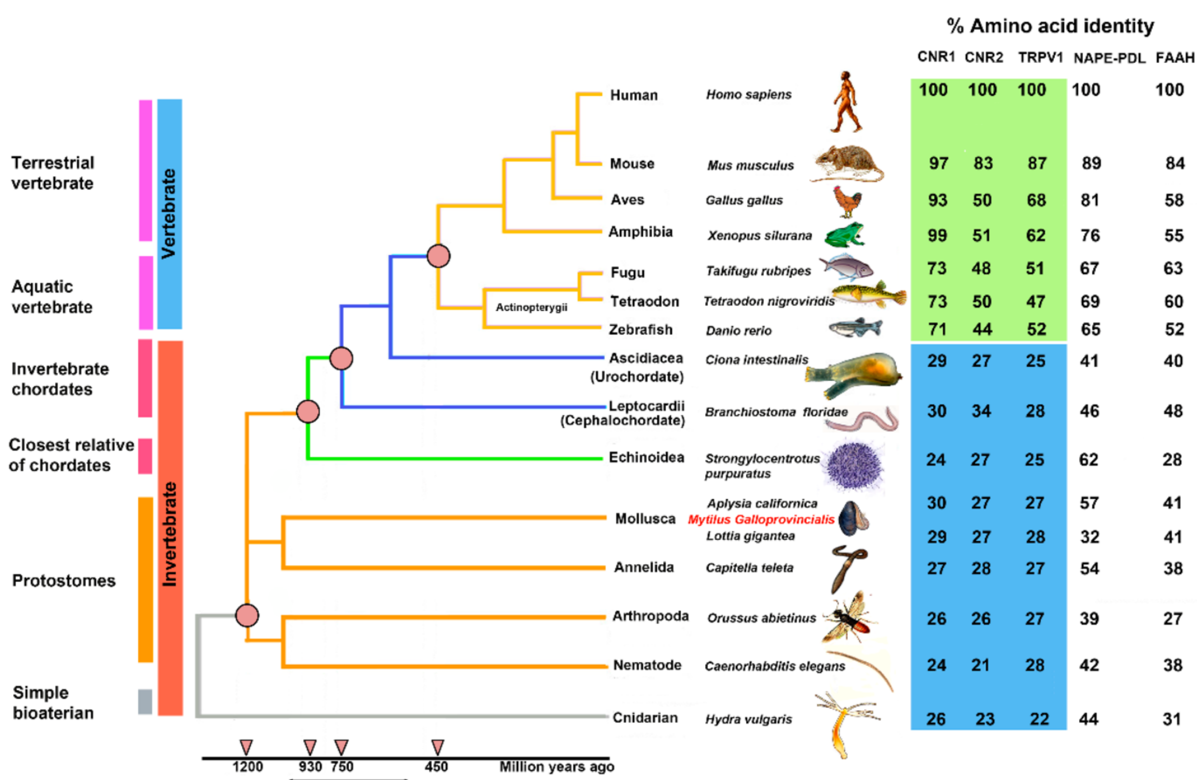


Figure 1.4: Phylogenetic tree of the endocannabinoid system in 15 species. The similarity of receptor and enzyme genetic coding associated with the endocannabinoid system expressed as a percentage compared to humans. Image obtained from (Mosca *et al*., 2021).

Cannabinoids can be classified as endogenous (produced naturally within humans) and exogenous (synthesised externally to humans). Exogenous cannabinoids can further be classified into phytocannabinoids, synthesised by *Cannabis* spp., and synthetic cannabinoids, laboratory made which exhibit binding affinity for cannabinoid and putative cannabinoid receptors (Table 1.2).

Table 1.2: Overview of cannabinoid compounds. Structural images sourced as follows: THC (Dawidowicz *et al.,* 2021), CBD (Kim *et al*., 2021), 7-OH-CBD (Merck, n.d.), abn-CBD (Kim *et al*., 2021) and O-1918 (Pertwee, 2001).

	Phytocannabinoid			Synthetic cannabinoid	
Compound	THC[1]	CBD[2]	7-OH-CBD[3]	Abn-CBD[4]	O-1918[5]
Molecular formula	$C_{21}H_{30}O_2$	$C_{21}H_{30}O_2$	$C_{21}H_{30}O_3$	$C_{21}H_{30}O_2$	$C_{19}H_{26}O_2$
Molar mass (g mol^{-1})	314.47	314.47	330.47	314.47	286.42
Structure					

1 Δ⁹-Tetrahydrocannabinol

2 Cannabidiol

3 7-hydroxycannabidiol

4 4-[(1*R*,6*R*)-3-Methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol

5 1,3-dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methyle thenyl)-2-cyclohexen-1-yl]benzene

1.3.2. Phytocannabinoids

1.3.2.1. Δ^9-Tetrahydrocannabinol

THC is the primary psychoactive phytocannabinoid associated with cannabis and is a volatile and liphophilic compound (How & Gamal El-Din, 2021). Due to its lipophilic nature, THC exhibits the tendency to accumulate in fat tissues where it can then slowly be released back into the bloodstream (Chayasirisobhon, 2020). THC acts as a partial agonist for both CB_1 and CB_2 receptors, with a higher efficacy at CB_1 (Howlett *et al*., 2002). The therapeutical potential of THC has showed promise with THC isolate being investigated for the inhibition or slowing

of the hallmark characteristics associated with Alzheimer's disease (Cao *et al*., 2014). THC was effective at lowering levels of the peptide amyloid-b and with direct interactions was shown to inhibit aggregation in N2a/bAPPswe cells. Furthermore, dose-dependent concentrations of THC was effective in lowering GSK-3b levels and phosphorylated GSK-3b at low concentrations. A report published by Lynch & Clark (2003) detailing three separate patients suffering with chronic non-cancer pain which were able to reduce opioid medications after beginning cannabis treatment. However, there are conflicting studies suggesting limitations to the efficacy of THC analgesia. Sativex, the THC/CBD oromucosal treatment, were shown to be more effective than THC isolate treatments in pain relief (Johnson *et al.* (2010). Furthermore, in a randomised double-blind, placebo-controlled trial with THC (5 mg), there were no significant differences with the placebo group in scores for postoperative pain in humans (Buggy *et al.,* 2003). However, the analgesic properties of THC, in the form of a synthetic version dronabinol, have shown success in the treatment of neuropathic pain from conditions such as multiple sclerosis (Schimrigk *et al.,* 2017; Ueberall *et al.,* 2022). A study by Schimrigk *et al.* (2017) revealed that dronabinol is a safe option for long-term treatment for neuropathic pain with similar number of side effects, low drug abuse and drug dependency potential. However, when researching cannabinoids as an add-on treatment for neuropathic pain, Ueberall *et al.* (2022) observed dronabinol as having inferior effectiveness when compared to the THC:CBD product, nabiximol. This suggests CBD may act to potentiate the analgesic effects of THC while modulating the adverse side effects. THC exhibits anticonvulsant properties, Wallace *et al.* (2001) investigated the mechanism of action using a maximal electroshock model in mice and determined it is receptor mediated through CB_1 by pre-treating mice with the CB_1 antagonist SR141716A which inhibited the anticonvulsant effects. THC has also been approved for treatment of chemotherapy-induced nausea/vomiting and weight loss/anorexia associated with advanced human immunodeficiency virus (HIV) (Madras, 2019).

1.3.2.2. Cannabidiol

CBD was first isolated from *Cannabis* in 1940 by Roger Adams, with its structure later identified in 1963 (Burstein, 2015). Furthermore, CBD and THC are isobaric isomers of each other, both having the molecular formula, $C_{21}H_{30}O_2$ (Table 1.2). Despite this, they are difficult

to differentiate even with mass spectrometry techniques (Golombek *et al*., 2020). CBD has a computed Log K_{ow} value between ~6-8 (Bragança *et al*., 2020; Tabboon *et al*., 2022). Furthermore, CBD has been detected in waste sewage sludge (0.1-1.5 µM), highlighting the importance of CBD research regarding its ecotoxicity (Mastroianni *et al.,* 2013). A study by Black *et al*. (2019) investigating endocrine active organic contaminants in Californian sewage sludge revealed CBD was detected in 43% of samples, while Mastroianni *et al*., (2013) saw an 80% detection rate from samples collected in Spain. CBD is emerging as a PIE and its use is increasing with direct-to-consumer products (Chesney et al., 2020; How & Gamal El-Din, 2021). The product market of CBD in the UK was valued at £690 million in 2022 (Delaney, 2022), furthermore, the global cannabis market is projected to increase from $57.18 billion (2023) to $444.34 billion (2030) (Fortune Business Insights, 2022). The high product market value of CBD and the increasing market value of cannabis suggests that with the higher rate of consumption, there will be a corresponding increase in product disposal, excretion and environmental occurrence. PIEs pose potential risks to non-target species as exogenous toxicants and despite the increasing use and prevalence of CBD, there is a paucity in the literature regarding its ecotoxicity and how it may impact aquatic species, microbial communities and sediment chemistry. It is therefore important to elucidate the ecotoxicity of CBD as there is the potential to bioaccumulate in the benthic organisms residing in the sediment it tends to partition to.

The pharmacodynamics of CBD is complex (Figure 1.5) and with the molecular targets and mechanism of action of CBD remaining to be fully elucidated. There are a number of molecular targets proposed for CBD and while it is widely accepted that CBD does not exhibit a high affinity for CB_1/CB_2 receptors, there are reports of its behaviour acting as an antagonist at both receptors (Almeida *et al*., 2014), a negative allosteric modulator (NAM) at CB_1 (Laprairie *et al*., 2015) and CB_2 (Martínez-Pinilla *et al*., 2017). An antagonist will bind to the orthosteric binding site competing with the receptors agonist while a NAM binds to the allosteric binding site causing a conformational change at the orthosteric site, both mechanisms of action result in reduced receptor activation. A review by Peng *et al*. (2022) reported CBD can act not only as an antagonist and a NAM, but also exhibits behaviour as an agonist, inverse agonist, indirect agonist, partial agonist and as an inhibitor.

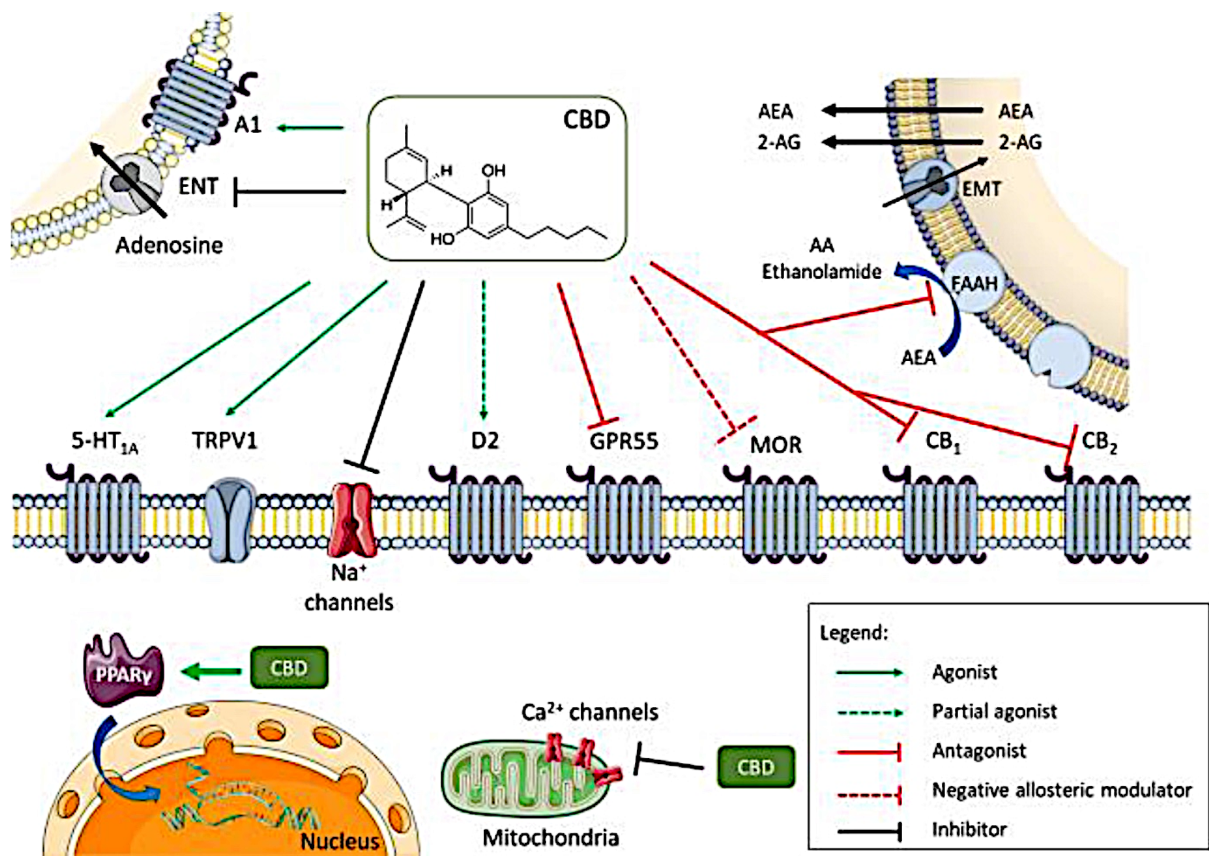


Figure 1.5: Proposed mechanisms of action of CBD. Image obtained from Almeida *et al*. (2014).

A previous study by Wallace *et al.* (2001) assessing the mechanisms of action of the anticonvulsant properties of cannabinoids revealed that while both CBD and THC exhibit this effect, CBD is not receptor mediated through CB_1, which is congruent with the low affinity CBD exhibits for cannabinoid receptors (Pertwee, 2008). The anxiolytic properties of CBD also show promise with a randomised, double-blind placebo controlled study by Crippa *et al.* (2011) observed 400 mg CBD significantly reduced anxiety states after 60 minutes, continuing for 140 minutes post-administration until observations ended. Furthermore, through nueralimaging Crippa *et al.* (2011) suggested that the anxiolytic effect of CBD was due to the the activity of CBD in the limbic and paralimibic areas of the brain. Shannon *et al.* (2019) used CBD doses in the range of 25-175 mg/day with anxiety scores reduced for the duration of the clinical trial. The dosing varied due to taking in to account the clinical preference of each participants practitioner. CBD has demonstrated antiemetic properties in an animal model in a study by Rock *et al.* (2012) where chemically induced vomitting was significantly reduced in shrews (*Sancus marinus*) with administration of CBD. The anticonvulsant effects of CBD have

been notable in people suffering from Lennox-Gastaut syndrome, a study by Thiele *et al.* (2018) assessed the efficacy of CBD as an add-on therapy to treatment resistant patients. Thiele *et al.* (2018) noted statistically significant and clinically meaningful reductions in the freuquency of seizures was observed when compared to the placebo, with three patients from the CBD-treated groups continued through the 12 week maintenance period with no drop seizures.

CBD was believed to only be produced by species within the *Cannabis* genus, however, it has been reported that the carboxylic acid precursors to CBD and THC, CBA and THCA respectively, have recently been detected within *Trema micrantha* (Ribeiro *et al*., 2024), a species within the family Cannabaceae (How & Gamal El-Din, 2021). Therefore, there is a potential of cannabinoid prospecting from non-*Cannabis* origins, although CBD yield from *T. micrantha* is significantly lower than that of medicinal strains of *Cannabis* (Ribeiro *et al*., 2024). CBD exhibits a low affinity for the orthosteric binding sites of the receptors CB_1 and CB_2 (Burstein, 2015; Peng *et al*., 2022), however, it does present allosteric activity on both cannabinoid receptors (Peng *et al*., 2022). While CBD can be metabolised to 7-OH-CBD or 7-COOH-CBD (Figure 1.6) after ingestion or inhalation, it is often excreted unmetabolised or in its glucuronide form (Pérez-Acevedo *et al*., 2020; Ujváry & Hanuš, 2016).

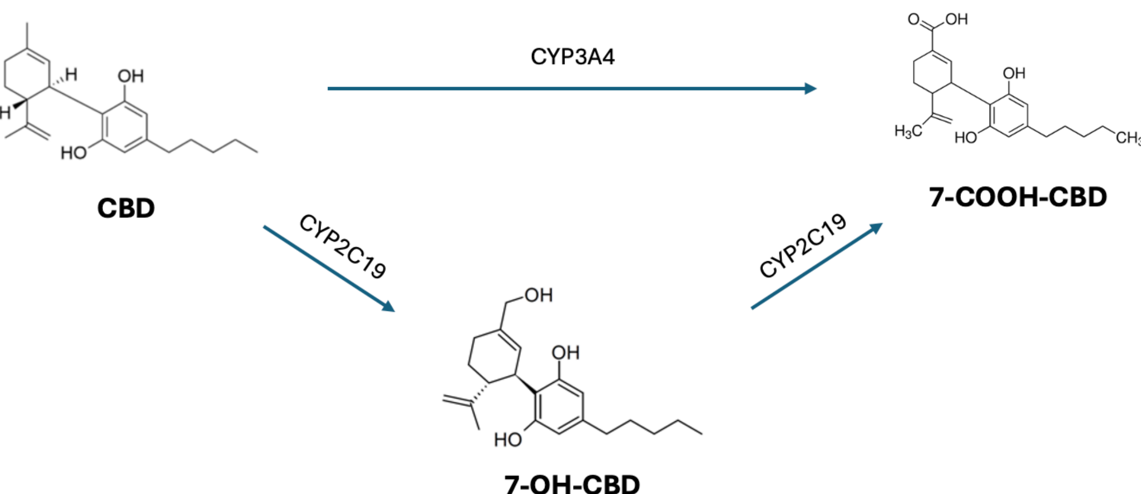


Figure 1.6: Metabolism pathways of CBD in humans. Image adapted from Austin *et al*. (2024).

1.3.2.3. 7-OH-CBD

7-OH-CBD is the primary metabolite of CBD. CBD is metabolised via hydroxylation at the 7C position (Figure 1.6) to produce the pharmacologically active metabolite, 7-OH-CBD, and is reported to be catalysed by cytochrome P450 enzymes (Beers *et al*., 2021; Jiang *et al*., 2011). Regarding the pharmacological potency of 7-OH-CBD there is conflicting information, support has been presented to suggest it has reduced (Nye *et al*., 1985), increased (Stott *et al*., 2015) or be equipotent (Beers *et al*., 2021; Nasrin *et al*., 2023) when compared to CBD. However, Beers *et al*. (2021) described that the area under the curve (AUC) of 7-OH-CBD which indicates the total drug exposure, is 38% lower than that of CBD and therefore limits exposure within the body before excretion or further metabolism to 7-COOH-CBD (Figure 1.6). The variability and diversity of molecular targets exhibited by CBD may be accountable for the discrepancy in potency comparisons between CBD and 7-OH-CBD. While the therapeutic uses of 7-OH-CBD are not as clear as CBD, it has been described as having anticonvulsant properties (Nasrin *et al*., 2023). 7-OH-CBD demonstrated anti-convulsant action and latency reduction in seizure onset in a pentylenetetrazole-induced seizure model using male Wistar rats (P24-29), furthermore, it was reported that 7-OH-CBD demonstrated greater potency than that of its parent compound, CBD (Scott *et al*., 2022). 7-OH-CBD has also been patented for use as a medication to treat non-alcoholic fatty liver disease (NAFLD) (Stott *et al*., 2015).

1.3.3. Synthetic cannabinoids

1.3.3.1. Abn-CBD

Abn-CBD is a synthetic, structural isomer of CBD having the same chemical formula (Table 1.2), differing by the transposition of the phenolic hydroxyl group and pentyl side chain (Romero-Zerbo *et al*., 2020). Abn-CBD, like CBD, does not exhibit psychoactive effects when consumed or administered, and is not associated with the CB_1 or CB_2 receptors but binds to GPR18 and GPR55 as an agonist (Matouk *et al*., 2018; Romero-Zerbo *et al*., 2020; Wang *et al*., 2023). Abn-CBD has demonstrated therapeutic promise in studies investigating the treatment of inflammation (González-Mariscal *et al*., 2022), cardiovascular (Matouk *et al*., 2018), diabetes (McCloskey *et al*., 2023) and Parkinson’s disease (Celorrio *et al*., 2017).

1.3.3.2. O-1918

O-1918 is a synthetic cannabinoid and an analog of CBD (Table 1.2), and similarly to CBD does not exhibit affinity for CB_1 or CB_2 receptors (Offertaler *et al*., 2003) suggesting it also does not produce a psychoactive effect, moreover it is a silent antagonist for the putative abn-CBD receptors, GPR18 and GPR55 (Pertwee, 2001) and has been observed to block GPR18 and GPR55 activation by abn-CBD (Caldwell *et al*., 2013).

1.4. Invertebrate models in research

1.4.1. The Three Rs

Invertebrates offer a more ethical alternative to *in vivo* experimental models in lieu of vertebrate studies which are confined by regulations such as the Animal (Scientific Procedures) Act (1986). Sentience is a multifaceted and subjective phenomenon with the ability to manifest into different degrees (Veit, 2023), with de Souza Valente (2024) examining definitions in the literature and concluding sentient organisms are those that hold a phenomenological experience of awareness. Sentient organisms are typically covered by legal protections that cover vertebrates, cephalopods and decapods (UK) (de Souza Valente, 2024). Furthermore, Horvath *et al.* (2013) discussed the literal regarding the general acceptance of invertebrates lacking the capacity to feel pain or suffer and their simplified nervous systems compared to vertebrates resulting in less ethical considerations. Therefore, invertebrates offer a practical and more ethical model alternative to vertebrates. Pain is described as a negative emotional response to an unpleasant stimulus associated with actual or potential damage to the recipient (Elwood, 2019). Nociception begins the process of pain following an activated central nervous system to bring awareness to the recipient, this awareness may be remembered possibly with anxiety to prevent future damage (Elwood, 2019). Oikawa et al. (2023) reported that the fruit fly, *Drosophila melanogaster,* exhibit a descending inhibitory mechanism of nociception through an evolutionary conserved cholecystokinin system present in mammals. This suggests that invertebrate models can offer mechanistic insight into complex biological systems which would require the use of whole organisms rather than *in vitro* studies. The principles of the Three Rs were originally coined by Russell and Burch in 1959 (Müller, 2024) of the Universities Federation for Animal Welfare (UFAW), it is an ethical

framework to tackle the controversy surrounding the use of sentient animals in research. The Three Rs were described in order of importance in which they should be addressed; Replacement, Reduction and Refinement.

Replacement: where appropriate, substitutions should be made to use lower organisms over those deemed to be sentient.

Reduction: the number of animals used as models in investigative research should be reduced

Refinement: inhumane practices should be minimised where possible when animal models are to be used.

In comparison to vertebrate models, invertebrate models offer several advantages in being typically low-cost to house and maintain, having simple behaviour patterns that are easily quantifiable, short lifespans and possessing genetic homologs of interest to human afflictions (Drewes, 1999; Kallarackal, 2023; Seeley *et al*., 2021; Wang *et al*., 2022; Zhang *et al*., 2020).

1.4.2. Invertebrates in CBD research

Given the ethical constraints and concern around using vertebrate models in science, invertebrate models have become vital tools in understanding the biological effects to exposure of emerging PIEs, including CBD. Combining the reduced regulations of use with ecological relevance, invertebrate models are highly suitable for the evaluation of sublethal and long-term toxicology of CBD. Wang *et al*. (2022) investigated the impact of CBD on autophagy, neuronal health and lifespan using *C. elegans* as a model. With continuous exposure of *C. elegans* to CBD (1 µm), it was revealed that CBD treatment increased lifespan and improved age-associated physiological functions. CBD treatment also promoted autophagy flux and highlighted that lifespan extension and neuronal health benefits were dependent on the autophagy-related genes, including SIRT1, highlighting the neuroprotective potential of CBD. Skowronek & Strachecka (2023) investigated the effects of CBD on the antioxidant system of the honeybee workers, *Apis mellifera*, through quantifying antioxidant

enzymes and ion concentrations via haemolymph analysis. Individuals were exposed to 30% CBD oil via sugar syrup or textile strips, with a control group receiving pure sugar syrup. CBD exposure revealed enhanced antioxidant enzyme activity and increased ion concentrations, particularly in the infused sugar syrup group. It was determined that CBD exposure can potentially support the immune system of *A. mellifera* by mediation of oxidative stress.

Casciato *et al*. (2020) examined the effects of CBD exposure on the short-term memory of young male fruit flies, *D. melanogaster*. Food was infused with CBD and the learning and memory of individuals were assessed against a control group. It was reported that CBD exposure did not enhance short-term memory as hypothesised, conversely, it had an adverse effect on individuals and appeared to impair short-term memory and therefore it was concluded that (male) *D. melanogaster* suffer an inhibitory effect to their short-term memory with CBD exposure.

1.5. Lumbriculus variegatus

Commonly referred to as the Californian black worm, the invertebrate *L. variegatus* is an aquatic endobenthic detritivore of the littoral zone in freshwater ponds, lakes and marshes and is primarily endemic to the Holarctic region (Figure 1.7A-B) (Coq *et al*., 2022; Drewes, 1999; Marchese *et al*., 2015). The occurrence records of *L. variegatus* in the temperate zone of the southern hemisphere (Figure 1.7B) are considered to be recent zone extensions, and likely due to species of the *Lumbriculus* genus to be able to undergo encystation when faced with ecological stressors or by its distribution as a commercial culture (Brinkhurst, 1989). It was proposed by Brinhust (1989) that due to *L. variegatus* occupying soft sediments there is potential for the uptake of organisms into ship bilges or in the collection and shipments of aquatic plants. Encystation is a survival mechanism where an organism will form a protective cyst to endure adverse environmental conditions until certain environmental conditions are met (Glasby *et al.,* 2021). In this cyst state, it is possible for the accidental transport and passive dispersion of cysts through animal and human mediated movements.

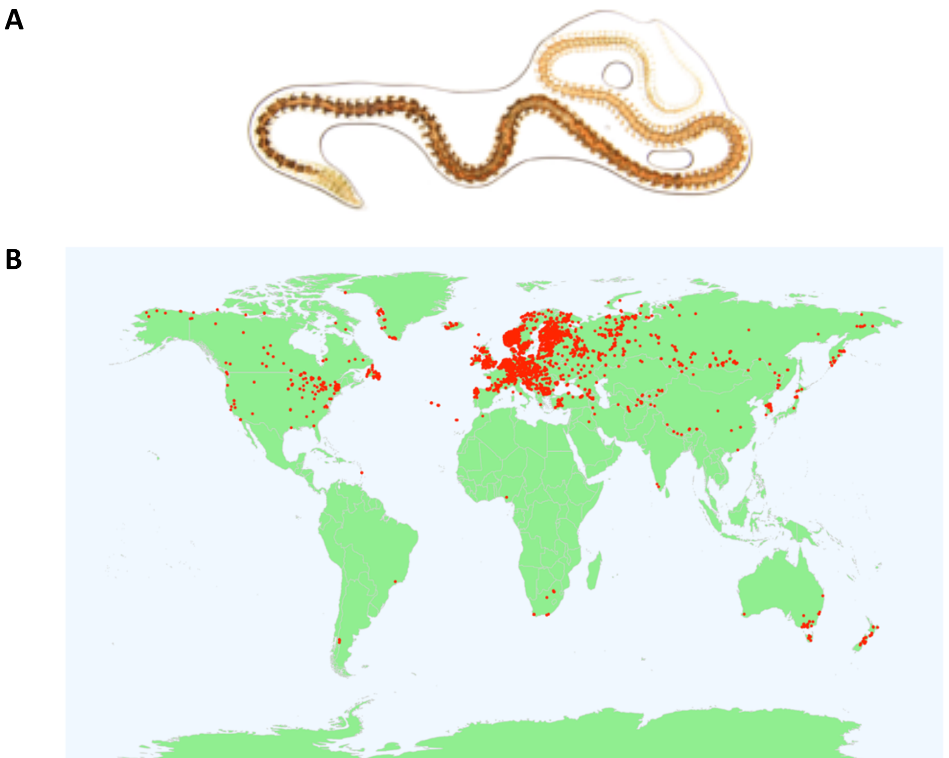


Figure 1.7: *Lumbriculus variegatus* and its geographical distribution. (A) *L. variegatus* under a Nikon SMZ1270i stereomicroscope. (B) Geographical distribution plotted using RStudio with occurrence records obtained from the Global Biodiversity Information Facility (GBIF.org, 2025). Code available in appendix A1.

Genetic variation was discovered in *L. variegatus* by Gustafsson *et al*. (2009), revealing cryptic speciation, described as when species that have been incorrectly classified as a single species due to being indistinguishable, especially when using morphology-based taxonomic methods (Bickford *et al.,* 2007; Knowlton, 1993). When comparing mitochondrial 16S, COI genes and the nuclear ITS region, three clades were determined with at least one clade being polypoid and another being diploid. This suggests at least one division of at least two lineages within the current taxon. *L. variegatus* has two forms based on their reproduction, those that undergo architomy (fragmentation and regeneration), and those that sexually reproduce, with the latter reportedly being scarce in population (Gustafsson *et al*., 2009). The rapid architomy of *L. variegatus* and the survival success rate of the resulting fragments able to regenerate into whole organisms is the evolutionary adaptive strategy in response to predatory pressures (Marchese *et al*., 2015). This presents *L. variegatus* as a suitable model in regenerative studies (Hill *et al*., 2018).

1.5.1. *Lumbriculus variegatus* physiology and behaviour

Naturally, *L. variegatus* will form a living collective, aptly named a worm blob in the literature (Nguyen *et al*., 2021; Tuazon *et al*., 2022), it is a compact and dense structure displaying properties of a non-Newtonian fluid when free of substrate or detritus (Tuazon *et al*., 2022). The worm blob arises from the thigmotactic survival behaviour of *L. variegatus* in response to environmental stress and to prevent desiccation (Tuazon *et al.*, 2022). While their tails have been reported to be used for oxygenation when in the form of a worm blob (Tuazon *et al*., 2022), it is not in the same fashion when they act individually. Other oligochaetes such as tubificids use undulatory action to facilitate gas exchange, however, observations by Drewes (1990) and O'Gara *et al.* (2004) were that *L. variegatus* would be anchored into the substrate with their anterior with their tails protruding into the water column, and if possible, would connect to the surface water flexing perpendicularly. *L. variegatus* have a large dorsal blood vessel (DBV) that acts to circulate blood with peristaltic contractions originating at the posterior (Drewes, 1990). Towards the posterior, the DBV lays juxtaposed to the epidermis possibly to facilitate gas exchange when the posterior is in contact with the surface water (Drewes, 1990). The cuticle of *L. variegatus* is transparent which allows for non-invasive and easy peristaltic observations of the DBV to measure their pulse rate (Figure 1.8) (Halfmann & Crisp, 2011). They have an eversible pharynx to feed within the sediment with a mucous-lined body wall for lubrication and respiration (Tuazon *et al*., 2023). *L. variegatus* have photoreceptor cells that are sparsely distributed through the organism that engage with the lateral giant nerve fibres to produce a shadow reflex effect (Drewes, 1990). It is believed this mechanism is for detecting decreases in illumination, or shadows, a possible predator-detection for surface predators such as birds. It was reported that this characteristic was observed in the newly hatched worms in sexually reproducing population suggesting this is an evolutionary trait derived from the selection pressure of predatory interactions.

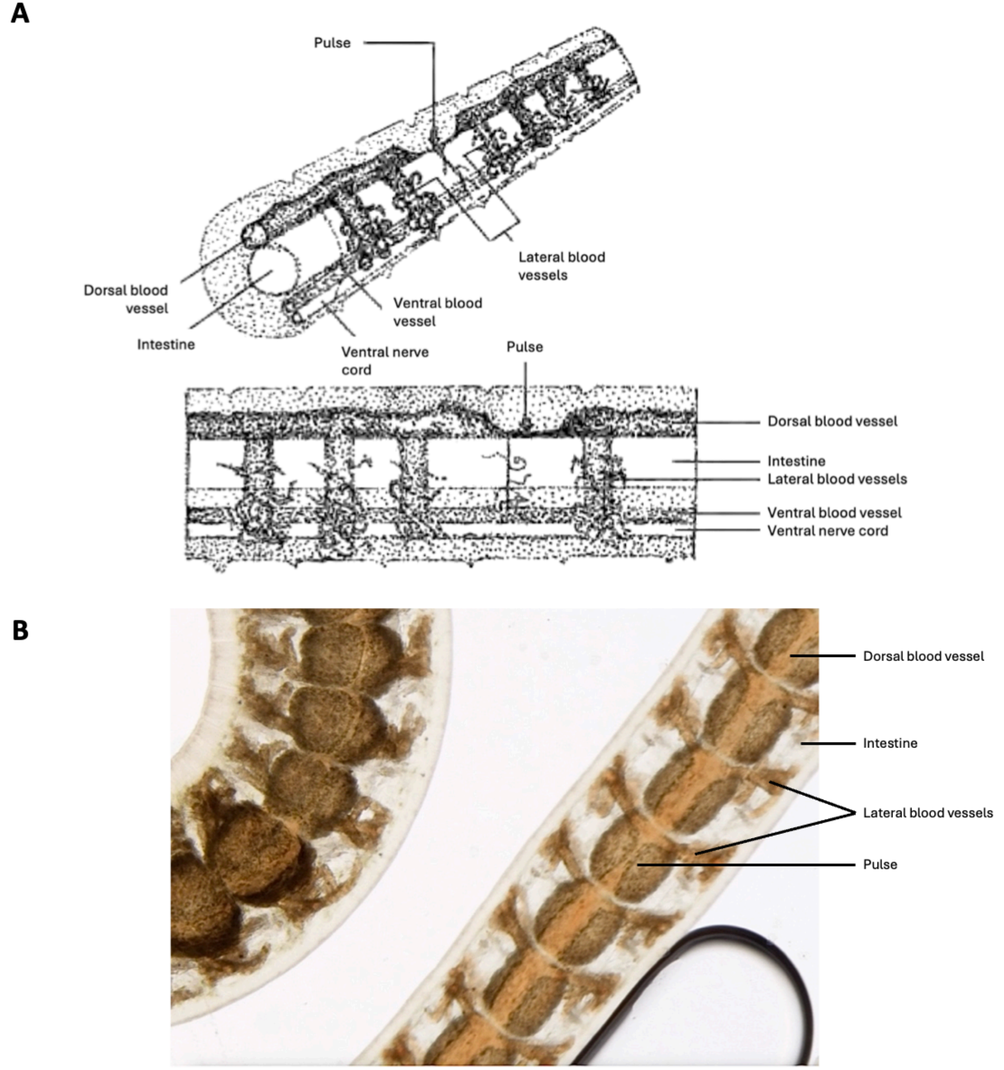


Figure 1.8: *Lumbriculus variegatus* circulatory system. (A) Annotated stipple drawing obtained and adapted from Halfmann and Crisp (2011) illustrating the internal anatomical arrange of *L. variegatus*. (B) Representative Nikon SMZ1270i stereomicroscope picture of *L. variegatus* highlighting the closed circulatory system and contraction of the dorsal blood vessel, however, due to viewing limitation, certain ventral structures are unable to be viewed.

Stereotypical locomotor movements, helical swimming and body reversal, of *L. variegatus* in response to tactile stimulation were first described by (Drewes, 1999) (Figure 1.9). Helical swimming is described as the response to tactile stimulation of the posterior of *L. variegatus,* it is the rhythmic and rapid bending of the body in the anterior-posterior direction to propel the individual forward. Body reversal is described as the response to tactile stimulation of the anterior, it is the rapid retraction of the body to reorientate the individual. Drewes (1999) noted that these locomotor responses are predatory evasion tactics whereby stimulating the anterior would result in reorientation in preparation of swimming away from a predator and

stimulation of the posterior would results in the rapid swimming away from a predator. These behavioural characteristics of *L. variegatus* allow for toxicokinetic and environmental studies to quantify changes in behaviour post-exposure (Gerhardt, 2007; O'Gara *et al*., 2004).

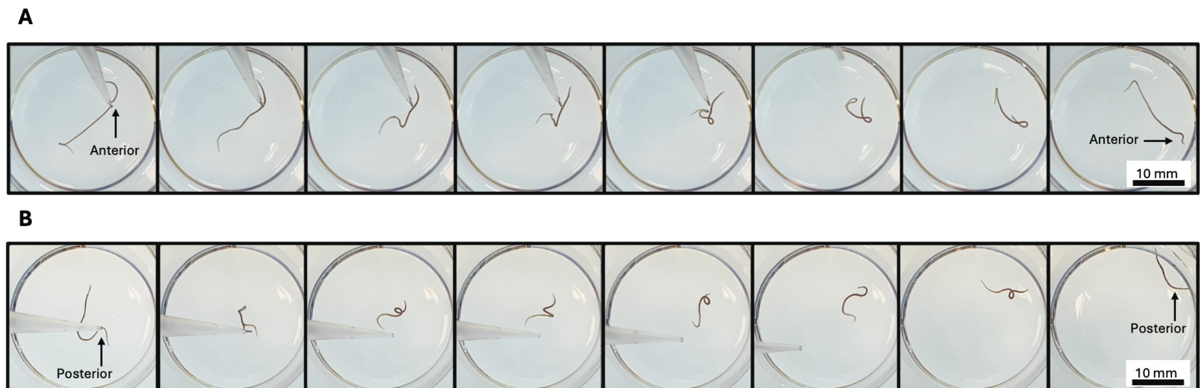


Figure 1.9: Stereotypical movements of *Lumbriculus variegatus* following tactile stimulation. Images show the stereotypical movement (A) body reversal, whereby bending movements reverse the head and tail positions, and (B) helical swimming, characterised by rapid helical body bends of *L. variegatus* following tactile stimulation with a 20-200 µL pipette tip on the anterior and posterior regions, respectively. Images show the stereotypical movements over a 2-3 second period following stimulation. Figure obtained from the Swansea Worm Integrative Laboratory.

1.5.2. Ecosystem services

As a detritivore and burrowing species they are involved in nutrient recycling through decomposition, bioturbation, act as prey to species in higher trophic levels and can act to restore organic pollutants and improve water quality (Daoud *et al*., 2022; Drewes, 1999; Ji *et al*., 2020; Kim *et al*., 2021; Kristensen *et al*., 2012; Tuazon *et al*., 2023).

1.5.3. Pharmacologically active compound research

The use of *L. variegatus* by the Swansea Worm Integrative Research Laboratory (SWIRL), Swansea University, was proposed as a novel model for studying pharmacologically active compounds over that of mammalian models (Seeley *et al*., 2021). The use of *L. variegatus* as an invertebrate model aligns with the more ethical approaches set by the Three Rs, while reducing culture costs and requiring a much lesser area to house than vertebrate models. *L.*

variegatus has served as a model indicator and keystone species that can be representative of freshwater ecosystems (Harkey *et al*., 1994; Vought & Wang, 2018) and used to investigate water pollutants such as copper (Ji *et al*., 2020; O'Gara *et al*., 2004), microplastics (Silva *et al*., 2021), bisphenols (BPA, BPS) (Vought & Wang, 2018), lead (Gerhardt, 2007), and in pharmacologically active compounds such as fluoxetine (Nentwig, 2007), ethanol (Seeley *et al*., 2024), nicotine (Davies *et al*., 2025), vinblastine and colchicine (Tweeten & Anderson, 2008) and galaxolide (Aikins *et al*., 2023). These studies have utilised behavioural parameters such as locomotor activity, stereotyped behaviours of body reversal and helical swimming, line body shape, energy reserves, cholinesterase activity, reproduction, biomass, regeneration, pulse rate, toxicity and growth as experimental endpoints. An indicator species is an ecological concept used to monitor a specific habitat and environmental changes, management efficacy of said habitat, and to provide insight into ecological shifts that may be occurring (Siddig *et al*., 2016). The endobenthic nature of *L. variegatus* allows for it to be utilised as an investigative model for environmental pollutants in sediment toxicity studies as a potential conduit for biomagnification, particularly those toxicants prone to sedimentation and bioaccumulation (Landrum *et al.,* 2002, 2004; Pakarinen *et al.,* 2011; Veltz *et al.,* 1996).

1.5.4. *Lumbriculus variegatus* and CBD research

The effects of CBD exposure on *Lumbriculus variegatus* remains understudied and there is paucity in the literature regarding its ecotoxicological impact. To date, one study has examined the effects of CBD (0.0 – 20.0 µM) after 10 minutes exposure where Williams *et al.* (2025) established that CBD significantly decreased the ability of *L. variegatus* to complete stereotypical movements, however, this was observed above environmentally relevant concentrations (Figure 1.10). CBD significantly inhibited body reversal (5.0 µM) and helical swimming (5.0 µM), however, it did not significantly impact the locomotor activity of *L. variegatus* (Figure 1.10) (Williams *et al*., 2025). Given that CBD has been observed to be present in the environment, it is likely its metabolites may also enter the aquatic ecosystem through human excretion. The ecological important of *L. variegatus*, the tendency of CBD to partition to the sediment where *L. variegatus* reside, its sensitivity to acute CBD exposure, and the ethical and practical advantages of using an invertebrate deem it as a suitable model

for investigating the ecotoxicological impact of chronic CBD exposure. This is of particular importance in light of the dominant global use of cannabis, being the third most commonly used controlled substance globally (Connor *et al.,* 2021) and the increase in interest of cannabis-derived treatments (Johnson & Colby, 2023). Given the established use of the endobenthic organism *L. variegatus* in sediment toxicity and bioaccumulation studies, the emerging presence of CBD in aquatic ecosystems with the limited research using this species a clear knowledge gap exists. This reliance on using few, well established invertebrate models such as *C. elegans*. This can cause issues with creating a limited biological representation and assumption of results, potential bias in biological and ecological insights and a narrow focus that could potentially result with missed novel discoveries.

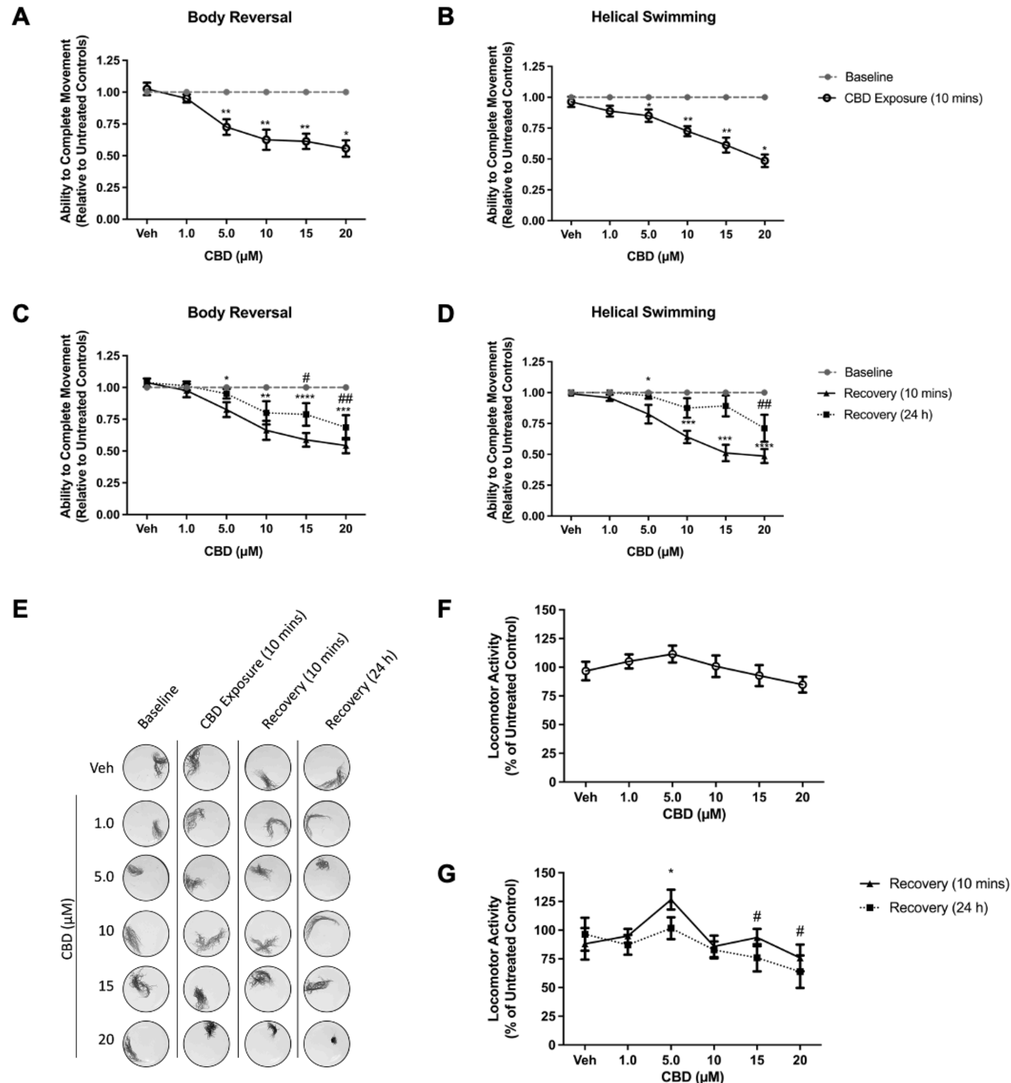


***Figure 1.*10: The effect of 10 minutes exposure to 0 – 20 µM CBD on *Lumbriculus variegatus* behaviour.** *L. variegatus* were exposed to CBD (0 – 20 µM) for 10 minutes and tested for the ability of tactile stimulation to elicit (A) body reversal or (B) helical swimming. Following removal of CBD, the ability of *L. variegatus* to perform (C) body reversal or (D) helical swimming was tested after 10 minutes and 24 hours. Data are expressed as a ratio of the movement score after exposure relative to the movement score at baseline. (E) Representative superimposed images analysed in ImageJ showing the effect of 10 minutes of exposure to CBD on locomotor activity measured before CBD exposure (Baseline), at 10 minutes exposure to 0 – 20 µM CBD (CBD Exposure (10 mins)), 10 minutes after CBD removal (Recovery (10 mins)) and 24 hours after CBD removal (Recovery (24 h)). Quantification of the area covered by *L. variegatus* following (F) 10 minutes of exposure to 0 – 20 µM CBD and (G) removal of CBD for 10 minutes (Recovery (10 mins)) and 24 hours (Recovery (24 h)), expressed as a percentage of the locomotor activity at baseline. Analyses were conducted by comparing CBD exposure conditions to baseline conditions by paired non-parametric two-tailed t-test for stereotypical movement assays and paired parametric two-tailed t-test for locomotor activity. A two-way ANOVA with Dunnett's post-test was used to analyse 10-minute and 24-hour recovery time points compared to baseline conditions for *L. variegatus*. */# p<.05, **/## p<.01, *** p<.001, **** p<.0001; where * refers to statistical significance between Baseline and CBD Exposure (10 mins) or statistical significance between Baseline and Recovery (10 mins), # refers to statistical significance between Baseline and Recovery (24 h). Error bars represent the standard error of the mean, n=8 with a single *L. variegatus* exposed to each concentration. Veh = 0.5 % (v/v) dimethyl sulfoxide (DMSO) in artificial pond water; CBD = Cannabidiol. Figure obtained from Williams *et al*. (2025).

1.6. Research aims

CBD prevalence is increasing globally through the dominant use of cannabis and increasing interest in cannabis-derived treatments, including the synthetic cannabinoids abn-CBD and O-1918. This is resulting in environmental contamination of CBD, and though it has yet to be identified, possibly its metabolites including 7-OH-CBD. The effects of these compounds on the environment have not yet been fully elucidated. Given the prevalent use of aquatic invertebrates in ecotoxicological research, the detection of CBD in sewage sludge (Mastroianni *et al*., 2013), reports of the environmental disposal of raw sewage (*Raw Sewage in Our Rivers*, 2025), and the probable accumulation of CBD in sediments due to its lipophilic nature, *L. variegatus* emerges as an appropriate model invertebrate species suitable for investigating the ecological impact of CBD exposure in the environment. 7-OH-CBD has been detected in urine following CBD administration, and due to its pharmacological activity, may represent a potential environmental contaminant of concern in aquatic ecosystems, thereby warranting investigation in the present study (Pérez-Acevedo *et al*., 2020). Additionally, Abn-CBD and O-1918 were selected as a complementary pair as Abn-CBD is a structural isomer of CBD, while O-1918 is an analog of CBD that functions as an antagonist at putative Abn-CBD receptors. In this study, the Log K_{ow}, toxicity and behavioural effects of CBD, 7-OH-CBD, abn-CBD and O-1918 were measured at environmentally relevant concentrations, with further investigation into the effects of CBD and 7-OH-CBD on the physiological and biochemical properties of *L. variegatus*.

2. Materials and Methods

2.1. Safety

All waste products were disposed of via hazardous waste routes in accordance with product SDS provided by the manufacture and in line with Swansea University policies. Product SDS were used to complete internal Control of Substances Hazardous to Health (COSHH) forms which were used when designing experimental methodology for correct laboratory conduct.

2.2. Reagents and solutions

Table 2.1: Inventory of reagents and solutions. The products listed here are those used in the experiments performed within this thesis, they include the supplier and product code information with the temperature at which they were stored in the laboratory.

Reagent	Supplier	Product Code	Storage temperature (°C)
7 hydroxy cannabidiol (7-OH-CBD)	Sigma-Aldrich	C-180	-20
Abnormal CBD (Abn-CBD)	Tocris	1297	-20
Bovine serum albumin (BSA)	ThermoFisher Scientific	B14	2-4
Bradford reagent	ThermoFisher Scientific	1856209	2-4
Calcium nitrate tetrahydrate	Duchefa Biochemie	C0505	Room temperature
Cannabidiol (CBD)	Tocris	1570	-20
Chloroform	Thermo Scientific	158210010	Room temperature
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D4540	Room temperature
Distilled water	Mill-RO		Room temperature
Ethanol	Fischer Chemical	E/0650DF/17	Room temperature
Glucose	Sigma-Aldrich	1003601722	Room temperature
Glyceryl trioleate (triolein)	Sigma-Aldrich	T7140	-20
HEPES buffer	Melford Laboratories	H75030	Room temperature
Magnesium sulfate heptahydrate	Duchefa Biochemie	M0513	Room temperature
Methanol	Fisher Chemical	M/4056/17	Room temperature
Methyl acetate	Sigma-Aldrich	296996	Room temperature
O-1918	Tocris	2288	-20
Potassium chloride	Melford Laboratories	P41000	Room temperature
Phenol	Sigma-Aldrich	102559056	-4
Phosphoric acid	Sigma-Aldrich	49685	Room temperature
Sodium chloride	Melford Laboratories	S23020	Room temperature
Sulphuric acid	Sigma-Aldrich	1.08131	Room temperature
Tris hydrochloride	Melford Laboratories	T60040	Room temperature
Vanillin	Sigma-Aldrich	V1104	Room temperature

2.3. Storage and preparation of drugs and solutions

Artificial pond water

Artificial pond water (APW) was used as the culture medium for *L. variegatus* as described by O'Gara *et al*. (2004) it was produced as a 1 L x100 concentration and diluted to 1 L x1 as required with ddH_20 to give a composition of: 1 mM sodium chloride; 4 µM calcium nitrate tetrahydrate; 13 µM potassium chloride; 17 µM magnesium sulfate heptahydrate; 71 µM HEPES buffer. APW solutions were stored at room temperature.

Spirulina

A 100 ml solution was prepared by dissolving 1000 mg of spirulina in 100 ml of APW and stored at 2-4°C before use.

Ethanol (70%)

Ethanol (99%) was diluted down to 70% with ddH_20.

CBD

CBD was supplied in powder form at 10 mg and was dissolved in 100% DMSO to generate a master stock solution of 5 mM which was aliquoted and stored at -20°C. The master stock of CBD was then diluted in APW and DMSO to give a final concentration of 0.5% DMSO and 25.0 µM CBD, as required.

7-OH-CBD

7-OH-CBD was supplied pre-dissolved in methanol as a 1 mg/mL solution resulting in a 3 mM master stock which was stored at -20°C. The master stock of 7-OH-CBD was then diluted as required for experimentation in APW and methanol to give a final concentration of 0.5% methanol and 15.0 µM 7-OH-CBD.

Abn-CBD

Abn-CBD was supplied pre-dissolved in methyl acetate as a 10 mg product to give a 15.9 mM solution. A 15 mM master stock was produced using methyl acetate and the solution was stored at -20°C. The master stock was then diluted in methyl acetate and APW during experiments to give a final concentration of 0.1% methyl acetate and 15 µM abn-CBD.

O-1918

O-1918 was supplied in powder form as 10 mg. A master stock of 100 mM was created by dissolving O-1918 in DMSO. Stocks of 10 mM were then produced with DMSO and stored at -20°C. When required, the 10 mM stock would be diluted during experimentation to give a final concentration of 0.5% DMSO and 50.0 µM O-1918.

2.4. *Lumbriculus variegatus* culture

L. variegatus is not subject to ethical approval due to not being covered by the Animal (Scientific Procedures) Act 1986 and the species is not currently covered by UK conservation designations. A culture of *L. variegatus* had been obtained from ALFA Fish Food and reared for use in the laboratory. Before any experiment was conducted, the cultures were maintained for a minimum of 3 months to ensure each individual had completed at least one asexual division. The culture was housed in aquaria containing aerated artificial pond water, aquarium substrate and a time-sensitive light as previously described by O'Gara *et al.* (2004) and Seeley *et al.* (2021). Aeration (via air stones) and filtration was continuous, and the aquaria were kept at room temperature and subject to a 16:8 light-dark cycle. Maintenance of the *L. variegatus* aquaria would be carried out weekly and included a change in the artificial pond water, cleaning the filtration system, disturbance to the algae accumulated on the glass and feeding of the organisms with TeraMin flakes and 10 mg/L spirulina. Individuals were randomly selected and were screened to ensure there were no apparent morphological anomalies present, this is to say they were not observed to have recently undergone fragmentation. Individuals were removed from the aquaria 18-24 before any experiment and placed into the appropriate experimental container with artificial pond water.

2.5. *Lumbriculus variegatus* disposal

Following assay endpoints, samples were either snap frozen in liquid nitrogen for future experiments investigating chronic exposure to the tested cannabinoid or sampled organisms were euthanised with 70% ethanol exposure (Seeley *et al.,* 2021). Samples were then aspirated and disposed of via appropriate hazardous waste routes.

2.6. Log K_{ow}

To determine estimates of the Log K_{ow}, EPI Suit^{TM} v4.1 (US EPA) was used to infer physiochemical properties and its potential to produce contamination in aquatic environments. The chemical structure of each compound was inserted using the KOWWIN^{TM} evaluation model to calculate the log K_{ow} estimation as an output. The program uses an atom/fragment contribution method to assess the physiochemical properties of the compound as a whole.

2.7. *In vivo* toxicity assay

To determine the no observable adverse effect level (NOAEL), an *in vivo* toxicity assay was performed with each of the cannabinoid compounds to determine the concentration which resulted in toxicity of 50% of the test population (Figure 2.1). *L. variegatus* were transferred to a CELLSTAR 24-well plate, one per well with 1 ml APW, from the aquaria 18-24 hours before exposure. The APW was then removed and replaced with one of the following compounds: CBD (0-25 µM), 7-OH-CBD (0-15 µM), abn-CBD (0-15 µM) or O-1918 (0-50 µM), or with their vehicle controls (represented by 0 µM concentrations) as 0.5% DMSO in APW, 0.5% methanol in APW, 0.1% methyl acetate in APW and 0.5% DMSO in APW, respectively, and subjected to 24 hour exposure. The worms were then counted for those that displayed signs of toxicity. Toxicity was described as individuals displaying pallor or decomposition (partial or full) and each assay was conducted with six experimental repeats in triplicate.

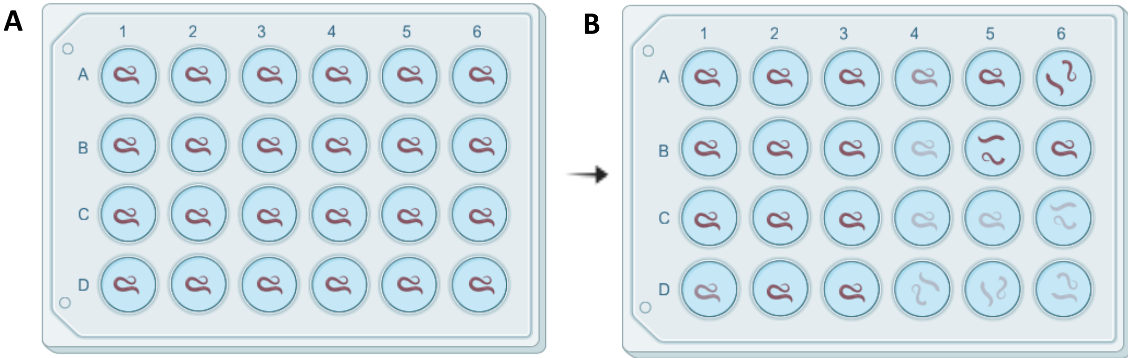


Figure 2.1: ***In vivo* toxicity assay procedure for *Lumbriculus variegatus*.** (A) Worms would be left to acclimate for 24 hours with 1 worm per well in triplicate. (B) Increasing concentrations of the tested compounds would be administered and left for 24 hours. Worms would then be counted for signs of toxicity, described here as those exhibiting pallor or decomposition (partial or full). Created with BioRender.com.

2.8. Behaviour

2.8.1. Stereotypical movement

To determine the effect in *L. variegatus* response to tactile stimulation, stereotypical movement assays (Figure 2.2) were conducted as described by Drewes (1999). *L. variegatus* were transferred from the aquaria 18-24 hours before exposure to a CELLSTAR 6-well plate with 4 ml of APW for acclimation. The APW was then replaced and baseline measurements were recorded by tactile stimulation alternatively on the anterior and posterior of *L. variegatus* using a 20 µl plastic pipette tip with stimulation occurring five times per end. At least five seconds were left between each stimulation event. Stimulation of the anterior area induced a body-reversal movement while stimulation of the posterior induced a helical swimming response. The responses were then scored as: 1 (no stereotypical movement), 2 (partial stereotypical movement) or 3 (full stereotypical movement). The APW was then removed and replaced with 4 ml of one of the following compounds: CBD (0-5 µM), 7-OH-CBD (0-5 µM), abn-CBD (0-5 µM) or O-1918 (0-5 µM) or with their vehicle controls (represented by 0 µM concentrations) as 0.5% DMSO in APW, 0.5% methanol in APW, 0.1% methyl acetate in APW and 0.5% DMSO in APW, respectively, for 24 hours. Tactile stimulation was again carried out using the same procedure and recorded. The worms were then "rescued" with removal of the cannabinoid or vehicle solution, washing of the well with APW

and then replenishing the well with 4 ml of APW. The worms were then again subjected to tactile stimulation after 10 mins (Rescue 10 mins) and 24 hours (Rescue 24 h).

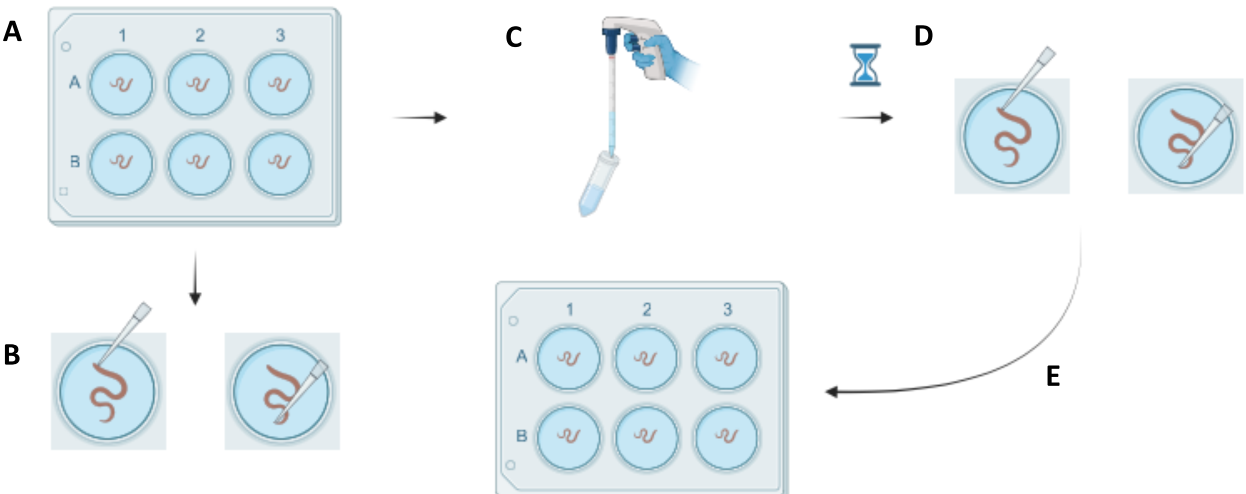


Figure 2.2: Stereotypical movement procedure for Lumbriculus variegatus. (A) *L. variegatus* would be left to acclimate for 18-24 hours and then individuals would be subjected to (B) tactile stimulation using a pipette tip to gently tap the posterior and anterior sections to invoke a behavioural response, recorded as a baseline. Stimulating the anterior and posterior results in the body reversal or helical swimming movements, respectively. These movements are then scored 1 (no movement), 2 (partial movement) and 3 (full movement). (C) *L. variegatus* are then exposed to the tested compounds for 10 minutes (acute exposure) or 24 hours (chronic exposure). (D) Stereotypical movement was then assessed and recorded. (E) Rescued period where the wells were washed of the tested solutions and replenished with APW to enter the recovery period where stereotypical movements were assessed after 10 minutes recovery (Rescue 10 mins) and 24 hours (Rescue 24 h). The data was then normalised to baseline and averaged. Created with BioRender.com

2.8.2. Free locomotion

To determine if the CBD, 7-OH-CBD, abn-CBD and O-1918 affect the unstimulated, free movement of *L. variegatus* resulting in hyper- or hypo-kinesis, free locomotion assays (Figure 2.3) were conducted following previously described methodology by Seeley *et al*. (2021). 18-24 hours before conducting the assay, *L. variegatus* were transferred from the aquaria to a CELLSTAR 6-well plate and left to acclimate in 2 ml of APW. After acclimation, the wells were washed and then replaced with 2 ml of APW. Baseline locomotion measurements were then recorded by placing the plate on a lightbox with a ruler-marked edge and a 13-megapixel camera fixed above. A series of pictures were then taken every second for 50 seconds. These photos inputted to Image J for the area of movement to be analysed. 2 mL was used to reduce movement in the z-axis. The APW was then removed and replaced with 2 ml of one of the following cannabinoid compounds, CBD (0-5 µM), 7-OH-CBD (0-5 µM), abn-CBD (0-5 µM) or

O-1918 (0-5 µM), or with their vehicle controls (represented by 0 µM concentrations) as 0.5% DMSO in APW, 0.5% methanol in APW, 0.1% methyl acetate in APW and 0.5% DMSO in APW, respectively, and exposed for 24 hours. Using the above procedure, measurements were again recorded. The worms were then "rescued" with removal of the cannabinoid or vehicle solution, washing of the well with APW and then replenishing the well with 2 ml of APW. The worms were again measured using the above procedure after 10 mins (Rescue 10 mins) and 24 hours (Rescue 24 h).

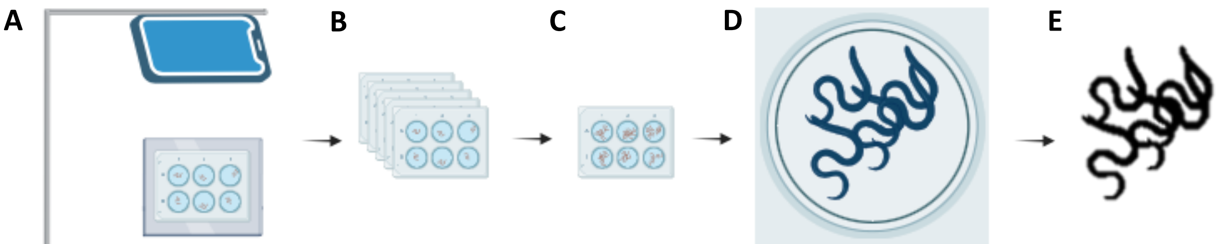


Figure 2.3: Free locomotion procedure for *Lumbriculus variegatus*. (A) *L. variegatus* were prepared on a light box with a 13-megapixel camera. (B) Sequential images were taken every 1 second for 50 seconds. (C) Images were then used to form a z-stack of superimposed images to create a single image showing movement. (D) Each well was then isolated for individual analysis. (E) Images were then manipulated using a colour threshold function to isolate pixels activated by *L. variegatus* to calculate movement as area covered. Picture manipulation conducted using ImageJ. Data is obtained at baseline (pre-exposure) and then 24 hours post-exposure, then rescue periods where the wells are washed with artificial pond water to remove the tested solution and then measured at Rescue (10 mins) and Rescue (24 h). Created with BioRender.com.

2.9. Regeneration

The initial experimental design included CBD, 7-OH-CBD, abn-CBD and O-1918. Following the completion of the behavioural experiments, further testing of abn-CBD and O-1918 was discontinued. Preliminary observations indicated variable and inconsistent response to abn-CBD between replicates which limited confidence in the interpretation of treatment effects. As O-1918 was included as a pharmacological counterpart to abn-CBD, both compounds were excluded from subsequent experiments.

Methodology previously described by Tweeten & Anderson (2008) was modified to determine the regenerative capacity of *L. variegatus* with exposure to CBD or 7-OH-CBD. *L. variegatus* were transferred from the aquaria to CELLSTAR 6-well plate and left to acclimate for 18-24 hours in APW. *L. variegatus* were then bisected at the midpoint using dissecting

scissors with the posterior and anterior ends separated into individual wells to observe head and tail growth, respectively. The bisections were then continuously exposed to CBD (0-5 µM) or 7-OH-CBD (0-5 µM), or with their vehicle controls (represented by 0 µM concentrations) as 0.5% DMSO in APW or 0.5% methanol in APW, respectively. With use of the stereomicroscope Nikon SMZ1270i, the amputation site of each fragment was imaged to determine the area of the blastema ($µm^2$) using the imaging software NIS-Elements. Imaging occurred at 0-, 24-, 48- and 72 hours post-amputation (hpa). This procedure is visualised in Figure (2.4). Blastema measurements were then expressed as a fold change ratio for statistical analysis where the data was subject to a mixed-effects analysis with Dunnet's test. There were six experimental repeats conducted in triplicate.

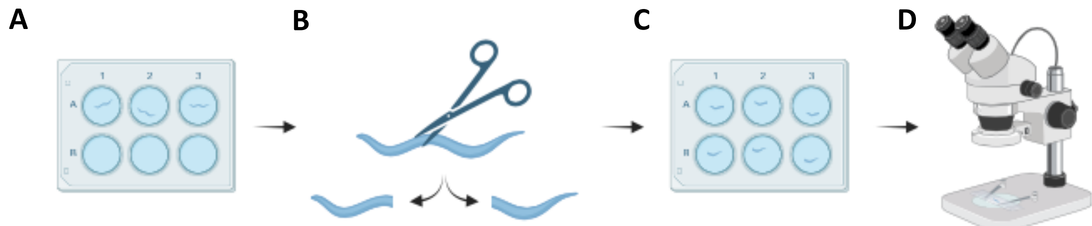


Figure 2.4: Regeneration procedure for *Lumbriculus variegatus*. (A) One six-well plate was used with 3 worms left to acclimatise for 24 hours. (B) Worms were then amputated at the midpoint and (C) separated by anterior sections on the top and posterior sections on the bottom of the plate. (D) Amputation sites were then analysed for their baseline surface area using a stereomicroscope. Worms were then exposed to the tested cannabinoid and left for 24 hours to then have the blastema's surface area calculated every 24 hours for 72 hours. Created with BioRender.com.

2.10. Respiration

The respiration of *L. variegatus* was investigated using adapted methodology described by Tuazon *et al*. (2022). The change in dissolved oxygen (DO_2) of the water column was used as a measure for respiration of *L. variegatus* to determine if exposure to CBD or 7-OH-CBD had physiological repercussions. 10 *L. variegatus* were transferred from the aquaria to a Sterilin™ 30 mL sample tube per tested concentration and left to acclimate for 18-24 hours in 10 ml of APW. A negative control (10 ml APW only) and a positive control (10 worms with 10 ml APW only) was also set up. DO_2 was measured using the Jenway 9500 oxygen meter and calibrated

following manufacturer's instructions before each experiment. After acclimation, the APW was then removed and replaced with 10 ml of CBD (0-5 µM) or 7-OH-CBD (0-5 µM), or with their vehicle controls (represented by 0 µM concentrations) as 0.5% DMSO in APW or 0.5% methanol in APW, respectively, or APW for the negative and positive controls. DO_2 was measured after replenishment at 0 hours, with further measurements being recorded at 1, 24, 48 and 72 hours post exposure. The oxygen meter probe was submerged to the same depth above the worm blob for each recording and timed for 2 minutes for the meter to acclimate before taking a recording. The probe was rinsed in ddH_2O between each recording. Protocol is visualised in Figure 2.5. There were three experimental repeats.

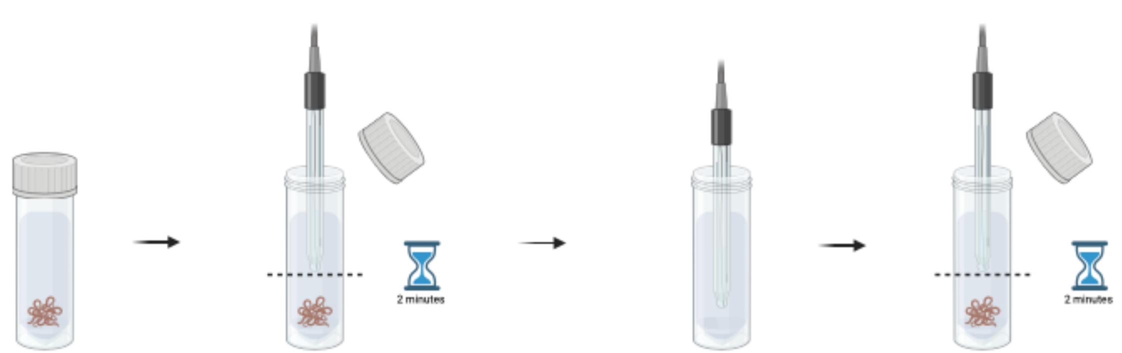


Figure 2.5: Respiration protocol for *Lumbriculus variegatus*. Worms were collected and left to acclimate for 24 hours. Baseline dissolved oxygen (DO_2) was recorded for each condition, leaving the probe to acclimate for 2 minutes and then rinsing the probe in ddH_2O between samples. The probe was inserted to the same depth ensuring not to penetrate the worm blob. Created with BioRender.com.

2.11. Pulse rate

Methodology described by Crisp *et al*. (2010) was adapted to determine the effect of cannabinoid exposure on the pulse rate of *L. variegatus* (Figure 2.6). *L. variegatus* were removed from the aquaria and left to acclimate in a CELLSTAR 12-well plate, one per well, for 18-24 hours in 3 ml of APW. Washing of the wells to remove aquaria debris was conducted prior to acclimation. Baseline pulse rate recorded were then conducted using the Nikon SMZ1270i7 stereomicroscope by mounting individual worms on a SuperFrost™ microscope slide, absorbing residual solution with an absorbent tissue and placing a glass coverslip on top to further aid in retarding worm locomotion. Using the NIS software, a MP4 video recording

of the midpoint of the worm was obtained of 30-60s for later analysis. The APW from the wells were then removed and *L. variegatus* were then subjected to exposure of 3 ml CBD (0, 1-5 µM) or 7-OH-CBD (0, 1-5 µM), or with their vehicle controls (represented by 0 µM concentrations) as 0.5% DMSO in APW or 0.5% methanol in APW, respectively, and left for 24 hours. Exposure recordings were then obtained using the above procedure. The videos were then analysed using QuickTime software to record the time for two contractions of the DBV at the midpoint and extrapolating this to determine an estimated beats per minute per worm. There were 3 experimental repeats per tested concentration conducted in triplicate.

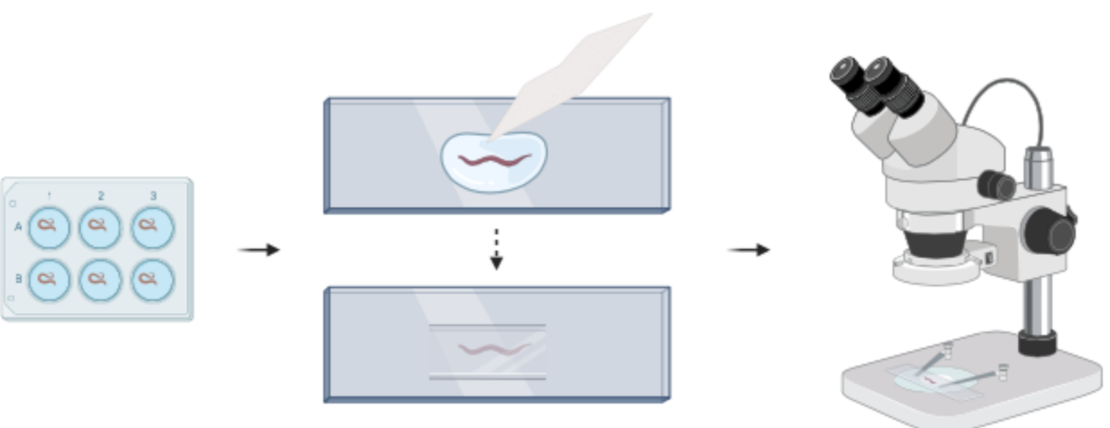


Figure 2.6: Pulse rate procedure for *Lumbriculus variegatus*. Individual worms would be transferred from their wells and dabbed carefully with tissue paper to absorb excess water to retard their movement with a coverslip gently placed over them. Handling time would be minimised to prevent ectopic pulsations with a low light setting used under the microscope. Created with BioRender.com.

2.12. Cholinesterase activity

To determine if cannabinoid exposure effected the cholinesterase activity of *L. variegatus*, methodology described by Komersová *et al*. (2007) was employed using a commercial acetylcholine quantification colorimetric assay (MAK056, Sigma=Aldrich, Dorset, UK). *L. variegatus* were transferred from the aquaria and acclimated in Sterilin™ 30 mL sample tubes containing 10 ml of APW for 24 hours before conducting the experiment with 20 worms used for each condition.

Free choline within *L. variegatus* homogenate was used to determine acetylcholine levels through a coupled enzyme reaction which produces a colorimetric result (570 nm). Samples of matched homogenate were treated with acetylcholinesterase, which when hydrolysed produces choline and acetate, with total choline levels being determined. Acetylcholine levels were determined by subtracting free choline levels from total choline levels with acetylcholine concentrations of *L. variegatus* determined according to manufacturer's instructions.

The cholinesterase activity of *L. variegatus* was then determined following Ellman's method (Komersová *et al*., 2007) using a commercial colorimetric assay (ab138871, Abcam, Cambridge, UK). This method used 5,5-dithio-bis-(2-nitobenzoic acid) (DTNB) for thiocholine quantification resulting from acetylthiocholine hydrolysis by cholinesterase. The absorbance intensity of DTNB (410 nm) was then used to quantify thiocholine production which is proportional to cholinesterase activity.

Once acclimated, the APW was removed and replaced with 10 ml CBD (5 µM), 7-OH-CBD (5 µM), or with their vehicle controls as 0.5% DMSO in APW or 0.5% methanol in APW, respectively, and left for 24 hours. *L. variegatus* from each condition were then transferred to 1.5 mL microcentrifuge tube Eppendorf tubes on ice with the APW removed. 100 µL of lysis buffer was added to each tube and with each sample then being homogenised using an Argos Tissue homogeniser. Samples were then transferred to a prechilled (4°C) centrifuge at 16.1 rcf and centrifuged for 15 minutes. In triplicate, 25 µL of homogenate was transferred to a Nunclon™ 96-well plate and quantification was conducted following manufacturer's instructions. Plates were mixed using a horizontal shaker at room temperature for 30 minutes or 10 minutes, respectively. Absorbances were read at 570 nm and 410 nm for acetylcholine quantification and cholinesterase activity quantification, respectively, using a FLUOstar Omega microplate reader (BMG Labtech). There were three experimental repeated conducted in triplicate.

2.13. Total energy

To determine if CBD or 7-OH-CBD affected the total energy available (E_a) on *L. variegatus*, previously described methodology by Silva *et al*. (2021) was adapted to quantify the levels of protein, carbohydrate and lipid. Per condition, 10 *L. variegatus* were transferred from the aquaria to Sterilin™ 30 mL sample tubes with 10 mL of APW and left to acclimate for 18-24 hours. After which, the APW was removed and replaced with CBD (0 µM, 1 µM, 5 µM), 7-OH-CBD (0 µM, 1 µM, 5 µM), or with their vehicle controls (represented by 0 µM concentrations) as 0.5% DMSO in APW or 0.5% methanol in APW, respectively, and left for 72 hours to acclimate. *L. variegatus* were then removed, dried with on filter paper and then weighed using a microbalance, and then homogenised using an Argos Tissue homogeniser in 100 µL ice-cold APW. A 30 µL aliquot was transferred to an Eppendorf 1.5 mL microcentrifuge tube and stored at -80°C in preparation for lipid quantification. The remaining homogenate was then centrifuged (prechilled) at 4°C, 16.1 rcf for 15 minutes. Two separate aliquots of 30 µM of the supernatant with two 30 µL aliquots of the supernatant transferred to 1.5 mL microcentrifuge Eppendorf tubes for protein and carbohydrate quantification. This process is visualised in Figure 2.7.

Protein quantification was conducted using the Bradford method (Bradford, 1976). 200 µL of Bradford reagent was administered to each well of a Nunclon™ 96-well plate with 1 µL of the protein aliquots were transferred in triplicate. This was left for 10 minutes under gentle agitation on a plate rocker at room temperature. Each well was then mixed using a pipette for gentle uptake and dispelling of the mixture. Each well was then transferred to a clean Nunclon™ 96-well plate using the reverse pipetting technique to ensure no bubbles are present. The absorbance was then read at 595 nm using the FLUOstar Omega Microplate Reader (BMG Labtech). BSA was used as a standard curve.

Carbohydrate quantification used methodology previously described by DuBois *et al*. (1956). A 5% phenol solution was produced using 0.25 g phenol in 5 mL ddH2O. 10 µL of the carbohydrate aliquot was combined in a 1.5 mL microcentrifuge Eppendorf tube with 100 µL of the phenol solution and 500 µL sulfuric acid (≥ 98%) and incubated for 10 minutes. The tubes were then vortexed for 30 seconds and placed in a heat block at 90 ^{o}C for 5 minutes. Samples were then removed and then left for a further 5 minutes at room temperature for

colour development. Samples were then transferred to a Nunclon™ 96-well plate, in triplicate, and the absorbance was measured at 492 nm using the FLUOstar Omega Microplate Reader (BMG Labtech). Glucose was used as a standard curve.

Lipid extraction utilised the Bligh & Dyer (1959) method whereby the 30 µL aliquot was transferred to a 2 mL GC glass vial and resuspended in 300 µL ddH_2O with 500 µL of chloroform and 500 µL methanol. After several inversions and being left to stand for 60 minutes at room temperature, the organic phase was then removed using a glass pipette to clean glass GC vials. The vials were then placed into a glass test tube and transferred to a SpeedyVac until complete dryness. Lipid quantification was conducted using adapted methodology described by (Men *et al.*, 2019). The samples were then acidified with 150 µL sulfuric acid (≥ 98%) and incubated at 90°C for 20 minutes. A phospho-vanillin reagent was produced in a ratio of 6:1:2 of 30 mg vanillin, 5 mL ddH_2O and 10 mL of 85% phosphoric acid, respectively. Vanillin was dissolved in ddH_2O in a 40°C heat bath with periodic vortexing and then the phosphoric acid was added. 450 µL of the phospho-vanillin reagent was then added to each sample, with the samples then left for 10 minutes at room temperature. Samples were then transferred to a Nunclon™ 96-well plate, in triplicate, and the absorbance was measured at 530 nm using the FLUOstar Omega Microplate Reader (BMG Labtech). Triolein was used as the standard curve.

Representative diagrams of the colorimetric analyses of proteins, lipids and carbohydrates can be viewed in Figure 3.8. Absorbances of the fractions of E_a (protein, carbohydrate, lipid) were then converted to energetic values using the corresponding energy of combustion obtained from De Coen & Janssen (1997) as follow: 24,000 mJ/mg protein, 17,500 mJ/mg glycogen, and 39,500 mJ/mg lipid. Energy data was expressed as mJ per mg of organism and E_a was then calculated using the following equation:

$$E_a = \Sigma (E_{protein} + E_{carbohydrate} + E_{lipid})$$

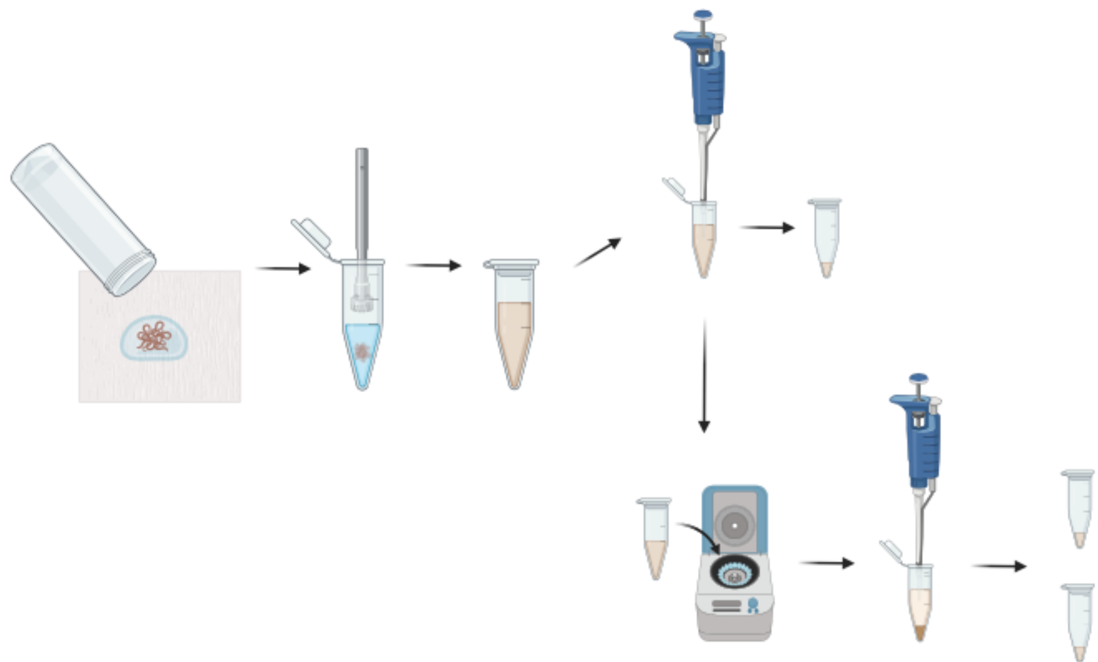


Figure 2.7: Methodology for the preparation of samples for energy analysis. After exposure, the worms are homogenised and part of the sample is removed for lipid analysis. The remainder is centrifuged with the supernatant then removed for separate protein and carbohydrate analysis. Created with BioRender.com.

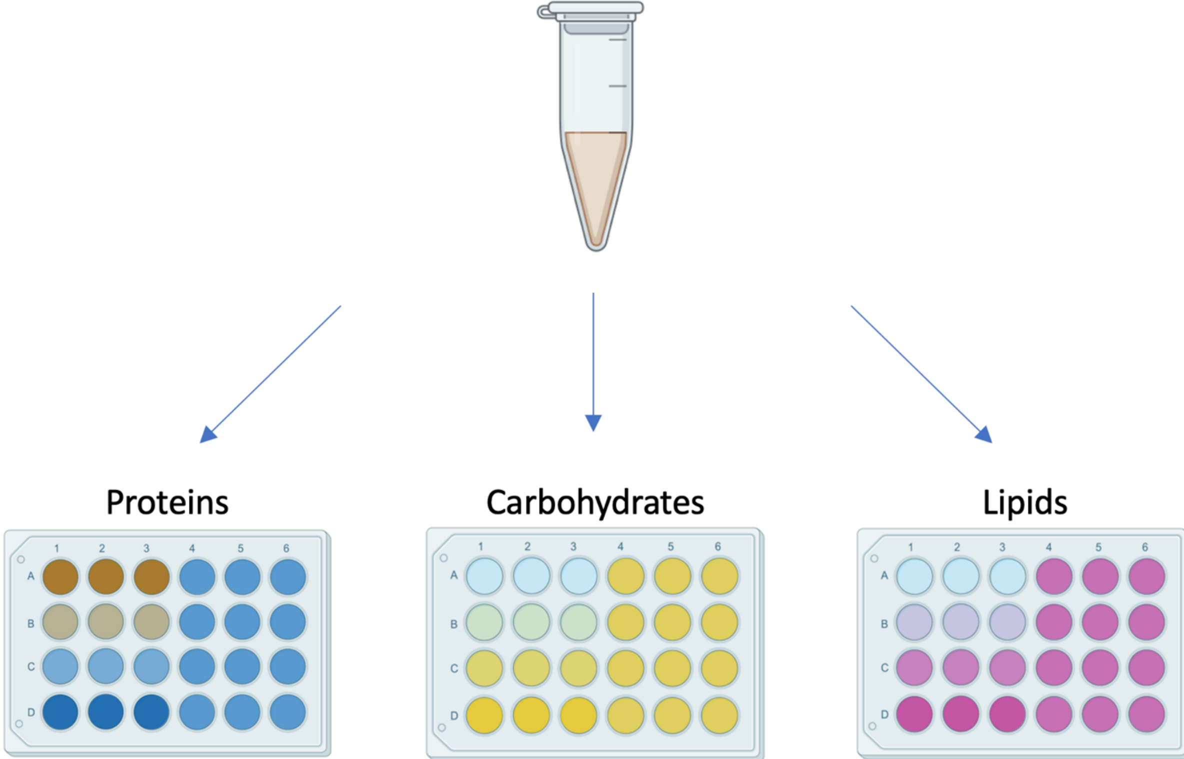


Figure 23.8: Representative colorimetric analyses of *Lumbriculus variegatus* sample for proteins, carbohydrates and lipids. Columns 1-3 represent standard curves and columns 4-6 represent unknown samples, visualised as triplicate measures. Protein analysis used Bradford reagent to produce a brown-blue colour change. Carbohydrate analysis used sulfuric acid followed by phenol to produce a colourless-yellow colour change. Lipid analysis used phosphovanillin reagent to produce a colourless to pink colour change. Images are not true depictions of plate arrangements. Created with Biorender.com.

2.14. Statistical analysis

All data analysis was performed using GraphPad Prism (Version 10.2.3). A non-linear regression was used to determine the toxicity (concentration at which 50% of the test population exhibited signs of toxicity) for the *in vivo* toxicity assays. Paired t-tests (Wilcox, two-tailed) were used for baseline and exposure, and two-way ANOVA for baseline and both rescue time points in the stereotypical movement. Paired t-tests (two-tailed) were used for baseline and exposure, and two-way ANOVA for baseline and both rescue time points in the free locomotion assays to determine significance. A mixed-effects analysis with Dunnet's test was used to compare against the baseline to determine significant effects for the regeneration assay. A mixed-effects analysis with Dunnet's test was used to compare against the baseline to determine significant effects for the CBD in the respiration assay, however, a two-way ANOVA with Dunnet's test was used 7-OH-CBD analysis. Paired t-tests (two-tailed) were used to determine significance in the pulse rate and cholinesterase activity assays. Analysis of the lipid, carbohydrate and protein energy quantification used paired t-tests (two-

tailed), except when comparing the vehicle and CBD 2.5 μM which required an unpaired t-test (two-tailed). The threshold for statistical significance was set at $p < 0.05$.

3. Results

3.1. Log K_{ow}

Using the KOWWIN^{TM} model in the EPI Suite^{TM} software, each investigated compound's molecular structure was digitally drawn to determine with the SMILES formula and the estimated Log K_{ow} values. The SMILES can be viewed in appendix A2. The calculated estimated Log K_{ow} values suggest that lipophilicity increases from O-1918, 7-OH-CBD, abn-CBD to CBD (Table 3.1).

Table 3.1: Estimates of the Log K_{ow} constant of the tested compounds as determined by EPI Suite^{TM} (v4.1).

Name	CBD	7-OH-CBD	Abn-CBD	O-1918
Log K_{ow} estimate	8.0198	7.5758	8.0131	7.1709

3.2. *In vivo* toxicity assay

Toxicity is determined here as the concentration at which 50% of the test population exhibits a toxic effect to exposure of an investigated compound. A toxic effect is described here as pallor or decomposition (partial or full). 50% toxicity was observed for CBD at 14.12 µM (95% CI: 12.28-15.90 µM), 7-OH-CBD at 11.29 µM (95% CI: 10.53-12.09 µM) and O-1918 at 15.84 µM (95% CI: 12.25-18.82 µM). However, the 50% toxicity concentration for abn-CBD was unable to be determined.

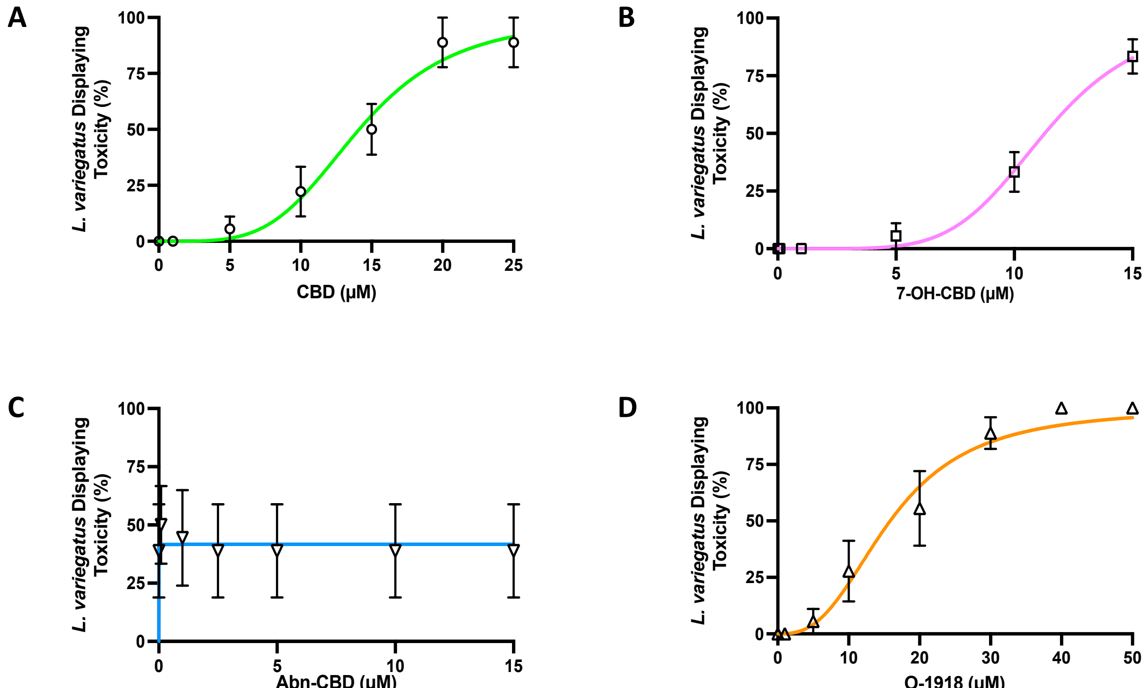


Figure 3.1: Toxicity of *Lumbriculus variegatus* with compound exposure. *L. variegatus* were subjected to increasing concentrations of **(A)** CBD (0 - 25 µM), **(B)** 7-OH-CBD (0 - 15 µM), **(C)** abn-CBD (0 – 15) and **(D)** O-1918 (0 - 50 µM) over 24 hours. Each worm was inspected for signs of toxicity (pallor or partial/full decomposition) and counted. Error bars represent the standard error of the mean, n=6 with three worms used per condition.

3.3. Behaviour

3.3.1. CBD

It was observed that chronic exposure (24 hours) to CBD (≤5 µM) was sufficient to elicit significant effects in the stereotypical movements of *L. variegatus* in response to tactile stimulation (Figure 3.2). *L. variegatus* displayed a reduced capacity for body reversal movements with exposure to ≥2.5 µM CBD ($p < 0.01$, Figure 3.2A, N = 8) and a reduced capacity for helical swimming at ≥0.5 µM CBD ($p < 0.05$, Figure 3.2B, N = 8). Removal of the CBD solution and incubation in APW was not sufficient to reverse these effects with body reversal behaviour inhibited at 0.5, 2.5 and 5.0 µM CBD ($p < 0.01$, Figure 3.2C, N = 8) and helical swimming inhibited at ≥2.5 µM CBD ($p < 0.0001$, Figure 3.2D, N = 8). After Rescue (24 h), 24 hours incubation in APW, body reversal remained inhibited at 5.0 µM CBD ($p < 0.0001$, Figure 3.2C, N = 8) and helical swimming inhibited at 1.0 µM CBD and continuing at 5.0 µM CBD ($p < 0.001$, Figure 3.2D, N = 8). Conversely, 24-hour exposure to 0.0 – 2.5 µM CBD did not inhibit free locomotion ($p > 0.05$, Figure 3.2E-F, N = 8) but hypokinetic effects were produced at 5 µM CBD ($p < 0.01$, Figure 3.2F, N = 8) with movement decreased to 45.12 ± 11.23%. Incubation in APW for 10 minutes was insufficient to reverse this with hypokinetic effects observed at 2.5 and 5.0 µM CBD ($p < 0.05$, Figure 3.2G, N = 8) with movements reduced to 74.20 ± 9.10% and 42.41 ± 6.17%, respectively. After 24-hours incubation in APW, hypokinetic effects continued at 5.0 µM CBD ($p < 0.01$, Figure 3.2G, N = 8) with movements reduced to 68.01 ± 11.05%, however, hyperkinetic effects were observed after 24 hours incubation in APW at 0.1 µM CBD ($p <.05$, Figure 3.2G, N = 8) with movements increasing to 103.20 ± 9.51%.

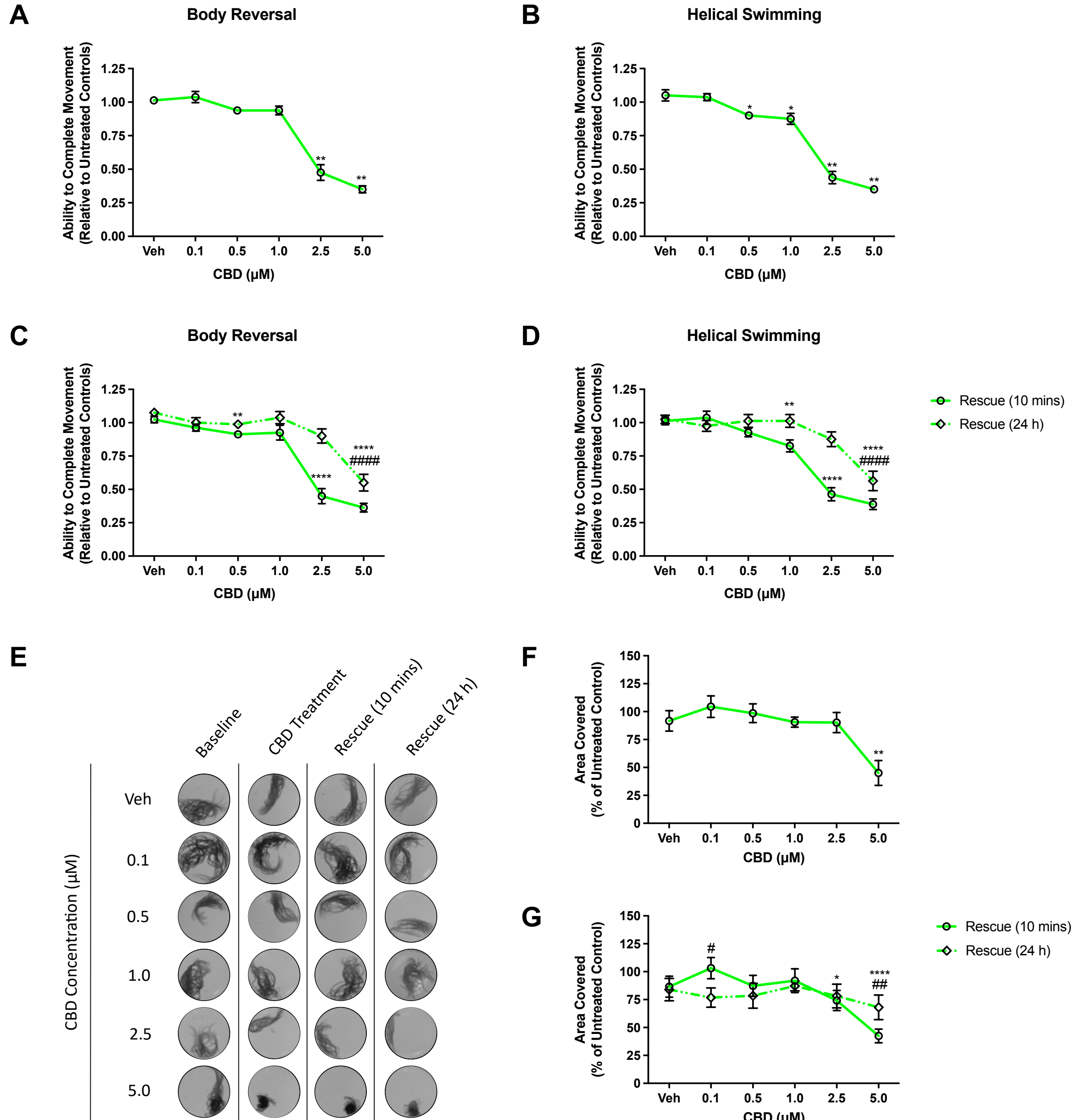


Figure 3.2: The effect of CBD exposure on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to CBD and a vehicle for 24 hours at concentrations 0.1 – 5.0 mM and their response to tactile stimulation of the anterior and posterior was recorded for **(A)** body reversal and **(B)** helical swimming respectively. CBD was then removed and the response of tactile stimulation was again measured at Rescue (10 mins) and Rescue (24 h) time points for **(C)** body reversal and **(D)** helical swimming. The response is scored (1) no movement, (2) partial movement) or (3) full movement with CBD Treatment, Rescue (10 mins) and Rescue (24 h) time points for body reversal and then expressed as a ratio relative to the baseline response. Unstimulated movement of *L. variegatus* was quantified to calculate the total area covered and expressed relative to the baseline movement as a percentage shown in **(E)** representative images. Unstimulated movement was measured before (Baseline) and after **(F)** CBD Treatment with **(G)** Rescue (10 mins) and Rescue (24 h) time points. Veh: 0.5% DMSO in artificial pondwater. */# $p < 0.05$, **/## $p < 0.01$, ****/#### $p < 0.0001$; where * refers to statistical significance between Baseline and CBD Treatment or Rescue (10 mins) and # refers to statistical significance between Baseline and Rescue (24 h). Error bars represent the standard error of the mean, n=8 for each condition.

3.3.2. 7-OH-CBD

With 24-hour exposure to 7-OH-CBD (0.0 – 5.0 μM) it was observed to be sufficient to elicit significant effects on the ability of *L. variegatus* to respond to tactile stimulation (Figure 3.3). Following exposure, there was a reduced capacity for *L. variegatus* to perform body reversal ($p < 0.05$, Figure 3.3A, $N = 8$) and helical swimming ($p < 0.01$, Figure 3.3B, $N = 8$) at 5.0 μM 7-OH-CBD exposure. Following the removal of 7-OH-CBD and incubation in APW, these effects were not reversed after 10 minutes with body reversal inhibited observed at 2.5 and 5.0 μM ($p < 0.01$, Figure 3.3C, $N = 8$) and helical swimming continuing to be inhibited at 5.0 μM ($p < 0.0001$, Figure 3.3D, $N = 8$). However, 24-hours incubation in APW was sufficient at reversing these effects for both body reversal and helical swimming ($p > 0.05$, Figure 3.3C-D, $N = 8$). Conversely, exposure to 1.0 μM and 5.0 μM 7-OH-CBD did inhibit the free locomotion of *L. variegatus* with hypokinetic effects observed, reducing movements to 72.94 ± 9.63% and 70.0 ± 12.62%, respectively ($p < 0.05$, Figure 3.3F, $N = 8$). After the removal of 7-OH-CBD and incubation in APW for 10 minutes, significant reduction in free locomotion was observed at 0.1 μM while also continuing at 1.0 μM and 5.0 μM ($p < 0.05$, Figure 3.3G, $N = 8$) with hypokinetic effects reducing movement to 64.58 ± 13.84%, 70.59 ± 11.53% and 69.10 ± 11.51% compared to baseline, respectively. After 24-hours incubation in APW, hypokinetic effects were only observed at 5.0 μM ($p < 0.05$, Figure 3.3G, $N = 8$) with movement reduced to 74.83 ± 9.49% compared to baseline.

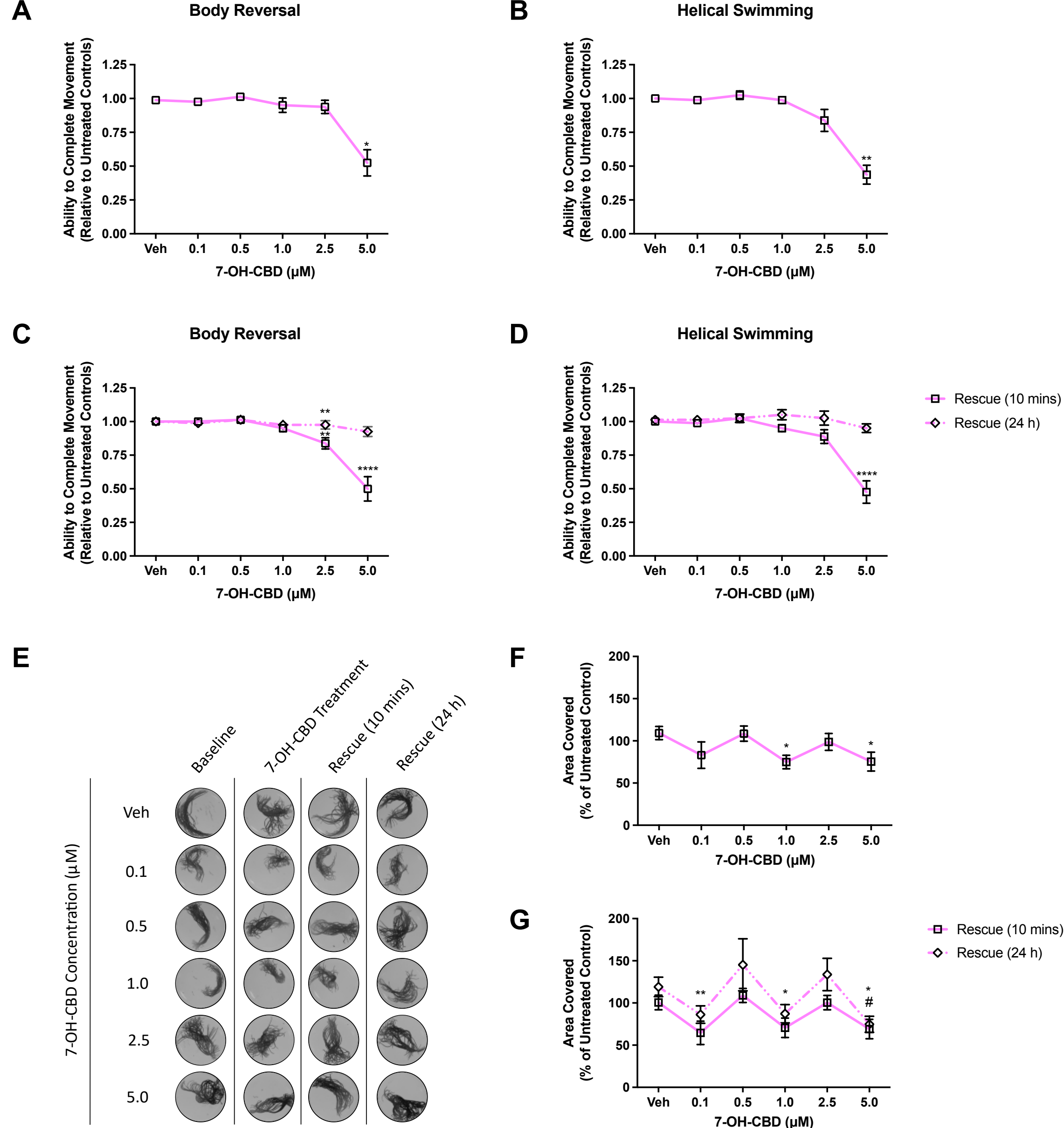


Figure 3.3: The effect of 7-OH-CBD exposure on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to 7-OH-CBD and a vehicle for 24 hours at concentrations 0.1 – 5.0 mM and their response to tactile stimulation of the anterior and posterior was recorded for **(A)** body reversal and **(B)** helical swimming respectively. 7-OH-CBD was then removed and the response of tactile stimulation was again measured at Rescue (10 mins) and Rescue (24 h) time points for **(C)** body reversal and **(D)** helical swimming. The response is scored (1) no movement, (2) partial movement) or (3) full movement with CBD Treatment, Rescue (10 mins) and Rescue (24 h) time points for body reversal and then expressed as a ratio relative to the baseline response. Unstimulated movement of *L. variegatus* was quantified to calculate the total area covered and expressed relative to the baseline movement as a percentage shown in **(E)** representative images. Unstimulated movement was measured before (Baseline) and after **(F)** 7-OH-CBD Treatment with **(G)** Rescue (10 mins) and Rescue (24 h) time points. Veh: 0.5% methanol in artificial pondwater. */# $p < 0.05$, ** $p<0.01$, **** $p < 0.0001$; where * refers to statistical significance between Baseline and 7-OH-CBD Treatment or Rescue (10 mins). Error bars represent the standard error of the mean, n=8 for each condition.

3.3.3. Abn-CBD

Exposing *L. variegatus* to abn-CBD for 24 hours was insufficient to elicit significant effects in the ability of *L. variegatus* to perform the stereotypical behaviours of body reversal and helical swimming ($p > 0.05$, Figure 3.4A-B, $N = 8$). However, incubation in APW for 10 minutes did yield significant effects in body reversal and helical swimming inhibition at 5 µM ($p < 0.01$, Figure 3.4C-D, $N = 8$) which continued to persist after 24 hours incubation in APW ($p < 0.05$, Figure 3.4C-D, $N = 8$). Conversely, exposure to 0.1 µM and 2.5 µM abn-CBD was sufficient to inhibit the free locomotion of *L. variegatus* ($p < 0.05$, Figure 3.4F, $N = 8$), with movement reduced to 82.71 ± 6.33% and 74.93 ± 7.19% when compared to baseline, respectively. Following incubation in APW for 10 minutes and 24 hours, it was observed that this was sufficient at reversing these effects with no significant difference observed in the free locomotion of *L. variegatus* observed ($p > 0.05$, Figure 3.4G, $N = 8$).

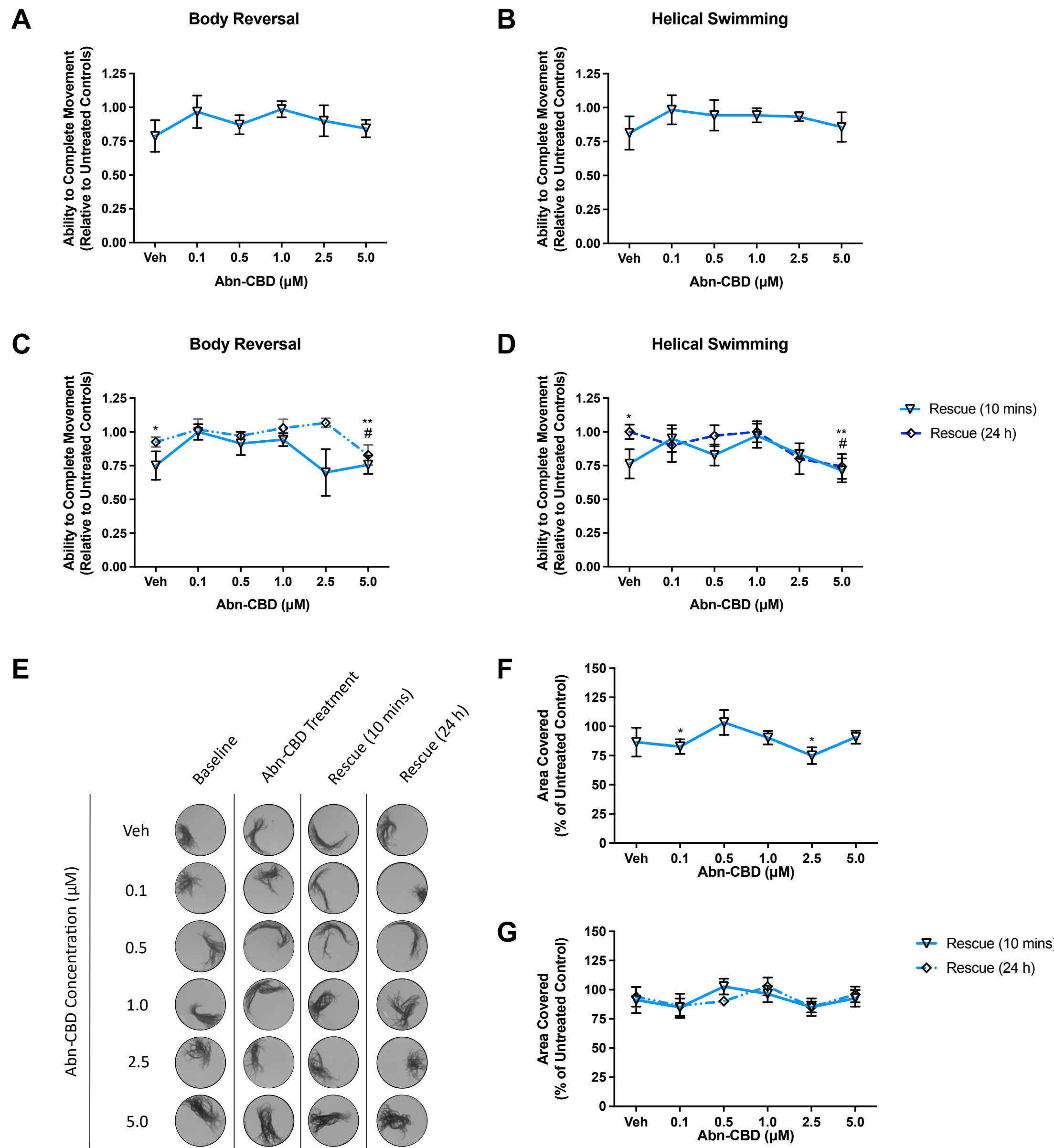


Figure 3.4: The effect of abn-CBD exposure on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to abn-CBD and a vehicle for 24 hours at concentrations 0.1 – 5.0 µM and their response to tactile stimulation of the anterior and posterior was recorded for **(A)** body reversal and **(B)** helical swimming respectively. Abn-CBD was then removed and the response of tactile stimulation was again measured at Rescue (10 mins) and Rescue (24 h) time points for **(C)** body reversal and **(D)** helical swimming. The response is scored (1) no movement, (2) partial movement) or (3) full movement with CBD Treatment, Rescue (10 mins) and Rescue (24 h) time points for body reversal and then expressed as a ratio relative to the baseline response. Unstimulated movement of *L. variegatus* was quantified to calculate the total area covered and expressed relative to the baseline movement as a percentage shown in **(E)** representative images. Unstimulated movement was measured before (Baseline) and after **(F)** abn-CBD Treatment with **(G)** Rescue (10 mins) and Rescue (24 h) time points. Veh: 0.1% methyl acetate in artificial pondwater. */# $p < 0.05$, ** $p < 0.01$; where * refers to statistical significance between Baseline and abn-CBD Treatment or Rescue (10 mins) and # refers to statistical significance between Baseline and Rescue (24 h). Error bars represent the standard error of the mean, n=8 for each condition.

3.3.4. O-1918

Exposing *L. variegatus* to O-1918 (0.0 – 5.0 µM) for 24 hours was sufficient to inhibit stereotypical movements in response to tactile stimulation (Figure 3.5). Significant inhibition of body reversal behaviour was observed at 5 µM O-1918 exposure ($p < 0.05$, Figure 3.5A, $N = 8$) and helical swimming at 2.5 µM exposure ($p < 0.05$, Figure 3.5B, $N = 8$). The removal of O-1918 and incubation of *L. variegatus* in APW for 10 minutes was insufficient at reversing these effects with significant inhibition observed at 2.5 µM exposure for body reversal ($p < 0.05$, Figure 3.5C, $N = 8$) and helical swimming inhibition ($p < 0.01$, Figure 3.5D, $N = 8$). After 24 hours of incubation in APW inhibition of stereotypical movements was observed at 5 µM for body reversal ($p < 0.01$, Figure 3.5C, $N = 8$) and helical swimming ($p < 0.001$, Figure 3.5D, $N = 8$). Conversely, 24-hour exposure to O-1918 did not inhibit free locomotion activity of *L. variegatus* ($p > 0.05$, Figure 3.5F, $N = 8$) with no significant inhibition observed following 10 minutes incubation in APW ($p > 0.05$, Figure 3.5F, $N = 8$). However, after 24 hours of incubation in APW, a significant inhibition in free locomotion was observed at 0.5 µM with hypokinetic effects reducing movement to 76.16 ± 5.11% when compared to the baseline ($p > 0.05$, Figure 3.5G, $N = 8$).

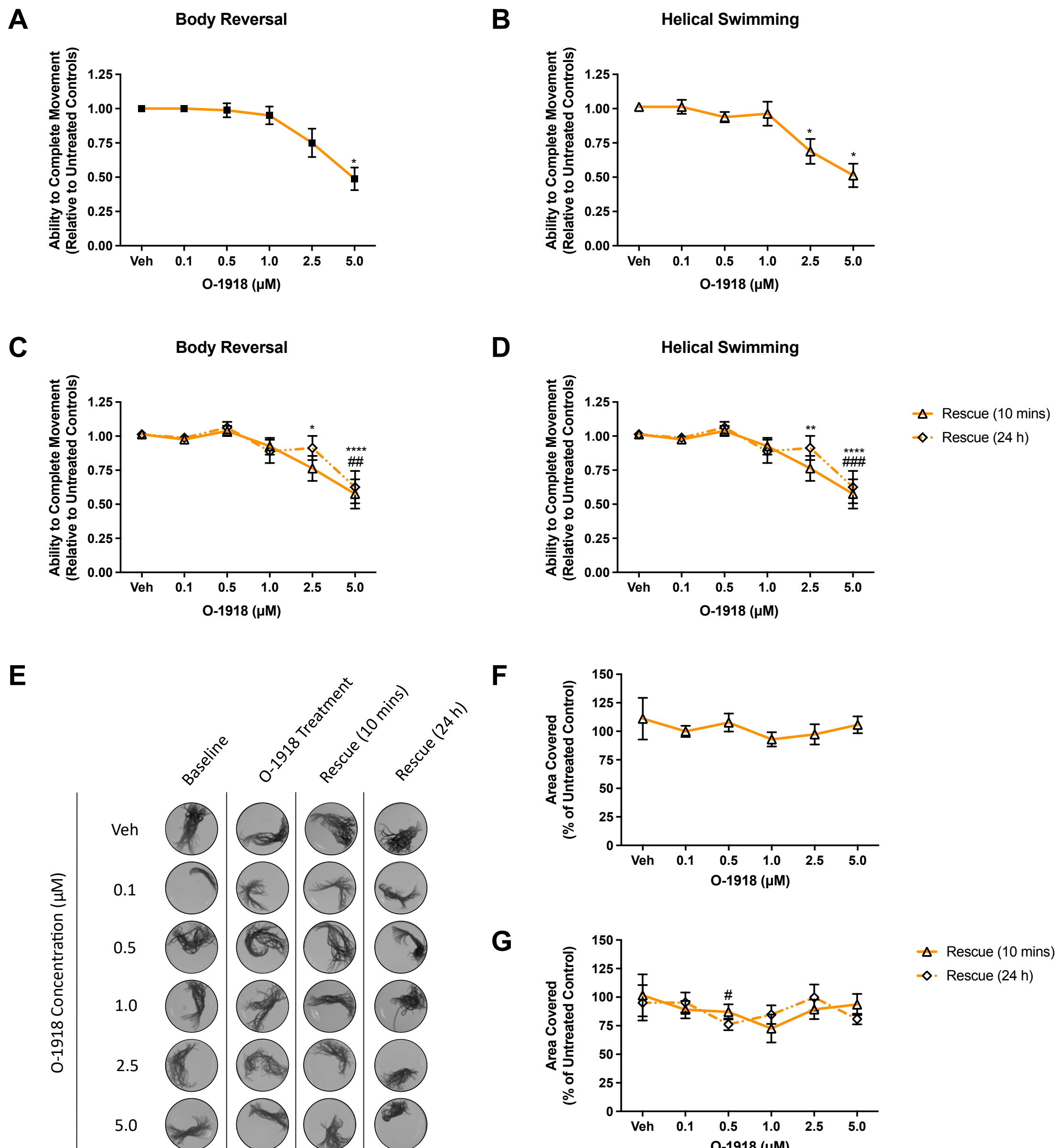


Figure 3.5: The effect of O-1918 exposure on *Lumbriculus variegatus* behaviour. *L. variegatus* were exposed to O-1918 and a vehicle for 24 hours at concentrations 0.1 – 5.0 µM and their response to tactile stimulation of the anterior and posterior was recorded for **(A)** body reversal and **(B)** helical swimming respectively. O-1918 was then removed and the response of tactile stimulation was again measured at Rescue (10 mins) and Rescue (24 h) time points for **(C)** body reversal and **(D)** helical swimming. The response is scored (1) no movement, (2) partial movement) or (3) full movement with O-1918 Treatment, Rescue (10 mins) and Rescue (24 h) time points for body reversal and then expressed as a ratio relative to the baseline response. Unstimulated movement of *L. variegatus* was quantified to calculate the total area covered and expressed relative to the baseline movement as a percentage shown in **(E)** representative images. Unstimulated movement was measured before (Baseline) and after **(F)** O-1918 Treatment with **(G)** Rescue (10 mins) and Rescue (24 h) time points. Veh: 0.5% DMSO in artificial pondwater. * $p < 0.05$, **/## $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; where * refers to statistical significance between Baseline and O-1918 Treatment or Rescue (10 mins) and # refers to statistical significance between Baseline and Rescue (24 h). Error bars represent the standard error of the mean, n=8 for each condition. The experiment was also conducted by Georgina Jomy, Megan Flanagan, Grace Hawkes and James McRobbie-Aston, data analysis conducted by Ben Williams.

3.4. Regeneration

The impact of CBD or 7-OH-CBD exposure to the regenerative capacity of *L. variegatus* was determined by measuring the blastema growth using a stereomicroscope (Figure 0.1, appendix A3) every 24 hours 72 hours post-amputation (hpa).

Exposure to CBD (0.0 – 5.0 µM) for 72 hours was sufficient to reduce the regenerative capacity of *L. variegatus* regarding head growth, but not tail growth, post-amputation (Figure 3.6A-B). Exposure to 0.0 – 1.0 µM CBD resulted in significant regeneration of the anterior (head growth) following 24, 48 and 72 hours hpa ($p < 0.01$, Figure 3.6A, $N = 6$), however, at 2.5 mM CBD exposure, significant regeneration was only observed at 72 hpa ($p < 0.05$, Figure 3.6A, $N = 6$) when compared to baseline measurements. Conversely, 0.0 – 5.0 µM CBD exposure was insufficient at reducing regeneration as significant posterior regeneration (tail growth) was observed following 72 hpa ($p < 0.05$, Figure 3.6B, $N = 6$).

Exposure to 7-OH-CBD (0.0 – 5.0 µM) for 72 hours was insufficient to reduce the regenerative capacity of *L. variegatus* regarding head growth and tail growth post-amputation (Figure 3.7A-B). Significant regeneration of the anterior (head growth) and posterior (tail growth) was observed for 0.0 – 5.0 µM 7-OH-CBD exposure following 72 hpa ($p < 0.01$, Figure 3.7A-B, $N = 6$).

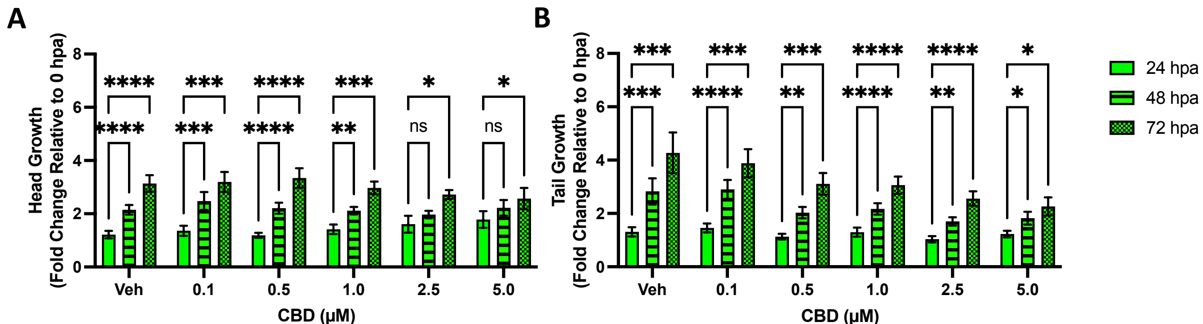


Figure 3.6: Regeneration of head and tail sections of *Lumbriculus variegatus* post CBD exposure. The amputation site was measured on the day of amputation and then blastema growth was measured for **(A)** head growth and **(B)** tail growth every 24 hours for 72 hours post-amputation (hpa) of *L. variegatus* post-exposure to CBD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; where * refers to statistical significance of blastema growth between 24 hpa and 48 hpa, or 24 hpa and 72 hpa. Error bars represent the standard error of the mean, n=6 conducted in triplicate.

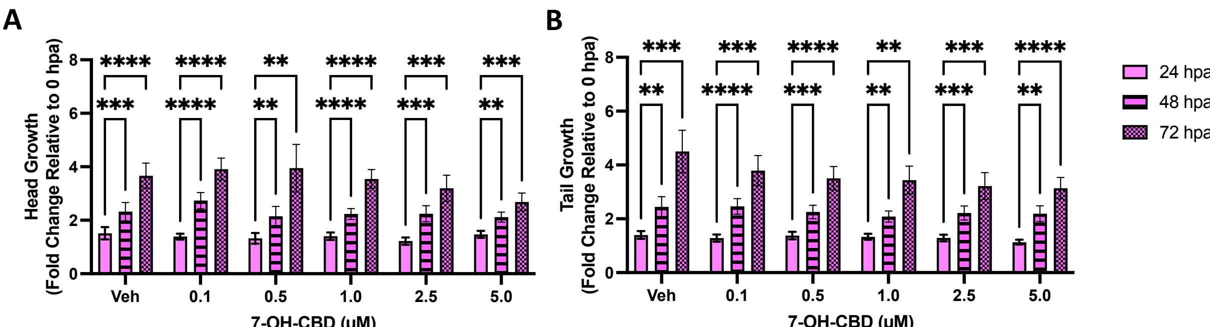


Figure 3.7: Regeneration of head and tail sections of *Lumbriculus variegatus* post 7-OH-CBD exposure. The amputation site was measured on the day of amputation and then blastema growth was measured for **(A)** head growth and **(B)** tail growth every 24 hours for 72 hours post-amputation (hpa) of *L. variegatus* post-exposure to 7-OH-CBD. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; where * refers to statistical significance of blastema growth between 24 hpa and 48 hpa, or 24 hpa and 72 hpa. Error bars represent the standard error of the mean, n=6 conducted in triplicate.

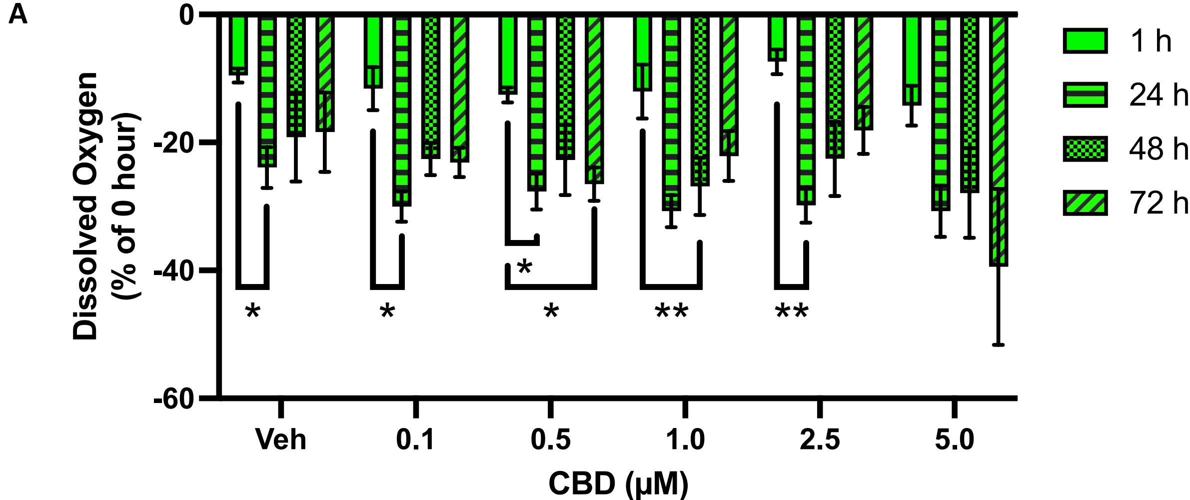
3.5. Respiration

To assess the effects of CBD (0.0 – 5.0 µM) or 7-OH-CBD (0.0 – 5.0 µM) exposure on the respiration activity of *L. variegatus,* the dissolved oxygen concentration (% of 0 hour) was measured at 1 h, 24 h, 48 h and 72 h intervals post-exposure (Figure 3.8A-B).

Regarding exposure to CBD, at 0.0 µM (Veh), there was a significant decrease in DO_2 between 1 h and 24 h (when compared to 0 h), where DO_2 decreased from -9.5 ± 1.1 % to 23.9 ± 3.2 % ($p < 0.05$, Figure 3.8A, $N = 3$). At 0.1 mM, there was a significant decrease in DO2 between 1 h and 24 h (when compared to 0 h), where DO_2 decreased from -11.6 ± 3.4 % to -30.0 ± 2.4 % ($p < 0.05$, Figure 3.8A, $N = 3$). At 0.5 µM , there was a significant decrease in DO_2 between 1 h and 24 h, and 1 h and 72 h (when compared to 0 h), where DO_2 decreased from -12.6 ± 1.2 % to -27.7 ± 2.8 % and -12.6 ± X % to -26.5 ± 2.6 %, respectively ($p < 0.05$, Figure 3.8A, $N = 3$). At 1.0 µM, there was a significant decrease in DO_2 between 1 h and 48 h (when compared to 0 h), where DO_2 decreased from -12.0 ± 4.2 % to -26.9 ± 4.5 % ($p < 0.01$, Figure 3.8A, $N = 3$). At 2.5 µM, there was a significant decrease in DO_2 between 1 h and 24 h (when compared to 0 h), where DO_2 decreased from -7.4 ± 1.9 % to -29.8 ± 2.7 % ($p < 0.01$, Figure 3.8A, $N = 3$).

Regarding exposure to 7-OH-CBD, at 0.5 µM, 1.0 µM and 5.0 µM there was a significant decrease in DO_2 between 1 h and 48 h (when compared to 0 h), where DO_2 decreased from -3.9 ± 2.0 % to 24.0 ± 10.1 % ($p < 0.05$, Figure 3.8B, $N = 3$), -5.6 ± 1.6 % to -29.7 ± 7.5 % ($p < 0.01$, Figure 3.8B, $N = 3$) and from -11 ± 1.0 % to -32.52 ± 9.6 % ($p < 0.01$, Figure 3.8B, $N = 3$), respectively.

A



B

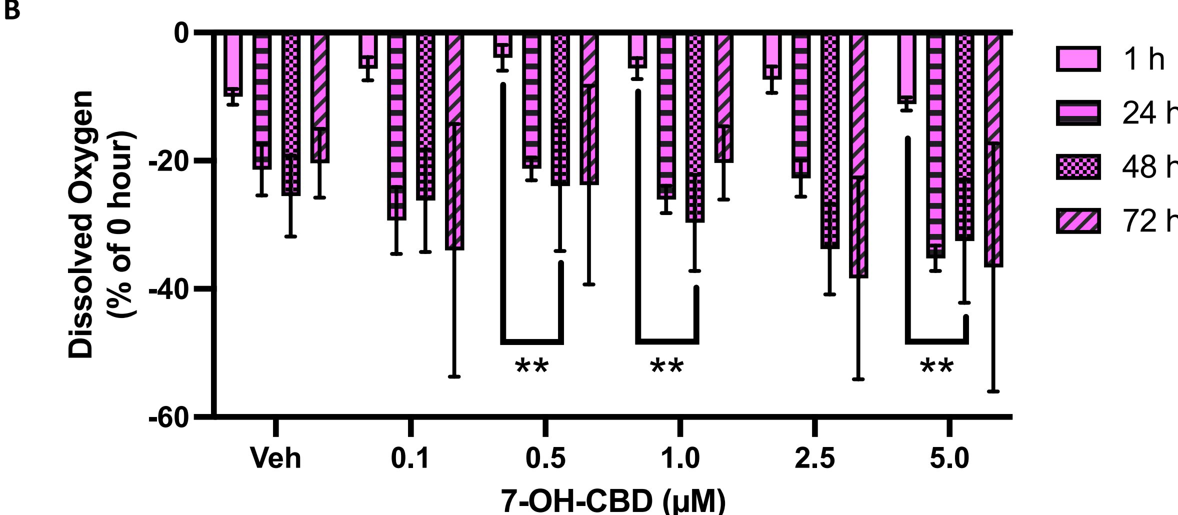


Figure 3.8. Dissolved oxygen percentage post-exposure of *Lumbriculus variegatus* to CBD and 7-OH-CBD. Dissolved oxygen was measured as a percentage change of 0 hour after exposure (72 h) to (A) CBD and (B) 7-OH-CBD. Veh composition is 0.5% DMSO and 0.5% methanol in artificial pondwater for CBD and 7-OH-CBD, respectively. * $p < 0.05$, ** $p < 0.01$ where * refers to statistical significance when compared to 0 h. Error bars represent the standard error of the mean, n=3 with 10 worms per condition.

3.6. Pulse rate

To assess the effects of CBD (0.0 – 5.0 µM) or 7-OH-CBD (0.0 – 5.0 µM) exposure on the pulse rate of *L. variegatus*, subjects had their baseline pulse rate (bpm) measured before being exposed for 24 hours with their pulse rates again measured. Contractions of the DBV can be viewed in Figure 0.2 in appendix A4. Exposure to CBD at 2.5 µM and 5.0 µM for 24 hours was sufficient to significantly reduce the pulse rate of *L. variegatus* by 7.2 ± 1.7 bpm ($p < 0.01$, Figure 3.9A, $N = 3$) and 8.0 ± 1.5 bpm ($p < 0.001$, Figure 3.9A, $N = 3$), respectively. There was no significant change in the pulse rate with 7-OH-CBD exposure after 24 hours ($p > 0.05$, Figure 3.9B, $N = 3$). However, there was a noticeable reduction in pulse rate by 4.4 ± 1.8 bpm at 2.5 µM.

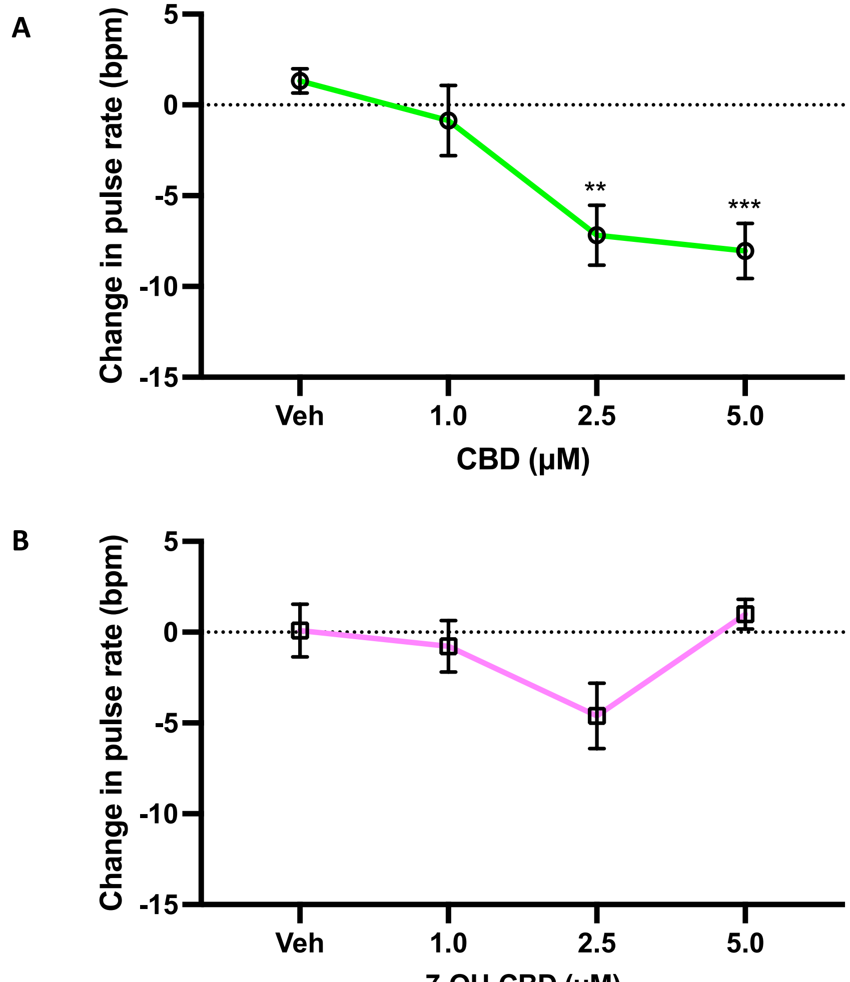


Figure 3.9. Change in pulse rate of the dorsal blood vessel of *Lumbriculus variegatus* with CBD and 7-OH-CBD exposure. Pulsations were observed at the midpoint of each organism under **(A)** CBD and **(B)** 7-OH-CBD exposure. Veh composition is 0.5% DMSO and 0.5% methanol in artificial pond water for CBD and 7-OH-CBD, respectively. **$p < 0.01$, *** $p < 0.001$. Average baseline pulse rate signified at 0 along the dotted line for CBD (12.11 bpm) and 7-OH-CBD (11.84). Error bars represent the standard error of the mean, n=3 in triplicate.

3.7. Cholinesterase activity

Determining the effects of cannabinoid exposure on the cholinesterase activity of *L. variegatus* revealed no significant difference in cholinesterase activity with exposure to CBD or 7-OH-CBD after 24 hours.

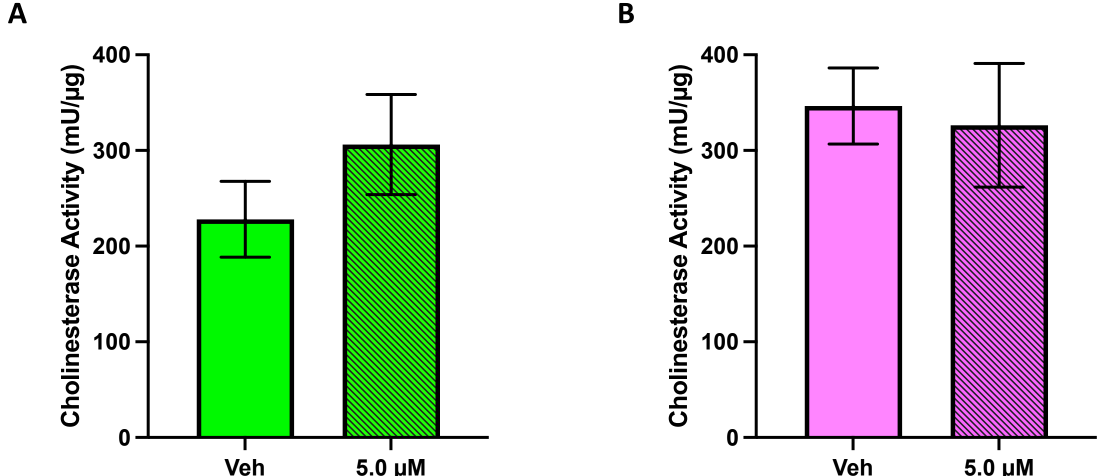


Figure 3.10: Change in cholinesterase activity with CBD and 7-OH-CBD exposure in *Lumbriculus variegatus*. No significance was observed post-exposure to **(A)** CBD and **(B)** 7-OH-CBD after 24 hours. Error bars represent the standard error of the mean, n=4 with 20 worms used per condition.

3.8. Total energy

Determining the effects on the total energy budget (Table 3.2) of *L. variegatus* revealed no statistical difference with both CBD and 7-OH-CBD after 72 hours. However, when reviewing the individual reserves, protein, carbohydrate and lipid reserves of *L. variegatus* with exposure to CBD (Figure 3.11) and 7-OH-CBD (Figure 3.12) it was revealed that CBD exposure after 72 hours was sufficient to induce significant effects. There was a significant decrease in carbohydrate reserves with CBD exposure to 372.38 ± 14.01 mJ / mg organism at 2.5 µM ($p < 0.05$, Figure 3.11B, $N = 4$), an approximate 21% decrease compared to the vehicle control. Conversely, there was a significant increase in the lipid reserves with CBD exposure to 265.53 ± 25.14 mJ / mg organism at 2.5 µM ($p < 0.05$, Figure 3.11C, $N = 4$), an approximate 42% increase compared to the vehicle control. Regarding the CBD results, $N = 4$ was due to the complete decomposition of organisms at 2.5 µM for two experiments. There was no significant effect with 7-OH-CBD exposure after 72 hours ($p > 0.05$, Figure 3.12, $N = 6$).

Table 3.3: Mean total energy budget of *Lumbriculus variegatus* with CBD or 7-OH-CBD exposure (72 h). Subjected concentrations of CBD and 7-OH-CBD were 0 µM (Veh), 1.0 µM and 2.5 µM under 72 h expsosure. E_c (the total available energy), is expressed as S $E_{protein} + E_{carbohydrate} + E_{lipid}$. There was no significant difference in E_c of *L. variegatus* after CBD or 7-OH-CBD exposure compared to their respective Veh controls. Values are reported as mean ± SEM.

Drug	Concentration (µM)	Mean E_c (mJ / mg organism)
CBD	Veh	1684 ± 49.35
	1.0	1704 ± 28.63
	2.5	1780 ± 102.50
7-OH-CBD	Veh	2161 ± 245.50
	1.0	1905 ± 89.58
	2.5	1722 ± 92.98

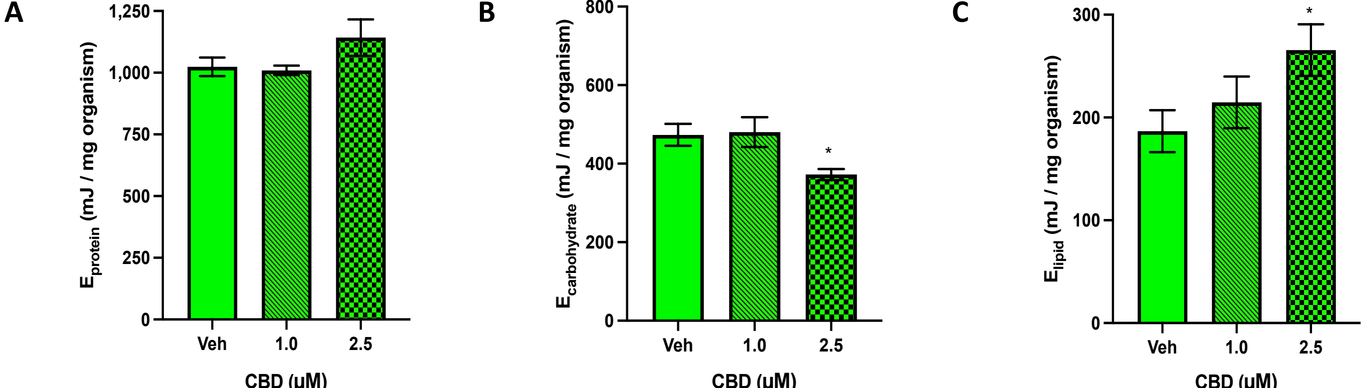


Figure 3.11: Energy quantification of *Lumbriculus variegatus* with CBD exposure. Subjected concentrations of CBD exposure (72 h) were 0 µM (Veh), 1.0 µM and 2.5 µM. Energy (E_c) expressed as mJ per mg of organism for **(A)** protein, **(B)** carbohydrate and **(C)** lipid. There was a significant decrease in $E_{carbohydrate}$ and a significant increase in E_{lipid} when compared to the Veh control at 2.5 µM CBD exposure ($p < 0.05$). Error bars represent the standard error of the mean, n = 4, conducted in triplicate.

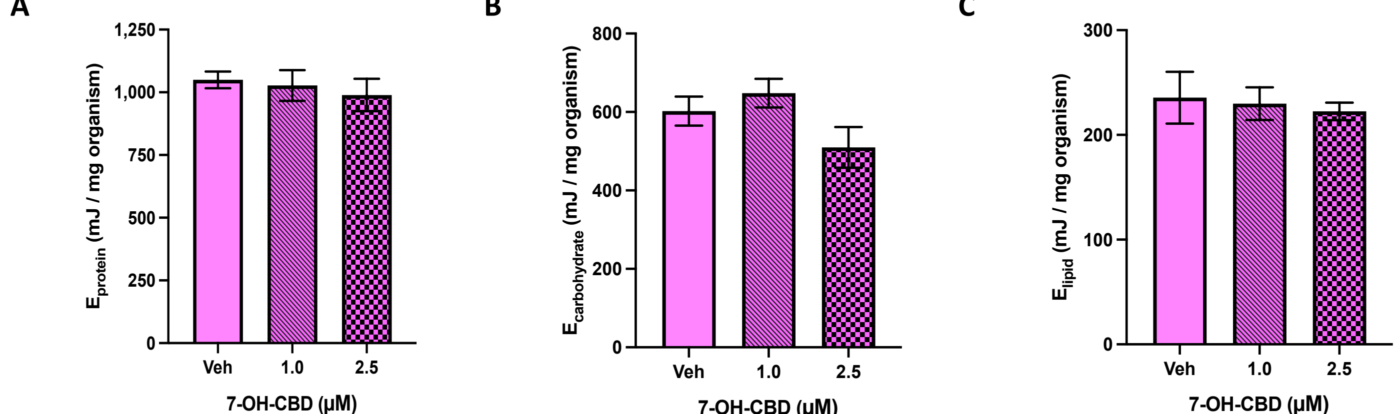


Figure 3.12: Energy quantification of *Lumbriculus variegatus* with 7-OH-CBD exposure. Subjected concentrations of 7-OH-CBD exposure (72 h) were 0 µM (Veh), 1.0 µM and 2.5 µM. Energy (E_c) expressed as mJ per mg of organism for **(A)** protein, **(B)** carbohydrate and **(C)** lipid. There was no significant difference in energy with exposure to 7-OH-CBD ($p > 0.05$). Error bars represent the standard error of the mean, n = 6 conducted in triplicate.

4. Discussion

4.1. Log K_{ow}

With rising awareness to the accumulating PIE, it is important to elucidate the fate of pharmaceuticals in the event of environmental contamination. Determining the Log K_{ow} is a key early indicator of a pharmaceutical's fate since revealing the lipophilicity, or lipophobicity, will reveal where the pharmaceutical is likely to partition, whether it will remain suspended in the water column or whether it will partition to the sediment. Ashauer *et al*. (2010) demonstrated with the freshwater invertebrate *Gammarus pulex* that the ability of a substance to bioaccumulate within an organism and the uptake rate constant of that substance is significantly related to the hydrophobicity (lipophilicity) of that substance. Therefore, determining the Log K_{ow} of a substance is highly valuable in the early insights of elucidating the ecotoxicology of a substance and, by extension, its potential for bioaccumulation.

The estimated Log K_{ow} values reveal that each evaluated cannabinoid is above the accepted values of >3 for compounds expected to exhibit lipophilicity and >5 for the potential to bioaccumulate (Arnot & Gobas, 2003; Mastroianni *et al*., 2013) (Table 3.1). Therefore, the Log K_{ow} values for CBD, 7-OH-CBD, abn-CBD and O-1918 suggest the potential to accumulate in the sediment with consequential bioaccumulation and biomagnification. Interestingly, the CBD metabolite, 7-OH-CBD, is expected to have a lower degree of lipophilicity than the parent compound. Further analysis was conducted for the Log K_{ow} values of the subsequent metabolite 7-COOH-CBD with an estimated Log K_{ow} of 6.7830. It appears that with each sequential metabolite, there is a decrease in the degree of lipophilicity. Although 7-OH-CBD and 7-COOH-CBD are still suggested to be lipophilic, the introduction of oxygen-containing functional groups from the metabolism of CBD increases the overall polarity of the metabolites when compared to CBD. Hydroxylation of CBD to 7-OH-CBD introduces an additional hydroxyl group and increases the compounds ability to hydrogen. Further oxidation to 7-COOH-CBD results in the formation of a carboxylic acid group which is more polar than a hydroxyl group. Consequently, the increasing polarity of successive metabolites results in decreasing lipophilicity as reflected in the decreasing Log Kow values from CBD to 7-COOH-CBD.

The results obtained herein align with studies by Jaipakdee *et al*. (2022) which used the KOWWIN^{TM} model for the determination of the Log K_{ow} of CBD, with a result of 8.01, which is congruent with the values obtained within this study. Furthermore, Kumer *et al*. (2019) used the HyperChem program to determine the Log P of CBD with a value of 3.41. Log P functions similarly to Log K_{ow} to determine partitioning behaviour between two immiscible solvents where positive assume lipophilicity and negative results assume hydrophilicity. Therefore, CBD was reported to be lipophilic supporting the results presented here. Furthermore, Muta *et al*. (2025) determined the partitioning behaviour of CBD by conducting high-performance liquid chromatography on mixtures of CBD in MilliQ Water or n-octanol to produce a Log P value of 7.54, which further supports the predictive results here. Although, the degree of lipophilicity was predicted to be lesser than what was experimentally determined.

There were no reports found of the Log K_{ow} of 7-OH-CBD, abn-CBD or O-1918 in the literature, however, the predictive modelling of CBD using two separate analytical modelling software were corroborated with the cited laboratory analysis. Therefore, the predicted results for 7-OH-CBD, abn-CBD and O-1918 here retain validity for their potential to partition to environmental sediment and bioaccumulate.

4.2. *In vivo* toxicity assay

With the partitioning behaviour described and revealing each compound is predicted to be lipophilic it was initially sought to determine the LD_{50} of phytocannabinoid and synthetic cannabinoid exposure to *L. variegatus* as this was absent in the literature. However, it was observed that some organisms were demonstrating signs of toxic effects with high degrees of decomposition and/or pallor while still being alive. Therefore, *in vivo* toxicity assays were used to assess an alternative endpoint of toxicity, determining the concentration at which 50% of the tested population exhibited toxicity after 24 hours exposure. To date, there has been no conducted research in determining the toxicity of CBD, 7-OH-CBD, abn-CBD or O-1918 exposure to *L. variegatus*, nor are there reports of lethal dose assays performed in invertebrate or mammalian models.

4.2.1. CBD

The no observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) of CBD was determined to be 1 µM and 5 µM, respectively. CBD has been extensively investigated using the nematode *C. elegans* which did not show signs of toxicity even with 4000 µM exposure for 6 hours, however, it should be noted that due to no adverse toxic effects being observed, further replicates were not conducted (Land *et al*., 2021). On the contrary, beneficial effects in thermotolerence of heat-induced molecular stress (resistance increased by 141% at 40 µM) was observed with no loss of life attributed to the exposure (Land *et al*., 2021). Furthermore, whole-life exposure (10 – 100 µM) to CBD resulted in 18% increase in the mean lifespan and late-stage motility increased by 206% when compared to the controls at 40 µM (Land *et al*., 2021). This is significant difference to the concentration at which 50% of the tested *L. variegatus* population exhibited toxic effects here at 14.12 µM (95% CI: 12.28-15.90 µM) (Figure 3.1A). *C. elegans,* however, are a terrestrial nematode species and is cultivated on solid nematode growth medium, while *L. variegatus* as previously mentioned, is an aquatic annelid and cultivated in liquid media. Therefore, differences in culture media could cause differences in permeability and accessibility to the subjected compound. Matta *et al*. (2007) discussed this issue concluding that equimolar concentrations of a drug delivered through solid and liquid medium are not experimentally comparable due to possible differences in total exposed surface area, or differences in osmoregulation or cuticular permeability.

4.2.2. 7-OH-CBD, abn-CBD and O-1918

While the NOAEL and LOAEL of 7-OH-CBD were equimolar to that of CBD, at 1 µM and 5 µM respectively, 7-OH-CBD achieved its 50% toxicity effects to the population at the lower concentration of 11.29 µM (95% CI: 10.53-12.09 µM) (Figure 3.1B). The NOAEL for O-1918 was determined to be 10 µM with the highest concentration exerting toxic effects to 50% of the tested population at 15.84 µM (95% CI: 12.25-18.82 µM) (Figure 3.1C), while it was inconclusive for abn-CBD since the control suffered toxic effects also with overall erratic results (Figure 3.1D). Due to the erratic nature of the abn-CBD *in vivo* toxicity assay it was assumed that the volatile nature of methyl acetate was producing vapours resulting in cross-contamination between wells where toxic effects were even seen in the control group. The vapour pressure of methyl acetate (Sigma-Aldrich, 296996) listing in the product description

is listed as 165 mmHg (20 °C) and condensation was observed on the lid of the experimental plates thus it is possible methyl acetate vapour was resulting in unaccounted for fluctuations in concentrations between wells. Due to the structural similarities of CBD to its metabolite 7-OH-CBD and its isomer abn-CBD, it is plausible that exposure of these compounds may induce toxicity in other invertebrate models and future elucidation would be favourable, given the excretion of 7-OH-CBD after human consumption of CBD (Pérez-Acevedo *et al*., 2020).

4.3. Behaviour

The stereotypical movements of body reversal and helical swimming were first described and characterised by Drewes (1999) after their initial paper determining the role of the lateral giant nerve fibres in the sensitivity of *L. variegatus* to mechanical stimuli (Drewes, 1990). The behaviour methodology and *L. variegatus* has since found merit with the proposal as a novel *in vivo* organism for pharmacological pedagogy (Seeley *et al.*, 2021) and in toxicological research (Davies *et al*., 2025; Ding *et al*., 2001; Seeley *et al*., 2024). Evaluating the response behaviour is an effective method of monitoring and determining the risk of environmental toxicants (Ji *et al*., 2020) when in conjunction with an indicator species such *L. variegatus* (Harkey *et al*., 1994).

4.3.1. CBD

The behaviour assays were conducted at sublethal concentrations (≤5 µM) informed by the LOAEL of CBD, 7-OH-CBD and O-1918. With 24-hour exposure to CBD, significant impairment of body reversal and helical swimming behaviours were observed at ≥2.5 µM and ≥0.5 µM, respectively, with both behaviours displaying markedly decreased ability to complete movements at 2.5 µM (Figure 3.2A-B). However, with increasing recovery time in artificial pondwater, the ability to complete these movements did increase with the exception at 5 µM where significant inhibition was still observed. The unstimulated movement of *L. variegatus* was only significantly impaired at 5 µM with effects of inhibition evidenced at ≥2.5 µM after 10 minutes recovery and at 0.1 µM and 5.0 µM after 24 hours. This suggests there was a period of latency in the onset of exposure effects to CBD. These results provide temporal insights into CBD exposure as even after 24 hours recovery in media devoid of CBD, inhibitory effects were still observed at concentrations lower than initial significant effects were

observed. At current environmental concentrations of CBD at 0.1 – 1.5 µM (Mastroianni *et al*., 2013; Pandopulos *et al*., 2021), CBD may exhibit toxic effects to aquatic invertebrate populations as evidenced by the significant inhibition of *L. variegatus* to complete helical swimming behaviours within this range.

While this study examined the impact of 24 h exposure to CBD, the effects of acute 10-minute CBD exposure (0 - 20 µM) has been studied (Williams *et al*., 2025). It was observed that after ≥ 5 µM CBD exposure for 10 minutes, there was a significant reduction in the stereotypical movement responses to tactile stimulation, however, after 24 hours recovery behavioural inhibition persisted at 20 µM. While these results show onset of exposure effects at concentrations present here, the time of exposure was 10 minutes compared to the 24-hour period *L. variegatus* were subjected to here. Therefore, it is possibly that the extended period allowed for a the bioconcentration of CBD in *L. variegatus* to increase for observable effects to be seen at lower concentrations. While it is unlikely that wild *L. variegatus* would only be subjected to cannabinoid toxicants for 24 hours, the results here detail the increasing bioaccumulation potential of CBD over time and at concentrations already present in the environment.

4.3.2. 7-OH-CBD

Exposure to 7-OH-CBD for 24 hours results in a rapid and significant impairment of body reversal and helical swimming at 5.0 µM (Figure 3.3. A-B), which persisted after 10 minutes recovery with a latent onset of impairment at 2.5 µM for body reversal (Figure 3.3. C-D). Furthermore, 7-OH-CBD did not exert inhibitory action on the unstimulated movement of *L. variegatus* (Figure 3.3.F-G). While there are inconsistencies with the potency comparison between CBD and 7-OH-CBD as mentioned previously, in the context of behavioural toxicology of *L. variegatus,* 7-OH-CBD displays reduced potency with higher concentrations required for toxicological behavioural effects to be observed. At present time, there are no behavioural studies investigating 7-OH-CBD exposure. With the lower Log K_{ow} value of 7-OH-CBD, compared to CBD, this may account for the decreased number of adverse effects observed with 7-OH-CBD as it is less able to accumulate internally. Furthermore, the

discrepancy in the literature regarding the potency of 7-OH-CBD and CBD it appears here that 7-OH-CBD is less potent than CBD, at least in the context of exposure to *L. variegatus*.

4.3.3. Abn-CBD

Abn-CBD exerted no significant effect to the stereotypical behaviours of *L. variegatus* after 24 hours exposure, however, there was apparent delayed-onset as inhibition of both stereotypical behaviours were observed at 5.0 µM after 10 minutes and 24 hours (Figure 3.4A-D). The delayed onset of toxicity may be due to the pharmacokinetics of abn-CBD, whereby the absorption, distribution, metabolism and excretion (ADME) of abn-CBD by *L. variegatus* may act differently to that of CBD which saw toxic effects earlier. The simulated Log K_{ow} of CBD and abn-CBD were nigh identical so absorption potential between the two may not have played a significant part. Since genomic sequencing of *L. variegatus* has not yet been completely performed as discussed by Martinez Acosta *et al*. (2021), there may be differential expression of CBD and abn-CBD targets in the organism with abn-CBD having reduced agonistic potential in *L. variegatus*. Dose-dependent effects were observed however regarding the unstimulated movement after exposure as hypokinesis was observed at 0.1 µM and 2.5 µM (Figure 3.4F), although recovery of the organism was successful as further inhibition was not observed (Figure 3.4G). Abn-CBD is described as behaviourally inactive (Caldwell *et al*., 2013), however, the context in which it was said was regarding murine research. While stereotypical movements did not see inhibition comparable to CBD, significant effects were observed as delayed onset. Thus, there is a discrepancy in the pharmacokinetics of abn-CBD between invertebrate and mammalian models. A study by Compton *et al*. (1990) did reveal that male ICR mice subjected to abn-CBD (100 mg/kg) saw a 30 ± 2% incidence of catalepsy. The apparent discrepancy between the lack of adverse locomotor effects observed in *L. variegatus* compared to the catalepsy reported in murine models suggests that there is a phylogenetic divergence of the endocannabinoid system between invertebrates and mammals. While invertebrates such as *C. elegans* have been discussed as possessing orthologous and homologous endocannabinoid components (Estrada-Valencia *et al*., 2023), functional differences between the endocannabinoid systems of invertebrates and mammals may account for the altered responses. Furthermore, administration of abn-CBD to the murine models was via injection while *L. variegatus* were

exposed to a solution, therefore, it is possible that with increased exposure time the bioconcentration of abn-CBD in *L. variegatus* may increase with resulting adverse effects.

4.3.4. O-1918

After 24 hours exposure O-1918 did exert significant inhibition of body reversal (5 µM) and helical swimming (≥2.5 µM) (Figure 3.5A-B) of *L. variegatus*. However, while there was no inhibition of unstimulated movement initially (Figure 3.5F) there was a delayed onset of inhibition after 24 hours in recovery at 0.5 µM (Figure 3.4G). At present, there are no studies in the literature using O-1918 to investigate behavioural effects. However, with O-1918 suggested to be a silent antagonist for putative abn-CBD receptors (Pertwee, 2001), it was expected to see effects opposite in nature to what was observed through abn-CBD exposure. However, while abn-CBD demonstrated delayed effects in stereotypical movements after 10 minutes in recovery and biphase hypokinesis in unstimulated movements, O-1918 demonstrated inhibitory effects in stereotypical movements which persisted through to the 24-hour recovery period and demonstrated delayed effects in unstimulated movements after 24 hours recovery. This suggests that abn-CBD and O-1918 are both acting agonistically. The pharmacokinetic differences between the two compounds suggests the two compounds likely have different ADME profiles resulting in differences in the rate at which the compounds are taken up and retained internally. O-1918 has been discussed by Simcocks *et al*. (2020) as being an atypical ligand displaying putative affinity to GPR18 and GPR55, which themselves are described as putative cannabinoid receptors, with GPR18 having low sequence homology compared to CB_1 and CB_2 while sharing a 21% amino acid identity with GPR55 (Morales *et al*., 2020). Phylogenetic analysis of *C. elegans* revealed neuropeptide receptor (NPR) NPR19 is related closest to human CB_1 and CB_2 and NPR24 and NPR32 are related to GPR18 and GPR55 (Pustuhov *et al*., 2016). If NPR are conserved and present in *L. variegatus*, then the modulation of the nervous system through NPR iterations mediated by O-1918 may account for the behaviour inhibition presented here.

The bioavailability of a drug describes the rate a drug is absorbed and can be affected by a range of factors including habitat preference (Meredith-Williams *et al*., 2012). It has been noted that the different routes of administration can make it difficult to compare between studies (Matta *et al*., 2007; Zheng *et al*., 2013) and is a key factor in the efficiency of a drugs

absorption to the target species, with the conclusion that drugs administered through liquid media yielded the highest drug absorption efficiency in *C. elegans*. Habitat preference could relate here to aquatic species being exposed to a uniform dispersion of the drug allowing for greater consistency in exposure and absorption to the target species. Conversely, terrestrial species that would be grown on solid media such as NGM would not be subject to uniformly distributed pharmaceutical, nor would it consistently have exposed to the pharmaceutical. Zheng *et al*. (2013) stated that due to the small nature of *C. elegans,* the methods of drug delivery were often indirect with their study typically mixing the pharmaceutical in with live or dead bacteria, while postulating that administration the pharmaceuticals topically onto the NGM media may incur a decrease in availability with the solution seeping into the agar. Furthermore, the methods utilising live bacteria performed poorly compared to methods with dead bacteria, attributing this to the live bacteria digesting the pharmaceutical with the conclusion that the main route of pharmaceutical uptake by *C. elegans* primarily is through the solution or the NGM surface. Regarding *L. variegatus*, with it being an aquatic annelid administration of compounds through liquid media aligns with its natural habitat and the point at which CBD ends up in the environment. The cuticle of invertebrates play a vital role in acting as a barrier between the organism and its environment and *L. variegatus* also is observed to have a smaller cuticle than *C. elegans* (Pakarinen *et al*., 2011; Peixoto *et al*., 1997), this difference compounded with the difference in habitat preference may account for the increased sensitivity of *L. variegatus* to CBD when compared to *C. elegans*. sensitivity.

The negative phototactic and thermotactic behaviour of *L. variegatus* has been discussed in the literature (Tuazon *et al*., 2022) where individuals subjected to excessive light or heat, respectively, will locomote away from these sources. The LED lightbox used in the free locomotion experiment makes direct contact with the plate housing the *L. variegatus* when collecting the images, and although LED machinery does produce heat (Lu *et al*., 2011) it is unlikely that the ~60 seconds of this contact it is enough to influence the ambient temperature of the solution and invoke negative thermotactic behaviour. However, the same cannot be assumed for the light produced and whether it would be sufficient to invoke negative phototactic behaviour and whether this would influence the unstimulated locomotory movement of *L. variegatus* by inducing hyperkinesis in efforts to locomote from these stressors. There is the question then of whether this experiment is truly unstimulated.

Though it was standard practice throughout all experiments to wash the wells before testing occurs, Tuazon *et al*. (2023) observed that the mucus secretions of *L. variegatus* resulted in 'clumping' of algae through thigmotaxis, causing a reduction in movement. This clumping was observed, but not noted in the results, and so it is unclear if this contributed to any significant reduction in the ability to carry out stereotypical behaviours. Understanding the behavioural toxicology is an important factor in assessing the ecological risk of xenobiotics, as changes in behaviour can act as initial indicators of toxicology prior to organism death. Furthermore, the lethality of a toxicant might not be best described by mortality and the determination of a lethal dose, as impaired behaviour could result in "ecological death" (Scott & Sloman, 2004), where the organism is unable to function appropriately within its niche and may result in indirect death from the inhibition of feeding, mating or predator avoidance strategies. It was suggested by Drewes (1999) that body reversal and helical swimming are means of predator avoidance, and so the impairment of these behaviours could result in prolonged exposure to predators, resulting in death by predation. Therefore, there is a rising urgency to quantify the behavioural toxicology of cannabinoids with the rising use and misuse of cannabinoids, leading to possible increasing environmental levels.

4.4. Regeneration

With cannabinoid exposure exerting significant behavioural effects it was then decided to explore the physiological effects of cannabinoid exposure on *L. variegatus* and the regenerative capacity of *L. variegatus* is has been discussed in the literature as a great example of morphallactic and epimorphic processes to regenerate body segments after injury or autotomy (Tweeten & Anderson, 2008). Morphallactic regeneration, or morphallaxis, involves the reorganisation or repatterning of existing tissues into new organised structures while epimorphic regeneration, or epimorphosis, involves blastema differentiation with cell proliferation to generate new body parts or tissues (Martinez Acosta *et al*., 2006). *L. variegatus* has the ability to regenerate both anterior and posterior segments after injury or transection with autotomy responses depending on segment location and follows a sequence of rapid sealing at the injury site, blastema formation and then segment regeneration (Lesiuk

& Drewes, 1999a). However, regenerative processes between anterior and posterior segments differ in *L. variegatus* with anterior regeneration employing cell differentiation and migration while posterior regeneration employs cell migration (Tweeten & Anderson, 2008). As *L. variegatus* occupy the littoral zone of freshwater bodies and thus are at greater risk of exposure to predators (Lesiuk & Drewes, 1999a) and habitat change than species that occupy deeper zones. Therefore, this may leave them more exposed to predators and at risk of predation or injury. If injury were to occur it is important to investigate their regenerative processes under cannabinoid exposure.

It was determined by Beinart & Gillen (2024) that regeneration of *L. variegatus* post-amputation requires upregulation of reactive oxygen species (ROS) which presents within 15 minutes after amputation. It is likely that this burst of ROS acts as a signalling mechanism to initiate the regenerative processes for blastema formation. Through chemical inhibition using the NOX inhibitor diphenyleneiodonium chloride, a delay in the regeneration of *L. variegatus* was observed with the inhibition of the endogenous ROS burst with regeneration capacity being partially rescued with the introduction of an exogenous reactive oxygen species. Usami *et al*. (2008) revealed that through CBD metabolism using mouse hepatic microsomes formed cannabidiol hydroxy-quinone which was suggested to be able to induce cell toxicity through the generation of ROS. However, Dawidowicz *et al*. (2021) revealed the antioxidant action of CBD, although this was dependent on the electron-accepting species, which theoretically may attenuate the ROS burst of *L. variegatus* in its initial regenerative process. Furthermore, CBD has been discussed in the literature for its anticancer properties through attenuation of cellular proliferation (Massi *et al*., 2013; Seltzer *et al*., 2020). The results here revealed that CBD exposure did significantly decrease anterior regeneration of the posterior segment at ≥2.5 µM. Therefore, the inhibition of anterior regeneration of the posterior segment by CBD may act by either by inducing an excessive ROS environment with sequential cellular toxicity or by suppressing cellular proliferation, possibly through attenuation of the initial ROS burst. Though future elucidation is required, CBD does not appear to inhibit cell migration processes involved in the posterior regeneration of the anterior segment, true for the concentrations in the range presented here. 7-OH-CBD does not appear to inhibit the regenerative ability of *L. variegatus* within the concentration range used here.

Vought & Wang (2018) demonstrated the inhibition of regeneration in *L. variegatus* using BPA and BPS, environmental contaminants from plastics. The number of new, whole regenerated

segments were counted over a comparable period of 60 hours and significant inhibition was observed with both drugs over this time. Although, while Vought and Wang (2018) examined whole new segments, here the total surface area of new growth was examined. The findings presented here support the precedent for how anthropogenic toxicants can disrupt critical regenerative processes. Though BPA and BPS act as endocrine disrupters, CBD is suggested here to act mechanistically different. The regenerative capacity of *L. variegatus* can act as a more sensitive endpoint to the effects of toxicant exposure over mortality where toxicants operating mechanistically different are able to have their resulting effects converge to suppress regenerative processes.

4.5. Respiration

CBD and 7-OH-CBD exerted concentration and time dependent effects on the DO_2 in the water column housing *L. variegatus* suggesting oxygen consumption of *L. variegatus* was significantly inhibited in a dose-response manner. The impact of toxicants on the respiration of aquatic invertebrates remains understudied in the literature, however. A previous study by Zhang *et al*. (2014) examined the impact of cadmium and copper on the oxygen consumption of juvenile ridgetail white prawn (*Exopalaemon carinicauda*) revealing significant inhibition. Though *E. carinicauda* is not closely related to *L. variegatus* and their habitats are saltwater and freshwater, respectively, both experiments support the increasing body of evidence that anthropogenic toxicants can cause adverse effects in non-target organisms. Tuazon *et al*. (2022), from which the methodology used here was derived, used a contactless oxygen sensor taking continuous measurements in a closed system for 60 minutes using 12 *L. variegatus* per repeat. Here, an insertable probe was used to collect measurements at intervals using 10 *L. variegatus* per repeat. A general trend of initial decreasing DO_2 with sequential increases was observed (Figure 3.8), whether this was due to the rate of oxygen diffusing into the solution being higher than the rate of respiration of *L. variegatus* however remains to be determined.

CB_1 receptors located in mouse neuronal mitochondria control energy production and cellular respiration and with activation by endogenous and exogenous cannabinoids there was a

resulting decrease in concentration of cyclic AMP, protein kinase A activity, complex I enzymatic activity and respiration in neuronal mitochondria (Bénard *et al*., 2012). This modulation of neuronal energy metabolism could mean that CBD may be exerting similar effects in *L. variegatus* with the decreasing mitochondrial activity resulting in compensatory metabolic action of increased respiration to maintain homeostasis under toxicant-induced stress of exogenous cannabinoid exposure. However, as previously mentioned invertebrates do not have the typical mammalian endocannabinoid system and so this remains speculative. The respiratory modulatory action of CBD and 7-OH-CBD presented here holds ecological implications. *L. variegatus* is a burrowing (Kristensen *et al*., 2012) detritivore so ecological services provided by this species may be adversely affected if compensatory metabolic action is occurring with potential inhibition of burrowing activity with subsequent sediment oxygenation decreasing, decreasing nutrient cycling in the benthic habitat and decreased available energy for reproduction, architomic regeneration and growth. Furthermore, increasing energy expenditure may also decrease the ability of *L. variegatus* to successfully complete predatory evasive behaviours, leading them to be more susceptible to predation.

4.6. Pulse rate

The results on effects of CBD and 7-OH-CBD on the pulse rate of *L. variegatus* reveal distinct physiological responses between the parent cannabinoid and its metabolite with novel insights into the pharmacodynamics of cannabinoids in aqautic invertebrates. CBD demonstrated a significant dose-dependent decrease in pusle rate of *L. variegatus* at ≥2.5 µM while 7-OH-CBD, although no significant effects were observed, a biphasic response was determined with a decrease in pulse rate at 2.5 µM.

Due to the negative phototactic and thermotactic behaviour of *L. variegatus*, it is possible that the use of excessive light, and resulting warmth produced, by a stereomicroscope could induce an ectopic pulse rate through increasing energy expenditure to escape the stressors. The stereomicroscope that was operated here uses oblique coherent contrast illumination and LED diascopic illumination. Though not noted in the methodology here, it was with this in mind that during the experimental design the stereomicroscope light was used on a low-

light setting to prevent over-stimulation and since the assessment was conducted outside of a liquid media, handling time was minimised to prevent desiccation and further ectopic pulsations (Bohrer, 2006; Halfmann & Crisp, 2011). Daoud *et al*. (2022) observed that the pulse rate of *L. variegatus* can be significantly affected by increased temperature. However, the use of a light microscope is common practice when conducting pulse rate analysis of *L. variegatus*, typically using a low-light setting (Bohrer, 2006; Daoud *et al*., 2022; Vought & Wang, 2018). Furthermore, the mean baseline pulse rate observed here (12 bpm) is comparable with the literature where it has been calculated in the range of 8-14 bpm (Halfmann & Crisp, 2011; Lesiuk & Drewes, 1999b; Wang & Wang, 2021) and thus the results remain consistent within context of the literature.

The midpoint of *L. variegatus* serves as a standard point to observe contractions of the DBV to record pulse rate in the literature (Crisp *et al*., 2010; Vought & Wang, 2018; Wang & Wang, 2021). While the pulses originate at the posterior and travel anteriorly, they can diminish as they travel and so recordings at the posterior would hypothetically be higher than recordings at the anterior (Wang & Wang, 2021). The decrease in pulse rate of *L. variegatus* observed here, while sublethal, could represent a decline in the subjected organisms fitness. Since *L. variegatus* have a closed circulatory system (Halfmann & Crisp, 2011), oxygen and nutrient transport through the organism may be reduced and thus compromising the ability of the exposure-subjected organisms to respond to environmental stressors, such as phototaxis, thermotaxis, predator evasive behaviours, burrowing behaviour, feeding and regeneration. Further analysis of tissue hypoxia may be beneficial to elucidate the impact of CBD on the respiratory activity of *L. variegatus*. Gorr *et al*. (2006) discussed the use of a hypoxia inducible factor (HIF) homolog which have been reported in *C. elegans, Drosophila* and *Daphnia magna*. HIFs are a highly conserved pathway in the animal kingdom and they accumulate in hypoxic cells with their activity and abundance in *Drosophila* measured by Gorr *et al*. (2006) through electrophoretic mobility shift assay (EMSA) and Western Blots, respectively. However, due to the limited genetic information of *L. variegatus*, it is unknown what HIF homolog they might possess but it provides a beginning framework for detecting hypoxia in *L. variegatus* through common laboratory techniques. Though mortality was not observed, these effects have the potential to translate to population level. Though the ≥2.5 μM concentrations that resulted in significantly reduced pulse rates of *L. variegatus* is above the concentrations observed in the

environment, the increasing use of cannabis and CBD products can allow for the safe assumption that environmental concentrations will increase if waste-water treatment policies continue as is.

4.7. Cholinesterase activity

Acetylecholinesterase (AChE) is an enzyme located in conductive tissues and acts to break down acetylcholine, a key neurotransmitter in the cholinergic system, both AChE and acetylcholine have been identified in *L. variegatus* (Davies *et al*., 2025; Kristoff *et al*., 2006; Puopolo *et al*., 2022). Furthermore, CBD has been shown to modulate the cholinergic system through exerting inhibitory effects on AChE and butylcholinesterase (BChE) by acting as a competitive inhibitor of both enzymes (Puopolo *et al*., 2022). ChE enzyme activity has been used as a biomarker for environmental contamination of pesticides to indicate neurotoxic effects (Kristoff *et al*., 2006). However, while the initial investigation was to determine AChE activity, the use of BChE inhibitors were not used in the quantification of cholinesterase activity and so comparison of the results here are limited to whole ChE activity. Based on the observations that CBD can modulate ChE activity, it was hypothesised that the modulatory activity of CBD on AChE activity may have caused the behavioural or physiological effects observed here and so it was sought to investigate this along with the metabolite 7-OH-CBD. This modulation of ChE may account for the previous inhibition in behaviour and pulse rate due to a disruption to motor neuron activation in the cholinergic system as findings by Davies *et al*. (2025) and Lesiuk & Drewes (1999b) suggested. However, while ChE activity was again confirmed in findings presented here, there was no significant difference observed with CBD, or 7-OH-CBD, despite the aforementioned modulatory properties of CBD which is likely due to the concentration of cannabinoids here being five times lower than the concentration used by Puopolo *et al.* (2022) which still did not show significant inhibitory effects. However, the study used *in vitro* techniques while here the experiment was conducted *in vivo*, therefore, the bioavailability of CBD to *L. variegatus* would be reduced as the toxicokinetic profile would be subject to a whole organism rather than cells.

Puopolo *et al*. (2022) used the Ellman method for evaluating ChE activity, as was done in this study. However, while CBD was found to modulate ChE enzyme activity, the same was not found here as there was no significant difference in ChE activity of *L. variegatus* after CBD exposure when compared to the control (Figure 3.10A), the same was for 7-OH-CBD exposure (Figure 3.10B). Sex-dependent differences were observed in behavioural alterations in the carabid beetle, *Pterostichus cupreus*, with the exposure to an organophosphorus insecticide revealing a correlation between locomotor behaviour and AChE inhibition (Jensen *et al*., 1997). The pesticide was applied topically to *P. cupreus* between the prothorax and elytra. The differences between studies may be due to species-specific sensitivity of the subjected compound and the route of administration. While there was continuous exposure of CBD to *L. variegatus* and topical administration to *P. cupreus* was limited, anatomical differences between these species and permeability differences between CBD and the pesticide to the respective species are likely due to the difference in ChE activity.

4.8. Total energy

As discussed by De Coen & Janssen (1997), the cellular energy allocation methodology was developed as a biomarker technique to evaluate the toxic stress on the energy reserves (protein, carbohydrate and lipid) of a species. Calow & Sibly (1990) previously proposed the idea of metabolic cost which describes that when an organism is subjected to toxic stress there will be alterations to its metabolism resulting in a decrease in its energy reserves. A decrease in energy reserves would hold serious repercussions at an individual and population level with decreased fitness. The metabolic shifts in energy reserves of *L. variegatus* under CBD exposure presented here (Figure 3.11B-C) represent metabolic disruption and potential energy reallocation as a compensatory measure, providing novel insight into the sublethal biochemical stress response in benthic oligochaetes to CBD. Energetic values were attempted to be gained at concentrations of 5 µM, however, exposure for 72 hours resulted in complete disintegration of some subjects with no ability to retrieve tissue for further experimentation, so further experimental repeats at 5 µM were neglected to remain within the practice of the 3R's. Future investigations into the effects of cannabinoids on total energy would benefit from using longer exposure times to observe the long-term effects of cannabinoid exposure. CBD

has demonstrated metabolic disruption through mitochondrial modulation with the intracellular decrease of lipid levels in murine 3T3-L1-derived adipocytes (CBD 10 µM) and *D. rerio* egg yolks (CBD ≥5 µM) (Silvestri *et al*., 2015). Furthermore, 7-OH-CBD demonstrated the enhanced ability to modulate lipid levels in 3T3-L1-derived adipocytes (7-OH-CBD ≥5 µM). However, neither CBD or 7-OH-CBD exhibited this decreasing effect of lipids in *L. variegatus*, suggesting that this process is not highly conserved in this model.

Carbohydrates are generally considered the primary and immediate source of energy across all animals (Wang *et al*., 2016), therefore, the decrease in carbohydrates may reflect a shift in the energetic demand of *L. variegatus* as a form of homeostasis due to stress induced by CBD exposure through detoxification processes or cellular repair. When compared to proteins and carbohydrates, lipids contain increased energy per unit mass (James *et al*., 2012), so the decrease in carbohydrates and increase in lipids may reflect compensatory action in energy storage or a dysregulation in lipid metabolism. Modulation of lipid metabolism by environmental stressors, including toxicants, has been recognised in the literature with lipids acting as crucial cellular components in homeostasis (Lee *et al*., 2018). Sokolova *et al*. (2012) discussed the effects of a species entering its pejus range, the range at which moderate stress is exerted onto it where fitness decreases but survival is still possible, homeostasis maintenance costs may increase, or metabolism may become impaired. This supports the findings here with CBD exposure as the concentration of 5 µM is sublethal as determined in Figure 3.1. The ecological consequences of these metabolic shift responses may result in the inability of *L. variegatus* to respond effectively to typical behavioural patterns in predatory evasion and its ability to regenerate after injury or during architomy.

4.9. General discussion & wider significance

The use of aquatic invertebrates such as *L. variegatus* has been highlighted in the literature as being crucial in ecotoxicological assessments for their low-cost maintenance, their significant role in the food chain, bioaccumulation potential and the ability to be representative of a diverse ecosystem (Rosner *et al*., 2024). Moreover, the phylum Annelida are distributed globally with characteristics such as multiple modes of reproduction,

regeneration processes, and typically endobenthic allowing for them to be novel models in numerous scientific fields (Rosner *et al*., 2024). The trophic transfer of toxicants through predation of *L. variegatus* by rainbow trout (*Oncorhynchus mykiss*) has been studied by Ng & Wood (2008) with findings that suggested that dietborne cadmium toxicity was higher than previous studies using commercial food diets, resulting in significant reductions in growth of *O. mykiss* of up to 50% due to cadmium-spiked *L. variegatus*. Amphibians are also considered appropriate bioindicators for ecotoxicological assessments for their split aquatic and terrestrial life stages, but also for their permeable skin making them susceptible to toxicants (Croteau *et al*., 2008). Amphibians have been showing declines for decades with accounting for toxicants as a contribution, though focus is held on pesticides (Brühl *et al*., 2013; Houlahan *et al*., 2000). It would be unwise to not consider wastewater contaminants as a potential risk potentiation factor for declining amphibian populations, considering when the larval stages are typically aquatic. Aquatic invertebrates and amphibians can both act as prey species in their environments, a decline in species fitness and population biomass holds serious implications for the ecosystem as a whole considering the further risk of bioaccumulation and biomagnification of toxicants through higher trophic levels.

There is also the potential to utilise *C. sativa* extract as an invertebrate vector insecticide (Fernandes *et al*., 2024), using different extract formulations on different life stages of medically important invertebrates acting as vectors for human disease (*Ctenocephalides felis felis*, *Aedes albopictus*, *Anopheles stephensi*, *Anopheles gambiae*, and *Culex quinquefasciatus*) and evidence this in mortality, fertility, birth rates and emergence of adult invertebrates. Consequently, the potential of *Cannabis* extracts emerging as an insecticidal agent could lead to elevated levels of CBD, and other cannabinoids, at rates in the environment that the exceed typical evolutionary selection pressures.

7-OH-CBD exposure revealed few incidences of significant effects when compared to CBD. Although the *in vivo* toxicity assay result for 7-OH-CBD was a lower concentration describing increased toxicity than that of CBD (Figure 3.1), the stereotypical behaviours and unstimulated locomotion experiments demonstrated a higher concentration of 7-OH-CBD (Figure 3.3) was required to exert inhibitory effects which did not persist after 24 hours in recovery, unlike CBD (Figure 3.2). This was again observed in the respiratory experiment

where lower concentrations of CBD exerted significant effects in the DO_2, although significant effects were observed in 5 µM of 7-OH-CBD where CBD did not exert these effects. Furthermore, where CBD exerted significant effects in regeneration, pulse rate and total energy experiments, 7-OH-CBD failed to do so. Beers *et al*. (2021) reported the AUC of 7-OH-CBD to be 38% lower than that of CBD which may account for the lower incidences of significant effects observed due to the decreased bioavailability despite its increased toxicity. Williams *et al*. (2025) investigated the effects of 7-COOH-CBD on the stereotypical behaviours and locomotor activity of *L. variegatus* with significant hypokinetic effects observed only at 24-hour exposure with 0.1µM and 24-hour post-recovery in APW with 0.5 µM. This further demonstrates the diminishing effect of CBD metabolites on these parameters suggesting future cannabinoid investigations should concentrate on CBD. While there is a paucity in the literature regarding the therapeutic potential of 7-COOH-CBD, it exhibits a higher affinity of binding to CB_2 receptors when compared to other CBD metabolites, including 7-OH-CBD (Tongkanarak *et al*., 2024), though it is reported to be inactive (Ujváry & Hanuš, 2016).

The polar hydroxyl functional group which is added during the metabolism of CBD to 7-OH-CBD is likely the cause for the decreased predicted lipophilicity determined from the Log K_{ow} values (Table 3.1). The shift in this partitioning behaviour, while still above the >5 limit for accepted bioaccumulation potential, may have affected the lipophilicity enough to lower the bioavailability of 7-OH-CBD resulting in the lack of statistical significance observed in regeneration, pulse rate and total energy experiments when compared to CBD. Both CBD and 7-OH-CBD are excreted in waste products after human consumption (Chayasirisobhon, 2020; Harvey & Mechoulam, 1990; Pérez-Acevedo *et al*., 2020), therefore, both can occur as contaminants concurrently and so there is merit in assessing the ecotoxicological impact of a pharmaceutical and its metabolites. There are numerous more metabolites of CBD (Austin *et al*., 2024), which are also not present in studies investigating environmental contamination. Therefore, with the absence of environmental and toxicological data of these metabolites there may be more cause for concern than what is presented here.

The findings presented here contribute to the growing body of evidence in the literature that pharmaceutical contamination in the environment can disrupt key behaviours, physiological and biochemical processes in non-target organisms through conserved biological pathways.

These results underscore the importance of including cannabinoids in environmental risk assessments and wastewater treatment consideration, this is pressing as the global consumption of cannabinoids continues to increase with evolving legalisation and the medicinal promises of cannabinoids are elucidated.

4.10. Future directions

While the determination of Log K_{ow} using Epi Suite ™ v4.1 can provide insight into the partitioning behaviour of a compound, the model does not consider environmental conditions, relying on the chemical and physical properties of the compound and its structure (Borrirukwisitsak *et al*., 2012). When assessing the effects of salinity, pH and temperature on the Log K_{ow} of BPA, it was observed that each factor did alter the Log K_{ow} of BPA with significant effects in pH (Borrirukwisitsak *et al*., 2012). This was further observed *in silico* with pH taken into account on the Log K_{ow} of fluoxetine and norfluoxetine with increasing pH increasing Log K_{ow} (Brooks *et al*., 2003). The environmental conditions at which CBD is released into the environment and the habitat of *L. variegatus* are not represented by the laboratory conditions here and so future experiments could look to account for these environmental parameters when further elucidating the ecotoxicology of CBD, and its associated compounds. This approach could use spiked-sediment methodologies previously established in the literature (Landrum *et al*., 2002; Liebig *et al*., 2005) to facilitate CBD partitioning to the sediment matrix as observed in the environment, with subsequent exposure of *L. variegatus* to the contaminated sediment.

There have been approximately 40 characterised Phase I metabolites of CBD from human metabolism (Ujváry & Hanuš, 2016) without considering the Phase II metabolites. With 7-OH-CBD being the primary active metabolite of CBD, it was easy to warrant its research, however, the most abundant excreted metabolites of CBD are hydroxylated 7-COOH-CBD derivatives (Chayasirisobhon, 2020) and so future research into cannabinoid toxicants should include these derivatives for a more representative view of the potential impact of excreted cannabinoids. There is also cause for concern with the increasing research into the polypharmacy applications of CBD. CBD has been investigated as an additive analgesic

alongside paracetamol for knee osteoarthritis (Pramhas *et al*., 2023) though the authors concluded there was no significant additive effect in pain relief. Furthermore, a review on the drug-drug interactions involving CBD revealed there is insufficient data in the literature that can be extrapolated to humans due to the use of *in vivo* and animal models, it was noted however that reduced efficacies might result with a possibility of certain antidepressants resulting in increased risks of toxicity (Vázquez *et al*., 2020). As discussed here previously, there are numerous pharmaceuticals present in the environment causing adverse effects in wild populations, *in vivo* and animal model data of CBD polypharmacy details above may prove useful in elucidating how CBD may interact with environmental pharmaceuticals and what effects may be seen to the exposed wildlife.

Further investigating the influence of CBD on the regenerative capacity of *L. variegatus* and to elucidate the inhibition results observed here would be beneficial. As discussed above, the diminished regenerative capacity may be due to induced cell toxicity from quinone derivatives of CBD or through the antioxidant action of CBD. To test these, post-exposure *L. variegatus* could be subjected to GC-MS analysis to determine if quinone-derivatives are present or adapting the methodology present by Dawidowicz *et al*. (2021) accounting of using *in vivo* models. Tellez-Garcia *et al*. (2021) conducted transcriptome analysis on *L. variegatus* during the first 72 hours of regeneration and produced a framework of 136 transcripts, of which 25 were downregulated and 111 were upregulated. Future transcriptome analysis of *L. variegatus* could be conducted to elucidate the mechanism of action in the inhibition of posterior regeneration with CBD exposure with comparisons to the mentioned study.

Since there could be concurrent contamination of CBD and 7-OH-CBD in the environment with 7-OH-CBD known to be excreted (Pérez-Acevedo *et al*., 2020), assessing the possible toxicological interactions is therefore merited through a mixed solution containing CBD and its metabolites to elucidate any additive or synergistic toxicity. The entourage effect is a popular field of study in cannabinoids that demonstrates positive synergy, therefore there is the possibility of a negative entourage effect. However, a review by André *et al*. (2024) on the entourage effect while discussing the exploratory evidence of terpene potential, concluded that synergy due to the entourage effects remains inconclusive. The entourage effects still remains discussed as an important facet of cannabinoid research and so research

is still warranted into this hypothesis. Concurrent exposure to abn-CBD and O-1918 may aid in elucidating the relationship of these compounds in *L. variegatus* as the results did not confer with the notion of O-1918 acting as a silent antagonist to putative abn-CBD receptors as observed in the behaviour experiments.

The erratic results gained from abn-CBD exposure experiments were surprising even when using sublethal concentrations obtained from the *in vivo* toxicity assay. It may have been possible that the vapours produced from methyl acetate would condense and redissolve throughout the solutions in the plates. However, the vehicle for 7-OH-CBD, methanol, is also described as producing vapours and there were no erratic results produced throughout the 7-OH-CBD exposure experiments. The vehicle for abn-CBD was limited to methyl acetate as it was sourced pre-dissolved in methyl acetate. Future directions to elucidate this, and to bolster the ecotoxicological impact of CBD analogues, would be to attempt sourcing abn-CBD that is either pre-dissolved in a different vehicle or in powder form for experimentation.

While the present findings here substantiate the detrimental ecotoxicological impact of CBD and its associated cannabinoids on *L. variegatus*, comprehensive GCMS analysis of *L. variegatus* post-exposure might elucidate the underlying mechanisms of toxicity. GCMS analysis could help characterise and distinguish between direct toxicological effects mediated by parental CBD and the toxicity induced by metabolite biotransformation. Such as the case of methanol, when metabolised in humans, formic acid accumulates and is responsible for methanol-induced blindness, morbidity and mortality (André *et al.*, 2024). Furthermore, it may provide insight into the endocannabinoid system in *L. variegatus* while informing risk assessment frameworks for cannabinoid toxicants in aquatic ecosystems.

The use of *L. variegatus* has been presented as an effective exposure vector for research into the trophic transfer of toxicants from prey to predator by Mount *et al*. (2006) due to the nutritional analysis of *L. variegatus* determining the protein and amino acid levels to meet fish nutritional guidelines and the acceptance of fathead minnows (*Pimephales promelas*) and *O. mykiss* as prey. It would be valuable to conduct research into the use of cannabinoid-spiked *L. variegatus* as food for predatory species such as *P. promelas* or *O. mykiss* to elucidate the

bioavailability and biomagnification potential of CBD and its resulting toxicological impact as previously seen by Ng & Wood (2008) using cadmium-spiked *L. variegatus.*

4.11. Limitations

When determining the effects of CBD (0.4 – 4000.0 µM) on the motility of *C. elegans*, (Land *et al*., 2021) observed a 43.5% decrease in motility at the highest concentration after 6 hours exposure when compared to the control. Due to the dominance of *C. elegans* as an invertebrate model, Land *et al.* (2021) were able to use the Infinity Screening System (Nema Life, Inc.), an AI powered high-throughput *in vivo* screening program for *C. elegans*. Though the number of experimental repeats was one, this method allowed for 53-65 organisms to be analysed per concentration compared to the eight experimental repeats here which allowed for greater alignment with the 3 R's principles and ethical research practices. *L. variegatus* does not have a dedicated program to conduct behaviour analyses at present time, the method of using ImageJ to manipulate the 50 pictures, one second apart, and quantify the locomotor activity (Davies *et al*., 2025; Seeley *et al*., 2021, 2024) and in burrowing activity (Pigneret *et al*., 2016) is peer-reviewed. However, due to the limited time in which movement is recorded there is the possibility of the data not being truly representative of the adverse effects to drug exposure conducted this way, though to what degree is uncertain. Ji *et al*. (2020) used a line body detection and a recurrent self-organising map and assessed the behavioural effects of copper sulfate on speed, stop duration, number of stops, turning rate and meander which appears to provide greater depth into the unstimulated behaviour of *L. variegatus*. The methodology used here is limited to data produced in a 2D image and does not capture movement in the 3D plane. Efforts are made however to limit movement in the z-axis through reducing the volume in the unstimulated movement assay compared to the stereotypical movement assays.

While this experiment provides insight into the sublethal effects of CBD on the respiratory action of aquatic invertebrates, the controlled laboratory conditions do not fully represent the complex natural system *L. variegatus* inhabit in the wild. The lack of sediment and organic matter prevents the sedimentation of CBD. This might alter the uptake and bioavailability of

CBD, and its associated cannabinoids. Furthermore, the previously discussed discovery of cryptic speciation in *L. variegatus* (Gustafsson *et al*., 2009) means that the truth for one population of *L. variegatus* may not be true for another. If genetic sequencing were to be conducted on a sampled population, the taxonomy of *Lumbriculus* spp. would have to be further characterised to account for the cryptic speciation as the genetic profiling would not be truly representative of the genus. Rosner *et al*. (2024) discussed the limitations of using laboratory reared aquatic invertebrates stating that due to environmental factors not being accounted for, extrapolation of laboratory results to the natural environment has limitations. Firstly, the inter-species variation in the sensitivity of toxicants rising from biomagnification and biotransformation potential. Secondly, synergistic effects that may be influenced by characteristics of the model organism. Thirdly, exposure to previous generations may result in epigenetic signatures.

5. Conclusion

The globally increasing use of CBD through recreational use in cannabis and as its growing promise as a therapeutic agent necessitates elucidation of its ecotoxicological impact, particularly given the observations of its presence in the environment. This investigation extended CBD to include its primary active metabolite, 7-OH-CBD, and the synthetic cannabinoid derivatives, abn-CBD and O-1918 using *L. variegatus*, an invertebrate model, supported here to be a sensitive bioindicator to toxicants for ecotoxicological assessments.

The findings presented here demonstrated different toxicological profiles of the cannabinoids investigated. *In silico* analysis of each cannabinoid revealed the potential to accumulate in the environment with hydrophobic characteristics, raising ecological concern. Abn-CBD and O-1918 demonstrated significant behavioural inhibition, while 7-OH-CBD exerted inhibitory effects in the behaviour and respiration of *L. variegatus*. CBD exhibited the most impactful profile with significant inhibition in the behaviour, regeneration, respiration, pulse rate and energy metabolism of *L. variegatus.* These findings established a hierarchy of cannabinoid toxicity and identified CBD as the compound of greatest ecotoxicological concern, among those tested, to the aquatic ecosystem.

CBD demonstrated significant impacts at environmentally relevant concentrations of which it has been found, suggesting there may be present and ongoing ecotoxicological impacts on benthic oligochaete populations. Individual level effects have the potential to translate to population level through the impaired regenerative capacity of *L. variegatus* which could greatly affect populations relying on reproduction via architomy. Furthermore, the comprised physiological function may disrupt critical ecosystem services carried out by benthic organisms may disrupt critical ecosystem services including nutrient cycling and bioturbation with cascading effects through the aquatic food web. Furthermore, the increased susceptibility to predation may facilitate biomagnification and bioaccumulation with potential consequences through higher trophic levels.

Future considerations should look to further elucidate CBD exposure at environmentally relevant concentrations potentially through whole life exposure and spiked sediment experiments to better represent conditions in the environment.

The findings presented here provide novel insights into the pharmacology and ecotoxicology of cannabinoids and the potential impact on aquatic ecology. It suggests evolutionary conservation of cannabinoid signalling pathways in *L. variegatus*, although the specifics remain to be elucidated. The observed behavioural, physiological and biochemical effects highlight the ecological risk associated with environmental cannabinoid contamination and underscores the need for further research into the impacts of cannabinoid compounds on aquatic ecosystems.

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Appendix

A1. Rstudio code

```
install.packages("ggplot2")
install.packages("maps")
library(ggplot2)
library(maps)

world_coordinates <- map_data("world")

map1<-ggplot() +
 geom_map(
  data = world_coordinates, map = world_coordinates,
  aes(long, lat, map_id = region),
  color = "lightgrey", fill = "lightgreen", linewidth = 0.2)

map2<-ggplot() +
 geom_map(
  data = world_coordinates, map = world_coordinates,
  aes(long, lat, map_id = region),
  color = "lightgrey", fill = "lightgreen", linewidth = 0.2 )+
 theme(panel.background = element_rect(fill = "aliceblue"),
    panel.grid.minor = element_line(color="aliceblue"),
    panel.grid.major= element_line(color="aliceblue"),
    axis.title.x=element_blank(),
    axis.text.x=element_blank(),
    axis.ticks.x=element_blank(),
    axis.title.y=element_blank(),
    axis.text.y=element_blank(),
    axis.ticks.y=element_blank())

Lv <- read.csv("~/Library/CloudStorage/OneDrive-SwanseaUniversity/Lv geo new.csv")
```

```
DistMap<-map2 +
  geom_point(
    data = Lv,
    aes(x=Long, y=Lat),  color = "red",
    size = .5, alpha = 1)

DistMap
```

A2. Log K_{ow} SMILES

CBD	c1(O)c(C2C=C(C)CCC{P-}2C(C)C)c(O)cc(CCCCC)c1
7-OH-CBD	c1(O)c(C{P-}2C{P-}(C(=C)C)CCC(CO)=C2)c(C)cc(CCCCC)c1
7-COOH-CBD	C(=O)(O)C1=CC(c2c(O)cc(CCCCC)cc2O)C(C(C)C)CC1
Abn-CBD	c1(O)c(C2C{P-}(C(=C)C)CCC(C)=C2)c(CCCCC)cc(O)c1
O-1918	C1(OC)c(C2C{P-}(C(=C)C)CCC(C)=C2)c(OC)cc(C)c1

A3. Regeneration panel

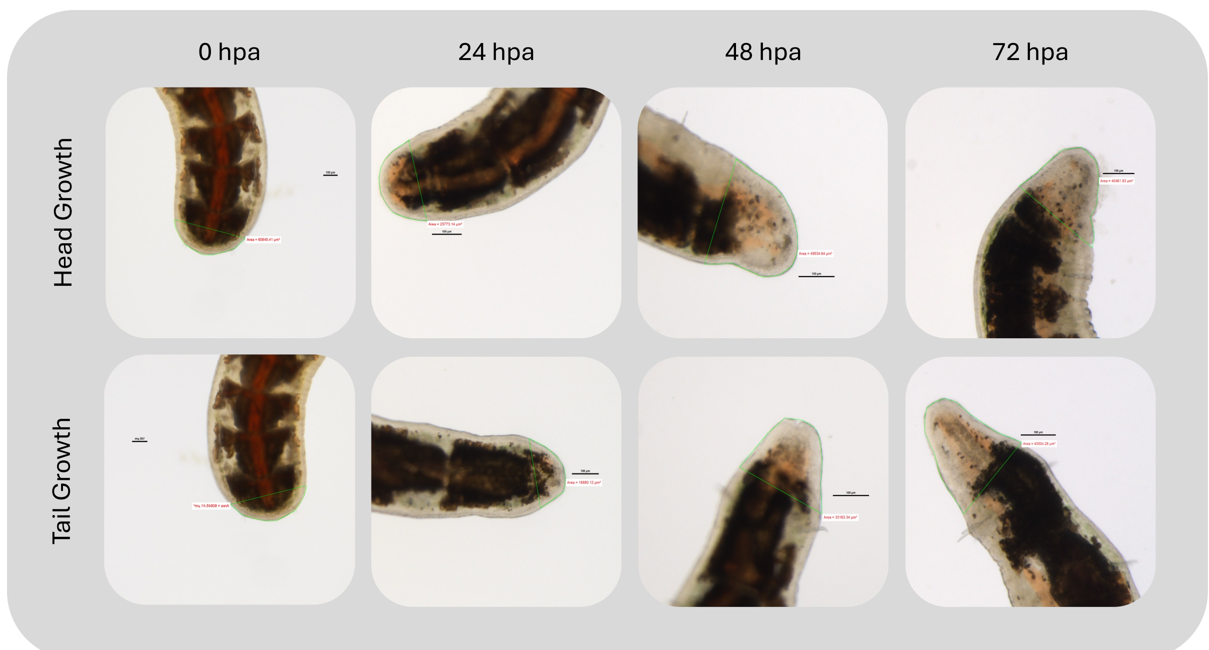

Figure A1: Representative images of the regeneration of *Lumbriculus variegatus* with exposure to 0.1 mM CBD. Pictures taken Nikon SMZ1270i stereomicroscope over 72 hours post-amputation (hpa).

A4. Contractions of the DBV

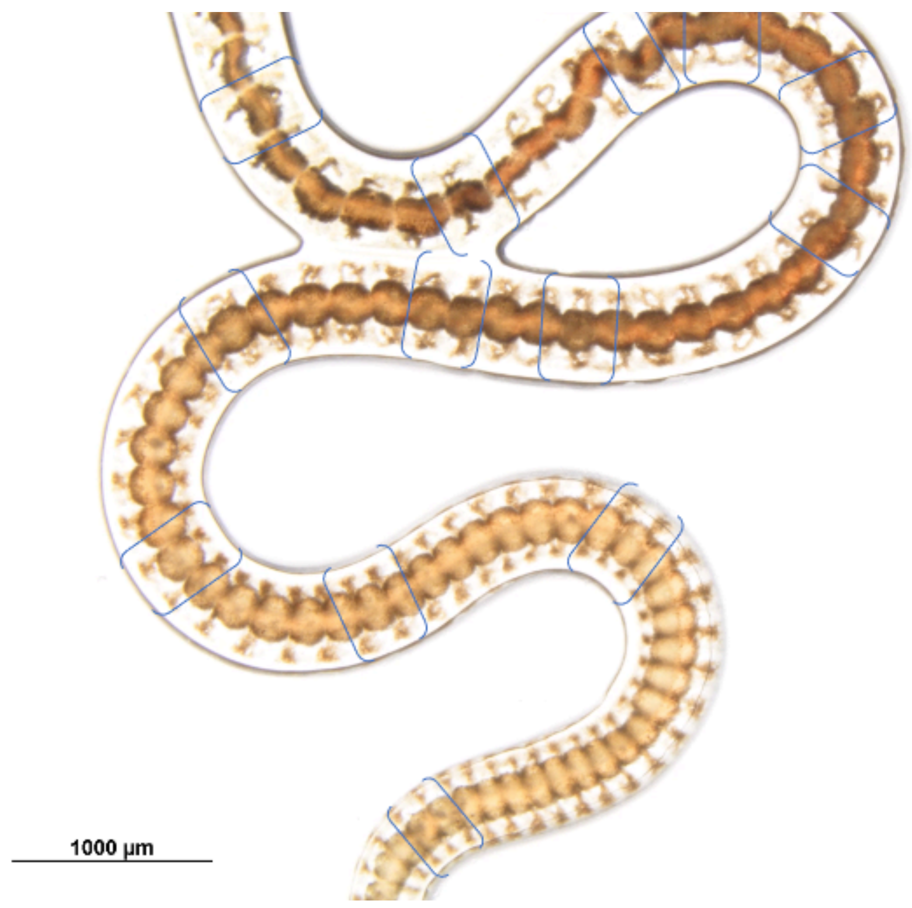

Figure A3: Muscular contractions of the dorsal blood vessel of *Lumbriculus variegatus*. Image taken using the Nikon SMZ1270i stereomicroscope from n=2, baseline, 2.5 mM, worm C.