Environmental Fate and Risk Assessment in Soil of RNAi-based biopesticide dsRNA V-ATPase subunit A against small hive beetle *Aethina tumida*

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Abstract

Pest control is essential for agricultural production. Many conventional pesticides present a risk for human/animal health and ecological biodiversity because of their environmental persistence and broad-spectrum action. Thus, the development of novel biopesticides which are less persistent and more selective in their action, and thus more eco-friendly, represent a promising avenue for more sustainable food production. However, European Union regulation like (EC) 1107/2009 requires to fully assess the risks of all new active substances.

Double stranded RNA (dsRNA) is a biomolecule that triggers the mechanism of RNA interference (RNAi), which is a protein synthesis disruptor mechanism in eukaryotic cells. dsRNA has considerable potential as a tool for selective insect pest control. Analytical protocols for its extraction, purification and quantification from soil are essential for the tests required to parameterize environmental fate assessments. This study systematically developed a procedure for recovering and quantifying dsRNA from loamy sand soil using RT-qPCR. During adsorption experiments following the OECD 106 Guidelines for Testing of Chemicals, it was found that pre-equilibrating the soil with $CaCl_2$ solution (0.01M) resulted in precipitation of dsRNA ($CaCl_2$ solution + dsRNA), because $CaCl_2$ might neutralize the charges on the phosphate backbone of dsRNA. This enhanced adsorption was compared to soil equilibration with distilled water. Thus, soil solution composition and ionic strength are important considerations when following OECD guidelines in assessing the sorption of a new generation of biopesticides consisting of nucleic acids. In adsorption tests, the dsRNA reached equilibrium within 1h in loamy sand soil with a sorption coefficient (K_d) 0.55 L kg^{-1}. Next, biodegradation tests in soil were carried out following the OECD 307 guidelines. The experimental data were fitted with three different biodegradation models (Single First-Order Rate Model, Double First-Order in Parallel, First Order Multi-Compartment). The results showed that dsRNA has a short half-life (DT_{50}) of 10h. This informed an environmental risk assessment in soil for dsRNA within European legislation EU 1107/2009 which is necessary for the registration of dsRNA as biopesticide. Furthermore, a new screening test procedure was proposed to identify sensitive species for biopesticide toxicity by using a bioinformatics tool (BLAST searching in the NCBI database) to find in the genome of non-target organisms regions of local similarity with the dsRNA sequence. Overall, it was concluded that even though the dsRNA had a low risk profile due to its ready biodegradability, considerable uncertainty remains around potentially high application rates and potential interference of the smaller siRNAs generated from the dsRNA with mRNA of non-target organisms.

Declaration

I hereby certify that this work is my own, except otherwise acknowledged, and that is has not been submitted for fulfilment of a degree at this or any other university.

Vincenzo Padricello

...to my family

Luigi, Antonietta, and Carla Padricello

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List of Abbreviations

AGO	Argonaute Protein
dsRNA	Double Stranded RNA
DT50	Half Life
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
ERA	Environmental Risk Assessment
EU	European Union
FP	Fusion Protein
GM	Genetic Modified
GMO	Genetic Modified Organism
GMP	Genetic Modified Plant
IPM	Integrated Pest Management
LC_{50}	Median Lethal Dose
miRNA	microRNA
mRNA	messenger RNA
NAP	National Action Plan
NTO	Non-target organism
OECD	Organisation for Economic Co-operation and Development
PIP	Plant Incorporated Protectants
piRNA	PIWI-interacting RNA
PO	Parent Only
PPP	Plant Protection Product
PTGS	Post-Transcriptional Gene Silencing
qPCR	Quantitative Polymerase Chain Reaction
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT	Reverse Transcriptase
siRNA	small (or short) interfering RNA
TER	Toxicity Exposure Ratio
TGS	Transcriptional Gene Silencing

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CHAPTER 1

Introduction

1.1. Introduction

Food security and environmental health are among the biggest challenges in the world. Since a growing global population requires more food production, the future challenges will be to improve the sustainability of crop production whilst preserving the natural resources, such as soil, water and biodiversity. Effective pest control still represents a necessary tool for the enhancement of agricultural production. In the past years, agricultural yields were improved to the detriment of environmental quality with excessive usage of chemicals for crop protection (i.e. DDT and organophosphates). To this day, conventional pesticides represent a risk for human and animal health because of their environmental persistence, mobility and broad-spectrum of action to non-target organisms. Thus, the development of novel pests control methods using less harmful, naturally occurring, selective and eco-friendly molecules, often referred to with the term "biopesticides", presents a valid alternative pathway to reducing these drawbacks (Glare *et al.*, 2012; Seiber *et al.*, 2014). Currently, the interest in biopesticides is growing globally (Cantrell, Dayan and Duke, 2012; Glare *et al.*, 2012). In the last years, the sector has increased from 3.5% to 5% by acquiring market share from conventional pesticides (Glare *et al.*, 2012; Olson, 2015). In addition, agrochemical companies are developing and marketing new "green pesticides" (Seiber *et al.*, 2014) due to the rising demands by consumers and farmers, for the use of less harmful products in the food sector. Therefore, with the growth of commercial biopesticides and their imminent placement on the market, it is crucial to study their behaviour in the environment for ecosystem security. The environmental fate of these novel macromolecules represents a new field of investigation. In fact, one of the big challenges in the agricultural and environmental sector is to achieve better knowledge of their behaviour within the abiotic and biotic sphere of the soil matrix. (Parker and Sander, 2017).

1.2. Pesticide Regulation in the European Context

Currently, the registration of plant protection products (PPPs) and their marketing is regulated by European Regulation (1107/2009). The aim of the regulation is to enhance agricultural yields by perusing a sustainable agriculture system without compromising the human and animal health, vulnerable population groups (children, infants, pregnant women) and agricultural communities. In addition, the European Union released National Action Plans (NAP) urging all European countries to commit and implement the EU directive 2009/128/EC which highlights a common pathway to achieve a sustainable use of pesticides. In this way, the EU directives outlined a specific direction for EU countries, but also for agrochemical manufacturers. Hence, the United Kingdom issued its NAP in February 2013, the "*UK National Action Plan for the Sustainable Use of Pesticides*", where it committed to "*adopting an integrated approach as described in the Directive, drawing on all available techniques to tackle pests, diseases and*

weeds". The strategy to implement the NAP relied on integrated pest management (IPM). It is an ecological-system strategy and essentially combines different agricultural practices to "*optimize the control of all classes of pests*" (Prokopy, 2003), whilst minimizing the risks for the environment and public health. In this context, the usage of biopesticides as alternative to, or in synergy with, conventional pesticides represent a valid tactic in limiting the use of persistent chemicals in agricultural fields (water bodies, sediments, groundwater), to achieve a more sustainable use of pesticides. Thus, their combination with IPM could contribute to a significant reduction in pesticide use by farmers (Srinivasan, 2012). The marketing authorization of pesticides (or PPPs) is an extensive evaluation procedure and involves European regulatory agencies and manufactures as outlined by the directive EU 1107/2009. Therefore, the agrochemical manufactures must provide a detailed environmental risk assessment study (ERA) identifying the risks related to the active substance of the PPP. The ERA represents the decision-making tool to establish whether a pesticide has a low risk profile. This characteristic is assessed through environmental fate studies, which evaluate the pesticide persistence in environmental media and capability to move through the different environmental compartments.

1.3. RNAi-based biopesticides

The use of RNA interference (RNAi) as a crop protection tool has gathered interest from many researchers (Baum *et al.*, 2007; Abd El Halim *et al.*, 2016; Joga *et al.*, 2016; Michelle E Powell *et al.*, 2017; Mehlhorn *et al.*, 2020). The molecular mechanism was discovered in the late 1990s (Fire *et al.*, 1998), and immediately captured the attention of the scientific community, which led to Dr. A. Fire and Dr. C. Mello being awarded the Nobel Prize in 2006. In general, RNAi is a highly conserved mechanism expressed by eukaryotic cells to regulate gene expressions (Meister and Tuschi, 2004). It is trigged by a double stranded RNA precursor (dsRNA), which are also used as the active substance of biopesticides. Once triggered, the RNAi happens at the messenger RNA (mRNA) level. It exploits the sequence-dependent mode of action, making it a powerful crop protection tool with potentially high species selectivity, establishing a limited spectrum of action to non-target organisms (NTO). In recent years, agrochemical companies adopted this novel molecular mechanism for pest control (San Miguel and Scott, 2016). Thus, the DvSnf7-dsRNA Maize MON 87411 was the first transgenic plant to express RNAi, and Monsanto was the first agrochemical company to market it in the United States. However, since the EU has in place a strict legal regime on transgenic plants and seeds, the future applications in the EU context of RNAi-based biopesticides are expected to be sprayable products as part of non-transgenic strategy.

1.4. PhD Research

The PhD Research was funded by the former Food and Environmental Research Agency, now FERA Science, in collaboration with the Institute for Agri-Food Research and Innovation (IAFRI) at the Newcastle University (UK). The research centred on the environmental fate study of a dsRNA macromolecule used as active substance of a RNAi-based biopesticide, by evaluating its sorption and degradation in agricultural soil. The choice in selecting the dsRNA molecule was made to consider the high impact that the biopesticide might have in terms of environmental benefits. Hence, the dsRNA selected was the *dsRNA V-ATPase subunit A* (Michelle E Powell *et al.*, 2017), which affects the *Aethina tumida*, a coleopteran parasite and serious pest of the European honey bee (*Apis mellifera*). This dsRNA molecule was already shown to be fully effective against the parasite in lab tests, with 100% mortality by injection and 50% by feeding application.

The research hypothesis tested three major assumptions underpinning the sustainability of biopesticides; that the dsRNA has (1) low mobility and (2) highly biodegradability in the soil matrix, and (3) does not affect non-target organism. Thus, the research had to investigate the fundamental mechanisms of sorption and biodegradation of the dsRNA molecule in soil, and potential interference with non-target organisms. To enable this investigation, it was necessary to first develop a reliable method of extraction, purification, concentration and quantification of the dsRNA (Chapter 3), which was achieved by the reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Using this method, it was possible to establish the sorption coefficient and the degradation rate for a reference soil (Chapter 4). Furthermore, using the measured data, a novel screening test methodology was proposed for the environmental risk assessment for a RNAi-based biopesticide (Chapter 5) to evaluate the environmental risk associated with the active substance within the European legislation framework (EU 1107/2009), including potential sensitivity of non-target species, as would be necessary for the registration of an active substance as biopesticide.

References Chapter 1

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CHAPTER 2

Literature Review

2.1. Biopesticides

Biopesticides are naturally occurring macromolecules used for pest management, which have become more popular in the last few years. They have specific features and properties which make them an alternative pest management product (Seiber *et al.*, 2014). Compared to synthetic pesticides, they are considered more eco-friendly and safer to humans and non-target organisms (NTOs) (Liu *et al.*, 2019). Within integrated pest management schemes (IPM), they can be used in synergy with or as substitutes for synthetic pesticides to reduce the usage of conventional pesticide in agriculture (Srinivasan, 2012). Currently, there is no common definition of the term "biopesticide". In general, it is possible to group them into two different categories; living organism and natural products (Glare *et al.*, 2012). The former acts by competition and inhibition with the pest, the latter have non-toxic actions (e.i. attraction or repulsion). The US Environmental Protection Agency (EPA) is the only environmental agency to provide a complete definition of biopesticides, as *"certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals"* (www.epa.gov). They fall into three categories:

a) Products derived from micro-organisms

Organisms like *Actinomycetes* can produce natural antibiotics like tetranectin (by *Streptomyces aureus*) which serve as miticide, or avermectins (by *Streptomyces avermitilis*) a potent acaricidal (Table 2.1). Furthermore, the most studied are the *B. thuringiensis* η-endotoxins ((Bt) toxins). Bt Toxins were isolated from different *B. thuringiensis* strains and proved to be effective against a wide range of pests such as Lepidoptera, Diptera, Coleoptera, as well as against nematodes (Beegle and Yamamoto, 1992). The mechanism of action is conducted by hydrolyzed Bt η-endotoxins, which bind to the receptors in the

Table 2.1 - Natural products used as active substances in PPP biopesticide.

Type	***Active Substance***	***Name Product***	***Target Pest***
insecticide	azadirachtin	Azatin XL	aphids, scale, thrips, whitefly, leafhoppers, weevils
fungicide	*Reynoutria sachalinensis* (extract)	Regalia	powdery mildew, downy mildew, *Botrytis*, late blight, citrus canker
herbicide	citronella oil	Barrier H	ragwort
nematicide	*Quillaja saponaria*	Nema-Q	plant parasitic nematodes
attractant	citronellol	Biomite	tetranychid mites

insect's gut cells causing ion leakage through the ion channels in the gut membrane (Tabashnik *et al*, 1990; Koziel *et al*, 1993; Estruch *et al* , 1996; Crickmore *et al* , 1998; E Schnepf *et al* , 1998; Bravo, Gill and Soberón, 2007). Thus, Bt toxin products became an

efficient tactic for pest management on fruit and vegetable crops. Furthermore, the fusion proteins (FP) are a novel class of biopesticides which became more popular in the last few years. FP are synthetic proteins designed to enhance their intrinsic properties. This novel class of biomolecules has more than one protein domain integrated into one molecule, joined by a linker peptide (Chen, Zaro and Shen, 2013; Yu *et al.*, 2015). These domains are naturally occurring, and synthesized by different organisms, encoded by the fusion of genes of different organism. The joining of more protein domains produces novel functional combinations with a wide range of biopesticide applications. For instance, the fusion proteins Pl1a/GNA and Hv1a/GNA contain the spider venom peptides PI1a or Hv1a respectively, linked to a carrier protein (GNA) extracted from snowdrop which improves the venom uptake in the pest's gut (Fitches *et al.*, 2004, 2012; Pyati, Fitches and Gatehouse, 2014; Yang *et al.*, 2014).

b) Plant-Incorporate Protectants (as PIPs):

Plant-incorporated protectants (PIPs) are genetic modified plants (GMPs) in which exogenous genetic sequences encoding for pest-controlling active substances are transferred to plants. Thus, GM crops are capable to produce the active substances conferring them resistance against pests (Lövei, Andow and Arpaia, 2009). Among transgenic plants, GM crops with *Bacillus thuringiensis* (Bt) toxin proved a success, therefore it was one of the most frequently used toxins in genetically engineered plants. There are different varieties of GM Bt crops which are capable to release crystal proteins (Table 2.2) as (Bt) toxins form root exudates (E. Schnepf *et al.*, 1998; Saxena, Flores and Stotzky, 1999). In addition, a new generation of GM plants with RNAi-based crop protection has been recently developed and commercialised (see paragraph below).

c) Biocontrol organisms

Some microbial insecticides are based on living organisms like bacteria, fungi and viruses (Table 2.3). For instance, baculoviruses (e.i polyhedroviruses and granuloviruses) are utilised as biopesticides on vegetable crops, cotton plants, and ornamental plants within IPM schemes. Arthropod-specific viruses are used to control lepidopteran pests (Inceoglu *et al.*, 2001). Furthermore, fungi are also a well-known category of pest control, including different strains such as; (1) *Trichoderma spp*., which is commonly used as biofertilisers and soil amendments, improving the soil microenvironment by degrading polysaccharides, hydrocarbons, and chlorophenolic compounds (Harman *et al.*, 2004), (2) *Candida oleophila* is a commercial yeast that acts in competition for nutrients against other fungi. It is used to enhance the resistance of citrus to decay (Bar-Shimon *et al.*, 2004), (3) *Ampelomyces quisqualis* is used as natural herbicide. In presence of the right amount of humidity, it grows

on host plant surfaces penetrating into plant's hyphae degenerating hyphae cells (Szentivanyi and Kiss, 2003).

Table 2.2 - Cry proteins expressed by GM plant based on B. thuringiensis.

Protein	***Insect Spectrum***	***Mass(kDa)***
CyIAa	Lepidoptera	133.2
CryIAb	Lepidoptera	131.0
CryIAc	Lepidoptera	133.3
CryIBa	Lepidoptera	138.0
CryICa	Lepidoptera	134.8
CryIDa	Lepidoptera	132.5
Cry2Aa	Lepidoptera /Diptera	70.9
Cry2Ab	Lepidoptera	70.8
Cry3Aa	Coleoptera	73.1
Cry3Ba	Coleoptera	74.2
Cry4Aa	Diptera	134.4
Cry4Ba	Diptera	127.8
Cry10Aa	Diptera	77.8
Cry11Aa	Diptera	72.4
Cry11Ba	Diptera	80
CytIAa	Diptera /others	27.4

Table 2.3 - Different microorganism used as commercial product biopesticide.

Microorganism	***Type***	***Strains***	***Name Product***	***Target Pest***
bacteria	insecticide	*Bacillus thuringiensis* var *kurstaki*	Dipel DF	caterpillars
	fungicide	*Bacillus subtilis* QST713	Serenade ASO	*Botrytis* spp.
	nematicide	*Pasteuria usgae*	*Pasteuria usgae BL1*	sting nematode
fungi	insecticide	*Beauveria bassiana*	Naturalis L	whitefly
	fungicide	*Coniothyrium minitans*	Contans WG	*Sclerotinia* spp.
	herbicide	*Chondrostereum purpureum*	Chontrol	cut stumps of hardwood trees and shrubs
	nematicide	*Paecilomyces lilacinus*	MeloCon WG	plant parasitic nematodes in soil
viruses	insecticide	*Cydia pomonella* GV	Cyd-X	codling moth
	anti-viral	zucchini yellow mosaic virus, weak strain	Curbit	zucchini yellow mosaic virus
oomycetes	herbicide	*Phytophthora palmivora*	DeVine	*Morenia orderata*

2.2. Biopesticide regulation within the European Union.

Currently, European Regulation (EC) No. 1107/2009 regulates the active substances in plant protection products (PPPs). The regulation does not provide a formal definition of biopesticides and doesn't discern between synthetic pesticides and biopesticides. Thus, it applies to all substances, including micro-organisms, with general or specific action against living organisms or plants as part of active substances of PPPs. Therefore, biopesticides also are covered by this regulation in the European Union. By contrast, GM plants encoding active substances are regulated under the GMO directive 2001/18/EC.

2.3. RNA Interference

RNA interference (RNAi) is a well-conserved mechanism that eukaryotic cells employ to regulate their gene expression (Meister and Tuschi, 2004). The complex mechanism is activated by double stranded RNA (dsRNA) precursors, operating via base-pairing with complementary sequences within targeted messenger RNA molecules (mRNA). This fascinating biological mechanism was discovered in the late 1990s in the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998). As consequence, the two scientists Fire and Mello shared the Nobel Prize in 2006. Further studies then demonstrated that the RNAi effect could also be activated in animals, plants and insects (Ratcliff, 1997; Terenius *et al.*, 2011; Kim *et al.*, 2015; Mitter *et al.*, 2017). The RNAi follows different pathways to silence the mRNA. The transcript can be degraded by an enzymatic complex (Castel and Martienssen, 2013), or it can be destabilised by inducing the inhibition of translation initiation and poly(A) shortening (Filipowicz, Bhattacharyya and Sonenberg, 2008). Thus, it was revealed to operate via post-transcriptional gene silencing (PTGS). However, further studies showed that the RNAi machinery can also operate by transcriptional gene silencing (TGS), for instance occurring through DNA methylation (Meister and Tuschi, 2004; Sampey *et al.*, 2012). In the following decade, the biomolecular mechanism was investigated more deeply revealing new insights. Studies showed that there were three different categories of dsRNAs, called non-coding RNAs, capable to trigger the RNAi effect; microRNA (miRNA), small (or short) interfering RNA (siRNA) and PIWI-interacting RNA (piRNA). The microRNA are endogenous RNA molecules, about 20–23 nucleotides in length, generated in the nucleus. Once transcribed as single strand RNA, microRNA folds back on itself to form dsRNA with a sterm-loop (Bartel, 2009). In general, they are responsible for the proliferation, death, and metabolism of eukaryotic cells. Furthermore, it has been discovered that the human genome may encode nearly one thousand miRNAs, and a single miRNA can regulate multiple mRNAs modulating more than half of the protein coding genes. Also, miRNAs have imperfect complementarity to mRNA targets that make it more difficult to predict the targeted transcript (Zheng *et al.*, 2013). PIWI-interacting RNAs (piRNAs) are another class of non-coding RNAs involved in nuclear processes of genome stability, repressing transposable elements in animal germ lines (Weick and Miska, 2014). Small interference RNA (siRNA) are instead derived by long sequence of dsRNA, assisted by an endoribonuclease called Dicer, which reduces the long sequence of dsRNA into segments of 20-22 nucleotides length (siRNA). Each siRNA has perfect complementarity to the mRNA-sequence, which means it modulates one specific transcript. The siRNA biogenesis differs according to different species, thus the endonuclease occurs in the nucleus or cytoplasm (Castel and Martienssen,

2013). Since the dsRNA-based biopesticides are exogenous long-sequences processed into siRNA, the further literature review is focused on this mechanism.

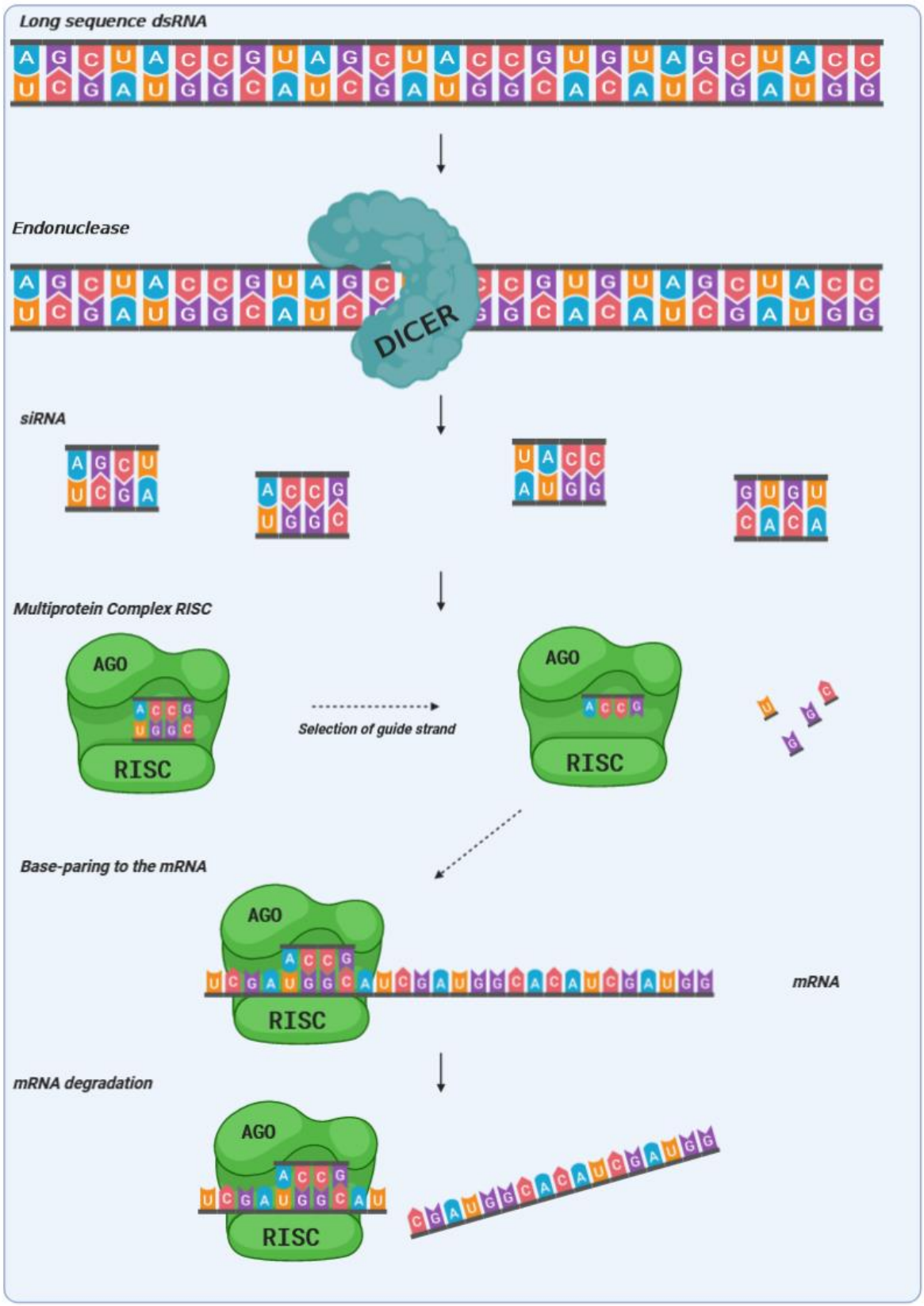
2.3.1. RNAi mechanism

To downregulate a specific gene expression, a dsRNA-sequence needs to be complementary to the mRNA target. Generally, the RNAi follows three major steps (Figure 2.1 – Mechanism of RNA interference.Figure 2.1). Once in the cytoplasm, (1) the dsRNA precursors are reduced into segments of 20-22 nucleotides length by the endoribonuclease Dicer, depending on the species (Santos *et al.*, 2019). It is a RNase III-like enzyme containing catalytic RNase III and dsRNA-binding domains, capable to cleave long dsRNA sequences into a RNAs duplex, called siRNAs (Bernstein *et al.*, 2001).Thus, the endonuclease cleaves from the 3'end of the sequence (Vermeulen, 2005), leaving phosphate groups at their 5' ends and 2 nucleotide overhangs at their 3' ends (Meister and Tuschi, 2004). Subsequently, (2) siRNAs are assembled into a multi-protein complex called RNA-induced silencing complex (RISC) (Meister and Tuschi, 2004). The RISC includes an Argonaute protein (AGO), which unwinds the siRNA selecting the complementary strand using it as a guide for recognising the sequence of mRNA targets by base-pairing. Once the mRNA has been thus identified, (3) the RISC performs the sequence-specific degradation of complementary RNA or mediates the translational repression (miRNA pathway).

2.4. RNAi-based biopesticide

RNAi is a biological mechanism well-conserved among species (plants, mammals, insects, fungi) which can nowadays be used for pest control (Fletcher *et al.*, 2020). In recent years, scientists have used RNAi as a tool to suppress gene functions in pests (Bellés, 2010). In 2007, the RNAi application was exploited for crop protection via artificial diet (Table 2.4), using transgenic plants. Thus, transgenic maize plants were engineered and able to exude dsRNAs from roots (*snf7* gene), which successfully tackled western corn rootworms (*Diabrotica* spp.) (Baum *et al.*, 2007). After ten years, the first RNAi-based GM maize plant was commercially produced by Monsanto in 2017, with the commercial name of SMARTSTAX PRO (Head *et al.*, 2017). Two years later, more products that were RNAi-based were approved by the US EPA and commercialised for the US market and crops such as potatoes and horticultural plants, expressing dsRNAs to reduce the level of several enzymes for crop quality enhancement (Waltz, 2015; Baranski, Klimek-Chodacka and Lukasiewicz, 2019).

Figure 2.1 – Mechanism of RNA interference.



Within the European Union, several RNAi products have been approved by EFSA, however they can only be used as food (or for feeding), but not for cultivation (Table 2.5). Many studies also focused on the foliar application of dsRNA, targeting plant viruses such as the pepper mild mottle virus (PMMoV), alfalfa mosaic virus (AMV), tobacco etch virus (TEV) and fungal pathogens (Lau *et al.*, 2014; Koch *et al.*, 2016; Mitter, Elizabeth A Worrall, *et al.*, 2017). The most demanding RNAi application is against arthropods (Fletcher *et al.*, 2020). The topical application and oral delivery of dsRNAs across invertebrates is very challenging because of variability of responsiveness (Joga *et al.*, 2016).

Table 2.4 – RNAi Mechanism identified in different species through dsRNA uptake. (Christiaens et al., 2020)

Order	***Species***	***Environmental RNAi***
Diptera	Drosophila melanogaster	+
	Bactrocera dorsalis	+
Coleoptera	Tribolium castaneum	+
	Diabrotica virgifera virgifera	+
	Leptinotarsa decemlineata	+
Lepidoptera	Spodoptera frugiperda	+
	Bombyx mori	–
Orthoptera	Schistocerca gregaria	–
	Locusta migratoria	–
Hymenoptera	Apis mellifera	+

Hence, the RNAi effectiveness depends by a variety of factors such as the enzymes in saliva's insects, difficulties to reach the organism's gut, and the fast degradation of dsRNA in weathering make the dsRNA uptake challenging. Therefore, scientists are seeking alternative answers for stabilising the dsRNA molecules. One solution might be nanomaterials, such as nanocarriers made of clay nanosheets (BioClay) which might help the dsRNA oral delivery, coating the molecule and gradually releasing it to overtake these environmental and biological barriers (Ghormade, Deshpande and Paknikar, 2011; Kuthati, Kankala and Lee, 2015; Mitter, Elizabeth A. Worrall, *et al.*, 2017).

Table 2.5 – EU ERA evaluation on RNAi-based GM Plants assessed as safety products.

Code Name	***Manufacture***	***Product Name***	***EU ERA evaluation (EFSA)***
Soybean 305423	DuPont	Treus™,	(EFSA, 2013)
Soybean 305423 × 40-3-2	DuPont	Plenish™	(EFSA, 2016)
Soybean MON87705	Monsanto	Vistive Gold™	(EFSA, 2012)
Soybean MON89788	Monsanto/Bayer	Intacta RR2 Pro™	(EFSA, 2015)
Maize MON87411	Monsanto	SmartStax Pro™	(Naegeli *et al.*, 2018)
Maize MON87427	Monsanto	Roundup Ready™	
Maize MON89034	Monsanto	YieldGard™ VT Pro™	(Naegeli *et al.*, 2019)
Maize MIR162	Syngenta	Agrisure™ Viptera	
Potato EH92-527-1	BASF	Amflora™	(EFSA, 2004)

2.5. Environmental Fate of RNAi-based biopesticides.

RNAi is a promising biological technique with an ongoing development of adapting it as crop management tool. Environmental risk assessment (ERA) is to evaluate whether agrochemical products are beneficial for agriculture and at the same time safe for the environment. Thus, when conducting an efficient ERA, an environmental fate study must be conducted for each of different environmental compartments such as soil, surface water, sediments, groundwater and air.

2.5.1. Extraction and quantification methods of dsRNA.

Soil is an extremely complex environment, and the nucleotide isolation is very challenging because of adsorption on soil minerals, humic substances, degradation by RNase or unsuccessful cell lysis (Franchi *et al.*, 1999). Therefore, an optimal extraction method aims to avoid these contaminations which could interfere with quantification techniques. Throughout these years, many nucleotide extraction and purification protocols from soil have been reported (Table 2.6). Unfortunately, there is no extraction method suitable for all types of soils. Furthermore, commercial kits are standardised methods which don't allow scientists to adapt the extraction procedure to their experimental conditions (Table 2.7). Thus, researchers have to develop different methodologies for their own purposes. Two extraction methods based on alkaline buffers are currently utilised for dsRNA extraction (Table 2.8). Moreover, three very different quantification techniques have been used, immunoassay, radiolabelling and RT-qPCR. The latter showed high sensitivity at 0.003 ng_{dsRNA}/g_{soil} (Fischer *et al.*, 2016; Parker *et al.*, 2019; Zhang *et al.*, 2020).

Table 2.6 – Selection of nucleotide extraction methods from soil in the current literature.

Published Methodologies	***References***	***Cited***
Flocculation (Al2(SO4)3) with alkaline conditions	(Peršoh *et al.*, 2008)	65
Cetyltrimethyl Ammonium Bromide (CTAB) buffer extraction & Precipitation of RNA by PEG	(Griffiths *et al.*, 2000)	1029
Adsorption by PVPP	(Mendum, Sockett and Hirsch, 1998)	22
Co-precipitation with guanidine hydrochloride	(Hahn *et al.*, 1990)	51
Adsorption with powdered activated charcoal (PAC)	(Desai and Madamwar, 2007)	82
Isolation RNA with CaCl2	(Sagova-Mareckova *et al.*, 2008)	26

2.5.2. Degradation in soil and aquatic systems.

Based on the review of the current literature, the environmental fate of dsRNA in soil has not yet been measured in field experiments (Zhang *et al.*, 2020). The first studies about the environmental fate of dsRNA-based biopesticides were conducted on GM plant MON87411 (SmartStax Pro™ by Monsanto) expressing the *DvSnf7* RNA. These laboratory tests showed a

rapid degradation in soil (Dubelman *et al.*, 2014; Fischer *et al.*, 2016). Dubelman *et al*, 2014 examined the degradation in three different agricultural soils, and found a DT_{50} of 27h (loamy sand), 19h (silt loam) and 15h (clay loam).

Table 2.7 - Selection of commercial nucleotide soil extraction kits

Soil Extraction Kits
PowerSoil™ Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA)
FastRNA® Pro Soil-Direct kit (MP Biomedicals, Solon, OH, USA)
FastRNA® Pro Soil-Indirect kit (MP Biomedicals, Solon, OH, USA)
E.Z.N.A.® Soil RNA kit (Omega Bio-tek, Norcross, GA, USA)
ISOIL for RNA, NIPPON GENE (Tokyo, Japan)
IT 1-2-3 Platinum PathTM Sample Purification kit (Idaho Technology, Salt Lake City, USA)
Soil Total RNA Purification Kit Norgen (Thorold, ON, Canada)
ZR Soil/Fecal RNA MicroPrep Zymo Research (Orange, CA, USA)

Table 2.8 – Procedures for dsRNA extraction and quantification from soil.

Extraction Method	***Quantification Method***	***Type***	***Reference***	***LoD (Limit of Detection)***
PBST(1) pH 7	QuantiGene Analysis	Immunoassay	(Dubelman *et al.*, 2014; Fischer *et al.*, 2016)	25 $ng_{dsRNA}\ g_{soil}^{-1}$
5mM MOPS(2) or MES(3), 10mM NaCl	^{32}Phospate	Radiolabelling	(Parker *et al.*, 2019)	1.5 $ng_{dsRNA}\ g_{soil}^{-1}$
Phenol: Chloroform: Isoamyl alcohol	RT-qPCR	Quantitative Polymerase Chain Reaction	(Zhang *et al.*, 2020)	0.003 $ng_{dsRNA}\ g_{soil}^{-1}$

(1) phosphate buffered saline-tween 20

(2) 4-morpholinepropanesulfonic acid (MOPS)

(3) 4-morpholineethanesulfonic acid (MES)

Thus, the half-life was reached in less than 30 h, and *DvSnf7* RNA was non-detectable after 48 h. Moreover, it was also demonstrated that the degraded molecule (at DT_{50} value) lost its functional activity against the target pest (western corn rootworms) during bioassay experiments. Similar degradation activity was also proven in Brazilian tropical soils, with a *DvSnf7* dsRNA dissipation characterized by DT_{50} = 22h (sand soil) and DT_{50}= 16 h (sand clay soil) (Joaquim *et al.*, 2019). Further studies showed that the degradation kinetic was independent of dsRNA length, sequence and structure (hairpin and linear) (Fischer *et al.*, 2016). In addition, environmental fate studies have been conducted in aquatic microcosm, which also confirmed a rapid degradation in the aquatic compartment (Albright *et al.*, 2017; Fischer *et al.*, 2017). Albright *et al.* 2017 investigated the partitioning of non-bioactive dsRNA between the water column and sediments, mimicking a spry-drift application or soil run-off in surface waters. The aquatic system was set up as pond water/pond sediment, and the dsRNA persisted in the microcosm for less than 60 h. Thus, the major degradation occurred in the water system

due to the biotic factors, because only 3% of the dsRNA was detected in the sediment. The fast degradation in aquatic systems was also verified in a different study, which confirmed the dsRNA degraded in less than 3 days (Fischer *et al.*, 2017). All these studies highlighted the low dsRNA persistence in soil and the aqueous phase.

2.5.3. Degradation of foliar-applied dsRNAs.

The foliar application of dsRNA-based biopesticides is the most relevant solution in terms of pest control. Sprayable pesticides are simpler to use and less expensive than GM plants, which undergo more restrictive ERA evaluation (under GMO regulation). Moreover, the formulation can incorporate more than one active substance. However, dsRNA studies of this application method reported to be inconsistent in terms of stability and insect oral delivery. A pilot study in an open field in Puerto Rico conducted by Bayern Crop Science tested the dsRNA foliar application, sprayed on leaf surfaces of soybean plants, under natural weathering (Bachman *et al.*, 2020). The study showed rapid degradation of dsRNA in a topical application (DT_{50} = 0.7 days, DT_{90} = 1.9 days). These results were in contrast with (Mitter, Elizabeth A. Worrall, *et al.*, 2017), where under lab condition the topical application lasted 5 days, and 28 days in greenhouse experiments (San Miguel and Scott, 2016). The inconsistency of these studies suggests that natural weathering conditions, such as the photo-degradation (UV light), wash-off due to rain and microbial activity, accelerate the dsRNA instability.

2.6. PhD Research and Gaps

In recent years, the use of novel dsRNA-based biopesticides as pest management tools has provided new research opportunities but basic questions still need to be addressed, such as (1) the environmental safety, (2) non-target organism (NTO) exposure and effects, and (3) risk for human health (Mendelsohn *et al.*, 2020). The double strand RNA is a macromolecule, which differs substantially from synthetic pesticides in terms of molecular weight. These macromolecules are made up by long chains of nucleotides with high molecular weight, containing a much larger number of chemical moieties that could contribute to sorption and degradation than conventional pesticides. In the area of environmental risk, there are several unanswered questions regarding sorption, transformation and exposure to NTO (Parker and Sander, 2017; Mendelsohn *et al.*, 2020). For conventional pesticides, the sorption in water-soil systems usually is mainly due to two mechanisms, *adsorption* and *absorption* into organic matter sorbents, due to the pesticides low size and molecular weight. Macromolecules such as dsRNA may have reduced absorption, by having a major interaction on the soil surface with mineral colloids which might then lead to their mobility and degradation in soil (Pietramellara *et al.*, 2009; Yu *et al.*, 2013). Therefore, developing appropriate sorption models that take into

account these interactions is still an area of research. Furthermore, the efficiency of extraction and quantification are additional areas to improve testing protocols in future years. GM Plants exude low amount of active substances which are arduous to detect. Currently, most studies conducted on the environmental fate utilised QuantiGene assays having a limit of detection of 25 ng g^{-1}. Developing a methodology which allows scientist to extract and detect lower amounts held in the soil matrix such as quantitative polymerase chain reaction, would improve the quality of the environmental risk assessment. Furthermore, at EU regulation level the approval of new active substance for the use of plant protectant product (PPP) follows standardised procedures (OECDs), which are well-established for pesticides, but less for macromolecular biopesticides. Therefore, the reliability of these procedure needs more investigation, seeking and avoiding any bias that might occur in the standardised methods.

References Chapter 2

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CHAPTER 3

Method Development

3.1. Introduction

Nucleic acids extraction is a well-known tool applied to different fields of environmental studies. Because of the complexity of environmental samples, the extraction yield and purity of nucleic acids change drastically according to the diverse chemical-physical features, mineralogy, and amount of organic matters of soil samples. Furthermore, the downstream processes, like (RT) PCR or (RT) qPCR are very sensitive techniques that can be inhibited by the sample content of humic substances, polysaccharides, proteins or lipids co-extracted with nucleic acids. Many methods have been published with different procedures (R. I. Griffiths *et al.*, 2000; Takada Hoshino and Matsumoto, 2005; Wang *et al.*, 2009; Yankson and Steck, 2009; Paulin *et al.*, 2013; Lever *et al.*, 2015a), with the aim to improve the nucleic acid yield and quality, but none of them have been adopted as universal standard procedure. This suggests that the extraction procedure needs to be optimised for each type of sample.

Generally speaking, the extraction protocol employs three specific steps. The first one is the cell lysis, in order to release the nucleic acids from within the cell wall to the environment, and it can be performed by mechanical or enzymatic processes. During the extraction, buffer solutions assisted by organic or detergent substances, like phenol, chloroform or cetyl trimethylammonium (CTAB) can help to reduce the concentration of humic substances, or proteins in the aqueous phase. An alternative procedure can be achieved through filtration with silica or ion exchange columns. At this point the sample is precipitated using different chemicals, like sodium acetate or ammonium acetate in order to neutralise the negative charges of the nucleic acid phosphate groups making the acids less soluble in water. The last step is to wash the pellets from salt residuals using a solution with a low dielectric constant (ε) like ethanol or isopropanol. Only few studies have investigated the environmental fate of dsRNA in soil and sediments (Dubelman *et al.*, 2014; Fischer *et al.*, 2016, 2017; Parker *et al.*, 2019), suggesting different extraction and quantification procedures. (Dubelman *et al.*, 2014; Fischer *et al.*, 2016, 2017) proposed the extraction in 1x PBST at pH 7.0 (pH 12.0 for clay soil), and filtering the aqueous phase through a 0.22 μm vacuum filter. The samples were purified with a buffered phenol-chloroform solution in order to eliminate the humic substances. To quantify the dsRNA, they used a QuantiGene 2.0 assay for gene expression quantification. Their procedure has similarity with an ELISA-like workflow, setting a target-specific probe to hybridize the dsRNA sequence. This can explain the choice in using the PBST buffer, usually utilised as wash buffer for ELISA, western blotting and other immunoassays. On the other hand, (Parker *et al.*, 2019) have utilised a different approach, labelling the dsRNA with 32-phosphorus (^{32}P), and pre-incubating the samples in organic buffer.

In order to follow the OECD 106 guideline for the evaluation of the environmental fate of crop protection products, we decided to adopt another approach, and quantify the dsRNA by performing RT-qPCR (RT-PCR) which is a well-known technique utilised to quantify gene copies and gene expression. Regarding the extraction procedure, our attention was drawn to the phenol, chloroform and phosphate buffer based procedure which is modified from the (R. I. Griffiths *et al.*, 2000) protocol, due to the ability of phosphate buffer to desorb the acids nucleic for clay minerals (Yankson and Steck, 2009) and the cationic surfactant to remove part of the humic acids in water samples (Brum and Oliveira, 2007). In this chapter, we will describe the method to extract and quantify the dsRNA from a standard soil, loamy sand, in order to be able to apply the OECD 106 guideline.

3.2. Material and Methods

3.2.1. Reagents, kits, equipment and laboratory environment

Table.3.1 lists the solutions, reagents, chemical kits, and laboratory equipment used to assess the sorption and biodegradation of double stranded RNA extracted from the soil. All experiments were performed in a Rnase and Dnase free environment, using DEPC-water, and molecular biology grade reagents. All solutions were prepared fresh, on the day of experiment, from the stock solutions and filtered through 0.22 µm filters (Millipore, UK).

3.2.2. Double stranded RNA sequence.

The dsRNA selected for the environmental fate assessment was previously evaluated by (Michelle E. Powell *et al.*, 2017) as an effective biopesticide against *Aethina tumida*, a small beetle, leading to 100% larvae mortality if injecting 5mg, whereas 50% mortality was achieved with oral delivery. The present dsRNA tackles the V-ATPase subunit A, a conserved evolutionarily enzyme complex which generates a proton gradient used for transport processes in eukaryotic organisms. The sequence (305 bp, Table 3.2) was synthetized by Genelution Ltd (South Korea) with a final concentration of 9.945 µg uL^{-1} (Nanodrop) according to them. The dsRNA standard was aliquoted in 2 mL tubes and stored at -80 °C. For everyday experiments, the standard was diluted 20 folds and stored at -20 °C.

3.2.3. Nanodrop quantification and copies numbers

The dsRNA standard concentration was verified by UV-Spectrophotometry (Devonix DS-11 FX) according to (Nwokeoji et al., 2017) who have evaluated the hypochromicity for the dsRNA duplex RNA/RNA. According to them, to accurately quantify the dsRNA, the absorbance A_{260} was multiplied by the average of the extinction coefficient (46.18–47.29

μg/mL/A_{260}). To express the concentration in gene copies per uL^{-1} we used equation (eq.3.2.1.1), taking into account the average molecular weight of each purine (Table 3.3).

Table.3.1 - List of chemical reagents

Stock Solution, CTAB 20% (w/v)	**Amount**	**Supplier**
Hexadecyltrimethylammonium bromide	4.00g	Sigma-Aldrich
DEPC-Water	20.00 mL	VWR
Stock Solution, KH_2PO_4, 1M		
Potassium dihydrogen phosphate	1.36g	Sigma-Aldrich
DEPC-water	10.00 mL	VWR
Stock Solution, K_2HPO_4, 1M		
Potassium phosphate dibasic $3H_2O$	2.28g	Sigma-Aldrich
DEPC-water	10.00 mL	VWR
Stock Solution PEG6000 in 1.6M NaCl		
Polyethylene glycol 6000	6.00g	Fischer Scientific
Sodium chloride	1.87g	
DEPC-water	20.00 mL	VWR
Ethyl Alcohol 70% (v/v)		
Ethyl alcohol 100%	7.00 mL	Sigma-Aldrich
DEPC-water	3.00 mL	VWR
Soil Buffer Extraction		
(CTAB 5%, 0.35M NaCl, 120 mM, pH 8.0 Phosphate Buffer)		
Vol. CTAB 10% (w/v) in 0.7M NaCl		
CTAB 20% (w/v)	5 mL	Sigma-Aldrich
Sodium chloride	0.41g	Sigma-Aldrich
DEPC-Water	5mL	VWR
Potassium phosphate buffer 240 mM, (pH 8.0)		
KH_2PO_4, 1M	0.12 mL	Sigma-Aldrich
K_2HPO_4, 1M	2.28 mL	Sigma-Aldrich
DEPC-Water	7.60 mL	VWR
Supernatant Buffer Extraction		
(CTAB 10%, 0.7M NaCl, 240 mM, pH 8.0 phosphate buffer)		
CTAB 20% (w/v)	5.00 mL	Sigma-Aldrich
Sodium chloride	0.41g	Sigma-Aldrich
KH_2PO_4, 1M	0.33 mL	Sigma-Aldrich
K_2HPO_4, 1M	2.06 mL	Sigma-Aldrich
DEPC-water	2.60 mL	VWR

Table 3.2 - Double Stranded RNA sequence (305bp)

5'GGUGUAACAGUUGGUGAUCCGGUGUUGCGUACCGGUAAACCCUUGUCCGUCGAAUUGGGACC
UGGUAUUAUGGGUUCAAUUUUCGACGGUAUCCAACGUCCGUUGAAAGACAUCAACGAUUUGAC
CCAGAGCAUUUACAUUCCCAAGGGUGUGAACGUGCCCGCCCUUUCGAGGACGGCCAAAUGGGAA
UUCAAUCCGUGGAACAUCAAAUUGGGAGCUCACUUAACGGGAGGUGACAUCUACGGUAUCGUC
CACGAAAACACCCUGGUGAAACACAAAAUCGUCCUGCCACCUAAAGCCAAGGG **3'**

Table 3.3 - Molecular weight of each base

Base	***g mol^{-1}***	***n. bases***	***Average for each base (g/mol)***
A	347.2	81	28123.20
C	323.2	75	24240.00
G	363.2	78	28329.60
U	324.2	71	23018.20
Average			**207581.00**

$$\frac{\frac{ng}{uL} * Avogrado\ number}{Lenght\ gene * Average\ of\ each\ bases\ \frac{g}{mol} * 10^9} \quad \text{(eq.3.2.1.1)}$$

3.2.4. Standard soil samples

A standard soil (loamy sand) was purchased from LUFA Speyer (Germany) with the following characteristics (Table 3.4). Part of the soil used for the sorption experiments was stored at 4°C and the soil stock was stored at -20°C for further experiments. Before proceeding with the experiments, a portion of soil was autoclaved twice at 121°C for 30 min, and incubated at 37°C overnight in between, then dried overnight at 105°C before the sorption experiment. The live soil stock used for the biodegradation experiment was stored at 4°C.

Table 3.4 - Standard soil; This table represents the mean values of different batch analyses ± standard deviation. All values refer to dry matter

Standard Soil Type	***n. 2.1***
Sampling Data	
Organic Carbon (%C)	0.71 ± 0.08
Nitrogen (%N)	0.06 ± 0.01
pH value (0.01M $CaCl_2$)	4.9 ± 0.3
CEC (meq/100g)	4.3 ± 0.6
Particle size (mm) distribution according to USDA (%):	
<0.002	3.0 ± 0.9
0.002 - 0.05	11.0 ± 1.3
0.05 - 2.0	86.0 ± 0.9
Soil type	Loamy Sand
Maximum water holding capacity (g/100g)	32.1 ± 1.7
Weight per volume (g/1000ml)	1437 ± 41

3.2.5. Mass balance assays

Nucleic acids extraction involves laborious procedures to recover and clean up as much DNA (or RNA) as possible from samples. Chemicals, enzymes and commercials kits are utilised to extract, preserve and purify the molecules from environmental inhibitors like organic and inorganic compounds that might inhibit downstream processes. All these harsh and long procedures might result in losses and have a negative effect on dsRNA recovery. Therefore, a mass balance of known dsRNA additions was carried out for each extraction step in order to identify what chemical or commercial kits might improve dsRNA recovery. All mass balance experiments were performed in triplicate, using the loamy sand standard soil, and dsRNA was quantified with the Qubit assay.

3.2.5.1. Qubit quantification

The Qubit™ microRNA HS Assay Kit (Invitrogen, UK) was used to quantify the dsRNA recovered during the mass balance experiments. The measurements were performed according to the manufacturer protocol, and taking into account the range of tolerated substance by the

assay kit. The measurements were performed utilising the Qubit 2.0 Fluorometer (Invitrogen, UK).

3.2.5.2. Precipitation assay

The precipitation assay was optimized by comparing three chemicals; sodium acetate, ammonium acetate, and polyethylene glycol 6000 at different temperatures, incubation times and glycogen amount (Table 3.5).

Polyethylene glycol; eight hundred microliter of PEG6000 (30%) in 1.6 M NaCl solution were mixed in 400 µL dsRNA solution containing ~170 ng dsRNA. Two different glycogen amounts were tested, 100 ug and 20 ug. Then, samples with different glycogen amounts were stored either at room temperature for 3 h, or overnight in ice. Afterwards they were centrifuged at 20000 x g for 20 min. The pellets were resuspended in 70 µL DEPC-water and quantified.

Sodium and ammonium acetate; Forty microliter of 3M sodium acetate was mixed with 400 µL of dsRNA solution at concentration of 0.425 ng μL^{-1}. Then 900 µL of ethanol 100% (v/v) (Sigma Aldrich - UK) was added to the vial. One-hundred micrograms of glycogen were added. Triplicates were stored at room temperature for 3 h, and another identical set of triplicates in ice overnight. Afterwards samples were centrifuged at 20000 x g for 20 min at 4 °C and the pellets were resuspended in 100 µL DEPC-water. The same procedure was followed for 5 M ammonium acetate (Invitrogen, UK).

Table 3.5 - Permutations of the precipitation assay.

		Sodium Acetate (3M)	***Ammonium Acetate (5M)***	***PEG (30% in 1.6M NaCl)***
T (°C)	**20**	✓	✓	✓
	4	✓	✓	✓
Incubation	**3h**	✓	✓	✓
	Overnight	✓	✓	✓
Glycogen (ug)	**100**	✓	✓	✓
	20	-	-	✓

3.2.5.3. Glass beads assay

The glass beads assay was performed in triplicate as follows; test assay (soil + dsRNA + beads), blank sample (soil + beads), known control (soil + dsRNA, no glass beads).

One-hundred and ten nanograms of dsRNA were spiked into soil and 10 glass-beads (3 mm, VWR), sterilized at 200 °C for 1 h, were introduced in microtube samples. The glass beads test followed the dsRNA extraction procedure mentioned below. The samples were resuspended in 100 µL DEPC-water and quantified.

3.2.5.4. Phase lock gel assay

Phase Lock Gel (PLG) microtubes enable a higher recovery of acids nucleic in water solution mixed with an organic phase (Phenol:Chloroform), avoiding interphase contamination during the separation of the two phases. This mass balance assay was performed to comprehend what

type of PLG most suitable for dsRNA recovery. Two types of PLG ware tested; PLG high salt content (Qiagen,UK), and PLG low salt content (VWR, UK). Both were compared with manually pipetting. For the latter, 1.5 mL low DNA binding polypropylene (PP) microtubes were used.

Three-hundred and twenty nanograms dsRNA were added in 250 µL buffer extraction and 250 µL PCI solution, each in triplicates. The solution was mixed manually and then centrifuged at 16000 x g for 10 min at 4 °C. The upper aqueous phase was transferred into a new PGL tube, or in the case of the manual pipetting test in a new 1.5 mL tube, and mixed with an equal volume of Chloroform:Isomayl Alcohol (24:1). The samples were centrifuged at 16000 x g for 5 min at 4 °C, recovering different volumes as follows; ~100 µL from manually pipetting, ~260 µL from PLG high salt content, and ~200 µL from PLG low salt content.

3.2.5.5. Purification assay

Acids nucleic purification is the last step before performing any molecular biology analysis. It is important to remove all inhibitors that might impede or reduce the efficiency of downstream processes.

Two commercial kits (RNeasy Mini Kit (Qiagen, UK) and QIAquick Nucleotide Removal Kit (Qiagen, UK)) were compared with ethanol 70% (v/v) in order to evaluate the procedure with the best dsRNA recovery.

RNeasy mini kit: The precipitated samples from the precipitation recovery assay using polyethylene glycol (glycogen final concentration at 73.5 µg mL^{-1}) were used. Ninety-nine microliter of dsRNA-sample were purified according to the manufacturer protocol and eluted in 20 µL.

QIAquick kit: The precipitated samples from the glass beads assay were used. Ninety microliter were purified according to the manufacturer protocol and eluted in 100 µL.

Ethanol 70% (v/v): One-hundred and eighty nanogram dsRNA were added in 400 µL DEPC-water. Eight-hundred microliter of PEG6000 in 1.6 M NaCl were added to the dsRNA solution with 100 µg of glycogen (Invitrogen, UK). The samples were incubated in ice overnight and then centrifuged at 20000 x g for 20 min at 4 °C. The supernatant was carefully removed and 1 mL cold ethanol 70% (v/v), was added to wash the pellet. They were briefly vortexed and centrifuged at 20000 x g for 20 min at 4°C. Afterwards, the ethanol solution was carefully removed by pipetting, without disturbing any pellets. The samples were air-dried for 15 min and resuspended in 100 µL DEPC-water.

3.2.6. Double stranded RNA extraction

The dsRNA was extracted mainly following the protocol of (R.I. Griffiths et al., 2000), and improvements were carried out following the mass balance experiments (see paragraph 3.2.5). For the sorption and biodegradation of loamy sand soil, ten 3mm glass-beads (VWR, UK) were sterilized at 200 °C for 1 h, and placed in a 2 mL low binding protein polypropylene (PP) microtube with 0.2 g soil sample. Then 0.2 mL of soil extraction buffer (CTAB 5% (w/v), 0.35M NaCl, 120 mM potassium phosphate buffer at pH 8.0 and 0.2 mL of phenol:chloroform:isomayl alcohol (25:24:1), pH 8.0 (VWR, UK) were added to the microtube. The vials containing the soil samples were vortexed with a vortex adaptor (MoBio, UK) for 5 min at max speed and centrifuged at 16000 x g for 10 min at 4 °C. The upper aqueous phases were transferred to 2 mL phase lock gel tubes (Qiagen, UK) adding an equal volume of chloroform:isomayl alcohol (24:1) and centrifuged at 16000 x g for 5 min at 4 °C. Afterwards the extracted dsRNA was precipitated with 2 volumes of PEG6000 30% (w/v) in 1.6 M NaCl solution and 10 mM $MgCl_2$, followed by the addition of glycogen (Invitrogen, UK) to reach the concentration of ~80 µg mL^{-1}. The samples were incubated in ice overnight and then centrifuged at 20000xg for 20 min at 4 °C. Afterwards, the supernatant was carefully removed without disturbing the pellets that were washed with 0.6 mL of ethanol 70% (v/v) and centrifuged again at 20000 x g for 20 min at 4 °C. The ethanol solution was discarded and the pellets air-dried for 30 min. The extracted-dsRNA was resuspended in 100 µL or 80 µL of DEPC-water. For extraction of the supernatant, 200 µL of supernatant were mixed with 200 µL of supernatant extraction buffer (CTAB 10% (w/v), 0.7 M NaCl and potassium phosphate buffer, 240 mM at pH 8.0) and 200 µL phenol:chloroform:isomayl alcohol (25:24:1) pH 8.0 (VWR, UK). Then the extraction followed the same procedure described above.

3.2.7. Amplification and quantification of dsRNA-fragments

For all sorption and biodegradation experiments, the dsRNA-fragment was quantified by Reverse transcriptase - Real Time Polymerase Chain Reaction (RT-qPCR).

3.2.7.1. Design and synthesis of oligonucleotides for PCR and qPCR

For the quantification of gene copies, two set of different primers were used to assess the sorption and biodegradation (Table 3.6). The sorption qPCR oligonucleotides were designed using the Primer 3 web version 4.1.0 part of services provided by ELIXIR - European research infrastructure for biological information. Criteria for choosing primers were set as follows: Primer length (bp); minimum 18, optimum 20, maximum 23. Melting point; minimum 57 °C, optimum 59 °C, maximum 62 °C. GC% – minimum 30, optimum 50, maximum 70. The primers were selected in order to amplify a length of 109 bp, ranging between 100 bp and 150 bp

according to MIQE ('Minimum Information for Publication of Quantitative real-time PCR Experiments'). The biodegradation qPCR primers were selected according to (Michelle E. Powell *et al.*, 2017) to amplify the whole dsRNA. Primers were synthesised by Thermofisher (UK) and diluted according to the supplied protocol to a final concentration of 100 µM.

Table 3.6 - List of Primers

PCR and qPCR Biodegradation (Michelle E. Powell *et al.*, 2017)	**Sequence**	**Amplicon Size**
V-type proton ATPase (F)	GGTGTAACAGTTGGTGATC	305bp
V-type proton ATPase (R)	CCCTTGGCTTTAGGTGGCA	
PCR and qPCR Sorption		
V-type proton ATPase (F)	TTCGACGGTATCCAACGTCC	109bp
V-type proton ATPase (R)	ATTCCCATTTGGCCGTCCTC	

3.2.7.2. Reverse transcriptase (RT)

The dsRNA molecules were synthesised in cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, United Kingdom). Before performing the RT, 2 µL dsRNA were denaturised in ssRNA at 98 °C for 5 min in 8 µL of reaction volume (DEPC-water). At the end of the denaturation process, the ssRNA were snap-chilled in ice for 2 min. Then the reverse transcription was performed according to the manufacturer protocol. At the end of RT, the cDNA was treated with 3.5U RNase H (New England BioLabs, UK) for 20 min at 37 °C and deactivated for 20 min at 65 °C. Reactions were performed on the Alpha Cycle Thermocycle (PCRmax, UK).

3.2.7.3. Polymerase Chain Reaction (PCR)

The cDNA was amplified with the Phusion™ Hot Start II High-Fidelity DNA Polymerase (Invitrogen, UK). PCR was performed in 20 µL of volume reaction, adding 2 µL cDNA, 0.6 µL DMSO, 1 µL of each PCR primer at 10 µM. All PCR reactions were conducted on the Alpha Cycle Thermocycle (PCRmax, UK) following the thermal cycling program; denaturation 98 °C for 30 s; amplification (35 times) at 98 °C for 10 s, annealing at 60 °C for 30 s, extension at 72 °C for 10 s; and final extension at 72 °C for 10 min. The amplicons were visualized by agarose gel electrophoresis (1.5%).

3.2.7.4. Real-time polymerase chain reaction (qPCR)

The Real Time PCR reactions were performed in the CFX96 Real-Time System (BioRad) using the SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green (BioRad). All reactions were prepared in triplicate with 2 µL cDNA in 10 µL reaction volume with a final concentration of 1X SsoAdvanced™ Universal Inhibitor Master Mix and 0.5 mM of each reverse and forward qPCR primer. The same procedure was followed for qPCR standards. At the end of the assay the fragments were evaluated by agarose gel electrophoresis (1.5%).

The qPCR thermal cycling program was set as follows: 98 °C for 3 min followed by 35 cycles of 15 s at 98 °C, 1 min at 56.4 °C. A specific melting curve of the amplified product was generated at the end of each qPCR program by including a cycle of 95 °C, 65 °C for 5 s, and incremental increases of 0.5 °C/s to reach 95 °C.

3.2.7.5. qPCR standards and standard curve

qPCR standards were synthetized by amplifying the dsRNA (whole length, 305bp) by RT-PCR as describes in the previous section. The amplicons ware cleaned-up by a MiniElute PCR Purification kit (QIAGEN, UK) following the manufacturer's protocol. The eluted solution was quantified by the Quant-iT™ PicoGreen™ dsDNA Assay Kit according to the manufacturer's protocol in a 96-wells plate, using the SpectraMax M3 (UK).

After running out of the previous stock, a new set of qPCR standards were purchased this time from ThermoFisher (UK) with the same length. Four-hundred nanogram of qPCR standards were diluted in 80 µL, obtaining a stock of $1.01*10^{10}$ copies μL^{-1}. In order to create a standard curve, 7-fold serial dilutions of the cDNA template was prepared and used to determinate their threshold cycle values, efficiency and reproducibility.

3.2.7.6. Optimization of the annealing temperature of qPCR primers.

When performing RT-qPCR it is ideal to optimize the primers annealing temperature (T_a). The optimization was carried out on the two set of primers for the sorption and biodegradation experiments. Two microliter of cDNA was dispensed in a 10 µL reaction volume, containing 1X SsoAdvanced™ Universal Inhibitor Master Mix and 0.5 mM of each qPCR primer. The optimization was carried out on the CFX96 Real-Time System (BioRad) in a 96-wells plate. From each plate, a temperature gradient was set up on each row between 50 °C and 70 °C and a specific melting curve of the amplified product was generated at the end of the qPCR program by including a cycle of 95 °C, 65 °C for 5 s, and incremental increases of 0.5 °C/s to reach 95 °C. Then, the optimization was repeated by reducing the range to 55 °C and 60 °C.

3.2.7.7. Primer Tests

Before any sorption and biodegradation experiments, the two sets of primers were tested on the soil, performing blank experiments, to check whether the oligonucleotides might amplify any nucleotide sequence similar to the dsRNA. The extraction and RT-PCR was performed for 0.2 g of soil as described before. Furthermore, a RT-PCR test was carried out, using the sorption and biodegradation primers. Then, the samples were visualised with agarose gel (1.5%).

3.2.7.8. Double stranded RNA denaturation assay

The dsRNA denaturation was also evaluted. Four samples were prepared adding 1ug dsRNA in 6 µL of water-DEPC, with a final volume of 8 µL. Then, all samples were denaturated as

follows (Table 3.7), using Alpha Cycle Thermocycle (PCRmax, UK). Each of the samples were visualized in agarogel (1.5%).

Table 3.7 - dsRNA denaturation program

Temperature	***Time***
95° C	5 min
95° C	2 min
98° C	5 min
98° C	2 min

3.3. Results and Discussion

3.3.1. dsRNA Denaturation, Primer Test and qPCR standard calculations

dsRNA Denaturation: A higher RT yield can be achieved by denaturing the dsRNA into ssRNA, before the reverse transcription (Lee et al., 2004; Freeman et al., 2008; Mijatovic-Rustempasic et al., 2013). Nucleic acids with a high GC content have higher melting temperatures (T_m) due to the purines triple bond. In our case, the dsRNA had 50% of GC content, for this reason a denaturation assay needed to be performed.

From the agarose gel electrophoresis (Fig.3.1) each band from each permutation shows a clear denaturation, where the bands are located below the marker 300bp ($dsRNA_{STD}$ is 305bp). It means that ssRNA, which has a lesser molecular weight than dsRNA, migrated through the agarose pores faster, validating the denaturation. Considering some environmental samples might have different amounts of dsRNA or low concentrations of chemicals co-extracted, we choose to denature for a longer time with the highest temperature tested (T= 98°C, t= 5min.) for all experiments.

Primers Test: Each set of primers (Table 3.6) was tested on soil in a blank experiment. The agarose gel electrophoresis for sorption (Fig. 3.2) and biodegradation (Fig. 3.3) primers showed no band in each blank sample, meaning that the primers selected for both experiments did not amplify any intrinsic soil dsRNA/ssRNA/dsDNA.

qPCR standard calculations: The quantification of the qPCR standard was obtained with a UV-spectrophotometer (Table 3.8). For each sample, the blank fluorescence was subtracted as a noise background to be erased from each sample. Plotting the fluorescence average against the known concentration of the DNA standard (Fig. 3.4), we calculated the qPCR standard concentration from the regression line, and quantified it as 0.08 ng μL^{-1}, corresponding to $5.23*10^{10}$ gene copies/µL.

Figure 3.1 - Program of dsRNA denaturation at different temperature and time. (+) represents the permutation applied, (-) represents the permutation excluded. M= marker, STD= dsRNA

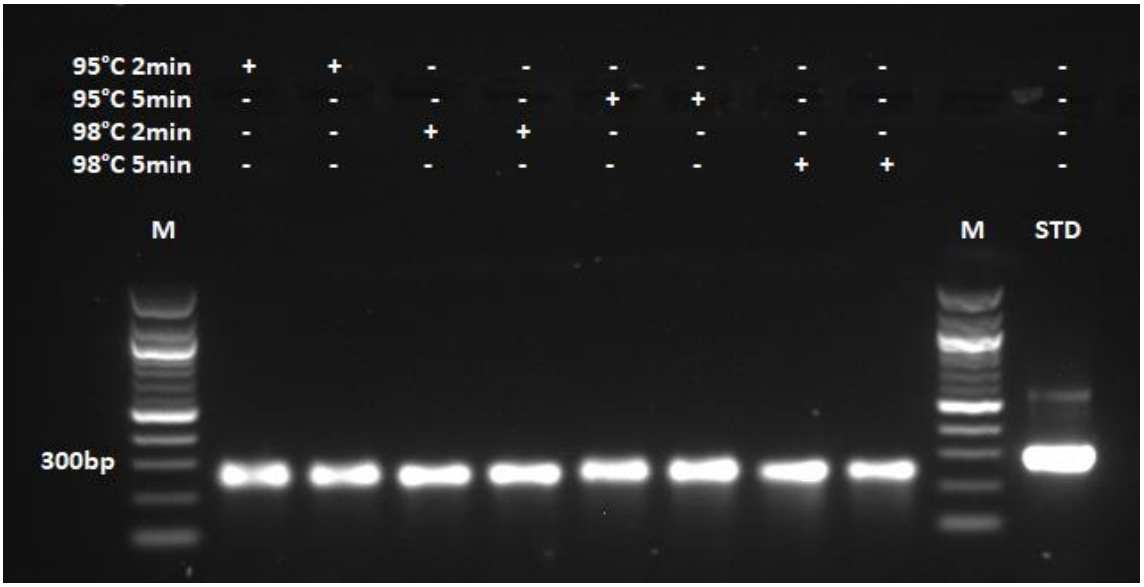


Figure 3.2 - Primer sorption test; S1, S2, S3 = Soil sample extraction. Sup1, Sup2, Sup3= Soil supernatant extraction. Sb = Soil Blank. Supb = Supernatant Blank. M=Marker, NTC= No Template Control.

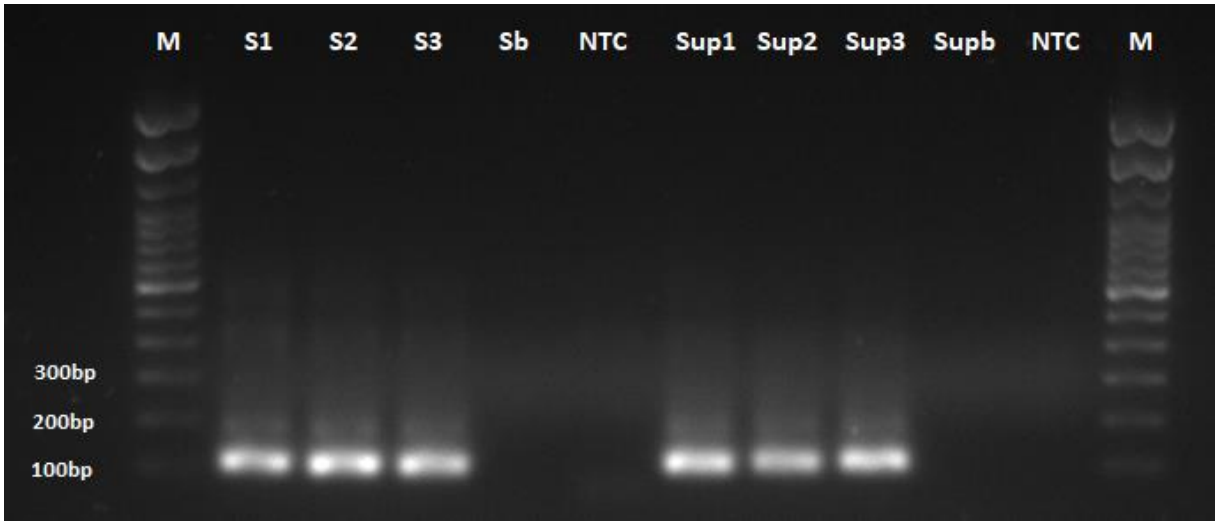


Figure 3.3 - Primer test biodegradation; 2.1 = Loamy Sand Soil, 2.3 = Sandy Loam Soil, 2.4 = Loam, 6S = Clay. M= Marker. NTC = No Template Control. STD = dsRNA Control.

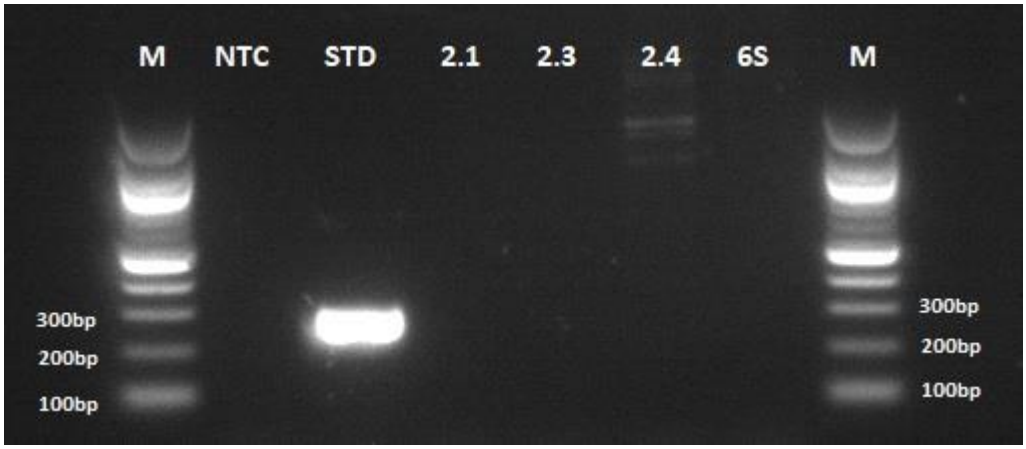
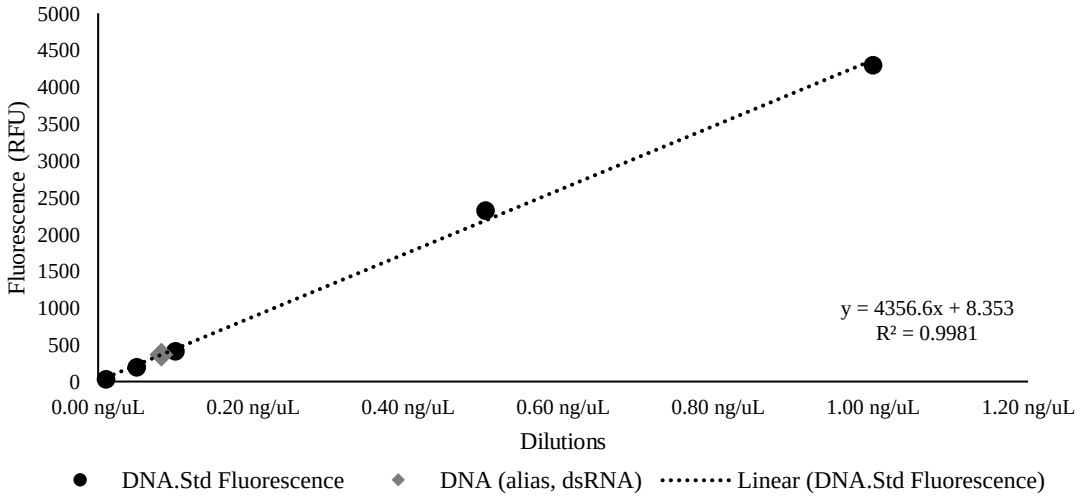


Table 3.8 - UV quantification of the qPCR standard. DNA [STD] = DNA Standards

	DNA,std	***Plate (96 wells) (RFU)***			***Average (RFU)***
DNA[std]	1.00 ng/uL	4200.69	4280.45	4417.51	4299.55
DNA[std]	0.50 ng/uL	2341.95	2344.21	2293.81	2326.65
DNA[std]	0.10 ng/uL	406.50	426.05	409.35	413.966
DNA[std]	0.05 ng/uL	198.44	199.35	198.82	198.868
DNA[std]	0.01 ng/uL	35.93	34.45	33.89	34.756
qPCR Standard	**0.08 ng/uL**	**316.76**	**389.44**	**389.54**	**365.247**

Figure 3.4 – UV spectrophotometer: Calculation of the qPCR standard concentration from the standard curve.



3.3.2. Mass Balance and dsRNA quantification

The extraction method was based mainly on (R.I. Griffiths *et al.*, 2000), with improvements obtained via the mass balance assays (paragraph 3.2.5). It was decided to not use commercial kits, because they gave a lesser degree of freedom in testing different chemicals and tools in order to improve the recovery. In order to do so, four mass balance experiments were carried out (Table 3.9); the glass beads assay (Figure 3.5, a), the precipitation assay (Figure 3.5, b), the PLG assay (Figure 3.5, c) and the purification assay (Figure 3.5, d).

Glass beads assay: Usually, the glass or ceramic beads are utilised in different size with lysis solutions inside pre-sterilized vials, to increase the efficiency of breaking the bacteria cell walls and obtaining higher nucleic acid yields. Since in our case, the biopesticide is spiked into the soil, adding the glass beads had the propose to shatter the soil and organic aggregates, and exposing more soil surface with attached dsRNA to the extraction buffer (Lever *et al.*, 2015b). Consequently, glass beads also might help to reduce the hysteresis effect in sorption experiments which is due to intraorganic diffusion processes of biopesticides within soil aggregates (Huang, Yu and Weber, 1998). In our assay (Fig.3.5**Error! Reference source not found.**, a), we found no significant differences in no beads/beads in vials, likely due to the low organic particulates and large size particles, ~ 89% ranging 0.05 - 2.0 (mm). Consequently, the recovery yield was achieved mainly by the extraction buffer which led to the desorption process.

Phase Lock Gel assay: The phase lock gel (PLG) is a useful tool to minimise nucleic acid loss and interphase-protein contamination during phenol extraction. We tested two types of PLG, for high (a) and low (b) salt concentration (Fig.3.5**Error! Reference source not found.**, b) to understand which one could maximise the recovery in this step. From the experiments, we found that the main loss was obtained by the manually pipetting method with almost 55% loss. Instead, we recovered ~ 63% of dsRNA from PLG High salt concentration having the highest recovery in terms of mass.

Precipitation Assay: The use of salts in alcoholic solutions is a well-known procedure to precipitate and concentrate nucleic acids. Cations assist the precipitation by neutralizing the negative charges of backbone phosphate, while the alcohol solution reduces solubility by disrupting the hydration shell around the DNA (RNA) molecules that would occur in water solution. Usually, the common salts utilised for this propose are sodium acetate and ammonium acetate. In contrast, a straight-chain polymer like, polyethylene glycol (PEG), is widely used as well. When evaluating them (Figure 3.5, c), the PEG (30%) showed the best recovery (~100%), both at 3 hours and overnight incubation times (Figure 3.5, c) and with the same amount of glycogen. In contrast, the two salts showed lesser recovery, which was especially affected by the incubation time. The glycogen concentration had a high impact on the recovery in this step. In fact, when comparing the two samples, PEG 1 (25 µg/mL, glycogen) and PEG 2 (80 µg/mL, glycogen), the recovery was improved by ~ 40%.

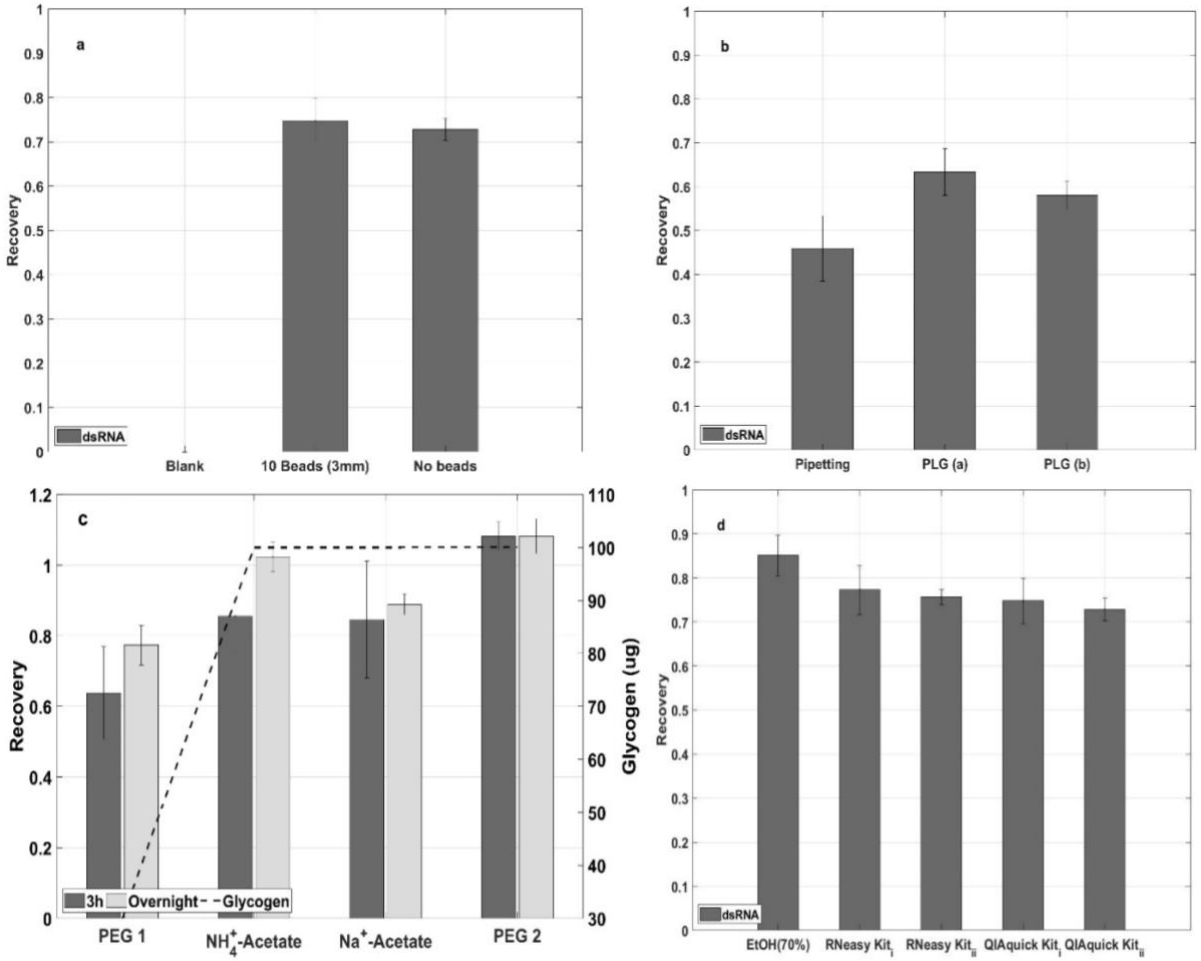
Purification assay: We also examined the efficacy of commercial kits for the final purification, in removing salts residual from the precipitation step before downstream processes. At the moment, there are no kits available for cleaning-up dsRNA molecules. Comparing those available for DNA and ssRNA with ethanol (Figure 3.5, d), we observed the maximum recovery

was obtained by ethanol (70%). This likely, because the commercial kits were designed for dsDNA and ssRNA, and the silica-membrane used might have denaturated or held onto part of the nucleic acid. So, washing the pellet with ethanol represented the best solution.

Table 3.9 – Recovery Data for the Mass Balance Assays (1.00 =100% recovery of the amount added).

Glass Beads Assay	***Recovery***	***Recovery (Std dev)***			***Phase Lock Gel Assay***	***Recovery***	***Recovery (Std dev)***
Beads	0.75	0.05			Pipetting	0.46	0.07
No Beads	0.73	0.02			PLG(a)	0.63	0.05
Blank	0	0			PLG(b)	0.58	0.03
Precipitation Assay	***Recovery***	***Recovery (Std dev)***	***Glycogen (ug)***	***Glycogen (ug/mL)***	***Purification Assay***	***Recovery***	***Recovery (Std dev)***
PEG 1 (3h)	0.64	0.13	30	24.88	Ethanol (70%)	0.851	0.045
PEG 1 (overnight)	0.77	0.06	30	24.88	RNAesy (3h)	0.773	0.056
PEG 2 (3h)	1.08	0.04	100	81.97	RNAesy (Overnight)	0.756	0.017
PEG 2 (overnight)	1.08	0.05	100	81.97	QIAquick Kit (no Beads)	0.748	0.051
NH_4^+ Acetate (3h)	0.86	0.10	100	73.53	QIAquick Kit (Beads)	0.728	0.025
NH_4^+ Acetate (overnight)	1.02	0.04	100	73.53			
Na^+ Acetate (3h)	0.85	0.17	100	73.53			
Na^+ Acetate (overnight)	0.89	0.03	100	73.53			

Figure 3.5 - Mass Balance Assay Charts; (a) Glass Beads; (b) Phase Lock Gel – PLG, (a) high salt content - PLG(b) low salt content; Precipitation Assay; Purification Assay.

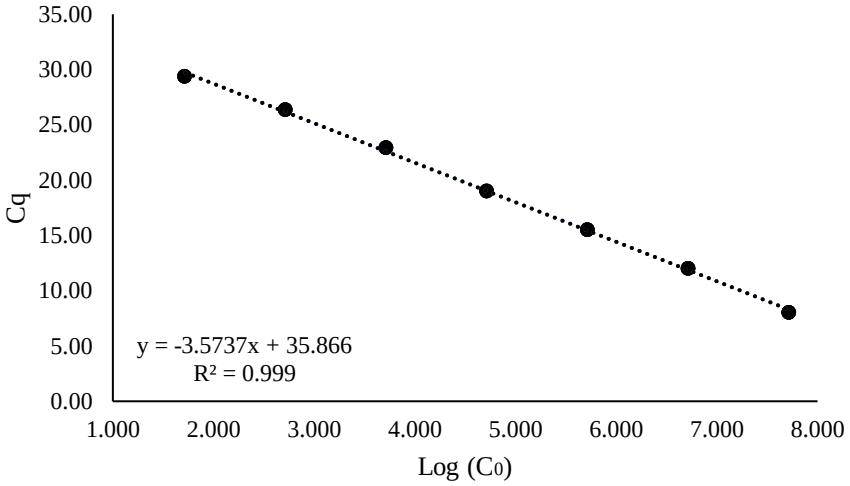


Standard curve and limit of detection: Before any qPCR assay, it is essential to validate the standard curve (Table 3.10, Figure 3.6), and establishing the Limit of Detection (LoD). The LoD is assumed to be the highest Cq value of the positive sample, and indicates the acceptable Cq values that can be used for the assay. In this case, it is $5.23*10^1$ gene copies uL^{-1}, where it represents also the Limit of Quantification (LoQ), considering that the assay is still linear (see Melting Peak, Appendix 3-1).

Table 3.10 - Data standard curve and limit of detection.

Sample	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log Starting Quantity	SQ Mean
Std 10^7	8.14	0.033	5.23E+07	7.719	5.23E+07
Std 10^6	11.67	0.071	5.23E+06	6.719	5.23E+06
Std 10^5	14.09	0.018	5.23E+05	5.719	5.23E+05
Std 10^4	17.94	0.054	5.23E+04	4.719	5.23E+04
Std 10^3	20.43	0.023	5.23E+03	3.719	5.23E+03
Std 10^2	23.57	0.088	5.23E+02	2.719	5.23E+02
Std 10^1	26.41	0.077	5.23E+01	1.719	5.23E+01

Figure 3.6 - dsRNA qPCR standard curve

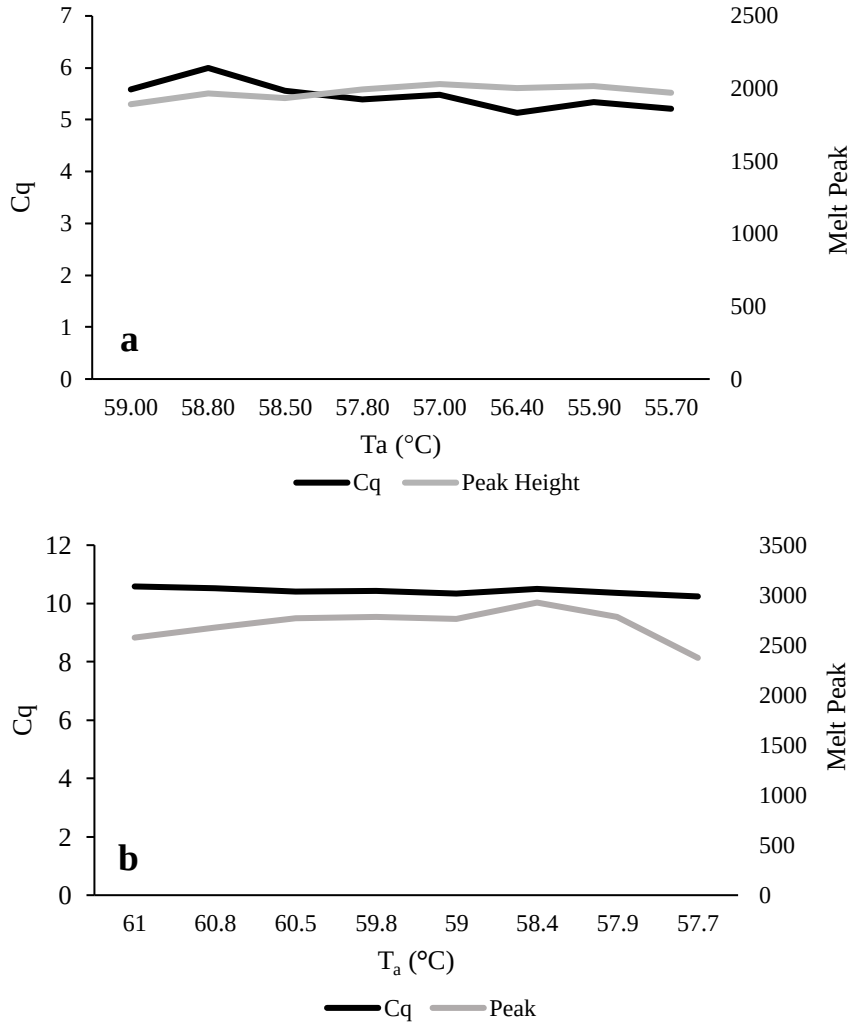


Test of annealing temperatures: An RT-qPCR assay was performed to test the annealing temperature (T_a) of the two sets of primers (Table 3.11). The range of T_a allowed us to detect the best temperature to amplify the target dsRNA. In choosing the T_a, we considerated the higher gap between the lower Cq value, that gives the higher gene copy number, and higher peak of the melting curve (Figure 3.7, a,b). Thus, for the sorption primer we obtained T_a=56.40 (°C) and for the biodegradation primers T_a = 57.7 (°C). Furthermore, looking at the melting curve for both, the assay gave us a really sharp peak, indicating that the primers have specifically amplified the target nucleic acid, avoiding any primer dimer (see Appendix 3-2, Appendix 3-3)

Table 3.11 - Annealing Temperature, Cq and melting curve values for sorption and biodegradation primers

Sorption Primers	***Ta (°C)***	***Cq***	***Peak Height***
#1	59.00	5.58	1891.99
#2	58.80	6.00	1967.55
#3	58.50	5.56	1936.01
#4	57.80	5.39	1996.19
#5	57.00	5.48	2030.85
#6	56.40	5.13	2001.98
#7	55.90	5.34	2019.03
#8	55.70	5.21	1969.37
Biodegradation Primers	***Ta (°C)***	***Cq***	***Melt Peak***
#1	61	10.58	2577.49
#2	60.8	10.52	2675.067
#3	60.5	10.42	2772.297
#4	59.8	10.43	2784.223
#5	59	10.33	2762.173
#6	58.4	10.49	2925.88
#7	57.9	10.36	2782.617
#8	57.7	10.24	2373.13

Figure 3.7 - Annealing Temperature in function of Cq and melt peak gap for the sorption (a) and biodegradation (b) primers.

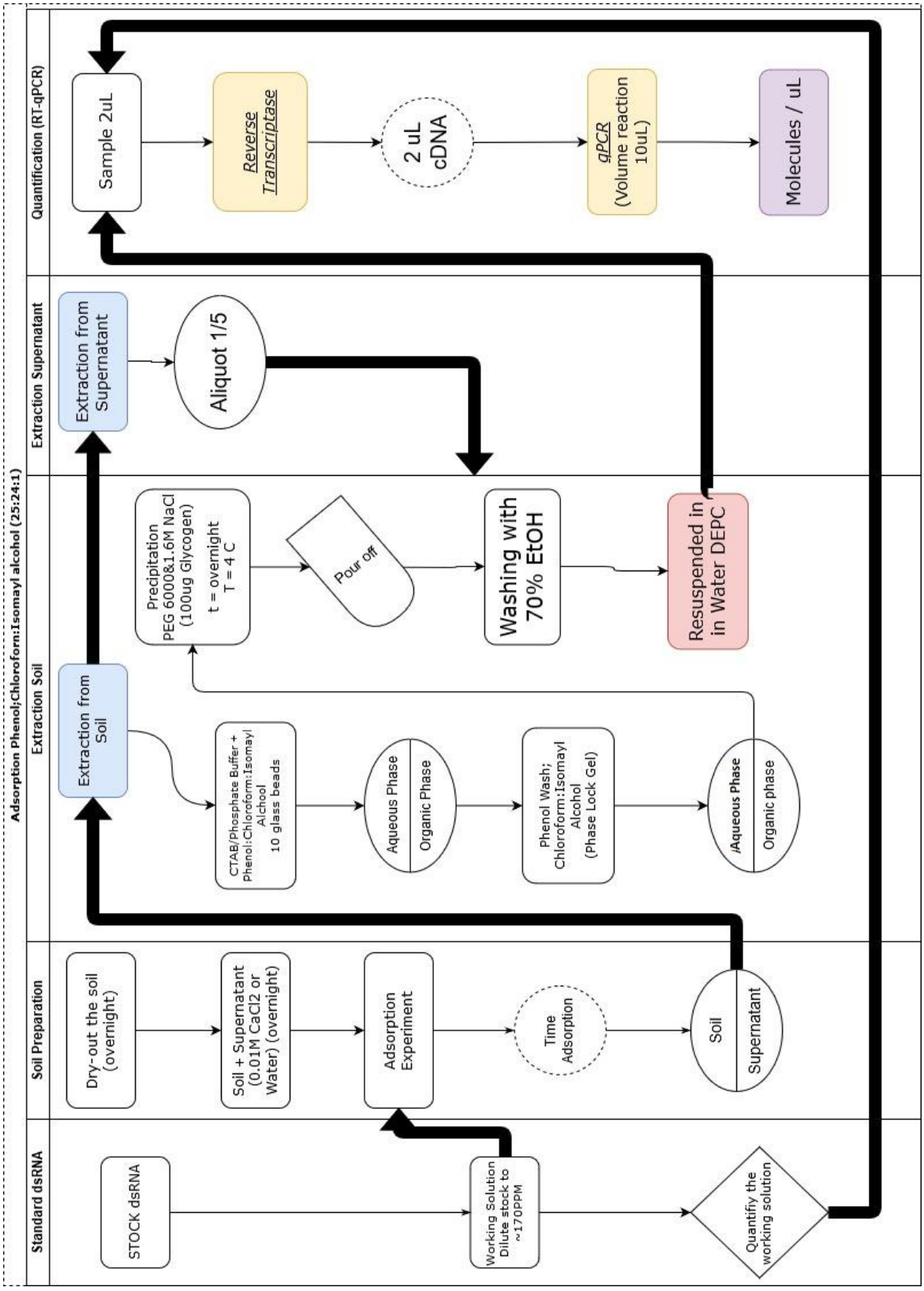


3.4. Conclusions

The extraction method for dsRNA was based mainly on (R.I. Griffiths *et al.*, 2000). The improvements (paragraph 3.2.5) targeted to enhance recovery as much as possible for the dsRNA spiked into the soil. We found that the precipitation assay was the step where most of the dsRNA was lost in terms of mass balance, when the glycogen was not used. The introduction of glycogen minimized these losses, and enabled recovery of most of the biopesticide. We also found that the commercial kits for cleaning–up nucleic acid samples, were not well designed for double stranded RNA, and washing with 70% Ethanol solution represented the best choice for residual salt removal. Furthermore, the glass beads did not improve the dsRNA yield, and the desorption from soil was mainly driven by the phosphate buffer. Nevertheless, we suggest to utilise glass beads with organic soil in order to break up organic soil aggregates and expose more colloid surfaces to the buffer solution. In addition, the introduction of Phase Lock Gel facilitated the pipetting off of the aqueous phase by avoiding the interphase contamination with

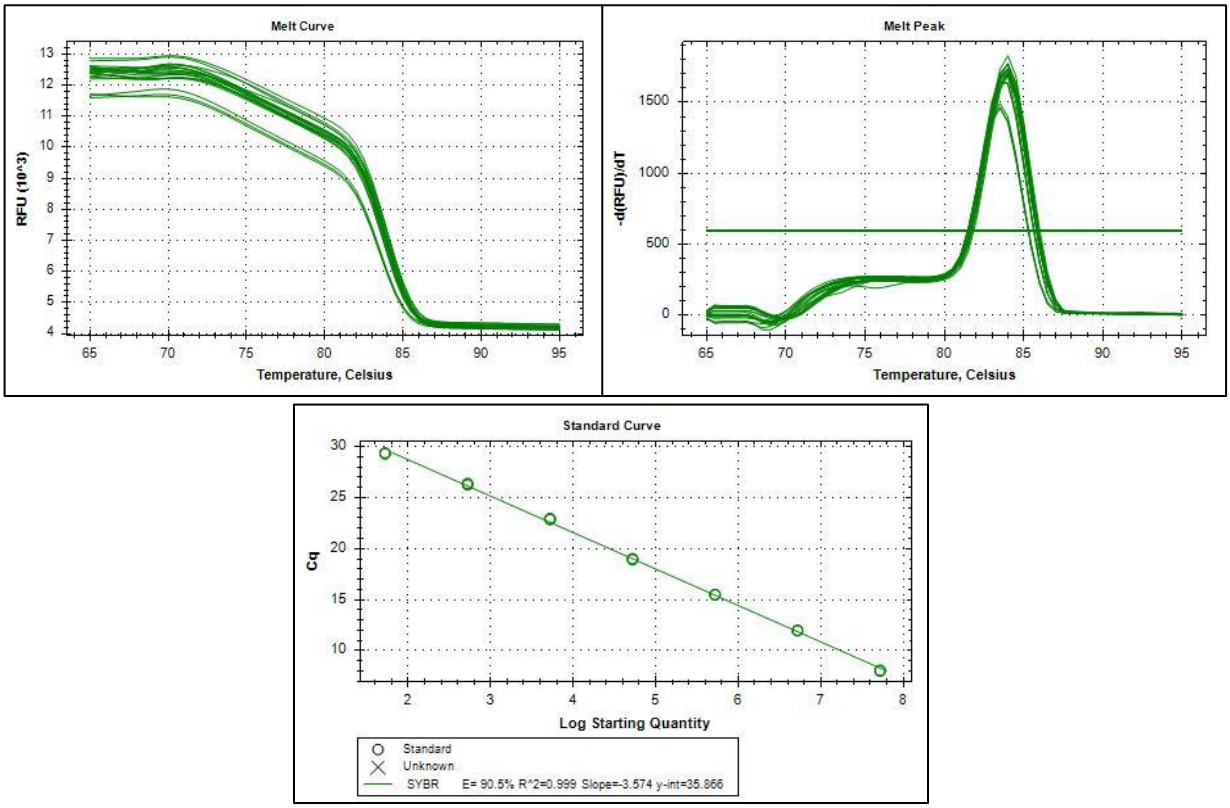
the organic solutions. Figure 3.8 presents a flow chart of the final protocol for the dsRNA extraction from loamy sand soil.

Figure 3.8 - Flow chart of the final protocol of dsRNA extraction from loamy sand soil.

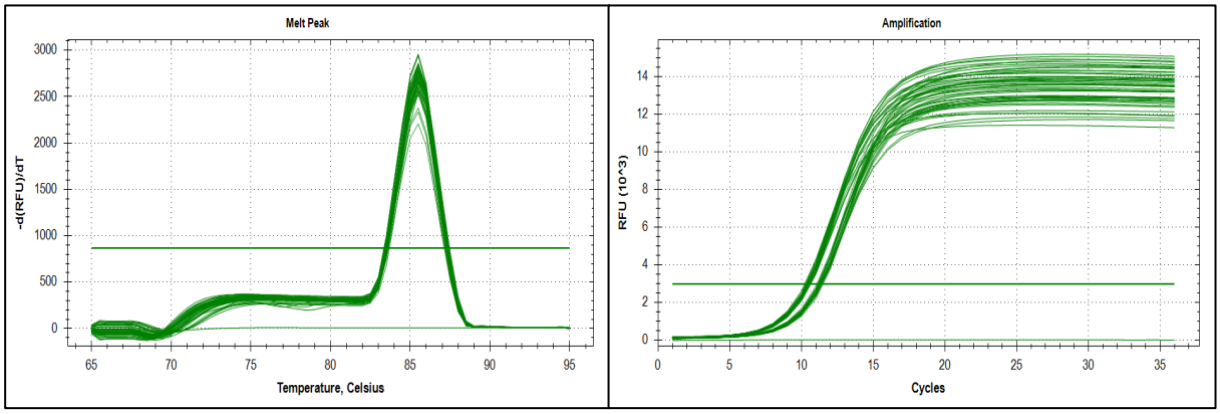


Appendices Chapter 3

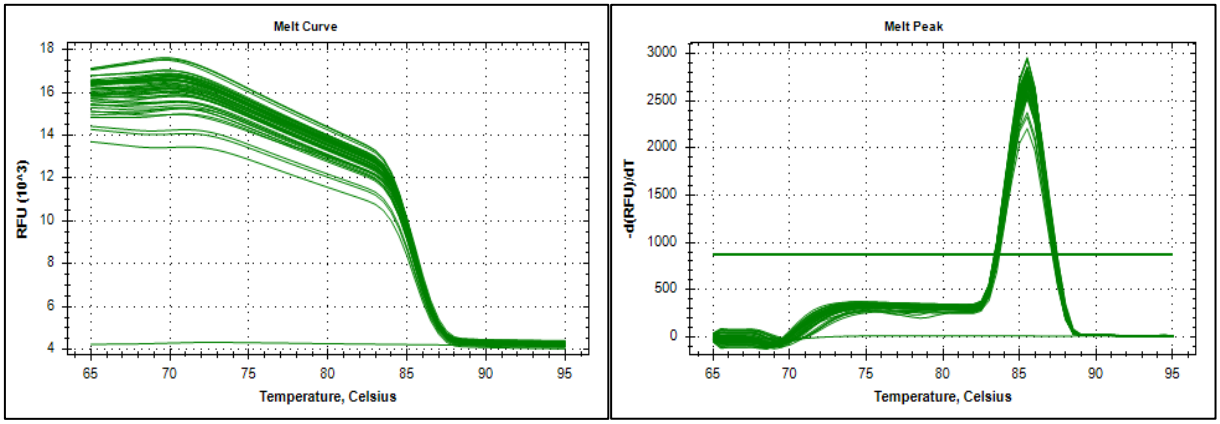
Appendix 3-1 - Melt curve, melt peak and standard curve of the qPCR standard.



Appendix 3-2 - Melt Peak and amplification curve of Ta for the sorption primers.



Appendix 3-3 - Melt Peak and amplification curve of Ta for the biodegradation primers.



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CHAPTER 4

Environmental Fate Assessment

4.1. Introduction

The market of plant protection products (PPPs) is regulated by EU legislations (Regulation (EC) No 1107/2009). Before any PPP can be marketed within the European Union, it must be approved by the European Commission. Thus, the chemical products undergo a rigorous environmental risk assessment which is then reviewed by the European Food Safety Authority (EFSA). The environmental fate study is an essential tool for the evaluation of chemical mobility in environmental compartments. The application of agricultural pesticides occurs mainly to soil (Helling, Kearney and Alexander, 1971) and it is affected by adsorption and degradation mechanism, which measure the mobility and persistence of the applied chemical in the soil matrix. The adsorption coefficient *(K_d)* quantifies the extent of pesticide repartitioning between the aqueous and solid phase, while its breakdown is estimated by the degradation rate *(k_{deg})* through kinetic models. The K_d and k_{deg} are evaluated by environmental scientists through laboratory and field experiments following specific guidelines with standardised procedures following principles of good laboratory practices. Consequently, these two parameters are utilised by regulatory agencies in evaluating the environmental risk, and to create a "conceptual model" useful in describing the pesticide mobility in the soil, the degradation pathways and metabolites or degradation products (Wauchope *et al.*, 2002; Weber, Wilkerson and Reinhardt, 2004). In this way, the adsorption coefficient and degradation rate represent inputs for modelling software used to simulate the exposure to chemicals in different ecosystems (Phelps, Winton and Effland, 2002).

4.2. Aim and Objectives

This chapter had the aim to evaluate the adsorption coefficient *(K_d)* and the degradation rate *(k_{deg})* of dsRNA in soil for a preliminary environmental risk assessment. For this propose, we followed the OECD guidelines for Testing Chemicals which are normalised lab protocols used by governments, industries and regulatory agencies to ascertain the hazard of chemical products. Thus, the environmental fate of dsRNA was assessed following the OECD Guidelines n.106 "*Adsorption/Desorption Using a Batch Equilibrium Method"* (OECD, 2000) and n.307 *"Aerobic and Anaerobic Transformation in Soil"*(OECD, 2002), ensuring that the two parameters (K_d and k_{deg}) were estimated through standardised procedures to minimise any bias in the scientific findings when reviewed by regulatory agencies. Furthermore, to calculate the dsRNA degradation kinetics (k_{deg} , DT_{50} and DT_{90}) we followed the *Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides* in EU Registration (FOCUS, 2006).

4.3. Materials and Methods

4.3.1. Ion Analysis

Ion analysis was performed to characterise the composition of cations and anions released from the soil to the supernatant. Four grams of soil were added to a 50 mL polypropylene centrifuge tubes (VWR, UK) with 20 mL of deionized water (ratio 1:5). The duplicate samples were vertically shaken (Heidolph, UK) at 20 rpm overnight. The day after an aliquot of supernatant was filtered with 0.45 µm syringe filter (Millipore, UK) and analysed (soil pH_{CaCl_2} = 6.77). Anion analysis was performed using a Dionex DX320 ion chromatography. Cations analysis was conducted with Varian Vista-MPX ICP-OES equipment. The metal concentrations (Appendix 4-1) were converted into equivalent per Litre following eq. 0.1, whilst the ionic strength was calculated according to eq. 0.2, where c_i are both cations and anions (mol L^{-1}) and z_i is the number of charges of the specific ion.

$$Eq.\ L^{-1} = \frac{mg\ L^{-1}}{MW_{ion}} * (n.charges) \qquad \text{eq. 0.1}$$

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2 \qquad \text{eq. 0.2}$$

4.3.2. XRD Analysis

Six grams of soil sample was analysed by a PANalytical X'Pert Pro MPD, powered by a Philips PW3040/60 X-ray generator and fitted with an X'Celerator* detector. Diffraction data was acquired by exposing the soil to Cu-Kα X-ray radiation, which has a characteristic wavelength (0.15418 nm) of 1.5418 Å. X-rays are generated from a Cu anode supplied with 40 kV and a current of 40 mA. Data sets were collected over a range of 10° to 80° 2θ with a step size 0.334° 2θ and nominal time per step of 100 seconds, using the scanning X'Celerator detector and a secondary graphite crystal monochromator in the diffracted beam path. The raw data were analysed by HighScore Plus Software.

4.3.3. OECD Guidelines for the Testing of Chemicals n.106

The *OECD 106 Adsorption/Desorption Using a Batch Equilibrium Method* is a standard procedure to identify physical-chemical properties of a test substance, with the aim to estimate its adsorption/desorption mechanism in different types of soil, determining its mobility and distribution between soil and supernatant. Briefly, the soil is pre-equilibrated in 0.01M $CaCl_2$ solution for 4h. Afterwards, a known volume of test substance is added to the pre-equilibrated sample (in an appropriate ratio soil : aqueous phase), and agitated for the required time. Then,

the two phases are separated, and the test substance is extracted and quantified by the appropriate methodology of detection.

4.3.3.1. Stability of dsRNA in $CaCl_2$ solution

The test substance was dissolved in a solution of 0.01M $CaCl_2$ used as aqueous solvent. To assess the stability of dsRNA molecules in the solvent, 792 ng of dsRNA was spiked in a solution of (1) 0.01M $CaCl_2$ and (2) DEPC Water (0M $CaCl_2$) (see Table 4.1). The triplicate samples were briefly vortexed and static-incubated for 30 min at 4°C and then quantified with MicroRNA Qubit (Invitrogen, UK).

Table 4.1 - Set up to assess dsRNA stability in $CaCl_2$ solution.

	0.01M $CaCl_2$	***DEPC-Water (control)***	***DEPC-Water (blank)***	***0.01M $CaCl_2$ (blank)***
Solutions	1 mL	1 mL	1 mL	1mL
Spiked dsRNA	792 µg	792 µg	0 µg	0 µg

4.3.3.2. Adsorption Test in $CaCl_2$ Solutions

Like the stability test described above, a sorption test was set up by pre-equilibrating the soil with different concentrations of $CaCl_2$. The test had the aim to evaluate any matrix effect that could affect the dsRNA sorption in a solution of variable ionic strength. The experiment followed the sorption assay described in the paragraph below (4.3.3.3), with the only difference being pre-equilibration of the loamy sand soil with a solution of 0.01 M, 0.001 M and 0 M $CaCl_2$ (Table 4.2). After the pre-equilibration, the dsRNA was added to the vials and vertically shaken (Heidolph, UK) at 20 rpm for 30min.

Table 4.2 - Sorption experiment (t=30min) with soil after pre-equilibration with different calcium chloride solutions.

	Pre-eq. (0.01M$CaCl_2$)		***Pre-eq. (0.001M$CaCl_2$)***		***Pre-eq. DEPC-Water (0M$CaCl_2$)***		
	Soil	***Supernatant***	***Soil***	***Supernatant***	***Soil***	***Supernatant***	***dsRNA***
Three Adsorption Replicates	0.2 g	1 mL	0.2 g	1 mL	0.2 g	1 mL	1 µg
Two Soil Extraction Controls	0.2 g	-	0.2 g	-	0.2 g	-	1 µg (*)
Two Supernatant Controls	-	1 mL	-	1 mL	-	1 mL	1 µg (*)
Blank Control	0.2 g	1 mL	0.2 g	1 mL	0.2 g	1 mL	-

(*) The dsRNA was spiked immediately before the extraction procedure.

4.3.3.3. Adsorption Experiments

The sorption experiments were assessed following a modified OECD 106 Guideline by pre-equilibrating the soil in DEPC-Water instead of 0.01 M $CaCl_2$. The experiments were carried out in batch equilibrium tests in parallel. The assay was set up as follows (Table 4.3). Before the start of any adsorption test, a portion of loamy sand soil was dried and sterilized at 105 °C overnight. Consequently, 0.2g of soil were placed in 2 mL low binding protein polypropylene microcentrifige tubes (Eppendorf, UK).

Table 4.3 - Scheme of Adsorption Experiments

	Pre-equilibrated in DEPC- Water		
	Soil	***Supernatant***	***dsRNA***
Three Adsorption Replicates	0.2 g	1 mL	1 µg
Two Soil Extraction Controls	0.2 g	-	1 µg (*)
Two Supernatant Extraction Controls (DEPC-Water)	-	1 mL	1 µg (*)
Blank Control	0.2 g	1 mL	-

(*) The dsRNA was spiked immediately before the extraction procedure.

Then, one millilitre of DEPC-Water was added to the three-adsorption replicates and blank control (ratio 1:5), and they were pre-equilibrated by vertically shaking (Heidolph, UK) at 20 rpm for 4h. Consequently, the samples were briefly centrifuged, one microgram of dsRNA was spiked into the vials (see Table 4.3), and they were vertically shaken (Heidolph, UK) at 20 rpm for the desired time. Subsequently, the samples were centrifuged at 5000 g for 5 min to settle the soil at the bottom of the tube, removing particles as much as possible from the supernatant. Carefully, ~ 980 µL of supernatant were pipetted off into a new vial and stored at -80 (°C) while the soil samples were extracted. The dsRNA was recovered following the extraction method described in Chapter 3 (Figure 3.8). The adsorption coefficient (K_d) and K_{OC} was determined by (eq. 4.3).

$$K_d = \frac{C_{soil}}{C_{sup}} \quad ; \quad K_{OC} = \frac{K_d * 100}{\%\ o.c.} \qquad \text{eq. 4.3}$$

C_{soil} and C_{sup} refer to the concentration of dsRNA extracted from the soil and supernatant.

4.3.4. OECD Guidelines for the Testing of Chemicals n.307

The OECD n.307 *Aerobic and Anaerobic Transformation in Soil* is a standard procedure to identify the rate of persistence of chemicals in soil and their metabolites. The test substance is incubated in the dark at a specific temperature. After appropriate time-intervals, the test substance/transformation products are extracted and analysed by an appropriate methodology of detection.

4.3.4.1. Biodegradation Experiments

The biodegradation experiments were carried out in aerobic condition. The assay was set up as described in Table 4.4.

Table 4.4 - Scheme of Biodegradation Assay

	Soil	***dsRNA***
Three Degradation Replicates	0.2 g	2.41 µg
Three Replicates Soil Extraction Control	0.2 g	2.41 µg (*)
Blank Control	0.2 g	-

(*) The dsRNA was added to the soil immediately before the extraction.

In order to acclimate the soil bacterial community, the day before the assay 0.2 g of live loamy sand soil were placed in 2 mL low binding protein polypropylene vials (Eppendorf, UK), and

static-incubated overnight at 20 ± 1 (°C). The vials were left open, covered by a thin layer of parafilm provided with five punctures, to exchange oxygen between the soil and the external environment. The day after the dsRNA was spiked into the vials and incubated for 1 d, 3 d, 10 d, 16 d, 39 d, and 69 d. During the biodegradation period, the soil moisture content was kept constant, considering the initial weight of the soil.

4.3.5. Data Analysis

The total amount of recovered dsRNA was given by the sum of the amounts extracted from the soil and supernatant (eq. 4.4). All experiments (adsorption and biodegradation) were performed in triplicates. The adsorption coefficient (K_d) was calculated as the average of the replicated batches (eq. 4.3) whereas the standard deviation (eq. 4.5) was calculated to consider the variation of measurements around the average.

$$Amount_{tot} = Amount_{soil} + Amount_{sup} \quad \text{eq. 4.4}$$

$$\sigma = \sqrt{\frac{\sum(x_i - u)^2}{N}} \quad \text{eq. 4.5}$$

It is unlikely that the extraction efficiency (Ext_{eff}, eq. 4.6) for environmental samples reaches 100% due to the extreme complexity of environmental adsorbents. To normalise this discrepancy and improve the data accuracy, three different approaches of recovery correction were investigated.

a) The recovery correction (RC) was applied to all samples. The amount of dsRNA extracted from the soil and supernatant were corrected taking in account the extraction efficiency for the control soil and supernatant samples (eq. 4.7).

$$Ext_{eff} = \frac{C_{tot}^{ext}}{C_{spiked}} \quad \text{eq. 4.6}$$

$$C_{soil}^{corrected} = \frac{C_{soil}}{Ext_{eff}^{soil}} \quad ; \quad C_{sup.}^{corrected} = \frac{C_{sup}}{Ext_{eff}^{sup}} \quad \text{eq. 4.7}$$

b) No recovery correction (NC). The measured dsRNA concentration was not corrected for the control extraction efficiency.

c) A Mass Balance Correction (MB) was applied to the samples. The dsRNA extracted from soil was corrected by the soil control extraction efficiency (eq. 4.6), whereas the dsRNA in the supernatant was calculated by mass balance (eq. 4.8),

$$C_{sup}^{ext} = C_{spiked} - C_{soil}^{corrected}$$

eq. 4.8

4.3.5.1. Q-Test

A Q-test was carried out to identify outliers in the dataset. The outliers were evaluated according to t (eq. 4.9).

$$Q_{exp} = \frac{x_i - x_n}{x_h - x_l}$$ eq. 4.9

The x_i refers to the possible outlier, whereas x_n is the nearest data to the x_i. Also, x_h and x_l represent the range of the dataset. In case the $Q_{exp} \geq Q_{tabulated}$, the outlier was rejected according to the level of significance listed in Q-test table (Appendix 4-30).

4.3.5.2. Kinetic Models

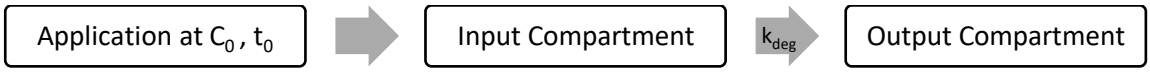
To evaluate the degradation of dsRNA in soil, we followed the European Framework *"Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration"*. To generate the degradation kinetics, the experimental data were analysed by Computer Assisted Kinetic Evaluation software (CAKE v3.3), which follows the FOCUS Kinetics guidance. Three different mathematical models were implemented in order to get the best fit model to the experimental data and calculate the constant degradation rate (k_{deg}) and consequent DT_{50} and DT_{90} with minimal error. The average of triplicates was used in curve fitting, and the analysis was conducted on the parent only compound (PO). The equations for the mathematical models are listed as follows;

a) Single First-Order Rate Model (SFO)

The concept model of the SFO is described by the Figure 4.1. It assumes that the degradation of an applied compound in soil is constant throughout the experiment (FOCUS, 2006). The degradation trend is described by the (eq. 4.10),

$$C_t = C_{t_0} e^{-kt} \quad ; \; DT_{50} = ln2/k_{deg} \quad ; \quad DT_{90} = ln10/k_{deg}$$ eq. 4.10

Figure 4.1 - Concept Model for the Single First Order Degradation Kinetic

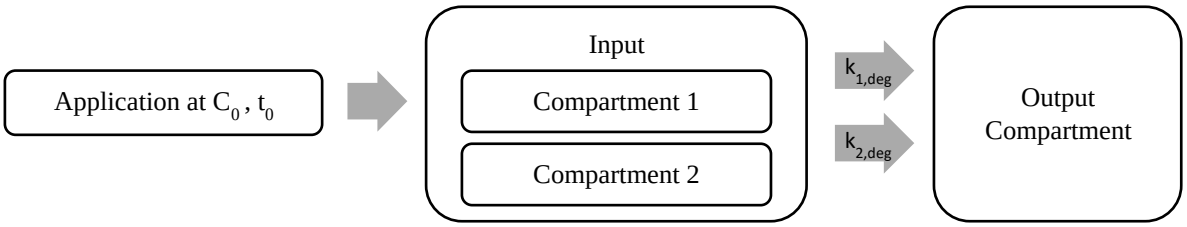


where the C_t is the dsRNA concentration at time t, C_0 is the dsRNA concentration at time t=0 and k_{deg} is the constant degradation rate. C_0 and k_{deg} are the parameters determined by the mathematical model.

b) Double First-Order in Parallel (DFOP) Rate Model

The DFOP model (Figure 4.2) is one of the bi-phasic models, which assumes that the concentration of the applied compound declined with different velocities, resulting in two constant rates, one slower than the other.

Figure 4.2 - Concept Model for the DFOP



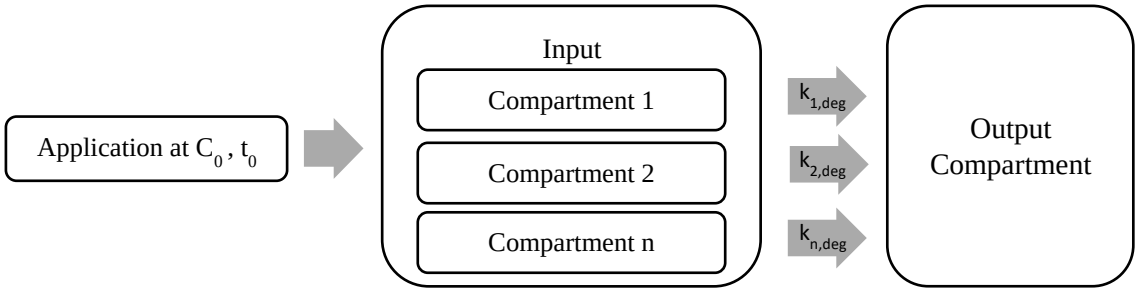
The DFOP trend is described by (eq. 4.11). The parameter *g* represents the fraction of C_0 applied to compartment 1, whereas $k_{1,deg}$ and $k_{2,deg}$ are the constant rate for the compartment 1 and 2, respectively. With three fitting parameters (g, $k_{1,deg}$ and $k_{2,deg}$), DT_x values can only be calculated by an iterative procedure.

$$C_t = C_{t_0}(ge^{-k_1 t} + (1-g)e^{-k_2 t}) \qquad \text{eq. 4.11}$$

c) First Order Multi-Compartment (FOMC) Rate Model

The FOCM (Figure 4.3) is the second bi-phasic model which describes how the degradation occurs not at constant rate. The α and β are not the degradation rates but parameters to shape the curve due to the coefficient of variation of k_n values (eq. 4.12).

Figure 4.3 - Concept Model for FOMC



$$C_t = \frac{C_{t_0}}{\left[\left(t/\beta\right)+1\right]^{\alpha}} \quad ; \quad DT_{50} = \beta\left(2^{1/\alpha}-1\right) \; ; \; DT_{90} = \beta\left(10^{1/\alpha}-1\right) \qquad \text{eq. 4.12}$$

4.3.5.3. Validation of kinetic models

The chi-square value (χ^2, eq. 4.13) is a statistical tool which evaluates the deviation between the experimental data (E) and the prediction (P) of mathematical model.

$$\chi^2 = \sum \frac{(P-E)^2}{err * \overline{E}^2} \quad \text{eq. 4.13}$$

If the $\chi^2 < \chi^2_{tabulated}$, the model can be accepted at the appropriate level of significance. Also, the chi-square (eq. 4.13) accounts for the uncertainty of the model (FOCUS, 2006), where the model error is described by (eq. 4.14).

$$err = \frac{\chi^2_{err}}{100} * \overline{E} \quad \text{eq. 4.14}$$

In order to define the best fit for our experimental data, the error for each mathematical model was calculated as chi-square error (χ^2_{err}, eq. 4.15) that represents the minimum error at which the model passes the confidence level tabulated (FOCUS, 2006). We selected a 0.05 level of significance (**Error! Reference source not found.**, Appendix).

$$\chi^2_{err} = 100 \sqrt{1/\chi^2_{tabulated} * \sum \frac{(P-E)^2}{\overline{E}^2}} \quad \text{eq. 4.15}$$

The model fit which resulted with the lowest χ^2_{err} represented for us the most appropriate model to evaluate the k_{deg}, DT_{50} and DT_{90}.

Furthermore, to assess the parameters' robustness as calculated by the model, we performed a t-test. The significance of *p-value* was evaluated reading the t-test table (Appendix 4-32) at the level of significance 0.05 (FOCUS, 2006)

4.4. Results and Discussion

The inconsistency regarded the K_d calculated at 0.01M and 0.001M $CaCl_2$, it is the result of interaction between the dsRNA molecules and the cations (Ca^{2+}) in solution at different ionic strength. These interdependences dsRNA-Ca^{2+} resulted then in a mass loss in the aqueous phase during the adoption tests (Figure 4.6). To overcome the K_d calculations affected by this matrix effect, we juxtaposed different mathematical correction methods to prevent any bias in analysing and comparing the data at different $CaCl_2$ concentration; recovery corrected (RC), no correction for recovery (NC) and mass balance (MB). The corrections resulted in good agreement in DEPC-Water experiments, which then has been used for the sorption experiments.

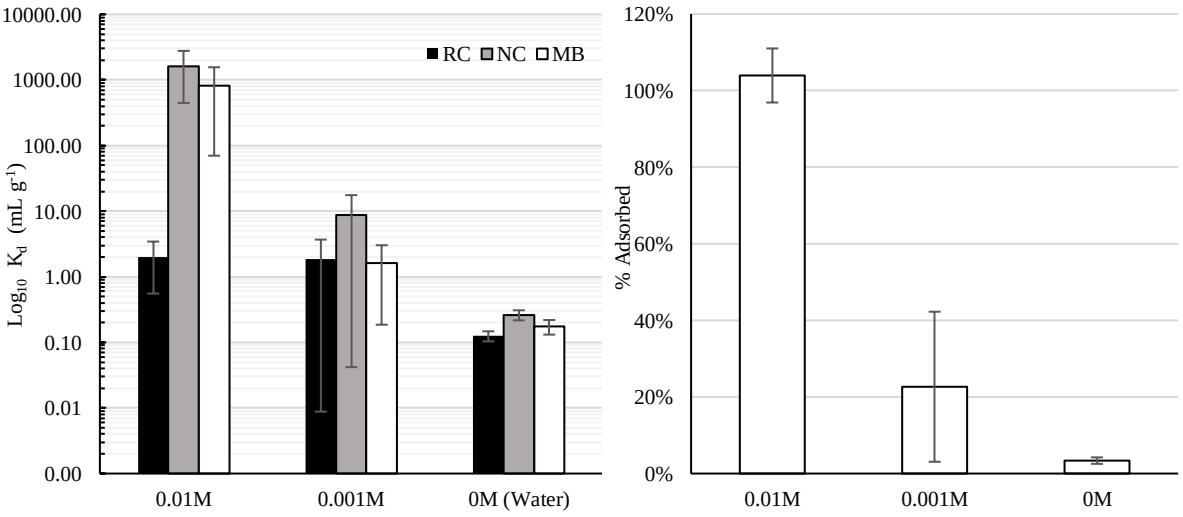
4.4.1. The Effect of Ionic Strength on the dsRNA Adsorption

When following the conventional and modified OECD 106 methods for studying adsorption by equilibrating soil with 0.01 M, 0.001 M and 0 M $CaCl_2$ solutions, respectively, we observed an extensive sorption coefficient (K_d) dependency on the variable calcium chloride concentrations (Figure 4.4 & 4.5). Also, it was observed from spiking experiments, that the recovery of dsRNA from aqueous solution was affected by ionic strength (Figure 4.4). For us, these results gave an

indication of the dependency of dsRNA recovery from aqueous solution on the solution ionic strength. We know from the literature that salty solutions, like sodium chloride, sodium or ammonium acetate are well known to concentrate and precipitate nucleotides from aqueous phases, and are widely utilised (Green and Sambrook, 2012). They also assist the precipitation of nucleic acids in alcoholic solvents. The cations, provided by salts, neutralise the negative charges of phosphate groups along the sugar phosphate backbone, reducing the nucleotide solubility. Higher precipitation rate occurs when utilising solvents with low dielectric constant (ξ), like ethanol (ξ = 24.5) or isopropanol (ξ = 17.9). These alcohols provide a less electrostatic interference between cations and (PO_3^-) phosphate groups, avoiding the hydration shells on the DNA structure, that would occur in water (ξ = 80.1) due to its strong dipole nature (Sissoëff, Grisvard and Guillé, 1978).

Figure 4.4 – Adsorption Test: dsRNA Adsorption Coefficient (K_d) with soil pre-equilibrated at different concentration of calcium chloride and incubated for 30min. The dsRNA concentration extracted was analysed with three different methods of data corrections; Recovery Correction (RC), No Data Correction (NC) and Mass Balance Correction (MB). The error bars represent the standard deviation of triplicate experiments.

Figure 4.5 – Adsorption Test: Amount of dsRNA adsorbed on the soil (t=30min) at different concentration of $CaCl_2$ effected by the divalent cation bridging. The dsRNA adsorbed was calculated by MB method. The error bars represent the standard deviation of triplicate experiments.



Furthermore in this particular case, alkaline metals like Ca^{2+} provided by calcium chloride solution, interact with phosphate groups on the sugar backbones of dsRNA like hydrated metals (Minasov, Tereshko and Egli, 1999; Chiu and Dickerson, 2000; Ahmad, Arakawa and Tajmir-Riahi, 2003). Thus, the dsRNA-complexes may have aggregated and precipitated (Franchi, Ferris and Gallori, 2003) under some conditions of our study making their recovery in the analysis of the aqueous phase more difficult.

Figure 4.6 - Recovery of dsRNA from calcium chloride solution and DEPC-Water (deionized water). Error bars stand for the standard deviation.

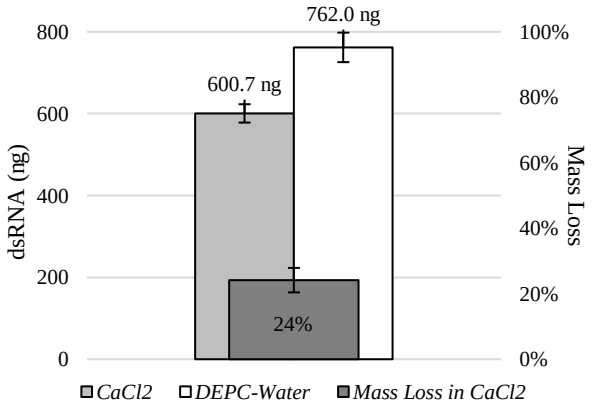


Table 4.5 - Results of dsRNA stability.

	dsRNA (ng)	***Std Dev* (ng)***
0.01M $CaCl_2$	600.6	22.4
DEPC-Water	762.0	36.0
0.01M $CaCl_2$ (Blank)	0	0
DEPC-Water (Blank)	0	0
dsRNA spiked	792	

* Standard Deviation

In order to compare different data sets it was thus crucially important to take into consideration an approach that would minimise the effect of mass loss during the analysis on the calculated K_d, especially at high $CaCl_2$ concentration (Figure 4.6, Table 4.5). When interpreting the data, using the recovery corrected (RC) supernatant and soil concentrations for the calculation of K_d at 0.01M $CaCl_2$ resulted in values 1000-fold magnitude lower than the values obtained with no correction of concentrations for recovery (NC) or by mass balance (MB) from the amount of dsRNA added and the recovery corrected soil concentration. The discrepancy originated from the very poor recovery of spiked dsRNA from the supernatant extraction control, which was only 0.14% (Appendix 4-3). Correcting for this very low recovery resulted in a significant overestimation of the total amount of dsRNA present in the systems (i.e. on average 459% of the amount added, see Appendix 4-3). This indicated that the measured, very poor recovery from the spiked supernatant extraction control of 0.14% was not a reliable basis for recovery correction of the batch sorption experiment results, which then *de facto* resulted in an underestimated K_d. By the contrast, the recovery from the soil extraction control was much better at all three $CaCl_2$ concentrations, ranging from 82-102 % on average. Thus, the mass-balance (MS) approach in which the dissolved amount of dsRNA was calculated as the difference between the amount added to the batches, and the recovery corrected amount of dsRNA measured after extraction of the soil matrix, provided in our judgement the most robust method in the K_d calculation with a smaller of uncertainty in the results especially for higher ionic strength of the solution (Figure 4.4). Furthermore, the three calculation methods tended to align at the same K_d, with a very low margin of error when the $CaCl_2$ concentration was reduced.

The data clearly show the importance of the soil solution ionic composition on K_d. 0.01M $CaCl_2$ had an ionic strength of 0.012 mol L^{-1}, 10-fold higher than ionic strength of 0.001M (0.004 mol L^{-1}) and DEPC-water (0.002 mol L^{-1}) after equilibration with the soil (Appendix 4-2). Not only will the ionic composition affect the dsRNA solubility, but in the presence of multivalent cations the nucleotide adsorption is also enhanced due to the cations bridge effect (Levy-Booth *et al.*, 2007; Beall *et al.*, 2009; Yu *et al.*, 2013). Thus, in our study the divalent charges of calcium (Ca^{2+}) may have facilitated the adsorption, by bridging the negative charges of soil colloids as well as the dsRNA phosphate groups and thereby increasing K_d. For the condition of our study with pH_{soil} = 6.6, the soil solids were negatively charged, since they mainly consisted of quartz minerals (pI=1.5, Adair, Suvaci and Sindel, 2001), as confirmed by XRD analysis (Appendix 4-42), and humic substances, while the nucleotide dsRNA was also negatively charged (pI=5.0). In fact, the dsRNA followed a similar trend to DNA in increased adsorption on soil colloids in the presence of divalent cations (Lorenz and Wackernagel, 1987; Saeki, Kunito and Sakai, 2010; Pedreira-Segade, Michot and Daniel, 2018). Similarly, Saeki, Kunito and Sakai (2010) observed a significant enhancement of nucleotides adsorption in magnesium chloride solution from 25% at 0.01M at 75% to 1M. Therefore, these insight must be considerate before assessing any environmental fate studies of dsRNA following the OECD 106 procedure. Since the actual ionic strength of water in soil is not readily known, but low K_d represents the highest dsRNA mobility and thus risk in environmental fate assessments, we decided to measure K_d at low ionic strength as a worst-case scenario. Therefore, based on these considerations we decided to further assess the adsorption coefficient (K_d) with the slightly modified OECD 106 procedure, i.e. by pre-equilibrating the soil in DEPC-Water and then calculating the K_d from the recovery corrected soil concentration, by using the mass balance method (MB).

4.4.2. Sorption kinetics

The sorption kinetics on loamy sand soil pre-equilibrated with DEPC-Water was rapid (Figure 4.7). The K_d ranged between 0.4 to 0.5 mL g^{-1} reaching equilibrium within 1h, similar to the results of a DNA adsorption study carried out by (Blum, Lorenz and Wackernagel, 1997) on sandy soils. Limited sorption at low ionic strength of the solution can be explained by the anionic nature of dsRNA at pH=6.77 (pI = 5.0). Since the silicon dioxide group of quartz (SiO_2) are negative charged at pH > pI=1.5 (Clunie and Ingram, 1983; Adair, Suvaci and Sindel, 2001), the negative electrostatic repulsion played a major role during the interaction between dsRNA and soil. As a result, the amount of dsRNA adsorbed at the equilibrium ranged around 8 % in the batches (Figure 4.8). Also, the poor soil organic content as well as low ionic strength did

not give favourable condition for stronger adsorption to soil. The low amount of total cations in aqueous phase after equilibration with DEPC-Water (1.48 meq L^{-1}) minimized the cation bridging effect described before. Therefore, at higher ionic strength of the soil porewater solution, and with more bivalent cations like Ca^{2+} (or Mg^{2+}), cation bridging would enhance the dsRNA binding to the soil solid surfaces, while precipitation would reduce the dsRNA solubility in the solution, hence resulting in higher K_d, and lower mobility. Therefore, soil porewater solution chemistry is critical for the mobility of dsRNA in soil. For the condition of our study, the dsRNA K_d depended strongly on ionic strength of the solution, potentially having a higher mobility for low ionic strength conditions, than the most utilised insecticides against the hive beetles (Table 4.6).

Table 4.6 – Comparison of dsRNA sorption coefficients with those of the most common active substances used against the small hive beetle, Aethina Tumida (Cuthbertson et al., 2013).

Insecticide	***Soil Type***	***K_d (mL g^{-1})***	***K_{OC}***	***pH***	***O.M. (%)***	***DT_{50} (days)***
(1)Permethrin						30
	Sandy Loam	217	20865	-	0.60	
	Sediment	401	44070	-	0.91	
	Sand	140	60870	-	0.13	
(1)Carbaryl						
	Sand	2.45	1054	7.7	0.4	17
	Loamy Sand	2.93	504	5.3	1.0	
(1)Methomyl						
	Sandy Loam	9.23	36	6.6	1.1	14-21
(2)Coumaphos	Soil	60 to 298	3994 to 11422	-	-	>365
dsRNA	Loamy Sand	0.45	63.38	6.77	-	0.041
	Loamy Sand	816.36(3)	1.14E5(4)	6.77	1.42	-

(1) Agricultural Research Service, U.S. Department of Agriculture (https://www.ars.usda.gov/)
(2) EPA, Reregistration Eligibility Decision Addendum and FQPA Tolerance Reassessment Progress Report
(3) and (4) are obtained considering the soil pre-equilibration in 0.01M $CaCl_2$

Figure 4.7 - Adsorption Coefficient (K_d) calculated with method RD and MB. The error bars represent the standard deviation of triplicate experiments.

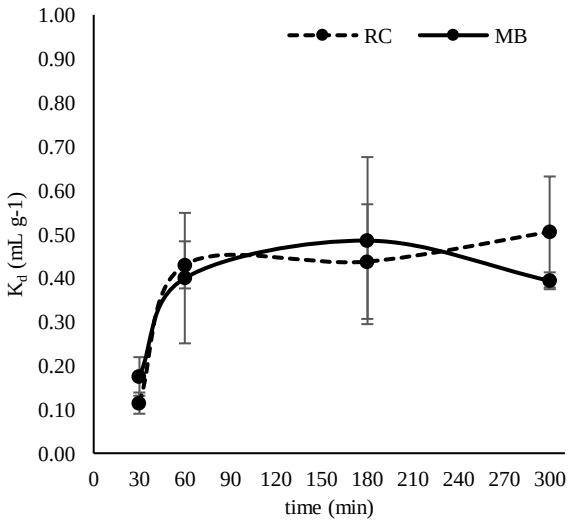
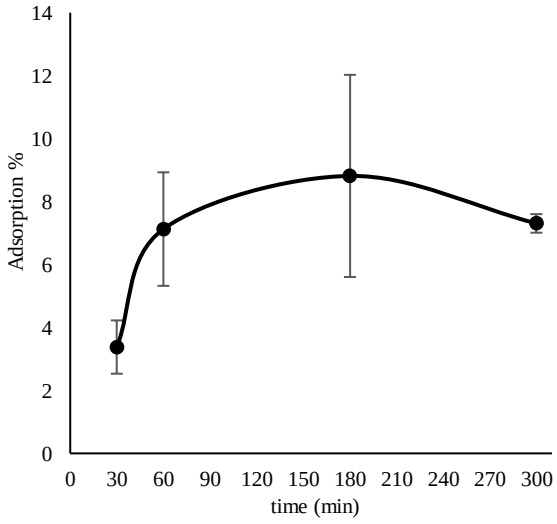


Figure 4.8 - Percentage of dsRNA adsorbed on the loamy sand soil over a period of 300min. The error bars represent the standard deviation of triplicate experiments.



4.4.3. Soil Degradation and DT50

The degradation of dsRNA in the loamy sand soil was very fast, occurring mainly during the first hours of the experiment (Figure *4.9*). The macromolecular breakdown was confirmed by the quantification of fragments with two primers, which annealed in different positions along the dsRNA sequence (Table 3.6, Chapter 3). Both primer sets used (i.e. for the full 305 bp dsRNA length, and for a 109 bp portion of it) gave similar results. The results of the degradation modelling showed that the SFO and FOMC model did not fit the data points well for the earliest days, whereas the DFOP fitted the data more closely (Figure 4.10). Furthermore, the DFOP fit was significantly better than the SFO and FOMC fits when considering the chi-square error of the three models (Table 4.7). First, the difference between the experimental data (E) and the predicted data (P) was very close to zero according to the DFOP fit, whereas the other two models were more likely to over or underestimate the predicted data between 0 day and 10th day, then getting closer to zero by the 39th and 69th day (Figure 4.12). The degradation rates calculated by the DFOP fit had higher level of significance (lower p-value, see Kinetic Model in Appendices).

Accordingly, the DFOP equation was the best model to describe the dsRNA degradation in our study and gave DT_{50} and DT_{90} with a level of confidence of 95% (Figure 4.11). In addition, this model described consistently the bi-phasic kinetics (FOCUS, 2006), describing the degradation kinetics with two rates; the fast degradation ($k_{deg(1)}$) occurred in the earlier hours reducing the dsRNA concentration by more than half in just one day, followed by a slower degradation ($k_{deg(2)}$). This consideration also highlights the differences between DFOP and SFO models. The latter could only fit the initial data (1st day) but not the long-term data due to the mathematical limits when having only one kinetic rate to fit from the data (eq. 4.10).

Table 4.7 - Half-lives and degradation rate derived by the three fit models

Model Fit	$k_{deg(1)}$	$k_{deg(2)}$	g	α	β	DT_{50} **(hours)**	DT_{90} **(days)**	χ^2_{err}
DFOP	16.91	0.28	0.64	-	-	9.8	4.54	2.66
SFO	1.18	-	-	-	-	13.9	1.94	20.30
FOMC	0.98$^{(1)}$	0.37$^{(2)}$	-	0.98	0.38	9.3	3.56	9.51

(1) and (2) do not represent the degradation rate, but the parameters α and β in the eq. 4.12

The DT_{50} was estimated as 9.8 h, showing low persistence in the soil matrix, in agreement with the dsRNA biodegradation (DT_{50}) estimate of 27.8 h by Dubelman *et al.*, which highlights the high degradability of dsRNA, with a 50% concentration reduction within about one day. We believe that the high degradation in the loamy sand soil is in accordance with the adsorption coefficient (K_d) described previously (paragraph 4.4.2). A weak adsorption (K_d^{eq} ~ 0.45 mL g^{-1}) left most of the dsRNA unbounded and readily accessible to the microorganisms,

endonucleases and ribonucleases (RNases) for degradation. The adsorbed fraction tends to be less accessible and slowly degradable (Lorenz and Wackernagel, 1987; Blum, Lorenz and Wackernagel, 1997; Crecchio and Stotzky, 1998; Levy-Booth *et al.*, 2007), resulting in bi-phasic kinetics, as was observed in this study. Although we observed a bi-phasic trend, the majority fraction of dsRNA was associated with the fast degradation rate.

4.5. Conclusion

In the present study, we showed that dsRNA adsorption and degradation in the loamy sand soil occurred rapidly. The sorption equilibrium was reached in 1h, and the macromolecule was highly degradable. The degradation experiments also showed bi-phasic kinetics which was faster in the latest hours and then slowed down after one day, resulting in a DT_{50}=9.8h and depleting the dsRNA concentration by 90% after 4.54 days. In the condition of our study, the adsorption coefficient was highly dependant on the ionic strength of the soil porewater. This is due to the anionic nature of nucleotide at alkaline pH, which likely resulted in an electrostatic repulsion to the quartz minerals, which are predominant in our soil.

Figure 4.9- dsRNA degradation in the loamy sand soil over the period of 69 days. The degradation (▼) was quantified using primers that amplified the amplicon size of 105bp, in the middle of the dsRNA sequence. The degradation (●) was assessed with primers that amplified the whole length of dsRNA (305bp). The error bars represent the standard deviation of triplicate experiments.

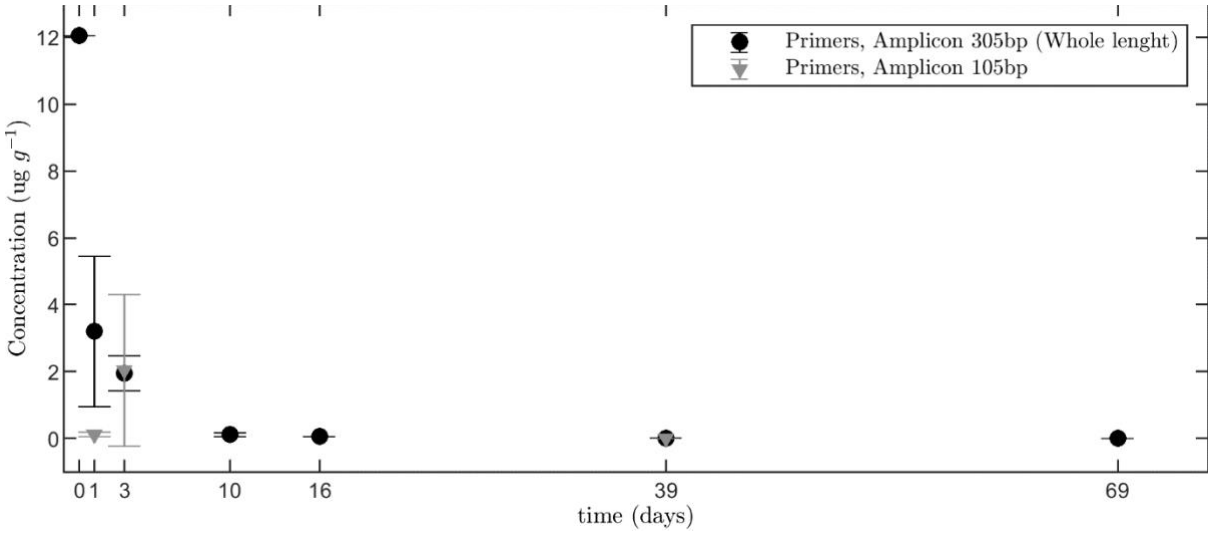


Figure 4.10 – Fit of the three mathematical models to the experimental data.

Figure 4.11 – Level of confidence 95% for the DFOP models.

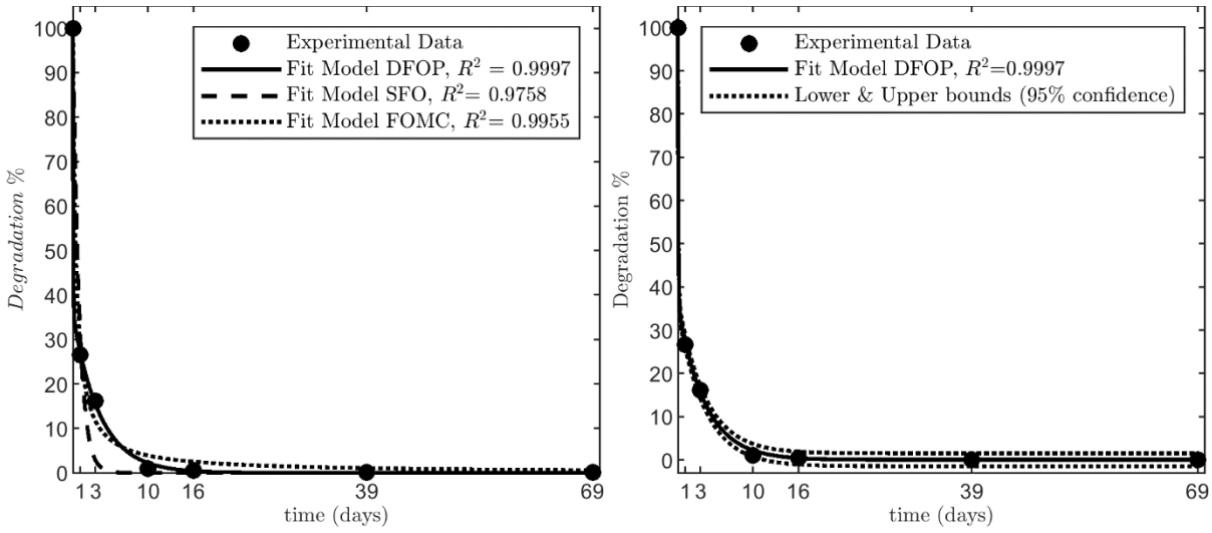
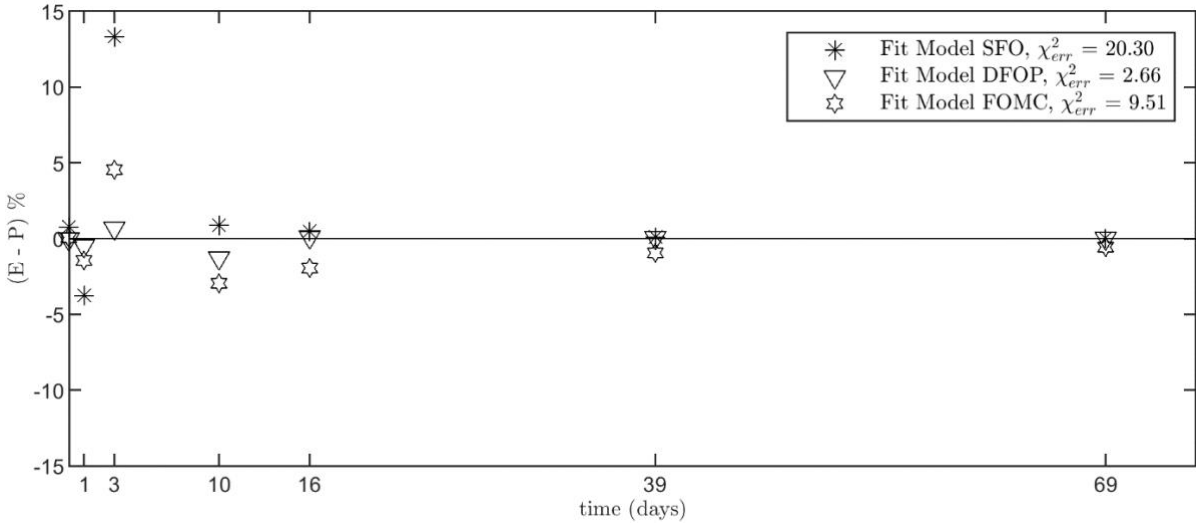


Figure 4.12 – Residuals of the three fit models representing the distance of the perdition models (P) to the experimental data (E).



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Appendices Chapter 4

Appendix 4-1- Ion analysis for loamy sand soil

Cations Concentration

	Total Ca	**Total Mg**	**Total Na**	**Total K**	**Total Ba**	**Total Fe**	**Total Mn**	**Total Al**	**Total Zn**
mg/L	7.3	2.2	0.4	3.0	0.0	1.2	2.3	2.0	0.031
meq./L	0.36	0.18	0.02	0.07	0.0	0.04	0.08	0.22	9.54E-04
	Total Pb	**Total Cu**	**Total As**	**Total Cd**	**Total Sb**	**Total Si**	**Total Ni**	**Total Cr**	**Total Sr**
mg/L	0.008	0.013	<0.005	<0.005	<0.005	3.589	0.004	0.006	0.023
meq./L	7.25E-5	2.05E-4	0.0	0.0	0.0	0.51	9.13E-08	3.46E-07	5.25E-07
								Tot. meq./L	**1.48**

***Anions Concentration*[(1)]**

Sample	**Total F**	**Total Cl**	**Total Nitrite**	**Total Bromide**	**Total Sulphate**	**Total Nitrate**	**Total Phosphate**
mg/L	1.045	1.552	0.138	0.367	2.548	8.004	6.840
meq./L	5.50E-02	4.38E-02	3.00E-03	4.59E-03	5.31E-02	1.29E-01	2.16E-01
						Tot. meq L^{-1}	**5.05E-01**

[(1)] Note that for the anions total amount, the carbonate ions were not included

Appendix 4-2 – Ionic strength at different $CaCl_2$ concentration 0M, 0.001M and 0.01M

	Total Ca	**Total Mg**	**Total Na**	**Total K**	**Total Ba**	**TotalFe**	**Total Mn**	**Total Al**
mg L^{-1}	7.264	2.177	0.391	2.983	0.022	1.209	2.307	1.989
MW	40.1	24.3	23.0	40.0	137.0	55.8	55.0	27.0
mol L^{-1}	1.81E-04	8.96E-05	1.70E-05	7.46E-05	1.57E-07	2.17E-05	4.19E-05	7.37E-05
n. Charge	2	2	1	1	1	2	2	3
$C_iz_i^2$	3.63E-04	3.58E-04	1.70E-05	7.46E-05	1.57E-07	8.66E-05	1.68E-04	6.63E-04
	Total Zn	**Total Pb**	**Total Cu**	**Total Si**	**Total Ni**	**Total Cr**	**Total Sr**	
mg L^{-1}	0.031	0.008	0.013	3.589	0.004	0.006	0.023	
MW	65.0	207.0	63.5	28.0	58.7	52.0	87.6	
mol L^{-1}	4.77E-07	3.62E-08	2.05E-07	1.28E-04	6.82E-08	1.15E-07	2.62E-07	
n. Charge	2	2	1	4	2	3	2	
$C_iz_i^2$	1.91E-06	1.45E-07	2.05E-07	2.05E-03	2.73E-07	1.04E-06	1.05E-06	
	Total F	**Total Cl**	**Total Nitrite**	**Total Bromide**	**Total Sulphate**	**Total Nitrate**	**Total Phosphate**	
mg/L	1.05E+00	1.55E+00	1.38E-01	3.67E-01	2.55E+00	8.00E+00	6.84E+00	
Mw	19.0	35.4	46.0	80.0	96.0	62.0	95.0	
mol/L	5.50E-05	4.38E-05	3.00E-06	4.59E-06	2.65E-05	1.29E-04	7.20E-05	
n. Charge	-1	-1	-1	-1	-2	-1	-3	
$C_iz_i^2$	5.50E-05	4.38E-05	3.00E-06	4.59E-06	1.06E-04	1.29E-04	6.48E-04	
I at 0M[(1)]	**0.002**							
I at 0.001M[(1)]	**0.003**							
I at 0.01M[(1)]	**0.012**							

[(1)] Note that for the anions total amount, the carbonate ions were not included

Appendix 4-3 - Results of 0.01M $CaCl_2$ calculated by RC Method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.61E+12	1.61E+12	82%	1.96E+12	4.65E+12	236%	0.1998		3.64
	$C_{supernatant}$	2.75E+09			2.70E+12				1	
#2	C_{soil}	1.62E+12	1.64E+12	83%	1.98E+12	1.25E+13	634%	0.2043		0.92
	$C_{supernatant}$	1.07E+10			1.05E+13				1	
#3	C_{soil}	1.82E+12	1.82E+12	93%	2.21E+12	9.98E+12	506%	0.1983		1.43
	$C_{supernatant}$	7.92E+09			7.77E+12				1	
#1	$C^{ext}_{soil\ control}$	1.74E+12		88%						
#2	$C^{ext}_{soil\ control}$	1.50E+12		76%						
#1	$C^{ext}_{sup.\ control}$	2.89E+09		0.15%						
#2	$C^{ext}_{sup.\ control}$	1.13E+09		0.06%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	6.35E+03							**Average**	2.00
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	1.44

Appendix 4-4 - Results of 0.01M CaCl2 calculated by NC Method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.61E+12	1.61E+12	82%				0.1998		2933.25
	$C_{supernatant}$	2.75E+09							1	
#2	C_{soil}	1.62E+12	1.64E+12	83%				0.2043		742.49
	$C_{supernatant}$	1.07E+10							1	
#3	C_{soil}	1.82E+12	1.82E+12	93%				0.1983		1156.23
	$C_{supernatant}$	7.92E+09							1	
#1	$C^{ext}_{soil\ control}$	1.74E+12		88%						
#2	$C^{ext}_{soil\ control}$	1.50E+12		76%						
#1	$C^{ext}_{sup.\ control}$	2.89E+09		0.15%						
#2	$C^{ext}_{sup.\ control}$	1.13E+09		0.06%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	6.35E+03							**Average**	1610.66
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	1163.93

Appendix 4-5 - Results of 0.01M $CaCl_2$ calculated by MB Method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.61E+12	1.61E+12	82%	1.96E+12	1.97E+12	100%	0.1998		865.31
	$C_{supernatant}$	2.75E+09			1.13E+10				1	
#2	C_{soil}	1.62E+12	1.64E+12	83%	1.98E+12	1.98E+12	101%	0.2043		1536.96
	$C_{supernatant}$	1.07E+10			6.29E+09				1	
#3	C_{soil}	1.82E+12	1.82E+12	93%	2.21E+12	2.45E+12	124%	0.1983		46.80
	$C_{supernatant}$	7.92E+09			2.38E+11				1	
#1	$C^{ext}_{soil\ control}$	1.74E+12		88%						
#2	$C^{ext}_{soil\ control}$	1.50E+12		76%						
#1	$C^{ext}_{sup.\ control}$	2.89E+09		0.15%						
#2	$C^{ext}_{sup.\ control}$	1.13E+09		0.06%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	6.35E+03							**Average**	816.36
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	746.28

Appendix 4-6 - Results of 0.001M $CaCl_2$ calculated by RC Method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	6.00E+11	9.06E+11	47%	5.61E+11	2.07E+12	108%	0.2017		1.84
	$C_{supernatant}$	3.06E+11			1.51E+12				1	
#2	C_{soil}	7.32E+11	9.18E+11	48%	6.84E+11	1.61E+12	84%	0.2010		3.69
	$C_{supernatant}$	1.86E+11			9.22E+11				1	
#3	C_{soil}	7.32E+09	4.99E+11	26%	6.84E+09	2.44E+12	127%	0.1984		0.01
	$C_{supernatant}$	4.92E+11			2.43E+12				1	
#1	$C^{ext}_{soil\ control}$	2.06E+12		107%						
#1	$C^{ext}_{sup.\ control}$	6.46E+11		34%						
#2	$C^{ext}_{sup.\ control}$	1.31E+11		7%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	1.85
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	1.84

Appendix 4-7 Results of 0.001M $CaCl_2$ calculated by NC Method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	6.00E+11	9.06E+11	47%				0.2017		8.75
	$C_{supernatant}$	3.06E+11							1	
#2	C_{soil}	7.32E+11	9.18E+11	48%				0.2010		17.58
	$C_{supernatant}$	1.86E+11							1	
#3	C_{soil}	7.32E+09	4.99E+11	26%				0.1984		0.07
	$C_{supernatant}$	4.92E+11							1	
#1	$C^{ext}_{soil\ control}$	2.06E+12		107%						
#1	$C^{ext}_{sup.\ control}$	6.46E+11		34%						
#2	$C^{ext}_{sup.\ control}$	1.31E+11		7%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	8.80
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	8.76

Appendix 4-8 - Results of 0.001M CaCl2 calculated by MB Method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	6.00E+11	9.06E+11	47%	5.61E+11	1.92E+12	100%	0.2017		2.04
	$C_{supernatant}$	3.06E+11			1.36E+12				1	
#2	C_{soil}	7.32E+11	9.18E+11	48%	6.84E+11	1.92E+12	100%	0.2010		2.75
	$C_{supernatant}$	1.86E+11			1.24E+12				1	
#3	C_{soil}	7.32E+09	4.99E+11	26%	6.84E+09	1.92E+12	100%	0.1984		0.02
	$C_{supernatant}$	4.92E+11			1.91E+12				1	
#1	$C^{ext}_{soil\ control}$	2.06E+12		107%						
#1	$C^{ext}_{sup.\ control}$	6.46E+11		34%						
#2	$C^{ext}_{sup.\ control}$	1.31E+11		7%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	1.61
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	1.42

Appendix 4-9 - Adsorption Assay at time = 30min. Data calculated by RC method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	4.82E+10	9.95E+11	51%	6.12E+10	2.57E+12	131%	0.1999	1	0.12
	$C_{supernatant}$	9.47E+11			2.51E+12					
#2	C_{soil}	4.19E+10	1.00E+12	51%	5.32E+10	2.61E+12	132%	0.1975	1	0.11
	$C_{supernatant}$	9.63E+11			2.55E+12					
#3	C_{soil}	6.72E+10	1.14E+12	58%	8.52E+10	2.93E+12	149%	0.2013	1	0.15
	$C_{supernatant}$	1.07E+12			2.84E+12					
#1	$C^{ext}_{soil\ control}$	1.51E+12		77%						
#2	$C^{ext}_{soil\ control}$	1.59E+12		81%						
#1	$C^{ext}_{sup.\ control}$	7.40E+11		38%						
#2	$C^{ext}_{sup.\ control}$	7.45E+11		38%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.13
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.02

Appendix 4-10 - Adsorption Assay at time = 30min. Data calculated by NC method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	4.82E+10	9.95E+11	51%				0.1999	1	0.25
	$C_{supernatant}$	9.47E+11								
#2	C_{soil}	4.19E+10	1.00E+12	51%				0.1975	1	0.22
	$C_{supernatant}$	9.63E+11								
#3	C_{soil}	6.72E+10	1.14E+12	58%				0.2013	1	0.31
	$C_{supernatant}$	1.07E+12								
#1	$C^{ext}_{soil\ control}$	1.51E+12		77%						
#2	$C^{ext}_{soil\ control}$	1.59E+12		81%						
#1	$C^{ext}_{sup.\ control}$	7.40E+11		38%						
#2	$C^{ext}_{sup.\ control}$	7.45E+11		38%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.26
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.05

Appendix 4-11 - Adsorption Assay at time = 30min. Data calculated by MB method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	4.82E+10	9.95E+11	51%	6.12E+10	1.97E+12	100%	0.1999		0.16
	$C_{supernatant}$	9.47E+11			1.91E+12				1	
#2	C_{soil}	4.19E+10	1.00E+12	51%	5.32E+10	1.97E+12	100%	0.1975		0.14
	$C_{supernatant}$	9.63E+11			1.92E+12				1	
#3	C_{soil}	6.72E+10	1.14E+12	58%	8.52E+10	1.97E+12	100%	0.2013		0.22
	$C_{supernatant}$	1.07E+12			1.88E+12				1	
#1	$C_{soil\ control}^{ext}$	1.51E+12		77%						
#2	$C_{soil\ control}^{ext}$	1.59E+12		81%						
#1	$C_{sup.\ control}^{ext}$	7.40E+11		38%						
#2	$C_{sup.\ control}^{ext}$	7.45E+11		38%						
#1	C_{soil}^{blank}	0.00E+00								
	C_{soil}^{blank}	0.00E+00							**Average**	0.18
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.04

Appendix 4-12 - Adsorption Assay at time = 60min. Data calculated by RC method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.54E+11	2.33E+11	12%	1.30E+11	3.91E+11	20%	0.197		$2.54^{(*)}$
	$C_{supernatant}$	7.98E+10			2.61E+11				1	
#2	C_{soil}	1.31E+11	4.89E+11	25%	1.11E+11	1.28E+12	65%	0.2029		0.47
	$C_{supernatant}$	3.58E+11			1.17E+12				1	
#3	C_{soil}	2.12E+11	9.18E+11	47%	1.80E+11	2.49E+12	126%	0.1991		0.39
	$C_{supernatant}$	7.06E+11			2.31E+12				1	
#1	$C_{soil\ control}^{ext}$	2.52E+12		128%						
#2	$C_{soil\ control}^{ext}$	2.13E+12		108%						
#1	$C_{sup.\ control}^{ext}$	5.94E+11		30%						
#2	$C_{sup.\ control}^{ext}$	6.12E+11		31%						
#1	C_{soil}^{blank}	0.00E+00								
	C_{soil}^{blank}	0.00E+00							**Average**	0.43
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.05

(*) The data has been excluded from the average with 95% of confidence (Q-test).

Appendix 4-13 - Adsorption Assay at time = 60min. Data calculated by NC method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.54E+11	2.33E+11	12%				0.197		9.77
	$C_{supernatant}$	7.98E+10							1	
#2	C_{soil}	1.31E+11	4.89E+11	25%				0.2029		1.80
	$C_{supernatant}$	3.58E+11							1	
#3	C_{soil}	2.12E+11	9.18E+11	47%				0.1991		1.51
	$C_{supernatant}$	7.06E+11							1	
#1	$C^{ext}_{soil\ control}$	2.52E+12		128%						
#2	$C^{ext}_{soil\ control}$	2.13E+12		108%						
#1	$C^{ext}_{sup.\ control}$	5.94E+11		30%						
#2	$C^{ext}_{sup.\ control}$	6.12E+11		31%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	1.65
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.21

Appendix 4-14 - Adsorption Assay at time = 60min. Data calculated by MB method.

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.54E+11	2.33E+11	12%	1.30E+11	1.97E+12	100%	0.197		0.36
	$C_{supernatant}$	7.98E+10			1.84E+12				1	
#2	C_{soil}	1.31E+11	4.89E+11	25%	1.11E+11	1.97E+12	100%	0.2029		0.29
	$C_{supernatant}$	3.58E+11			1.86E+12				1	
#3	C_{soil}	2.12E+11	9.18E+11	47%	1.80E+11	1.97E+12	100%	0.1991		0.50
	$C_{supernatant}$	7.06E+11			1.79E+12				1	
#1	$C^{ext}_{soil\ control}$	2.52E+12		128%						
#2	$C^{ext}_{soil\ control}$	2.13E+12		108%						
#1	$C^{ext}_{sup.\ control}$	5.94E+11		30%						
#2	$C^{ext}_{sup.\ control}$	6.12E+11		31%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.40
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.50

Appendix 4-15 - Adsorption Assay at time = 180min. Data calculated by RC method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	3.42E+10		64%	1.10E+11	1.96E+12	99%	0.2002		0.30
	$C_{supernatant}$	1.24E+12	1.27E+12		1.85E+12				1	
#2	C_{soil}	7.36E+10		91%	2.36E+11	2.79E+12	142%	0.2017		0.46
	$C_{supernatant}$	1.71E+12	1.78E+12		2.56E+12				1	
#3	C_{soil}	5.44E+10		56%	1.75E+11	1.74E+12	88%	0.2010		0.56
	$C_{supernatant}$	1.05E+12	1.10E+12		1.56E+12				1	
#1	$C^{ext}_{soil\ control}$	2.79E+11		14%						
#2	$C^{ext}_{soil\ control}$	9.48E+11		48%						
#1	$C^{ext}_{sup.\ control}$	2.24E+12		114%						
#2	$C^{ext}_{sup.\ control}$	3.96E+11		20%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.44
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.13

Appendix 4-16 - Adsorption Assay at time = 180min. Data calculated by NC method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	3.42E+10		64%				0.2002		0.14
	$C_{supernatant}$	1.24E+12	1.27E+12						1	
#2	C_{soil}	7.36E+10		91%				0.2017		0.21
	$C_{supernatant}$	1.71E+12	1.78E+12						1	
#3	C_{soil}	5.44E+10		56%				0.2010		0.26
	$C_{supernatant}$	1.05E+12	1.10E+12						1	
#1	$C^{ext}_{soil\ control}$	2.79E+11		14%						
#2	$C^{ext}_{soil\ control}$	9.48E+11		48%						
#1	$C^{ext}_{sup.\ control}$	2.24E+12		114%						
#2	$C^{ext}_{sup.\ control}$	3.96E+11		20%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.20
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.06

Appendix 4-17 - Adsorption Assay at time = 180min. Data calculated by MB method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	3.42E+10	1.27E+12	64%	1.10E+11	1.97E+12	100%	0.2002		0.29
	$C_{supernatant}$	1.24E+12			1.86E+12				1	
#2	C_{soil}	7.36E+10	1.78E+12	91%	2.36E+11	1.97E+12	100%	0.2017		0.68
	$C_{supernatant}$	1.71E+12			1.73E+12				1	
#3	C_{soil}	5.44E+10	1.10E+12	56%	1.75E+11	1.97E+12	100%	0.2010		0.48
	$C_{supernatant}$	1.05E+12			1.80E+12				1	
#1	$C^{ext}_{soil\ control}$	2.79E+11		14%						
#2	$C^{ext}_{soil\ control}$	9.48E+11		48%						
#1	$C^{ext}_{sup.\ control}$	2.24E+12		114%						
#2	$C^{ext}_{sup.\ control}$	3.96E+11		20%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.49
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.19

Appendix 4-18 - Adsorption Assay at time = 300min. Data calculated by RC method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.22E+11	3.73E+11	19%	1.46E+11	1.65E+12	84%	0.2007		0.48
	$C_{supernatant}$	2.51E+11			1.51E+12				1	
#2	C_{soil}	1.15E+11	2.93E+11	15%	1.37E+11	1.20E+12	61%	0.2015		0.64
	$C_{supernatant}$	1.78E+11			1.06E+12				1	
#3	C_{soil}	1.24E+11	4.43E+11	22%	1.49E+11	2.06E+12	105%	0.1991		0.39
	$C_{supernatant}$	3.19E+11			1.91E+12				1	
#1	$C^{ext}_{soil\ control}$	1.62E+12		82%						
#2	$C^{ext}_{soil\ control}$	1.68E+12		85%						
#1	$C^{ext}_{sup.\ control}$	3.94E+11		20%						
#2	$C^{ext}_{sup.\ control}$	2.63E+11		13%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.50
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.13

Appendix 4-19 - Adsorption Assay at time = 300min. Data calculated by NC method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.22E+11	3.73E+11	19%				0.2007		2.42
	$C_{supernatant}$	2.51E+11							1	
#2	C_{soil}	1.15E+11	2.93E+11	15%				0.2015		3.21
	$C_{supernatant}$	1.78E+11							1	
#3	C_{soil}	1.24E+11	4.43E+11	22%				0.1991		1.96
	$C_{supernatant}$	3.19E+11							1	
#1	$C^{ext}_{soil\ control}$	1.62E+12		82%						
#2	$C^{ext}_{soil\ control}$	1.68E+12		85%						
#1	$C^{ext}_{sup.\ control}$	3.94E+11		20%						
#2	$C^{ext}_{sup.\ control}$	2.63E+11		13%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	2.53
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.63

Appendix 4-20 - Adsorption Assay at time = 300min. Data calculated by MB method

Replicate	Sample	Copies	Tot. Copies	Recovery	Copies Corrected	Tot. Copies Corrected	Recovery Corrected	Soil (g)	Supernatant (mL)	K_d (mL g^{-1})
#1	C_{soil}	1.22E+11	3.73E+11	19%	1.46E+11	1.97E+12	100%	0.2007		0.40
	$C_{supernatant}$	2.51E+11			1.82E+12				1	
#2	C_{soil}	1.15E+11	2.93E+11	15%	1.37E+11	1.97E+12	100%	0.2015		0.37
	$C_{supernatant}$	1.78E+11			1.83E+12				1	
#3	C_{soil}	1.24E+11	4.43E+11	22%	1.49E+11	1.97E+12	100%	0.1991		0.41
	$C_{supernatant}$	3.19E+11			1.82E+12				1	
#1	$C^{ext}_{soil\ control}$	1.62E+12		82%						
#2	$C^{ext}_{soil\ control}$	1.68E+12		85%						
#1	$C^{ext}_{sup.\ control}$	3.94E+11		20%						
#2	$C^{ext}_{sup.\ control}$	2.63E+11		13%						
#1	C^{blank}_{soil}	0.00E+00								
	C^{blank}_{soil}	0.00E+00							**Average**	0.39
	$dsRNA_{spiked}$	1.97E+12							**Standard Deviation**	0.02

Appendix 4-21 - - Biodegradation Assay at time = 1 day.

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	1.63E+12	0.1989	34%	1.39E+12	6.98E+12
#2	C_{soil}	2.44E+12	0.1991	51%	2.07E+12	1.04E+13
#3	C_{soil}	3.80E+11	0.2016	8%	3.24E+11	1.61E+12
#1	$C_{soil,t0}$	7.40E+12	0.1961	155%		
#2	$C_{soil,t0}$	0.00E+00	0.1960	-		
#3	$C_{soil,t0}$	3.78E+12	0.2023	79%		
#1	C_{blank}	3.83E+05	0.1995	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	6.33E+12
					Standard Deviation	4.44E+12

Appendix 4-22 - Biodegradation Assay at time = 3 days.

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	5.50E+11	0.1989	12%	5.95E+11	2.99E+12
#2	C_{soil}	9.10E+11	0.1991	19%	9.85E+11	4.95E+12
#3	C_{soil}	6.70E+11	0.2016	14%	7.25E+11	3.60E+12
#1	$C_{soil,t0}$	6.05E+12	0.1961	127%		
#2	$C_{soil,t0}$	4.79E+12	0.1960	101%		
#3	$C_{soil,t0}$	2.35E+12	0.2023	49%		
#1	C_{blank}	7.45E+05	0.1995	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	3.85E+12
					Standard Deviation	1.00E+12

Appendix 4-23 - Biodegradation Assay at time = 10 days

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	2.57E+10	0.1979	1%	3.22E+10	1.63E+11
#2	C_{soil}	3.01E+10	0.2023	1%	3.77E+10	1.86E+11
#3	C_{soil}	5.75E+10	0.2013	1%	7.21E+10	3.58E+11
#1	$C_{soil,t0}$	3.17E+12	0.2025	66%		
#2	$C_{soil,t0}$	4.19E+12	0.2020	88%		
#3	$C_{soil,t0}$	4.04E+12	0.1992	85%		
#1	C_{blank}	2.97E+05	0.1970	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	2.36E+11
					Standard Deviation	1.07E+11

Appendix 4-24 - Biodegradation Assay at time = 16 days

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	1.02E+10	0.1979	0%	2.10E+10	1.06E+11
#2	C_{soil}	1.25E+10	0.2023	0%	2.59E+10	1.28E+11
#3	C_{soil}	1.22E+10	0.2013	0%	2.52E+10	1.25E+11
#1	$C_{soil,t0}$	2.61E+12		55%		
#2	$C_{soil,t0}$	2.75E+12		58%		
#3	$C_{soil,t0}$	1.55E+12		32%		
#1	C_{blank}	1.90E+05		0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	1.20E+11
					Standard Deviation	1.19E+10

Appendix 4-25 - Biodegradation Assay at time = 39 days

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	1.12E+09	0.1970	0%	3.30E+09	1.67E+10
#2	C_{soil}	1.24E+08	0.1974	0%	3.65E+08	1.85E+09
#3	C_{soil}	2.93E+09	0.2003	0%	8.63E+09	4.31E+10
#1	$C_{soil,t0}$	2.16E+12	0.2028	45%		
#2	$C_{soil,t0}$	7.05E+11	0.2025	15%		
#3	$C_{soil,t0}$	1.99E+12	0.2019	42%		
#1	C_{blank}	0.00E+00	0.2012	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	2.06E+10
					Standard Deviation	2.09E+10

Appendix 4-26 - Biodegradation Assay at time = 69 days

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	1.52E+09	0.1983	0%	1.37E+09	6.93E+09
#2	C_{soil}	1.35E+09	0.1956	0%	1.22E+09	6.24E+09
#3	C_{soil}	3.07E+09	0.2012	0%	2.78E+09	1.38E+10
#1	$C_{soil,t0}$	9.00E+12	0.2017	189%		
#2	$C_{soil,t0}$	3.06E+12	0.2003	64%		
#3	$C_{soil,t0}$	3.74E+12	0.1995	79%		
#1	C_{blank}	2.26E+05	0.2009	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	8.99E+09
					Standard Deviation	4.18E+09

Appendix 4-27 - Biodegradation Assay at time = 1 day

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	4.85E+10	0.1989	1%	7.03E+10	3.54E+11
#2	C_{soil}	4.09E+10	0.1991	1%	5.92E+10	2.97E+11
#3	C_{soil}	1.17E+10	0.2016	0%	1.69E+10	8.38E+10
#1	$C_{soil,t0}$	3.84E+12	0.1961	81%		
#2	$C_{soil,t0}$	4.45E+12	0.1960	93%		
#3	$C_{soil,t0}$	1.57E+12	0.2023	33%		
#1	C_{blank}	3.83E+05	0.1995	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	2.45E+11
					Standard Deviation	1.42E+11

Appendix 4-28 - Biodegradation Assay at time = 3 days

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	1.11E+12	0.1989	23%	1.83E+12	9.21E+12
#2	C_{soil}	1.78E+11	0.1991	4%	2.94E+11	1.48E+12
#3	C_{soil}	1.75E+11	0.2016	4%	2.89E+11	1.43E+12
#1	$C_{soil,t0}$	3.65E+12	0.1961	77%		
#2	$C_{soil,t0}$	2.76E+12	0.1960	58%		
#3	$C_{soil,t0}$	2.22E+12	0.2023	47%		
#1	C_{blank}	0.00E+00	0.1995	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	4.04E+12
					Standard Deviation	4.48E+12

Appendix 4-29 - Biodegradation Assay at time = 39 days

Replicate	Sample	Copies	Soil (g)	Recovery	Copies Corrected	dsRNA/Soil
#1	C_{soil}	7.00E+08	0.1989	0%	2.57E+09	1.29E+10
#2	C_{soil}	1.42E+11	0.1991	3%	5.20E+11	2.61E+12(*)
#3	C_{soil}	7.60E+08	0.2016	0%	2.79E+09	1.39E+10
#1	$C_{soil,t0}$	1.66E+12	0.1961	35%		
#2	$C_{soil,t0}$	4.78E+11	0.1960	10%		
#3	$C_{soil,t0}$	1.75E+12	0.2023	37%		
#1	C_{blank}	5.35E+05	0.1995	0%		
	$dsRNA_{spiked}$	4.76E+12				
					Average	1.34E+10
					Standard Deviation	6.51E+08

(*) The data has been excluded from the average with 99% of confidence (Q-test).

Appendix 4-30 - Level of confidence of Dixon's Q-Test

Number of Replicates:	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
$Q_{90\%}$:	0.941	0.765	0.642	0.560	0.507	0.468	0.437	0.412
$Q_{95\%}$:	0.970	0.829	0.710	0.625	0.568	0.526	0.493	0.466
$Q_{99\%}$:	0.994	0.926	0.821	0.740	0.680	0.634	0.598	0.568

Appendix 4-31 – Chi-square Table.

DoF(1)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
P = 0.05	3.84	5.99	7.82	9.49	11.07	12.59	14.07	15.51	16.92	18.31	19.68	21.03	22.36	23.69	25	26.3	27.59	28.87	30.14	31.41	32.67	33.92	35.17	36.42
P = 0.01	6.64	9.21	11.35	13.28	15.09	16.81	18.48	20.09	21.67	23.21	24.73	26.22	27.69	29.14	30.58	32	33.41	34.81	36.19	37.57	38.93	40.29	41.64	42.98
P = 0.001	10.83	13.82	16.27	18.47	20.52	22.46	24.32	26.13	27.88	29.59	31.26	32.91	34.53	36.12	37.7	39.25	40.79	42.31	43.82	45.32	46.8	48.27	49.73	51.18

(1) Degree of Freedom

Appendix 4-32 - Two Tails T Distribution Table.

df	**a = 0.2**	**0.10**	**0.05**	**0.02**	**0.01**	**0.002**	**0.001**
∞	t_a = 1.282	1.645	1.960	2.326	2.576	3.091	3.291
1	3.078	6.314	12.706	31.821	63.656	318.289	636.578
2	1.886	2.920	4.303	6.965	9.925	22.328	31.600
3	1.638	2.353	3.182	4.541	5.841	10.214	12.924
4	1.533	2.132	2.776	3.747	4.604	7.173	8.610
5	1.476	2.015	2.571	3.365	4.032	5.894	6.869
6	1.440	1.943	2.447	3.143	3.707	5.208	5.959
7	1.415	1.895	2.365	2.998	3.499	4.785	5.408
8	1.397	1.860	2.306	2.896	3.355	4.501	5.041
9	1.383	1.833	2.262	2.821	3.250	4.297	4.781
10	1.372	1.812	2.228	2.764	3.169	4.144	4.587
11	1.363	1.796	2.201	2.718	3.106	4.025	4.437
12	1.356	1.782	2.179	2.681	3.055	3.930	4.318
13	1.350	1.771	2.160	2.650	3.012	3.852	4.221
14	1.345	1.761	2.145	2.624	2.977	3.787	4.140
15	1.341	1.753	2.131	2.602	2.947	3.733	4.073
16	1.337	1.746	2.120	2.583	2.921	3.686	4.015
17	1.333	1.740	2.110	2.567	2.898	3.646	3.965
18	1.330	1.734	2.101	2.552	2.878	3.610	3.922
19	1.328	1.729	2.093	2.539	2.861	3.579	3.883
20	1.325	1.725	2.086	2.528	2.845	3.552	3.850
21	1.323	1.721	2.080	2.518	2.831	3.527	3.819
22	1.321	1.717	2.074	2.508	2.819	3.505	3.792
23	1.319	1.714	2.069	2.500	2.807	3.485	3.768
24	1.318	1.711	2.064	2.492	2.797	3.467	3.745
25	1.316	1.708	2.060	2.485	2.787	3.450	3.725
26	1.315	1.706	2.056	2.479	2.779	3.435	3.707
27	1.314	1.703	2.052	2.473	2.771	3.421	3.689
28	1.313	1.701	2.048	2.467	2.763	3.408	3.674
29	1.311	1.699	2.045	2.462	2.756	3.396	3.660
30	1.310	1.697	2.042	2.457	2.750	3.385	3.646
60	1.296	1.671	2.000	2.390	2.660	3.232	3.460
120	1.289	1.658	1.980	2.358	2.617	3.160	3.37

Results Kinetic Models

Data SFO Fit

Estimated Values:

Parameter	Value	σ	Prob. > t	Lower (90%) CI	Upper (90%) CI	Lower (95%) CI	Upper (95%) CI
Parent_0	99.24	6.207	N/A	86.73	111.7	83.28	115.2
k_Parent	1.185	0.206	0.001115	0.7697	1.6	0.6553	1.714

χ^2

Parameter	Error %	Degrees of Freedom
All data	20.3	5
Parent	20.3	5

Decay Times:

Compartment	DT50 (days)	DT90 (days)
Parent	0.585	1.94

Additional Statistics:

Parameter	r^2 (Obs v Pred)	Efficiency
All data	0.9791	0.9758
Parent	0.9791	0.9758

Parameter Correlation:

	Parent_0	k_Parent
Parent_0	1	0.2888
k_Parent	0.2888	1

Observed v. Predicted:

Compartment Parent

Time (days)	Value (%)	Predicted Value	Residual
0	100	99.24	0.7621
1	26.61	30.35	-3.738
3	16.16	2.837	13.33
10	0.91	0.0007099	0.9093
16	0.503	0	0.503
39	0.086	0	0.086
69	0.038	0	0.038

Data DFOP Fit

Parameter	Value	s	Prob. > t	Lower (90%) CI	Upper (90%) CI	Lower (95%) CI	Upper (95%) CI
Parent_0	100	0.8776	N/A	97.93	102.1	97.21	102.8
k1_Parent	16.91	0.5006	2.85E-5	15.73	18.08	15.31	18.5
k2_Parent	0.2802	0.0248	7.44E-4	0.2218	0.3386	0.2013	0.359
g_Parent	0.6434	0.01718	N/A	0.6029	0.6838	0.5887	0.698

χ^2

Parameter	Error %	Degrees of Freedom
All data	2.66	3
Parent	2.66	3

Decay Times:

Compartment	DT50 (overall days)	DT90 (overall days)	k1 DT50 (days)	k2 DT50 (days)
Parent	nd	4.54	0.041	2.47

Additional Statistics:

Parameter	r^2 (Obs v Pred)	Efficiency
All data	0.9997	0.9997
Parent	0.9997	0.9997

Parameter Correlation:

	Parent_0	k1_Parent	k2_Parent	g_Parent
Parent_0	1	-1.619E-06	9.695E-05	0.182
k1_Parent	-1.619E-06	1	2.003E-05	-1.996E-05
k2_Parent	9.695E-05	2.003E-05	1	-0.79
g_Parent	0.182	-1.996E-05	-0.79	1

Observed v. Predicted:

Compartment Parent

Time (days)	Value (%)	Predicted Value	Residual
0	100	100	-0.000317
1	26.61	27.1	-0.4942
3	16.16	15.47	0.6884
10	0.91	2.177	-1.267
16	0.503	0.4053	0.09775
39	0.086	0.0006434	0.08536
69	0.038	0	0.038

Data FOMC Fit

Estimated Values:

Parameter	Value	s	Prob. > t	Lower (90%) CI	Upper (90%) CI	Lower (95%) CI	Upper (95%) CI
Parent_0	99.94	3.019	N/A	93.51	106.4	91.56	108.3
alpha	0.9828	0.298	N/A	0.3474	1.618	0.1553	1.81
beta	0.3787	0.2346	N/A	-0.1214	0.8789	-0.2727	1.03

χ^2

Parameter	Error %	Degrees of Freedom
All data	9.51	4
Parent	9.51	4

Decay Times:

Compartment	DT50 (days)	DT90 (days)
Parent	0.388	3.56

Additional Statistics:

Parameter	r^2 (Obs v Pred)	Efficiency
All data	0.9957	0.9954
Parent	0.9957	0.9954

Parameter Correlation:

	Parent_0	alpha	beta
Parent_0	1	-0.02467	-0.08928
alpha	-0.02467	1	0.9749
beta	-0.08928	0.9749	1

Observed v. Predicted:

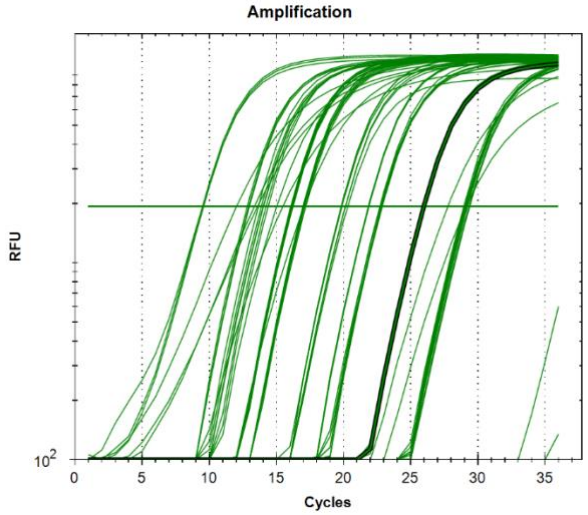
Compartment Parent

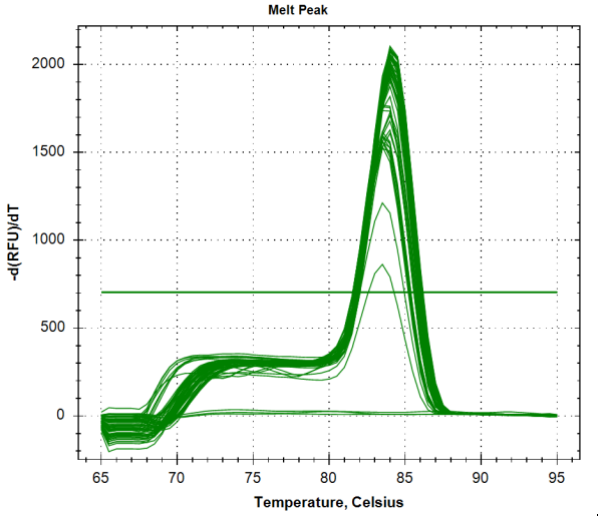
Time (days)	Value (%)	Predicted Value	Residual
0	100	99.94	0.05913
1	26.61	28.07	-1.463
3	16.16	11.63	4.531
10	0.91	3.861	-2.951
16	0.503	2.466	-1.963
39	0.086	1.041	-0.9551
69	0.038	0.5967	-0.5587

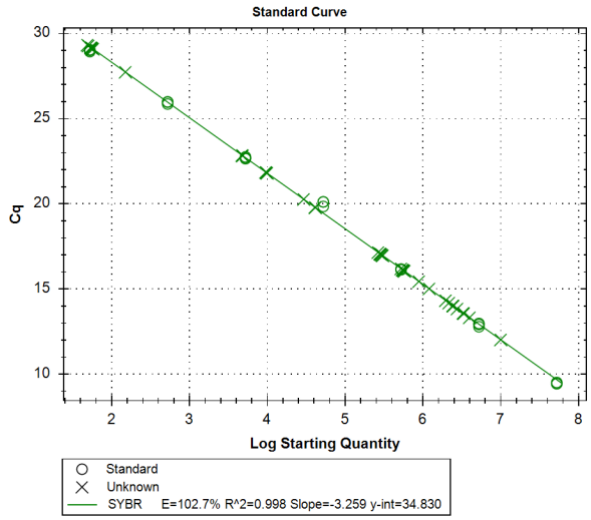
RT-qPCR

Adsorption Test

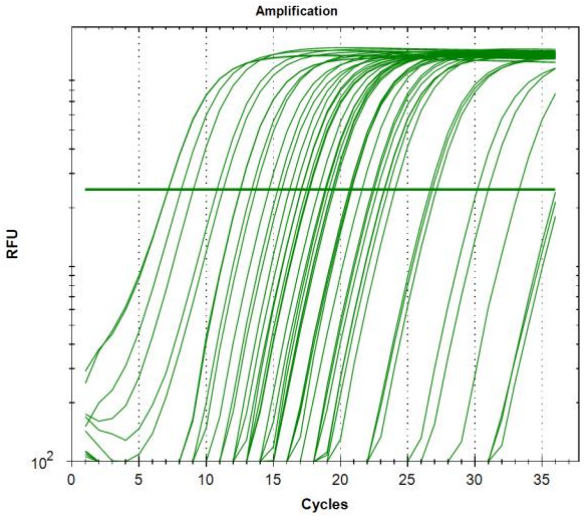
Appendix 4-33 - Amplification, Standard and Melting Curve of RT-qPCR at 0.01M $CaCl_2$

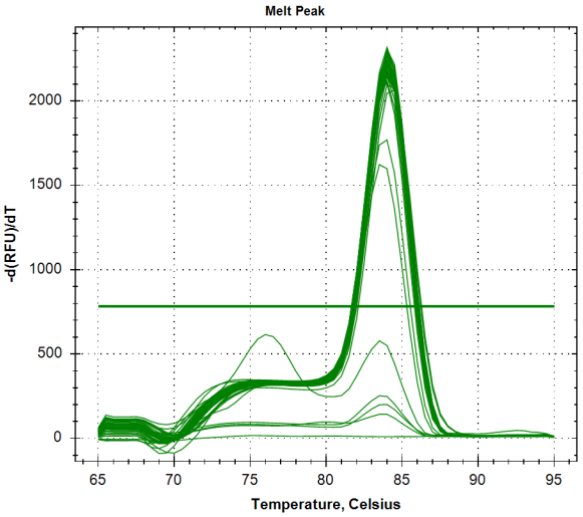


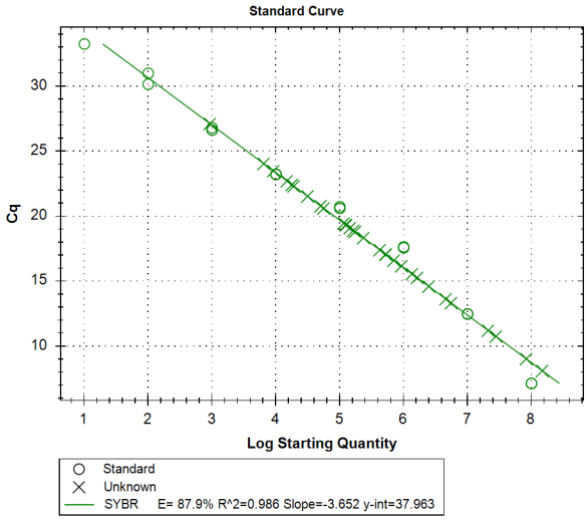




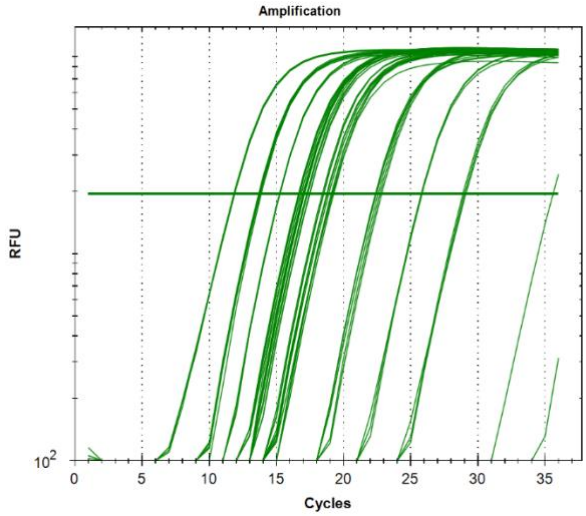
Appendix 4-34 - Amplification, Standard and Melting Curve of RT-qPCR at 0.001M $CaCl_2$

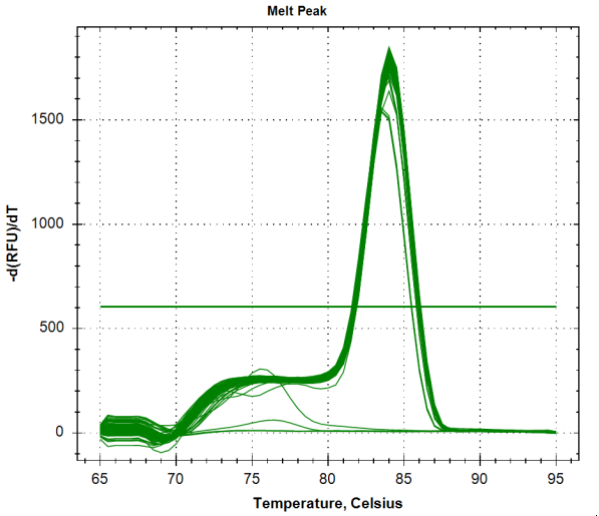


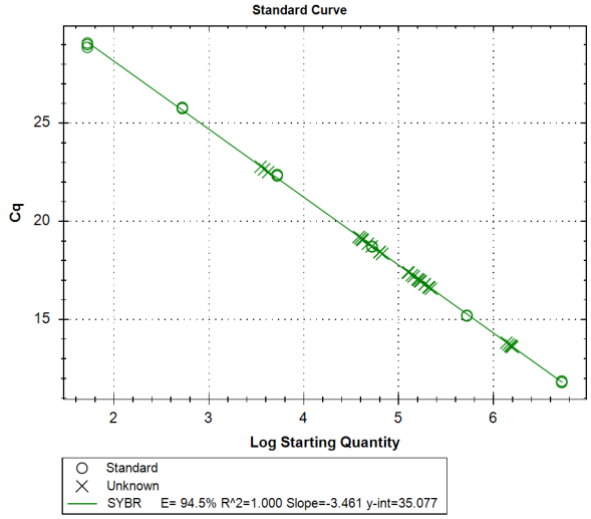




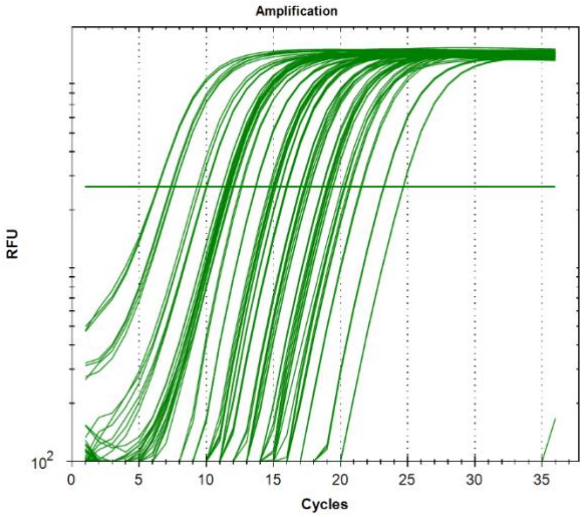
Appendix 4-35 - Amplification, Standard and Melting Curve of RT-qPCR at t = 30min

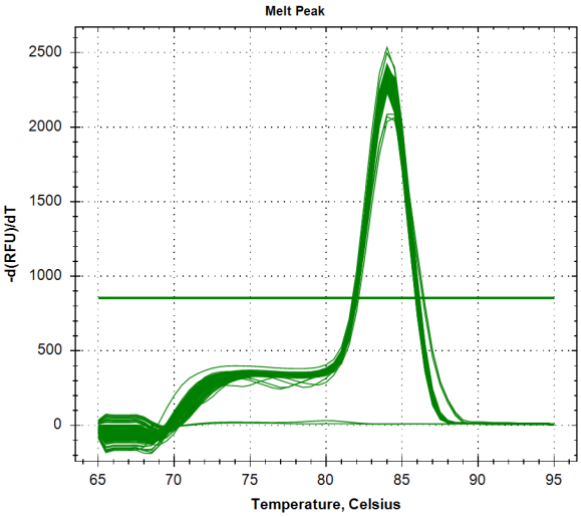


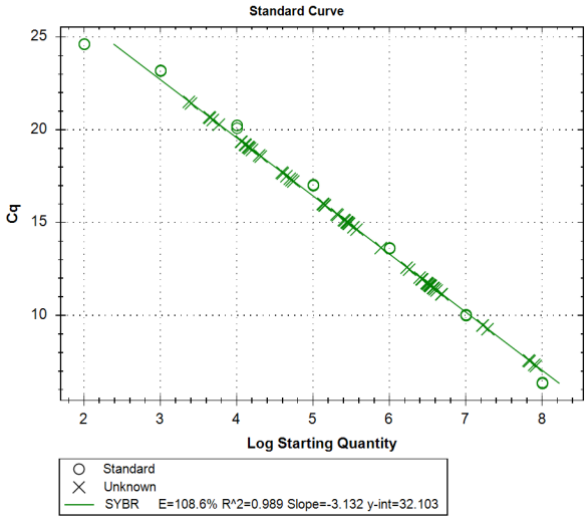




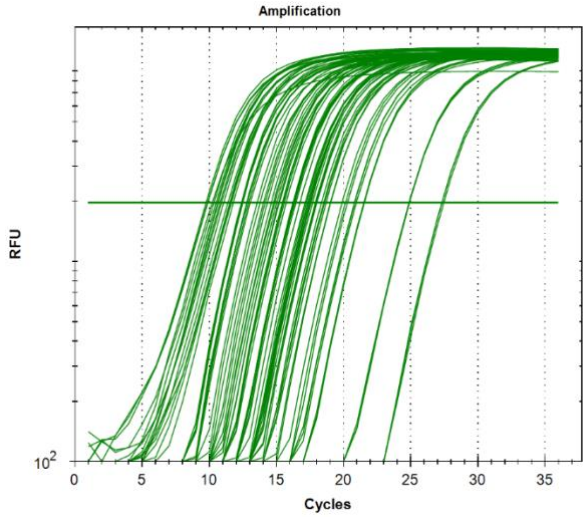
Appendix 4-36 - Amplification, Standard and Melting Curve of RT-qPCR at t = 60min

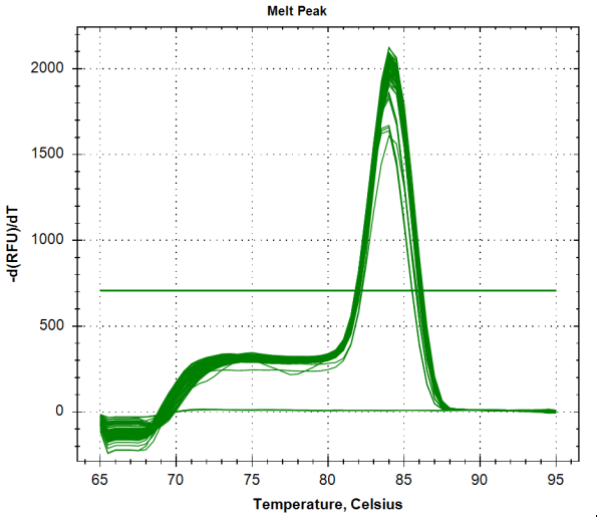


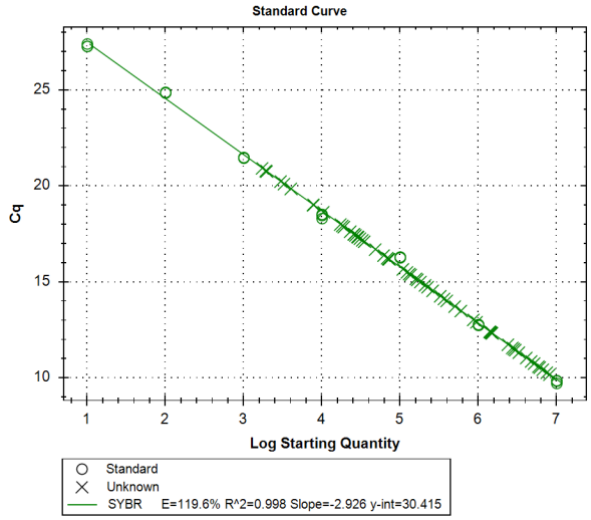




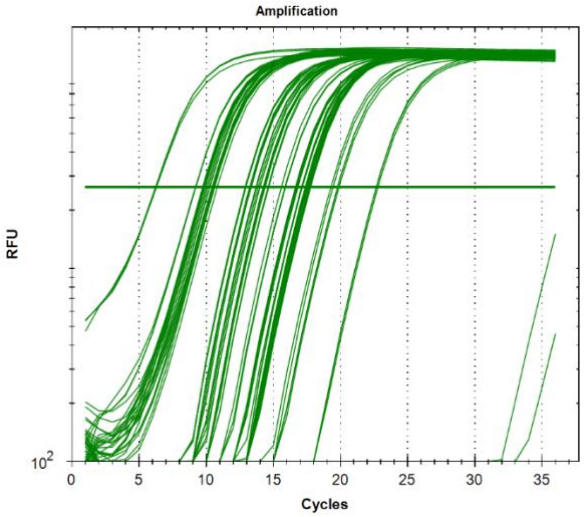
Appendix 4-37 - Amplification, Standard and Melting Curve of RT-qPCR at t = 180min

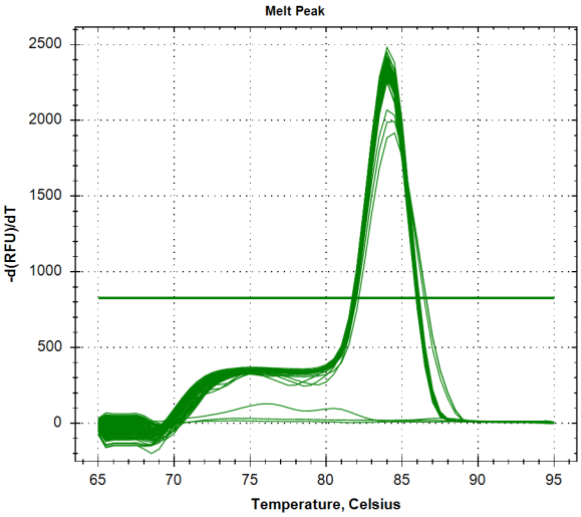


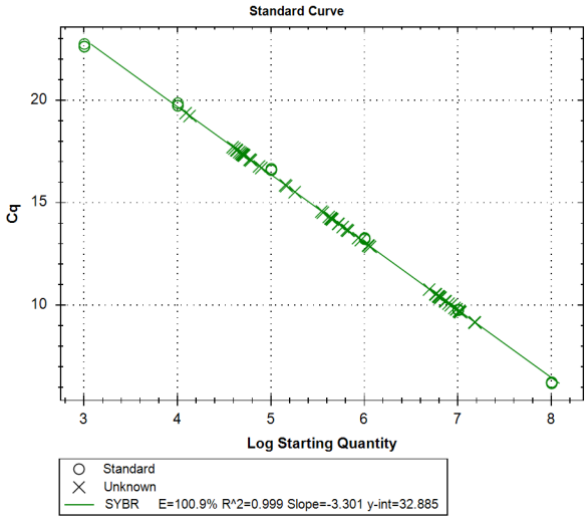




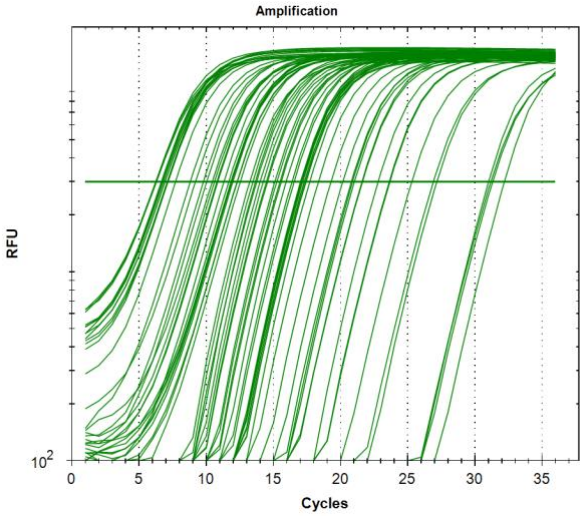
Appendix 4-38 - Amplification, Standard and Melting Curve of RT-qPCR at t = 300min

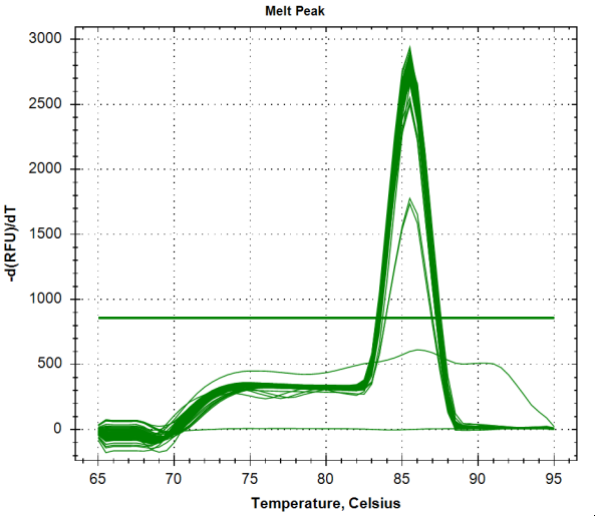


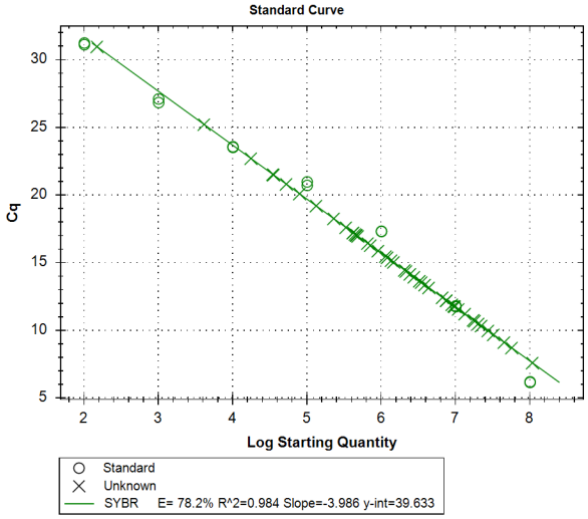




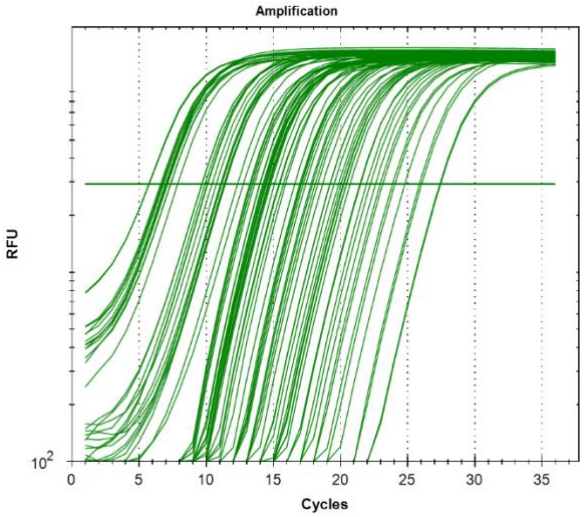
Appendix 4-39 - Amplification, Standard and Melting Curve of RT-qPCR at t = 1day and 3days

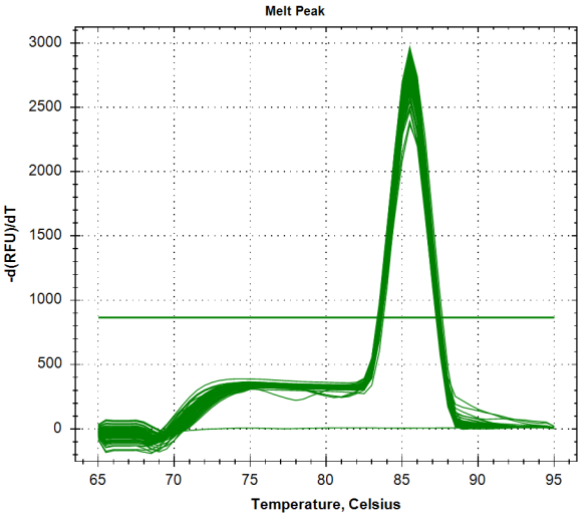


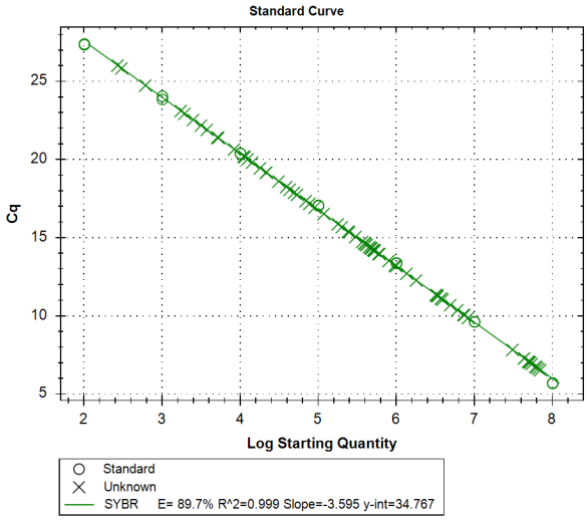




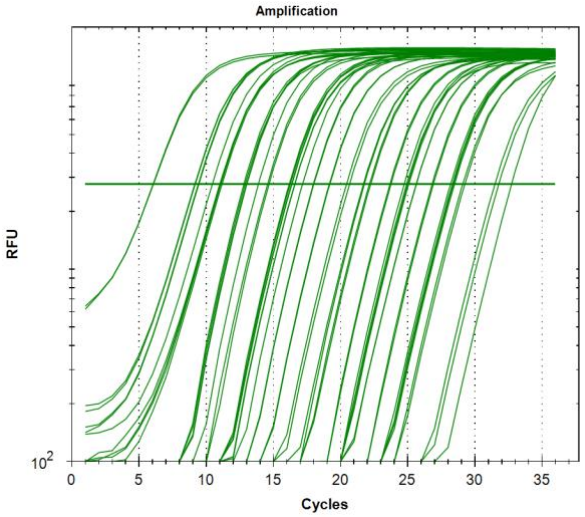
Appendix 4-40 - Amplification, Standard and Melting Curve of RT-qPCR at t = 10, 16 and 69days.

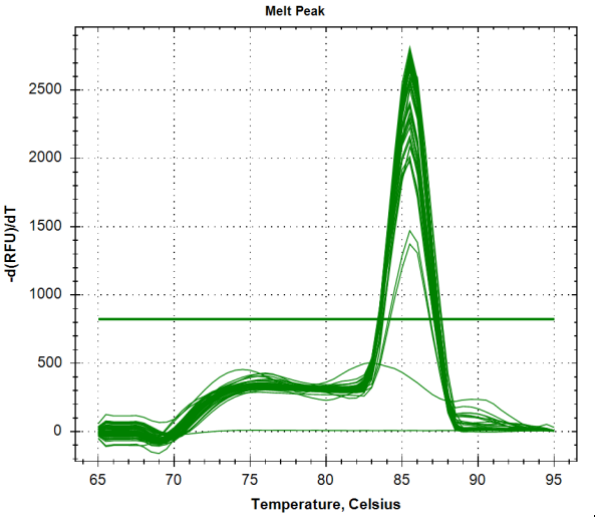


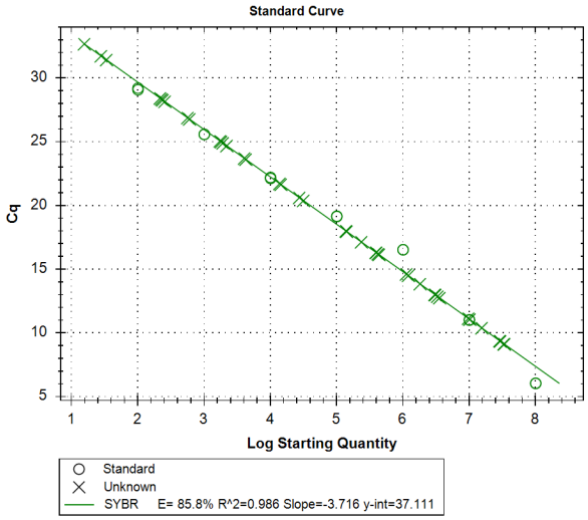




Appendix 4-41 - Amplification, Standard and Melting Curve of RT-qPCR at t = 39days.

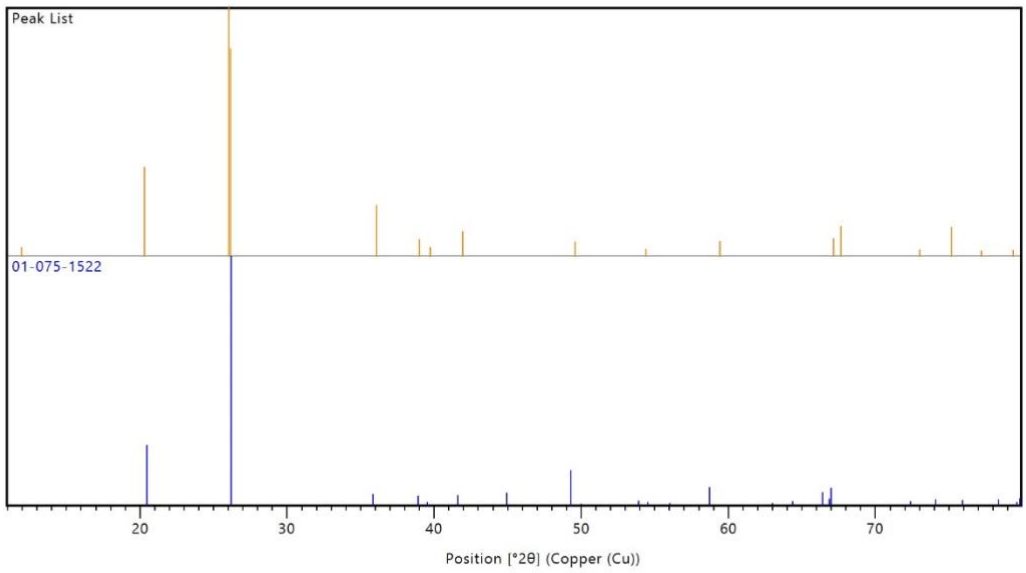


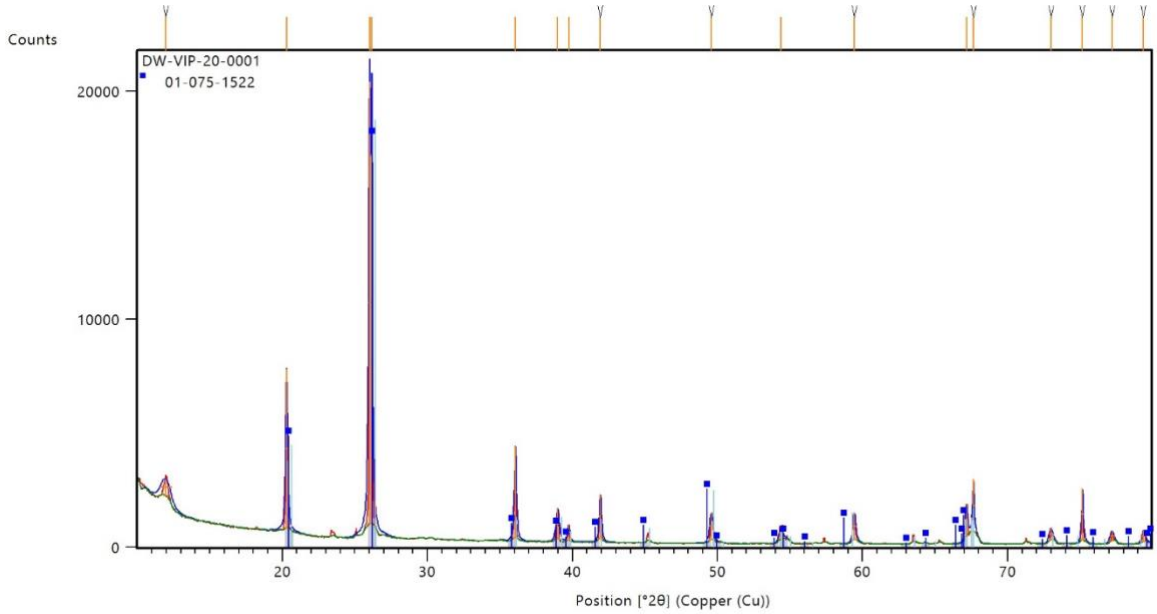




XRD

Appendix 4-42 - XRD Analysis of Loamy Sand Soil, (01-075-1522 Silicon Dioxide).





CHAPTER 5

Environmental Risk Assessment

5.1. Introduction

Environmental Risk Assessment (ERA) is a decision-making tool for environmental protection (Augustijn-Beckers, Hornsby and Wauchope, 1994). In the agrochemical sector, the goal is to predict risks associated with the use of chemicals before they are released into the environment. The aim of ERA is to reduce or eliminate detrimental long-term impacts to sensitive species and ecosystems. In the European Union, the pesticide market is regulated by legislation (EC) 1107/2009, which outlines the approval of active substances for plant protection products (PPPs). The regulatory process is an extensive evaluation procedure and involves manufacturers and regulatory agencies. Thus, the registrant (manufacturer) must provide a risk assessment study, which identifies the risk and hazard of a pesticide to human health, as well as environmental fate and toxicity to no-target organisms (World Health Organization, 2010). These risk assessments are then reviewed by European Agencies, i.e. the European Food Safety Authority (EFSA) and European Chemicals Agency (ECHA), to evaluate the risk assessments regarding the proprieties of the active substances. Based on these evaluations, which indicate the likelihood of the risk, the European Commission approves or disapproves the usage of active substances or, in a restricted form, can include them in the list of candidates for substitution. In this context, the approval of biopesticides follows the same EU regulation 1107/2009 which also applies to synthetic pesticides with relative risk evaluation, even if substances are considered lower risk (Villaverde *et al.*, 2014). The regulation discerns the active substances as standard, candidate for substitutions, low risk, or basic. The biopesticides may fall into low-risk or basic substances, thus the approval procedures might be less restrictive, and the approval may be up to 15 years or may even not need to be renewed (Villaverde *et al.*, 2014).

5.2. Risk Assessment within the EU.

The ERA for chemical compounds focuses on three main characteristics: persistence in environmental compartments, toxicity to non-target organisms and mobility. The mobility and persistence are estimated by the environmental fate assessments, whereas the toxicity is evaluated through eco-toxicological risk assessment. Thus, the Directive EU 546/2011 lays out the Uniform Principles for *decision-making* to ensure that evaluations regarding PPPs authorization provide a high level of protection of human and animal health and the environment. Accordingly, the European Regulatory Frameworks outline the environmental risk using tiered systems (EFSA, 2009). Each tier represents the gravity of the risk to non-target organisms (lower to higher tier), as identified by a risk indicator, the toxicity exposure ratio (TER), which assesses the pesticide toxicity to the species relative to their predicted

exposure in their media (Table 5.1). The TER is derived by the lethal concentration (LC_{50}) or lethal dose (LD_{50}) evaluated by eco-toxicological tests for specific organisms. TER is calculated by dividing the LC_{50} by the predicted environmental concentration (PEC) (Matthews, 2006; Damalas and Eleftherohorinos, 2011; Silva *et al.*, 2019). The latter is estimated by environmental fate modelling software, to determinate the persistence of the chemicals in environmental compartments and consequently estimates the environmental exposure of non-target organisms (Phelps, Winton and Effland, 2002). Thus, the PEC is evaluated considering the principal environmental compartments, soil, groundwater, surface water, sediments and air (EU 546/2011), and how the species can be exposed to the substances by secondary routes (SANCO/4145/2000, 2002). On the other hand, eco-toxicological risk assessment identifies the acute and chronical toxicity of test substances in long- and short-term contact to the organisms.

Table 5.1 – Generic overview on tiers triggered based on the TER (Damalas and Eleftherohorinos, 2011). However, the TER threshold depends on the species.

	TER	Tier 1	Higher Tiers
Representative Species	> 100	✓	
	< 100 (1)		✓
	< 10	Chronical Risk Assessment	
	< 5	Hypothesis of no authorization (2)	

(1) In general, at TER < 100 needs a more detailed risk assessment, which can fall into higher tiers.
(2) (SHORE *et al.*, 2005)

Depending on the environmental exposure, toxicity tests are carried out on the most sensitive relevant categories of organisms, which are considered to be representative of all those species potentially at risk. These species are listed in OECD and EU guidance referring to (1) birds and mammals, (2) aquatic organisms (3) honeybees (4) arthropods other than bees (5) earthworms (6) soil non-target micro-organisms and (7) non-target plants (Table 5.2).
The initial step for the environmental risk assessment is to carry out a *screening test* for the active substance. In a worst-case scenario and using indicator species[1], the preliminary test recognises whether the test substance can be identified as low risk before moving to higher tiers. If the screening test detects a case of risk (i.e. $PEC_{max} > RAC$[2]), it triggers higher tiers which have a more realistic exposure representation (EFSA, 2013). Thus at tier 1, the risk assessment is evaluated using generic focal species[3] and laboratory tests are no longer based on a single food approach but on a mixed diet, to identify the acute toxicity threshold (EFSA, 2009). Tier 2 has more complex scenarios, using focal species[4] in extended lab tests with

[1] *Indicator species* is not a real species, but considering its size and feeding habits are considered to have higher exposure than other species in that particular crop fields.
[2] *RAC*, Regulatory Acceptable Concentration.
[3] *Generic focal species* are not real species, but they are considered to be representative of all those species potentially at risk.
[4] *Focal species* are real species that occur in the crop fields.

complex population models. For higher tier, the risk is evaluated by full-scale experiments, semi-field studies or specific-case studies. Therefore, the realism and ecological complexity scales-up with increasing tiers.

Table 5.2 - OECD guidelines for eco-toxicological tests.

OECD	***Species***	***Tests***
205	Birds	Avian Dietary Toxicity Test
206	Birds	Avian Reproduction Test
223	Birds	Avian Acute Toxicity Test
203	Fish	Fish Acute Toxicity Test
204	Fish	Fish Prolonged Toxicity Test
210	Fish	Fish Early Life Toxicity Test
215	Fish	Fish Juvenile Growth Test
230	Fish	21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition
208	Terrestrial Plant	Seeding Emergence and Growth test
227	Terrestrial Plant	Vegetative Vigour Test
222	Earthworms	Earthworm Reproduction Test
232	Collembolan	Collembolan Reproduction Test in Soil
226	Soil Mites	Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil
213	Honeybee	Acute Oral Toxicity Test
214	Honeybee	Acute Contact Toxicity Test
201	Algae	Freshwater Algae and Cyanobacteria, Growth Inhibition Test
202	Daphnia	Daphnia sp. Acute Immobilisation Test

This chapter outlines a preliminary environmental risk assessment within the EU framework for the approval of an active substance based on dsRNA against the small hive beetle *Aethina tumida*. It proposes a new approach based on a bioinformatics tool (BLAST search of the NCBI database) as a decision-making procedure in *the screening test* of ERA. The evaluation of the risk to non-target organisms (NTOs) was based on the dsRNA-sequence alignment to the genomic sequence of NTOs queried in the NCBI database. In addition, we also evaluated the worst-case environmental fate assessment for an active substance based on dsRNA use against the small hive beetle *Aethina tumida* in soil, by calculating the predicted environmental concentration (PEC) for regulatory submission. These insights can be utilised as starting point for future studies to evaluate a complete ERA under EU and UK guidelines. Currently data gaps for complete ERA under EU and UK guidelines are also outlined in this chapter.

5.3. Materials and Methods

5.3.1. NCBI database

The critical assumption for the use of the dsRNA-based biopesticide as a pest control method is the specific knockdown of the target-gene in the target pest. The need for sequence alignment with the target makes dsRNA a potential low risk biopesticide against non-target

organisms (NOTs) without that target-gene in their genome. Thus, the dsRNA-sequence (see Chapter 3) was compared to the all genomic sequences of species in the NCBI database thought the basic local alignment search tool (BLAST) (Altschul, 1997) to identify potentially sensitive NOTs. The algorithm searching-tool was developed by National Center for Biotechnology Information (NCBI) and the database contained a wide range of species. However for greater transparency, it was also validated against all the taxa of the representative species for the environmental risk assessment which were included in the NCBI database (Appendix 5-1).

Double Stranded RNA Alignment.

The dsRNA ATPase catalytic subunit A was inquired in the BLAST engine, searching for any mRNA belonging to NTOs which would exhibit homology to the dsRNA sequence. The nucleotide query program was used for the alignment search within *reference RNA sequences (refseq_genomes)* database. The *blastn* algorithm was selected as alignment search engine, which operates correctly for cross-species searches. The queries were sorted by *expected value* (e-value), describing the quality and the significance of the searched alignment in the homology match. This value indicates the expected number of matched queries which may have occurred by chance in a randomized database. For instance, the e-value of 0.07 represents the chance of 7 in 100 that the alignment obtained occurred by chance. Thus, the lower the e-value, the more significant the alignment. Hence, we reported a range of significance thresholds for the e-values to assess the query significance (Table 5.3).

Table 5.3 – Level of significance of alignment between query sequence and sequences in the database.

Significance	***Category***
e-value < 10^{-100}	identical sequences
10^{-50}< e-value <10^{-100}	nearly identical (high related sequence)
10^{-5} < e-value < 10^{-50}	homologous sequence
10^{-1} < e-value < 10^{-5}	distantly homologous
e-value < 10^{-1}	likely random

Thus, the dsRNA sequence was assessed as homologous with a cut-off e-value < 10^{-5}. Furthermore, the BLAST engine returned the *query cover* describing whether the whole length was screened, and the *percent identity* which outlines the mismatching within Watson–Crick base pairing rule. As previously described (Chapter 2), during the RNAi mechanism the dsRNA is cleaved by the endonuclease DICER from the 3'end of the sequence (Vermeulen, 2005), generating smaller siRNAs segments of 20 - 22 nucleotides, depending on the species. Consequently, these short RNAs, assisted by the RISC complex, attach to the target mRNA by precise base-paring, while the multi-protein complex RISC then degrades the transcript or inhibits the protein translation. Thus, once the BLAST returned the best e-value of mRNAs for NTOs, these sequences were also aligned with siRNAs of 20 nucleotides produced from

the dsRNA ATPase catalytic subunit A (Table 5.4). Therefore, NTOs interference was considered based on an analysis of e-values to identify species with homologous genes, and then also analysing the extent of matches between the siRNAs produced by the endonuclease DICER and the mRNA of NTOs with homologous genes as potential responsiveness of dsRNA, elucidating whether the base-pair matching of siRNAs might also interfere with mRNAs of NTOs.

Table 5.4 – List of siRNA produced by endonuclease DICER from 3'end of dsRNA sequence

	siRNAs			Length
seq1	5'	CTGCCACCTAAAGCCAAGGG	3'	20bp
seq2	5'	TGGTGAAACACAAAATCGTC	3'	20
seq3	5'	GTATCGTCCACGAAAACACC	3'	20
seq4	5'	TAACGGGAGGTGACATCTAC	3'	20
seq5	5'	ACATCAAATTGGGAGCTCAC	3'	20
seq6	5'	AATGGGAATTCAATCCGTGG	3'	20
seq7	5'	CCGCCCTTTCGAGGACGGCC	3'	20
seq8	5'	TTCCCAAGGGTGTGAACGTG	3'	20
seq9	5'	ATTTGACCCAGAGCATTTAC	3'	20
seq10	5'	GTCCGTTGAAAGACATCAAC	3'	20
seq11	5'	CAATTTTCGACGGTATCCAA	3'	20
seq12	5'	TGGGACCTGGTATTATGGGT	3'	20
seq13	5'	GTAAACCCTTGTCCGTCGAA	3'	20
seq14	5'	GTGATCCGGTGTTGCGTACC	3'	20

5.3.2. Environmental risk assessment scenario

The underlying assumption for the environmental risk assessment was that the active substance dsRNA V-ATPase subunit A would be used as a spray on beehive frames in an open field, since the *Aethina tumida's* larvae grows and develops inside beehives (Hood, 2004; Cuthbertson *et al.*, 2013). The predicted environmental concentration (PEC) was estimated by MS spreadsheets models provided by the UK government agency (HSE). However, the Excel models required input parameters which at the time of our study were still unknown, such as the dsRNA application rate, which is defined as the amount of active ingredient applied on the target surface. Thus, we theoretically estimated this application rate with conservative assumptions to represent the worst-case scenario for a *screening test* of the risk assessment. Firstly, we considered that the honeybee colony would be made up of about 40'000 individual cells (British Beekeepers Association) with a requirement to protect each honeycomb cell from the pest, because it inhabits one cell. Since 10 μg of dsRNA V-ATPase subunit A resulted in 50% of beetle mortality (Powell *et al.*, 2017), we assumed application of this same amount to each honeycomb cell for a total of 400 mg_{dsRNA} per application. Secondly, our prediction assumed that the beehive was stood on a soil surface of 1 m^2. Based on these assumptions, we were able to estimate the application rate (eq.5.1), using it as reference for the worst-case scenario.

$$\text{applicatio rate [g Ha}^{-1}] = \frac{\text{honeycomb cells} * 10\mu g_{dsRNA}}{1m^2} * \left(\frac{g}{10^6 \mu g}\right) * \left(\frac{10^4 m^2}{\text{hectare}}\right) \quad \text{(eq.5.1)}$$

5.3.3. Predicted Environmental Concentration

According to the directive EU 546/2011 for the uniform principles for evaluation and authorisation of PPPs, the Member States shall evaluate the likelihood of the active substance reaching the environmental compartments, such as soil, surface water, groundwater and air regarding the environmental fate assessment. The environmental exposure modelling tools used to assess the PEC implemented the FOCUS guideline, (FOrum for the Co-ordination of Pesticide Fate Models and their USe) to develop standardised worst-case scenarios. Thus, the PEC_{soil} is used for the risk assessments in performing eco-toxicological assessments.

5.3.4. PEC soil

The PEC in soil was estimated for the parent only (PO) degradation using the *Nordic PECsoil calculator* released by the Sweden Chemical Agency which implement the document "*Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration*" (Sanco/10058/2005, version 2.0, June 2006) (FOCUS, 2006). The climatic and topographical parameters were selected according to the *Soil Persistence Models and EU Registration* guideline (FOCUS, 1997).

Table 5.5 - Worst-case scenarios: input parameters

Application rate (g Ha^{-1})	4000
Hive Interception (%)	50
Days until following application	7
Soil T (°C)	10
Soil depth (cm)	5
Soil density (g cm^{-3})	1.5
Baseline Soil T (°C)	5
Plateau Soil T (°C)	10
Degradation model	DFOP
Degradation in lab condition T (°C)	20
Number of application	10

Our worst-case scenario (Table 5.5) was assessed considering the dsRNA application rate derived from eq.5.1, with 10 applications per year, and with a target interception of 50%. The degradation was normalised by T = 20 (°C), which it was the temperature in the laboratory biodegradation experiment. The kinetic model was chosen as DFOP with degradation rates of k_1 = 16.91, k_2 = 0.28 and g = 0.64, as identified in the environmental fate study (Chapter 4).

5.4. Result and Discussion

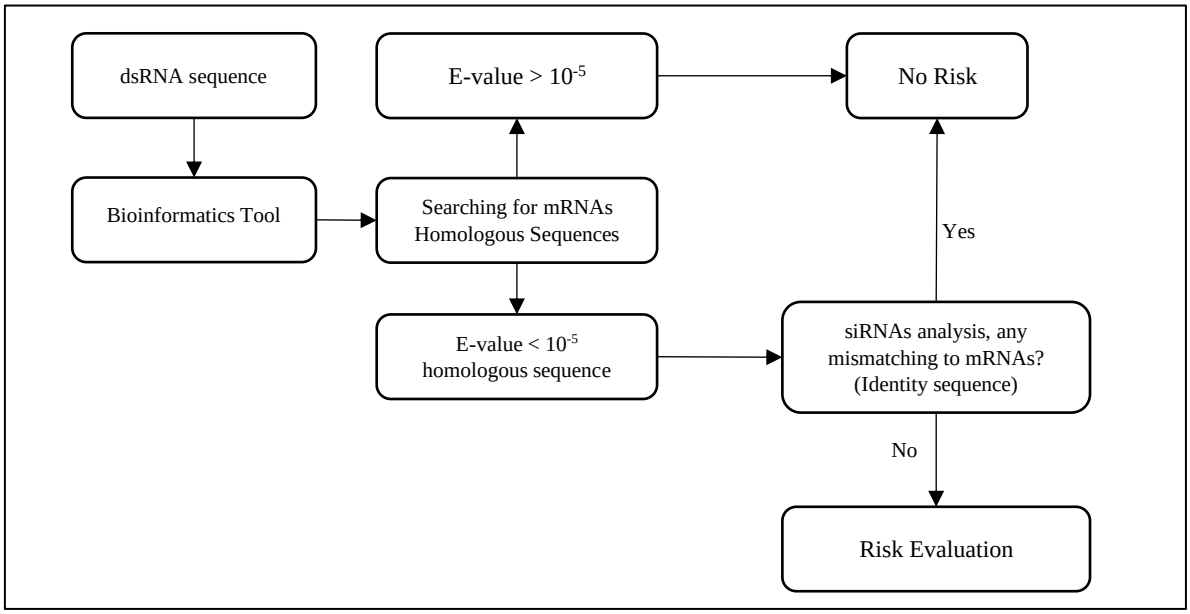
The dsRNA is a RNAi-based biopesticide targeting the pest based on its genetic sequence. We used a bioinformatics tool (BLAST database) to evaluate the potential responsiveness of the

dsRNA-sequence V-ATPase subunit A (sequence in Chapter 3) across non-target organisms (NTOs). Thus, the dsRNA-sequence was queried within the BLAST database to identify any NTOs genetic homology to the molecule sequence. This new approach in the ERA has the potential to expand the study of NTOs interference of a dsRNA-based biopesticide across the variety of living organisms, and not only limiting it to the representative species (Table 5.2). At the time of the current study, the BLAST database included half million of species, and most were Eukaryotas (Table 5.6). Firstly, the risk assessment (Figure 5.1) examined the alignment of the dsRNA sequence to mRNA homologues in the database.

Table 5.6 – Database BLAST: Taxonomy Statistics (www.ncbi.nlm.nih.gov ,2020)

Ranks:	***higher taxa***	***genus***	***species***	***lower taxa***
Archaea	396	197	715	0
Bacteria	4,525	3,907	19,928	900
Eukaryota	59,833	89,242	449,179	32,083
Fungi	5,250	6,687	47,260	1,461
Metazoa	43,441	62,811	232,993	16,113
Viridiplantae	7,580	16,108	155,935	14,149
Viruses	1,456	1,409	4,655	12

Figure 5.1 – Proposed scheme for screening t Environmental Risk Assessment of RNAi-based biopesticide



When querying the database, the dsRNA-sequence V-ATPase subunit A aligned to the V-ATPase subunit A mRNAs of sixty-one species (Table 5.7) showing a range of nearly identical or homologous sequences (10^{-42} < e-value <10^{-67}). This high significance should not surprise due to the highly conservative gene sequence of the V-ATPase subunit A through the invertebrates. Secondly, each transcript was then aligned to each siRNA of 20 bp (Table 5.4), as worst case scenario, looking for homologies and the grade of identity in the base-paring

between the siRNAs and homologous mRNA. The siRNA queries resulted in a total of thirty-first siRNA homologous sequences through all sixty-one V-ATPase subunit A mRNAs belonging to each organism identified as having a homologous target gene (cut-off, e-value $<10^{-5}$, Table 5.8 and Appendix 5-2). The siRNAs had a minimum of 0 to 3 mismatches in the mRNA alignments. Therefore, the sequence perfect alignment (0 mismatches) is considered at risk for the NTO. For a complete screening test, the process outlined here for siRNAs with 20 nt length should be repeated with 21 nt and 22 nt segments, as the exact length of siRNA produced by the DICER is currently uncertain.

A key potential benefit of sequence-based biopesticides is that their design can be targeted to achieve safe plant protection products (PPPs) which do not stress or interfere with NTOs. However, some uncertainties were revealed in our evaluation of environmental risk assessment which were due to the complexity of biological processes and practical knowledge gaps. Although RNA interference is a highly specific mechanism, some studies reported that siRNAs may silence unintended genes (Jackson and Linsley, 2010; Lundgren and Duan, 2013). Due to the relatively short length of siRNA molecules it is quite likely that homologous mRNA may be present in a non-target organism. Homologous mRNA with zero and few siRNA mismatches have been identified in our analysis, which prevent us to exclude for certain any side effects to NTOs. Indeed, the length and number of mismatches of siRNA which may be metabolically active for target or NTOs are still under discussion (Christiaens *et al.*, 2018). Moreover, the siRNAs length is species dependent. For instance, Coleopteran usually processes 21nt, Lepidoptera 20nt and Orthopteran 22nt (Santos *et al.*, 2019). Thus, how exactly the dsRNA is broken down into siRNA is uncertain and adds further uncertainty in the risk evaluation. Furthermore, even a full siRNA/dsRNA match to the target transcript does not necessary imply the biological activation of RNAi. In fact, physiological barriers, such as digestive processes (saliva, acid condition in organism's gut), endonuclease enzymes, thermodynamic parameters for the successful annealing of siRNA to mRNA (Naito and Ui-Tei, 2012), and weathering conditions (such as UV light) might present an obstacle for RNAi efficacy to both target and non-target organisms. However, for the screening test the generic approach outlined here for the toxicity assessment can use desk study to identify potentially sensitive NTOs, which can then also inform the selection of test species for higher tier toxicity testing.

Table 5.7 - No-Target organisms (NTOs) V-ATPase subunit A mRNAs homologues to the dsRNA V-ATPase subunit A sequence

Description	*Species*	*Gene Bank*	*Query Cover*	*E-value*	*% identy*
ants	Dinoponera quadriceps	XM_014616581.1	90.0%	1E-42	75.81
ants	Linepithema humile	XM_012374543.1	90.0%	8E-44	75.63
ants	Monomorium pharaonis	XM_012678073.2	97.0%	4E-47	75.33

Description	*Species*	*Gene Bank*	*Query Cover*	*E-value*	*% identy*
ants	Cyphomyrmex costatus	XM_018547879.1	97.0%	2E-45	75.25
ants	Trachymyrmex cornetzi	XM_018520527.1	97.0%	8E-44	74.92
ants	Nylanderia fulva	XM_029304025.1	97.0%	3E-43	74.83
ants	Trachymyrmex zeteki	XM_018444719.1	97.0%	1E-42	74.58
ants	Wasmannia auropunctata	XM_011693481.1	97.0%	1E-42	74.33
beetles	*Aethina tumida (Target)*	*XM_020020451.1*	*100.0%*	*2E-153*	*100*
beetles	Leptinotarsa decemlineata	XM_023156515.1	100.0%	1E-67	79.34
beetles	Diabrotica virgifera	XM_028294206.1	100.0%	6E-64	78.36
beetles	Nicrophorus vespilloides	XM_017920880.1	100.0%	3E-61	77.7
beetles	Agrilus planipennis	XM_018471259.1	91.0%	1E-53	77.42
beetles	Sitophilus oryzae	XM_030892164.1	100.0%	2E-58	77.05
beetles	Anoplophora glabripennis	XM_018724027.1	100.0%	7E-57	76.72
beetles	Tribolium castaneum	XM_971095.4	100.0%	7E-57	76.72
beetles	Dendroctonus ponderosae	XM_019909288.1	100.0%	5E-53	75.74
beetles	Onthophagus taurus	XM_023048932.1	96.0%	1E-48	75.25
beetles	Dendroctonus ponderosae	XM_019914326.1	100.0%	2E-50	75.08
bony fishes	Ictalurus punctatus	XM_017453679.1	94.0%	8E-44	74.83
bugs	Nilaparvata lugens	XM_022340038.1	100.0%	3E-43	73.86
butterflies	Vanessa tameamea	XM_026634533.1	100.0%	1E-48	74.75
butterflies	Papilio xuthus	XM_013317464.1	100.0%	5E-46	74.51
butterflies	Pieris rapae	XM_022257534.1	100.0%	6E-45	73.77
butterflies	Bicyclus anynana	XM_024084741.1	100.0%	3E-43	73.44
flies	Rhagoletis zephyria	XM_017626005.1	92.0%	3E-62	79.86
flies	Bactrocera dorsalis	XM_011211687.3	92.0%	1E-59	79.15
flies	Lucilia cuprina	XM_023443546.1	92.0%	4E-60	79
flies	Zeugodacus cucurbitae	XM_011180357.2	92.0%	7E-57	78.45
flies	Ceratitis capitata	XM_004533323.4	92.0%	9E-56	78.09
flies	Bactrocera latifrons	XM_018949399.1	92.0%	4E-54	77.74
flies	Drosophila eugracilis	XM_017216441.1	92.0%	7E-51	76.51
flies	Bactrocera oleae	XM_014244806.1	92.0%	1E-48	76.33
flies	Drosophila serrata	XM_020952305.1	92.0%	4E-48	75.8
flies	Musca domestica	XM_011292740.2	92.0%	4E-48	75.8
flies	Drosophila mauritiana	XM_033301989.1	92.0%	2E-46	75.44
flies	Drosophila grimshawi	XM_032736319.1	92.0%	2E-46	75.44
flies	Drosophila sechellia	XM_002042058.2	92.0%	2E-46	75.44
flies	Drosophila willistoni	XM_002065133.3	92.0%	2E-46	75.44
flies	Drosophila bipectinata	XM_017253334.1	92.0%	2E-46	75.44
flies	Drosophila ficusphila	XM_017205697.1	92.0%	2E-46	75.44
flies	Drosophila erecta	XM_001969660.3	92.0%	2E-45	75.09
flies	Drosophila obscura	XM_022360790.1	92.0%	2E-45	75.09
flies	Drosophila rhopaloa	XM_017121277.1	92.0%	2E-45	75.09
flies	Scaptodrosophila lebanonensis	XM_030527544.1	92.0%	2E-44	75
flies	Drosophila guanche	XM_034272616.1	92.0%	8E-44	74.73
flies	Drosophila pseudoobscura	XM_002132979.3	92.0%	8E-44	74.73
flies	Drosophila mojavensis	XM_002003736.3	92.0%	8E-44	74.73
flies	Drosophila virilis	XM_002051631.3	92.0%	8E-44	74.73
flies	Drosophila arizonae	XM_018004298.1	92.0%	8E-44	74.73
flies	Drosophila subobscura	XM_034810107.1	92.0%	1E-42	74.38
flies	Drosophila navojoa	XM_030386573.1	92.0%	1E-42	74.38
flies	Drosophila persimilis	XM_026987784.1	92.0%	1E-42	74.38
flies	Drosophila takahashii	XM_017157298.1	92.0%	1E-42	74.38
flies	Drosophila kikkawai	XM_017170473.1	92.0%	1E-42	74.38
moths	Galleria mellonella	XM_026897056.2	100.0%	2E-51	75.41
moths	Manduca sexta	XM_030172710.1	100.0%	1E-48	74.75
moths	Spodoptera frugiperda	XM_035581534.1	100.0%	2E-46	74.11
moths	Ostrinia furnacalis	XM_028300118.1	100.0%	5E-46	74.1
moths	Spodoptera litura	XM_022970792.1	100.0%	6E-45	73.79
wasps	Nasonia vitripennis	XM_001604635.6	100.0%	5E-53	76.38

Since interference with NTOs was not excluded, estimating the dsRNA exposure becomes important information. Therefore, to evaluate at what concentration the off-set organisms (NTOs) would be exposed, an exposure assessment was conducted calculating the predicted environmental concentration (PEC) in soil for a worst-case scenario. Unfortunately, it was not possible to estimate the PEC for surface water, sediment and groundwater because of the lack of physicochemical information regarding the molecule and DT_{50} in water required by the modelling software. However, considering that the biopesticide would only be applied very locally on beehives, the soil on which the beehives are stood would be the environmental compartment most likely impacted by the usage of this biopesticide. Thus, we evaluated the biopesticide concentration in soil below beehives with the related data to estimate the PEC_{max} based on our worst-case application scenario (Table 5.5). For the soil exposure assessment, the ERA dossiers require the PEC_{max} values after single or multiple applications and PECs plateaus (Silva *et al.*, 2019), which are calculated according to FOCUS (FOCUS, 1997). We found that the $PECsoil_{max}$ value in the first year of application was estimated as 4.11 mg $Kg^{-1}_{dry\text{-}soil}$ (Table 5.9). For a long term application, the $PECsoil_{acc}$ reached 5.33 mg $Kg^{-1}_{dry\text{-}soil}$, which is the highest predicted concentration during a period of 20 years. Besides, the $PECsoil_{plateau}$ was calculated as 1.2 mg $Kg^{-1}_{dry\text{-}soil}$ (PEC_{acc} –PEC_{max}, see User manual for Nordic PEC_{soil} calculator), which represents the contribution of $PECsoil_{max}$ to the PEC_{soil} value for each year, and how the $PECsoil_{acc}$ builds up every year (FOCUS, 1997). Based on the $PECsoil_{max}$ value we could not exclude the exposure of non-target organisms in soil to the biopesticide, despite of its ready biodegradability. Thus, the exposure values would need to be evaluated within the risk assessment study on earthworms and soil microorganism as described in the *Guidance Document on Terrestrial Ecotoxicology (Sanco/10329/2002 rev 2 final)* (Ockleford *et al.*, 2017). Furthermore, more input data and assessments are needed to support ERA in surface water, groundwater and sediment, such as DT_{50} in aquatic compartments (surface water and sediment) and spray drift. Also, we found some difficulties in evaluating the groundwater assessment using FOCUS PEARL, due to the lack of input parameters which are required by the software. Below is a list of parameters needed to complete a preliminary environmental risk assessment, which are currently unavailable (Table 5.10). Furthermore, limited knowledge about the intended application mode and dose adds considerable uncertainty to the ERA.

Table 5.8 - No-Target organisms (NTOs) V-ATPase subunit A mRNAs homologue sequences to the siRNAs sequences (20 nucleotides). Mismatching with NTO mRNAs.

Species	***Seq1***	***Seq2***	***Seq3***	***Seq4***	***Seq5***	***Seq6***	***Seq7***	***Seq8***	***Seq9***	***Seq10***	***Seq11***	***Seq12***	***Seq13***	***Seq14***
Aethina tumida	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Rhagoletis zephyria								3						
Leptinotarsa decemlineata			1					1						
Bactrocera dorsalis								1						
Lucilia cuprina										1				
Zeugodacus cucurbitae								1						
Diabrotica virgifera												2		
Ceratitis capitata						1		1						
Bactrocera latifrons														
Nicrophorus vespilloides														
Agrilus planipennis														
Sitophilus oryzae	1													
Anoplophora glabripennis														
Tribolium castaneum														
Drosophila eugracilis								2						
Nasonia vitripennis														
Bactrocera oleae														
Dinoponera quadriceps														
Drosophila serrata								2						
Musca domestica														
Dendroctonus ponderosae														
Linepithema humile														
Drosophila mauritiana								2						
Drosophila grimshawi														
Drosophila sechellia								2						
Drosophila willistoni														
Drosophila bipectinata								0						
Drosophila ficusphila								0						
Galleria mellonella														
Monomorium pharaonis														
Onthophagus taurus														
Cyphomyrmex costatus										0				
Drosophila erecta								2						
Drosophila obscura											3			
Drosophila rhopaloa								2						
Dendroctonus ponderosae														
Scaptodrosophila lebanonensis														
Trachymyrmex cornetzi										0				
Nylanderia fulva										0				
Ictalurus punctatus														
Manduca sexta								2						
Vanessa tameamea														
Drosophila guanche											3			
Drosophila pseudoobscura											3			
Drosophila mojavensis								2						

Species	*Seq1*	*Seq2*	*Seq3*	*Seq4*	*Seq5*	*Seq6*	*Seq7*	*Seq8*	*Seq9*	*Seq10*	*Seq11*	*Seq12*	*Seq13*	*Seq14*
Drosophila virilis														
Drosophila arizonae								2						
Trachymyrmex zeteki										0				
Papilio xuthus														
Drosophila subobscura														
Drosophila navojoa								2						
Drosophila persimilis											3			
Drosophila takahashii								2						
Drosophila kikkawai														
Wasmannia auropunctata														
Spodoptera frugiperda														
Ostrinia furnacalis														
Nilaparvata lugens														
Spodoptera litura														
Pieris rapae														
Bicyclus anynana														

5.5. Conclusion

This chapter had the aim to evaluate the potential role of bioinformatics tools (NCBI database) as a novel decision-making tool for a preliminary environmental risk assessment of dsRNA-based biopesticides. This new approach had the ability to screen a large variety of species that might by sensitive to an unintended RNAi due to their genetic homology to the dsRNA-sequence. With this approach, it was possible to assess the potential hazard to a very large number of NTO by querying the sequence information in the database. This procedure may benefit the *screening test* of ERA, and higher tier toxicity testing, by identifying those particular families of off-target species that would be receptive to the RNAi. Hence, risk assessors could more precisely design eco-toxicological studies. However, the analysis revealed several uncertainties related to the novelty of the RNAi mechanism and its current unknowns. The potential risk for NTOs and the efficiency of RNAi is related to the degree of homology between the dsRNA and the gene organism (Fletcher *et al.*, 2020). For the current state of the art, the scientific community has not yet agreed on the numbers of nucleotides that must match the target to trigger the RNAi and the transcript (mRNA) degradation (Christiaens *et al.*, 2018). Furthermore, there is uncertainty around the sequencing length of the siRNA molecules produced from dsRNA in different organisms. However, Kulkarni *et al.*, 2006 already underlined how perfect matches' $\geq$ 19 nucleotides can lead to the RNAi in NTOs. Also, several studies have reported off-target gene silencing effects (Birmingham *et al.*, 2006; Jackson, 2006; Chen *et al.*, 2015), which must be considerate during the ERA evaluation. These drawbacks are likely to occur because of differences in RNAi mechanism among organisms as well as

substantial barriers in the pathway of exposure (oral or dermal) (Fletcher *et al.*, 2020). Nonetheless, the bioinformatics approach cannot only be a tool for preliminary toxicity assessments without animal testing, but it can also be used in support of designing the dsRNA molecules in order to avoid any similarity with the NTOs' transcripts. When comparing these drawbacks to the ones of chemical pesticides, the dsRNA-based biopesticide still have a great potential to be low risk based on well-considered designs. The fact that the dsRNA is readily biodegradable in soil, as has been proven in the environmental fate study (Chapter 4), is often used to argue for these molecules presenting low risks to the environment. As a consequence of rapid biodegradation, the dsRNA may never be taken up and transported in the eukaryotic cells of non-target organisms. However, low environmental stability may then also imply

Table 5.9- PECs based on the worst-case scenario for the dsRNA application.

Soil	
PEC_{max} *(mg* Kg^{-1}*)*	4.117
PEC_{acc} *(mg* Kg^{-1}*)*	5.333
$PEC_{plateau}$ *(mg* Kg^{-1}*)*	1.216

Table 5.10 – Missing input parameters to complete the assessment in remaining environmental compartments.

Input Parameters	***Assessment***
DT_{50} *(water)*	*Surface Water, Sediments and sprydrift*
Molar enthalpy of vaporization, dissolution, and adsorption	*Groundwater (software FOCUS PEARL)*
Limited size of Molecular Mass (1)	

(1) The software does not support pesticide with molecular mass greater than 10'000 g mol^{-1} (our dsRNA is ~ 200'000 g mol^{-1})

that a high dose and frequent applications may be needed for such biopesticides to be effective. In the worst-case scenario of this study, high PEC were predicted for soil below beehives despite of the ready biodegradability of the dsRNA due to a high application dose. In support of the low risk profile of dsRNA, the New Zealand Environmental Protection Agency excluded any ERA, considering the foliar application (spray application) as safe (EPA, 2018) . However, the New Zealand EPA dossier raised criticisms, which led to an ongoing discussion in the scientific community on whether or not environmental dsRNA might lead to side effects on NTO (Mochizuki and Gorovsky, 2004; Heinemann, 2019).

References Chapter 5

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Appendices Chapter 5

Appendix 5-1 – Standard taxa test for the eco-toxicological assessments included in the BLAST Database

TaxonomyID	Organism_Name	Common_Name	Database	OECD	Species
8839	Anas platyrhynchos	Mallard Duck	YES	205	Birds
8839	Anas platyrhynchos	Mallard Duck	YES	206	Birds
8839	Anas platyrhynchos	Mallard Duck	YES	223	Birds
9014	Colinus virginianus	Bobwhite Quail	YES	205	Birds
9014	Colinus virginianus	Bobwhite Quail	YES	206	Birds
9014	Colinus virginianus	Bobwhite Quail	YES	223	Birds
8932	Columba livia	Rock Pigeon	YES	205	Birds
8932	Columba livia	Rock Pigeon	YES	223	Birds
93934	Coturnix japonica	Japanese quail	YES	205	Birds
93934	Coturnix japonica	Japanese quail	YES	206	Birds
93934	Coturnix japonica	Japanese quail	YES	223	Birds
9054	Phasianus colchicus	Ring necked pheasant	YES	205	Birds
9079	Alectoris rufa	Red Legged Partridge	YES	205	Birds
59729	Poephila guttata (Taenopygia guttata)	Zebra finch	YES	223	Birds
13146	Melopsittacus unduratus	Budgerigar	YES	223	Birds
7955	Danio rerio	Zebrafish	YES	203	Fish
90988	Pimephales promelas	Fathead Minnow	YES	203	Fish
7962	Cyprinus carpio	Common Carp	YES	203	Fish
8090	Oryzias latipes	Japanese medaka	YES	203	Fish
8081	Poecilia reticulata	Guppy	YES	203	Fish
13106	Lepomis macrochirus	Bluegill	YES	203	Fish
8022	Oncorhynchus mykiss	Rainbow trout	YES	203	Fish
69293	Gasterosteus aculeatus	Three-spined stickleback	YES	203	Fish
28743	Cyprinodon variegatus	Sheepshead Minnow	YES	203	Fish
13489	Dicentrarchus labrax	European Sea Bass	YES	203	Fish
143350	Pagrus major	Red sea bream	YES	203	Fish
8022	Oncorhynchus mykiss	Rainbow trout	YES	210	Fish
90988	Pimephales promelas	Fathead Minnow	YES	210	Fish

TaxonomyID	Organism_Name	Common_Name	Database	OECD	Species
7955	Danio rerio	Zebrafish	YES	210	Fish
8090	Oryzias latipes	Japanese medaka	YES	210	Fish
7962	Cyprinus carpio	Common Carp	YES	210	Fish
269057	Menidia beryllina	inland silverside	YES	210	Fish
8022	Oncorhynchus mykiss	Rainbow trout	YES	215	Fish
7955	Danio rerio	Zebrafish	YES	215	Fish
8090	Oryzias latipes	Japanese medaka	YES	215	Fish
7955	Danio rerio	Zebrafish	YES	230	Fish
90988	Pimephales promelas	Fathead Minnow	YES	230	Fish
8090	Oryzias latipes	Japanese medaka	YES	230	Fish
4039	Daucus carota	Carrot	YES	227	Terrestial Plant
4232	Helianthus annuus	Sunflower	YES	227	Terrestial Plant
4236	Lactuca sativa	Lettuce	YES	227	Terrestial Plant
3728	Sinapis alba	White Mustard	YES	227	Terrestial Plant
93385	Brassica campestris var. chinensis	Chinese cabbage	YES	227	Terrestial Plant
3708	Brassica napus	Oilseed rape	YES	227	Terrestial Plant
3716	Brassica oleracea var. capitata	Cabbage	YES	227	Terrestial Plant
51350	Brassica rapa	Turnip	YES	227	Terrestial Plant
33125	Lepidium sativum	Garden cress	YES	227	Terrestial Plant
3726	Raphanus sativus	Radish	YES	227	Terrestial Plant
161934	Beta vulgaris	Sugar beet	YES	227	Terrestial Plant
3659	Cucumis sativus	Cucumber	YES	227	Terrestial Plant
1298722	Glycine max (G. soja)	Soybean	YES	227	Terrestial Plant
3916	Phaseolus aureus	Mung bean	YES	227	Terrestial Plant
3885	Phaseolus vulgaris	Dwarf bean, French bean, Garden Bean	YES	227	Terrestial Plant
3888	Pisum sativum	Pea	YES	227	Terrestial Plant
78534	Trigonella foenum-graecum	Fenugreek	YES	227	Terrestial Plant
47247	Lotus corniculatus	Birdsfoot trefoil	YES	227	Terrestial Plant

TaxonomyID	Organism_Name	Common_Name	Database	OECD	Species
57577	Trifolium pratense	Red Clover	YES	227	Terrestial Plant
3908	Vicia sativa	Vetch	YES	227	Terrestial Plant
4006	Linum usitatissimum	Flax	YES	227	Terrestial Plant
3617	Fagopyrum esculentum	Buckwheat	YES	227	Terrestial Plant
4081	Solanum lycopersicon	Tomato	YES	227	Terrestial Plant
4679	Allium cepa	Onion	YES	227	Terrestial Plant
4498	Avena sativa	Oats	YES	227	Terrestial Plant
4513	Hordeum vulgare	Barley	YES	227	Terrestial Plant
4522	Lolium perenne	Perennial ryegrass	YES	227	Terrestial Plant
4530	Oryza sativa	Rice	YES	227	Terrestial Plant
4550	Secale cereale	Rye	YES	227	Terrestial Plant
4558	Sorghum bicolor	Grain sorghum, shattercane	YES	227	Terrestial Plant
4565	Triticum aestivum	Wheat	YES	227	Terrestial Plant
4577	Zea mays	Corn	YES	227	Terrestial Plant
4039	Daucus carota	Carrot	YES	208	Terrestial Plant
4232	Helianthus annuus	Sunflower	YES	208	Terrestial Plant
4236	Lactuca sativa	Lettuce	YES	208	Terrestial Plant
3728	Sinapis alba	White Mustard	YES	208	Terrestial Plant
93385	Brassica campestris var. chinensis	Chinese cabbage	YES	208	Terrestial Plant
3708	Brassica napus	Oilseed rape	YES	208	Terrestial Plant
3716	Brassica oleracea var. capitata	Cabbage	YES	208	Terrestial Plant
51350	Brassica rapa	Turnip	YES	208	Terrestial Plant
33125	Lepidium sativum	Garden cress	YES	208	Terrestial Plant
3726	Raphanus sativus	Radish	YES	208	Terrestial Plant
161934	Beta vulgaris	Sugar beet	YES	208	Terrestial Plant
3659	Cucumis sativus	Cucumber	YES	208	Terrestial Plant
1298722	Glycine max (G. soja)	Soybean	YES	208	Terrestial Plant
3916	Phaseolus aureus	Mung bean	YES	208	Terrestial Plant

TaxonomyID	Organism_Name	Common_Name	Database	OECD	Species
3885	Phaseolus vulgaris	Dwarf bean, French bean, Garden Bean	YES	208	Terrestial Plant
3888	Pisum sativum	Pea	YES	208	Terrestial Plant
78534	Trigonella foenum-graecum	Fenugreek	YES	208	Terrestial Plant
47247	Lotus corniculatus	Birdsfoot trefoil	YES	208	Terrestial Plant
57577	Trifolium pratense	Red Clover	YES	208	Terrestial Plant
3908	Vicia sativa	Vetch	YES	208	Terrestial Plant
4006	Linum usitatissimum	Flax	YES	208	Terrestial Plant
3617	Fagopyrum esculentum	Buckwheat	YES	208	Terrestial Plant
4081	Solanum lycopersicon	Tomato	YES	208	Terrestial Plant
4679	Allium cepa	Onion	YES	208	Terrestial Plant
4498	Avena sativa	Oats	YES	208	Terrestial Plant
4513	Hordeum vulgare	Barley	YES	208	Terrestial Plant
4522	Lolium perenne	Perennial ryegrass	YES	208	Terrestial Plant
4530	Oryza sativa	Rice	YES	208	Terrestial Plant
4550	Secale cereale	Rye	YES	208	Terrestial Plant
4558	Sorghum bicolor	Grain sorghum, shattercane	YES	208	Terrestial Plant
4565	Triticum aestivum	Wheat	YES	208	Terrestial Plant
4577	Zea mays	Corn	YES	208	Terrestial Plant
6396	Eisenia fetida	Brandling worm	YES	222	Earthworms
168636	Eisenia andrei	segmented worms	YES	222	Earthworms
158441	Folsomia candida	springtails	YES	232	Collembole
1387114	Folsomia fimetaria	springtails	YES	232	Collembole
704012	Gaeolaelaps aculeifer	mites & ticks	YES	226	Soil Mites
7460	Apis mellifera	Honeybee	YES	213	Honeybee
7460	Apis mellifera	Honeybee	YES	214	Honeybee
1180732	Pseudokirchneriella subcapitata	Algae	YES	201	Algae
104105	Desmodesmus subspicatus	Algae	YES	201	Algae

TaxonomyID	Organism_Name	Common_Name	Database	OECD	Species
913975	Navicula pelliculosa	Diatoms	YES	201	Algae
1166	Anabaena flos-aquae	Cyanobacteria	YES	201	Algae
32047	Synechococcus leopoliensis	Cyanobacteria	YES	201	Algae
6668	Daphnia	common water fleas	YES	202	Daphnia

Appendix 5-2 – Analysis of siRNA alignments to the NTO mRNAs (cut off, e-value >10^{-5})

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq1	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq1	XM_030892164.1	Sitophilus oryzae	95	20	1	1	20	6.70E-06	IN	1
sq1	XM_030892164.1	Sitophilus oryzae	100	12	0	8	19	0.003	OFF	8
sq1	XM_971095.4	Tribolium castaneum	90	20	2	1	20	7.65E-05	OFF	2
sq1	XM_001604635.6	Nasonia vitripennis	95	20	1	1	20	1.09E-05	OFF	1
sq1	XM_012678073.2	Monomorium pharaonis	100	11	0	1	11	0.013	OFF	9
sq1	XM_029304025.1	Nylanderia fulva	100	11	0	1	11	0.013	OFF	9
sq2	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq2	XM_011211687.3	Bactrocera dorsalis	93.75	16	1	1	16	0.001	OFF	5
sq2	XM_011180357.2	Zeugodacus cucurbitae	93.75	16	1	1	16	0.001	OFF	5
sq2	XM_028294206.1	Diabrotica virgifera	92.857	14	1	3	16	0.012	OFF	7
sq2	XM_018724027.1	Anoplophora glabripennis	100	14	0	3	16	2.90E-04	OFF	6
sq2	XM_026897056.2	Galleria mellonella	93.75	16	1	1	16	0.001	OFF	5
sq2	XM_026634533.1	Vanessa tameamea	93.75	16	1	1	16	0.001	OFF	5
sq2	XM_013317464.1	Papilio xuthus	93.75	16	1	1	16	0.001	OFF	5
sq3	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq3	XM_017626005.1	Rhagoletis zephyria	89.474	19	2	1	19	3.09E-04	OFF	3
sq3	XM_023156515.1	Leptinotarsa decemlineata	95	20	1	1	20	6.56E-06	IN	1
sq3	XM_011211687.3	Bactrocera dorsalis	92.857	14	1	6	19	0.013	OFF	7
sq3	XM_011180357.2	Zeugodacus cucurbitae	92.857	14	1	6	19	0.014	OFF	7
sq3	XM_004533323.4	Ceratitis capitata	92.857	14	1	6	19	0.013	OFF	7
sq3	XM_018949399.1	Bactrocera latifrons	92.857	14	1	6	19	0.013	OFF	7
sq3	XM_014244806.1	Bactrocera oleae	92.857	14	1	6	19	0.013	OFF	7

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq3	XM_022257534.1	Pieris rapae	94.737	19	1	1	19	2.19E-05	OFF	2
sq4	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq4	XM_011211687.3	Bactrocera dorsalis	100	11	0	12	2	0.013	OFF	9
sq4	XM_011180357.2	Zeugodacus cucurbitae	100	11	0	12	2	0.014	OFF	9
sq4	XM_004533323.4	Ceratitis capitata	100	11	0	12	2	0.013	OFF	9
sq4	XM_018949399.1	Bactrocera latifrons	100	11	0	12	2	0.013	OFF	9
sq4	XM_017216441.1	Drosophila eugracilis	100	11	0	12	2	0.012	OFF	9
sq4	XM_011292740.2	Musca domestica	100	11	0	12	2	0.012	OFF	9
sq4	XM_016180070.1	simulans uncharacterized	100	11	0	12	2	0.011	OFF	9
sq4	XM_033301989.1	Drosophila mauritiana	100	11	0	12	2	0.012	OFF	9
sq4	XM_032736319.1	Drosophila grimshawi	100	11	0	12	2	0.014	OFF	9
sq4	XM_017205697.1	Drosophila ficusphila	100	11	0	12	2	0.012	OFF	9
sq4	XM_001969660.3	Drosophila erecta	100	11	0	12	2	0.013	OFF	9
sq4	XM_017121277.1	Drosophila rhopaloa	100	11	0	12	2	0.012	OFF	9
sq4	XM_030527544.1	Scaptodrosophila lebanonensis	100	11	0	12	2	0.012	OFF	9
sq4	XM_029304025.1	Nylanderia fulva	88.889	18	2	3	20	0.001	OFF	4
sq4	XM_034272616.1	Drosophila guanche	100	11	0	12	2	0.012	OFF	9
sq4	XM_002132979.3	Drosophila pseudoobscura	100	11	0	12	2	0.012	OFF	9
sq4	XM_034810107.1	Drosophila subobscura	100	11	0	12	2	0.011	OFF	9
sq4	XM_026987784.1	Drosophila persimilis	100	11	0	12	2	0.013	OFF	9
sq5	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq5	XM_018471259.1	Agrilus planipennis	89.474	19	2	1	19	2.75E-04	OFF	3
sq5	XM_017157298.1	Drosophila takahashii	100	11	0	1	11	0.012	OFF	9
sq6	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq6	XM_017626005.1	Rhagoletis zephyria	100	15	0	2	16	8.84E-05	OFF	5
sq6	XM_023156515.1	Leptinotarsa decemlineata	93.75	16	1	1	16	0.001	OFF	5
sq6	XM_011211687.3	Bactrocera dorsalis	100	14	0	3	16	3.17E-04	OFF	6
sq6	XM_023443546.1	Lucilia cuprina	100	14	0	3	16	2.83E-04	OFF	6
sq6	XM_011180357.2	Zeugodacus cucurbitae	92.857	14	1	3	16	0.014	OFF	7
sq6	XM_004533323.4	Ceratitis capitata	100	15	0	2	16	8.68E-05	OFF	5
sq6	XM_018949399.1	Bactrocera latifrons	100	14	0	3	16	3.14E-04	OFF	6

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq6	XM_001604635.6	Nasonia vitripennis	92.857	14	1	3	16	0.02	OFF	7
sq6	XM_014244806.1	Bactrocera oleae	92.857	14	1	3	16	0.013	OFF	7
sq6	XM_020952305.1	Drosophila serrata	100	14	0	3	16	2.75E-04	OFF	6
sq6	XM_011292740.2	Musca domestica	92.857	14	1	3	16	0.012	OFF	7
sq6	XM_032736319.1	Drosophila grimshawi	100	15	0	3	17	9.27E-05	OFF	5
sq6	XM_002065133.3	Drosophila willistoni	100	14	0	3	16	2.66E-04	OFF	6
sq6	XM_026897056.2	Galleria mellonella	92.857	14	1	3	16	0.013	OFF	7
sq6	XM_012678073.2	Monomorium pharaonis	100	14	0	3	16	3.06E-04	OFF	6
sq6	XM_017121277.1	Drosophila rhopaloa	92.857	14	1	3	16	0.012	OFF	7
sq6	XM_029304025.1	Nylanderia fulva	100	15	0	3	17	8.89E-05	OFF	5
sq6	XM_030172710.1	Manduca sexta	92.857	14	1	3	16	0.013	OFF	7
sq6	XM_026634533.1	Vanessa tameamea	92.857	14	1	3	16	0.013	OFF	7
sq6	XM_002003736.3	Drosophila mojavensis	92.857	14	1	3	16	0.011	OFF	7
sq6	XM_002051631.3	Drosophila virilis	100	14	0	3	16	2.56E-04	OFF	6
sq6	XM_018004298.1	Drosophila arizonae	92.857	14	1	3	16	0.01	OFF	7
sq6	XM_030386573.1	Drosophila navojoa	92.857	14	1	3	16	0.01	OFF	7
sq6	XM_011693481.1	Wasmannia auropunctata	100	16	0	2	17	2.53E-05	OFF	4
sq6	XM_035581534.1	Spodoptera frugiperda	92.857	14	1	3	16	0.012	OFF	7
sq6	XM_022970792.1	Spodoptera litura	92.857	14	1	3	16	0.013	OFF	7
sq6	XM_022257534.1	Pieris rapae	93.75	16	1	1	16	0.001	OFF	5
sq7	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq8	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq8	XM_017626005.1	Rhagoletis zephyria	100	17	0	3	19	7.26E-06	IN	3
sq8	XM_023156515.1	Leptinotarsa decemlineata	95	20	1	1	20	6.56E-06	IN	1
sq8	XM_011211687.3	Bactrocera dorsalis	100	19	0	1	19	6.11E-07	IN	1
sq8	XM_011180357.2	Zeugodacus cucurbitae	100	19	0	1	19	6.15E-07	IN	1
sq8	XM_004533323.4	Ceratitis capitata	100	19	0	1	19	5.85E-07	IN	1
sq8	XM_018949399.1	Bactrocera latifrons	94.737	19	1	1	19	2.57E-05	OFF	2
sq8	XM_017216441.1	Drosophila eugracilis	100	18	0	3	20	1.91E-06	IN	2
sq8	XM_001604635.6	Nasonia vitripennis	93.333	15	1	3	17	0.006	OFF	6
sq8	XM_014244806.1	Bactrocera oleae	94.737	19	1	1	19	2.48E-05	OFF	2

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq8	XM_020952305.1	Drosophila serrata	100	18	0	3	20	1.85E-06	IN	2
sq8	XM_016180070.1	simulans uncharacterized	100	18	0	3	20	1.74E-06	IN	2
sq8	XM_033301989.1	Drosophila mauritiana	100	18	0	3	20	1.91E-06	IN	2
sq8	XM_032736319.1	Drosophila grimshawi	88.889	18	2	3	20	0.001	OFF	4
sq8	XM_002042058.2	Drosophila sechellia	100	18	0	3	20	1.90E-06	IN	2
sq8	XM_002065133.3	Drosophila willistoni	94.444	18	1	3	20	7.61E-05	OFF	3
sq8	XM_017253334.1	Drosophila bipectinata	100	20	0	1	20	1.51E-07	IN	0
sq8	XM_017205697.1	Drosophila ficusphila	100	20	0	1	20	1.60E-07	IN	0
sq8	XM_001969660.3	Drosophila erecta	100	18	0	3	20	2.06E-06	IN	2
sq8	XM_022360790.1	Drosophila obscura	94.444	18	1	3	20	8.34E-05	OFF	3
sq8	XM_017121277.1	Drosophila rhopaloa	100	18	0	3	20	1.94E-06	IN	2
sq8	XM_030172710.1	Manduca sexta	100	18	0	3	20	2.01E-06	IN	2
sq8	XM_026634533.1	Vanessa tameamea	94.118	17	1	3	19	2.95E-04	OFF	4
sq8	XM_034272616.1	Drosophila guanche	94.444	18	1	3	20	8.09E-05	OFF	3
sq8	XM_002132979.3	Drosophila pseudoobscura	94.444	18	1	3	20	7.89E-05	OFF	3
sq8	XM_002003736.3	Drosophila mojavensis	100	18	0	3	20	1.68E-06	IN	2
sq8	XM_002051631.3	Drosophila virilis	88.889	18	2	3	20	8.94E-04	OFF	4
sq8	XM_018004298.1	Drosophila arizonae	100	18	0	3	20	1.64E-06	IN	2
sq8	XM_034810107.1	Drosophila subobscura	94.444	18	1	3	20	7.71E-05	OFF	3
sq8	XM_030386573.1	Drosophila navojoa	100	18	0	3	20	1.64E-06	IN	2
sq8	XM_026987784.1	Drosophila persimilis	94.444	18	1	3	20	8.47E-05	OFF	3
sq8	XM_017157298.1	Drosophila takahashii	100	18	0	3	20	1.93E-06	IN	2
sq8	XM_035581534.1	Spodoptera frugiperda	94.118	17	1	3	19	2.85E-04	OFF	4
sq8	XM_022970792.1	Spodoptera litura	94.118	17	1	3	19	3.08E-04	OFF	4
sq9	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq9	XM_019909288.1	Dendroctonus ponderosae	89.474	19	2	1	19	2.87E-04	OFF	3
sq9	XM_017453679.1	Ictalurus punctatus	94.118	17	1	4	20	2.91E-04	OFF	4
sq10	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq10	XM_023156515.1	Leptinotarsa decemlineata	94.118	17	1	1	17	2.79E-04	OFF	4
sq10	XM_011211687.3	Bactrocera dorsalis	90	20	2	1	20	9.08E-05	OFF	2
sq10	XM_011211687.3	Bactrocera dorsalis	100	11	0	1	11	0.013	OFF	9

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq10	XM_023443546.1	Lucilia cuprina	95	20	1	1	20	6.66E-06	IN	1
sq10	XM_028294206.1	Diabrotica virgifera	93.75	16	1	1	16	0.001	OFF	5
sq10	XM_018949399.1	Bactrocera latifrons	100	11	0	1	11	0.013	OFF	9
sq10	XM_030892164.1	Sitophilus oryzae	89.474	19	2	1	19	2.85E-04	OFF	3
sq10	XM_018724027.1	Anoplophora glabripennis	94.118	17	1	1	17	2.90E-04	OFF	4
sq10	XM_001604635.6	Nasonia vitripennis	90	20	2	1	20	1.32E-04	OFF	2
sq10	XM_014244806.1	Bactrocera oleae	100	11	0	1	11	0.013	OFF	9
sq10	XM_011292740.2	Musca domestica	90	20	2	1	20	8.41E-05	OFF	2
sq10	XM_012678073.2	Monomorium pharaonis	94.444	18	1	3	20	8.78E-05	OFF	3
sq10	XM_018547879.1	Cyphomyrmex costatus	100	20	0	1	20	1.64E-07	IN	0
sq10	XM_018520527.1	Trachymyrmex cornetzi	100	20	0	1	20	1.65E-07	IN	0
sq10	XM_029304025.1	Nylanderia fulva	100	20	0	1	20	1.72E-07	IN	0
sq10	XM_018444719.1	Trachymyrmex zeteki	100	20	0	1	20	2.05E-07	IN	0
sq11	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq11	XM_017626005.1	Rhagoletis zephyria	94.118	17	1	3	19	3.09E-04	OFF	4
sq11	XM_011211687.3	Bactrocera dorsalis	94.118	17	1	3	19	3.17E-04	OFF	4
sq11	XM_023443546.1	Lucilia cuprina	88.889	18	2	3	20	0.001	OFF	4
sq11	XM_011180357.2	Zeugodacus cucurbitae	94.118	17	1	3	19	3.18E-04	OFF	4
sq11	XM_018949399.1	Bactrocera latifrons	94.118	17	1	3	19	3.14E-04	OFF	4
sq11	XM_971095.4	Tribolium castaneum	94.118	17	1	3	19	2.67E-04	OFF	4
sq11	XM_017216441.1	Drosophila eugracilis	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_014244806.1	Bactrocera oleae	94.118	17	1	3	19	3.03E-04	OFF	4
sq11	XM_020952305.1	Drosophila serrata	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_011292740.2	Musca domestica	94.118	17	1	3	19	2.94E-04	OFF	4
sq11	XM_016180070.1	simulans uncharacterized	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_012374543.1	Linepithema humile	88.889	18	2	3	20	7.94E-04	OFF	4
sq11	XM_033301989.1	Drosophila mauritiana	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_002042058.2	Drosophila sechellia	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_017253334.1	Drosophila bipectinata	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_017205697.1	Drosophila ficusphila	88.235	17	2	3	19	0.004	OFF	5
sq11	XM_026897056.2	Galleria mellonella	94.737	19	1	1	19	2.57E-05	OFF	2

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq11	XM_012678073.2	Monomorium pharaonis	88.889	18	2	3	20	0.001	OFF	4
sq11	XM_018547879.1	Cyphomyrmex costatus	94.118	17	1	3	19	2.97E-04	OFF	4
sq11	XM_001969660.3	Drosophila erecta	88.235	17	2	3	19	0.004	OFF	5
sq11	XM_022360790.1	Drosophila obscura	100	17	0	3	19	6.84E-06	IN	3
sq11	XM_017121277.1	Drosophila rhopaloa	88.235	17	2	3	19	0.004	OFF	5
sq11	XM_030527544.1	Scaptodrosophila lebanonensis	94.118	17	1	3	19	2.82E-04	OFF	4
sq11	XM_018520527.1	Trachymyrmex cornetzi	94.118	17	1	3	19	2.99E-04	OFF	4
sq11	XM_029304025.1	Nylanderia fulva	94.444	18	1	3	20	8.89E-05	OFF	3
sq11	XM_017453679.1	Ictalurus punctatus	94.444	18	1	3	20	8.34E-05	OFF	3
sq11	XM_030172710.1	Manduca sexta	88.235	17	2	3	19	0.004	OFF	5
sq11	XM_026634533.1	Vanessa tameamea	94.737	19	1	1	19	2.42E-05	OFF	2
sq11	XM_034272616.1	Drosophila guanche	100	17	0	3	19	6.64E-06	IN	3
sq11	XM_002132979.3	Drosophila pseudoobscura	100	17	0	3	19	6.48E-06	IN	3
sq11	XM_002003736.3	Drosophila mojavensis	94.444	18	1	3	20	7.15E-05	OFF	3
sq11	XM_002051631.3	Drosophila virilis	94.118	17	1	3	19	2.56E-04	OFF	4
sq11	XM_018004298.1	Drosophila arizonae	94.118	17	1	3	19	2.43E-04	OFF	4
sq11	XM_018444719.1	Trachymyrmex zeteki	94.118	17	1	3	19	3.71E-04	OFF	4
sq11	XM_030386573.1	Drosophila navojoa	94.118	17	1	3	19	2.43E-04	OFF	4
sq11	XM_026987784.1	Drosophila persimilis	100	17	0	3	19	6.95E-06	IN	3
sq11	XM_017157298.1	Drosophila takahashii	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_017170473.1	Drosophila kikkawai	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_011693481.1	Wasmannia auropunctata	88.235	17	2	3	19	0.004	OFF	5
sq11	XM_035581534.1	Spodoptera frugiperda	88.235	17	2	3	19	0.003	OFF	5
sq11	XM_022340038.1	Nilaparvata lugens	94.118	17	1	3	19	2.38E-04	OFF	4
sq11	XM_022970792.1	Spodoptera litura	94.118	17	1	3	19	3.08E-04	OFF	4
sq11	XM_024084741.1	Bicyclus anynana	88.235	17	2	3	19	0.004	OFF	5
sq12	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq12	XM_017626005.1	Rhagoletis zephyria	90	20	2	1	20	8.84E-05	OFF	2
sq12	XM_023156515.1	Leptinotarsa decemlineata	88.889	18	2	3	20	0.001	OFF	4
sq12	XM_011211687.3	Bactrocera dorsalis	90	20	2	1	20	9.08E-05	OFF	2
sq12	XM_023443546.1	Lucilia cuprina	89.474	19	2	1	19	2.83E-04	OFF	3

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq12	XM_011180357.2	Zeugodacus cucurbitae	90	20	2	1	20	9.12E-05	OFF	2
sq12	XM_028294206.1	Diabrotica virgifera	100	18	0	3	20	1.93E-06	IN	2
sq12	XM_004533323.4	Ceratitis capitata	90	20	2	1	20	8.68E-05	OFF	2
sq12	XM_018949399.1	Bactrocera latifrons	90	20	2	1	20	8.98E-05	OFF	2
sq12	XM_018471259.1	Agrilus planipennis	88.889	18	2	3	20	0.001	OFF	4
sq12	XM_030892164.1	Sitophilus oryzae	90	20	2	1	20	8.16E-05	OFF	2
sq12	XM_018724027.1	Anoplophora glabripennis	94.444	18	1	3	20	8.30E-05	OFF	3
sq12	XM_971095.4	Tribolium castaneum	90	20	2	1	20	7.65E-05	OFF	2
sq12	XM_001604635.6	Nasonia vitripennis	94.737	19	1	1	19	3.80E-05	OFF	2
sq12	XM_014244806.1	Bactrocera oleae	89.474	19	2	1	19	3.03E-04	OFF	3
sq12	XM_011292740.2	Musca domestica	89.474	19	2	1	19	2.94E-04	OFF	3
sq12	XM_019909288.1	Dendroctonus ponderosae	90	20	2	1	20	8.22E-05	OFF	2
sq12	XM_032736319.1	Drosophila grimshawi	89.474	19	2	1	19	3.24E-04	OFF	3
sq12	XM_002065133.3	Drosophila willistoni	89.474	19	2	1	19	2.66E-04	OFF	3
sq12	XM_019914326.1	Dendroctonus ponderosae	90	20	2	1	20	8.23E-05	OFF	2
sq12	XM_030527544.1	Scaptodrosophila lebanonensis	89.474	19	2	1	19	2.82E-04	OFF	3
sq12	XM_002003736.3	Drosophila mojavensis	89.474	19	2	1	19	2.50E-04	OFF	3
sq12	XM_018004298.1	Drosophila arizonae	89.474	19	2	1	19	2.43E-04	OFF	3
sq12	XM_034810107.1	Drosophila subobscura	89.474	19	2	1	19	2.69E-04	OFF	3
sq12	XM_030386573.1	Drosophila navojoa	89.474	19	2	1	19	2.43E-04	OFF	3
sq12	XM_022340038.1	Nilaparvata lugens	94.118	17	1	3	19	2.38E-04	OFF	4
sq13	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq13	XM_017626005.1	Rhagoletis zephyria	94.444	18	1	3	20	8.84E-05	OFF	3
sq13	XM_011211687.3	Bactrocera dorsalis	88.889	18	2	3	20	0.001	OFF	4
sq13	XM_023443546.1	Lucilia cuprina	94.444	18	1	3	20	8.11E-05	OFF	3
sq13	XM_011180357.2	Zeugodacus cucurbitae	88.889	18	2	3	20	0.001	OFF	4
sq13	XM_018949399.1	Bactrocera latifrons	88.889	18	2	3	20	0.001	OFF	4
sq13	XM_030892164.1	Sitophilus oryzae	88.889	18	2	3	20	0.001	OFF	4
sq13	XM_971095.4	Tribolium castaneum	89.474	19	2	1	19	2.67E-04	OFF	3
sq13	XM_011292740.2	Musca domestica	94.444	18	1	3	20	8.41E-05	OFF	3
sq13	XM_012374543.1	Linepithema humile	88.889	18	2	3	20	7.94E-04	OFF	4

siRNA	*Gene Bank*	*Organisms*	*% identity*	*Alignment length*	*Mismatches*	*seq. start*	*seq. end*	*e-value*	*cut-off*	*Total Mismatches*
sq13	XM_012678073.2	Monomorium pharaonis	88.889	18	2	3	20	0.001	OFF	4
sq13	XM_023048932.1	Onthophagus taurus	100	11	0	19	9	0.011	OFF	9
sq13	XM_030172710.1	Manduca sexta	88.889	18	2	3	20	0.001	OFF	4
sq13	XM_013317464.1	Papilio xuthus	88.889	18	2	3	20	0.001	OFF	4
sq13	XM_011693481.1	Wasmannia auropunctata	100	11	0	10	20	0.013	OFF	9
sq14	XM_020020451.1	Aethina tumida	100	20	0	1	20	1.59E-07	IN	0
sq14	XM_017626005.1	Rhagoletis zephyria	94.737	19	1	1	19	2.53E-05	OFF	2
sq14	XM_023156515.1	Leptinotarsa decemlineata	94.444	18	1	3	20	7.99E-05	OFF	3
sq14	XM_018949399.1	Bactrocera latifrons	94.737	19	1	1	19	2.57E-05	OFF	2
sq14	XM_014244806.1	Bactrocera oleae	94.118	17	1	3	19	3.03E-04	OFF	4
sq14	XM_002065133.3	Drosophila willistoni	94.118	17	1	3	19	2.66E-04	OFF	4

CHAPTER 6

Future Work

In the next years, European policies in support of sustainable agriculture could be promoting the usage of biopesticides. Currently, there is increasing demand for less harmful products in food production. In this work, an RNAi-based biopesticide was shown to have high potential as safe product in environmental systems due to its ready biodegradability. However, the dsRNA stability and its effective oral delivery to the targeted pest are challenges to be addressed by future research. Therefore, new nanomaterials need to be developed to delivery methods for biopesticides, allowing foliar application which would be the favourite usage. Then, our understanding of the environmental fate will also depend on understanding these novel interactions between nanomaterials and biopesticides in future formulations, and their fate in the various environmental compartments, and whether the scientific methods developed so far will be able to address these new questions. Another uncertainty to be addressed is the exposure of non-target organisms to RNAi-based pesticides, and by which pathway the exogenous application might cause interference with gene expression in these species. Currently, it is not possible to predict responsiveness across the species due to uncertainties about the exact functioning of the RNAi mechanisms. Furthermore, a standardization of analytical methods for the dsRNA quantification would benefit experimental work and help regulatory agencies in setting up robust thresholds and parameters and testing protocols for environmental risk assessment.

From the point of view of the regulatory framework clarification is needed for the use of the term *"biopesticide"*, and how the regulation of these novel biomolecules, with different methods of interaction with NTO, will be assessed in the EU 1107/09 framework. When the European institutions introduced EU 1107/09, the goal was to harmonise the regulation pathway for PPPs (and active substances), which at that time was fragmented between different EU directives. In addition, the new regulation had the aim to boost innovation and R&D of new sustainable active substances to replace the harmful ones. In 2018, the Policy Department for Economic, Scientific and Quality of Life Policies of European Commission issued a negative outlook on the effectiveness of EU 1107/09 to create a favorable pathway for innovation and development of alternatives and new active substances[5]. The reason for a negative impact relied on the fact that the EU1107/09 increased costs of R&D related to data requirements, test

[5] The impact of Regulation (EC) No 1107/2009 on innovation and development of alternatives and new plant protection products, Nazim Punja (2008) - ISBN 978-92-846-3854-3 | doi:10.2861/644498

guidelines with ill-defined terminologies, unrealistic endpoints and inadequate validation. Therefore, it introduced more uncertainty in the development processes. Nevertheless, the biopesticide market has grown in the last years showing a great potential in terms of market share for new "green" active substances.

At the current state of the art, no regulatory category for biopesticide has been introduced into the EU 1107/09, and their evaluation still falls into the pesticide categories of "basic substances" or "low risk". One should acknowledge the difficulties to insert a variety of different macromolecules and different methodology of interaction with NTOs into one category. However, providing a subcategory for basic (or low risk) substances (or biopesticide) might support manufacturers in designing and developing new molecules. For instance, in our case study, the dsRNA based-biopesticide might be evaluated as low risk substance and might fall into a proposed subcategory of *genetic active substance*.

Therefore, the author believes that the key point for a final success of new generation pesticides depends not only on scientific insights, but also on the efficiency of regulatory frameworks to support and guide the manufactures with key guidelines on how to produce scientific dossiers with validated methodologies that efficiently identify any environmental risk of new plant protection products.