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THE EFFECT OF NON-UV LIGHT ON CROP PROTECTION PRODUCT DEGRADATION AND SOIL MICROBIAL COMMUNITY STRUCTURE AND FUNCTION

LAWRENCE OLIVER DAVIES

A thesis submitted for the degree of Doctor of Philosophy

School of Life Sciences, University of Warwick

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Declaration

I declare that the work presented in this thesis was conducted by me under the direct supervision of Doctor Gary D. Bending, Doctor Hendrik Schäfer, Doctor Samantha Marshall, Doctor Irene Bramke, Doctor Emma France, and Doctor Robin Oliver, with the exception of those instances where the contribution of others has been specifically acknowledged. The work contained in this thesis has not been submitted previously for any other degree. Chapter II and III have been published in the peer-reviewed journals *Environmental Science & Technology* and *PLoS ONE*, respectively.

Lawrence Oliver Davies

List of Abbreviations

^{14}C – Carbon labelled with the radioactive isotope carbon 14

AI – Active ingredient

ASE – Accelerated solvent extraction

BBM – Bold's basal medium

BLAST – Basic local alignment search tool

BSC – Biological soil crust

cDNA – Complementary DNA

CPP – Crop protection product

CT – constant temperature

DAT – Days after treatment

$DegT_{50}$ – Time taken for 50% degradation

$DegT_{90}$ – Time taken for 90% degradation

DNA – Deoxy ribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

emPCR – Emulsion PCR

FOCUS – Forum for the co-ordination of pesticide fate models and their use

HPLC – High performance liquid chromatography

ITS – Internal transcribed spacer

Koc – Soil organic carbon-water partitioning coefficient

Kow – Octanol-water partition coefficient

LC-MS – Liquid chromatography - mass spectrometry

MEGAN – Metagenome analyser

MG-RAST – Metagenome rapid annotation using subsystem technology

MPC – Magnetic particle collector

mRNA - Messenger RNA

NER - Non-extractable residues

NMDS – Non-metric multidimensional scaling

OECD – Organisation for economic co-operation and development

OTU – Operational taxonomic unit

PCR – Polymerase chain reaction

PLFA – Phospholipid fatty acid analysis

Prot CRs – Protein coding regions

qPCR – quantitative PCR

QIIME – Quantitative insights into microbial ecology

rRNA – Ribosomal RNA

RNA – Ribonucleic acid

RT-PCR – Reverse transcription PCR

SOM – Soil organic matter

STAMP - Statistical analysis of metagenomic profiles

TRF – Terminal restriction fragment

TRFLP – Terminal restriction fragment length polymorphism

Summary

The use of crop protection products (CPPs) is essential to improve crop production levels and feed the rising global population. Safety testing of CPPs is also essential to ensure that their use does not adversely affect human health or the environment. Currently, laboratory-based studies typically over-estimate the environmental persistence of CPPs in the environment. The inclusion of environmental variables that are currently omitted from laboratory studies, such as non-UV light, could reduce the disparity between laboratory and field degradation studies.

The inclusion of light resulted in a significant reduction in extractable parent compound for benzovindiflupyr, chlorotoluron, prometryn, imidacloprid, and fludioxonil compared to dark conditions. In contrast, a significantly slower rate of cinosulfuron transformation was observed under light compared to dark conditions. In a separate experiment, terminal restriction fragment length polymorphism showed that under light conditions, the soil surface (0-3 mm) harboured distinct phototroph, bacterial and fungal communities compared to the underlying bulk soil (3-12 mm), or dark incubated soil. 454 pyrosequencing revealed that light selected for diazotrophs at the soil surface, including *Nostoc punctiforme*, in addition to heterotrophic bacteria, particularly within the phylum Firmicutes. Metatranscriptomic analysis showed that light also selected for actively transcribing photosynthetic eukaryotes at the soil surface, such as Chlorophyceae and Saccharomycetes, in addition to heterotrophs, such as *Nostocaceae*. Finally, light selected for major soil functions such as photosynthesis, and reduced the alpha and beta diversity of predicted protein coding regions at the soil surface. This work has important implications for CPP regulatory studies and soil surface management practices

1. CHAPTER I: INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. THE IMPORTANCE OF CROP PROTECTION PRODUCTS IN AGRICULTURE

1.1.1 World crop protection product use

The agricultural revolution of Western Asia took place ~11, 000 years ago (Childe, 1952), however, it was not until ~4, 500 years ago that crop protection products (CPPs) were first used in agriculture when Sumerians applied sulphur compounds to control insects (International Union of Pure and Applied Chemistry (IUPAC), 2010). Since then, CPPs and crop productivity have been intimately linked. A CPP has been defined as (Food and Agriculture Organisation (FAO), 2002):

"Any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food."

There are a wide range of CPPs and a classification system is used, which groups CPPs based on the organisms they are designed to target such as a fungicide or rodenticide, and/or their chemical structure such as an organochlorine or an organophosphate. In 2007, worldwide CPP use was estimated at 2, 367 million kg and expenditure was estimated at US$ 39, 443 million (U.S Environmental Protection Agency (U.S EPA), 2011). Herbicide use was estimated to account for

~39% of worldwide CPP use whereas insecticides, fungicides, and 'Other' CPPs accounted for 28%, 23% and 9% of CPP use, respectively (U.S EPA, 2011).

1.1.2. UK crop protection product use

In the UK, the total area of land treated with CPPs increased from 45.0 million hectares (ha) in 1990 to 68.8 million ha by 2010 (Figure 1.1). However, the design of more efficient and targeted CPPs has meant that the total mass of CPPs applied has decreased from 34.4 million kg in 1990 to 16.8 million kg by 2010 (Figure 1.1; FERA, 2012).

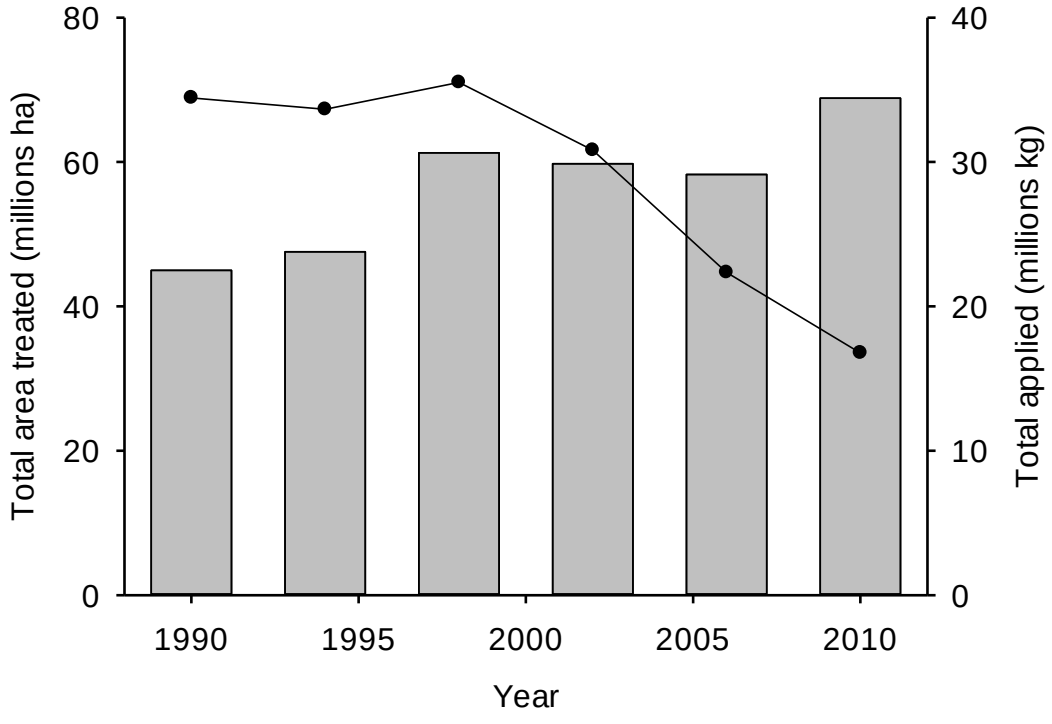


Figure 1.1: Total area treated (bars) and total mass applied (●) to all UK crops during the period 1990-2010 (Taken from FERA, 2012).

In 2010, ~50% of UK land treated with CPPs received fungicides, however, fungicide applications only accounted for ~33% of the total volume of applied CPPs. In contrast, herbicides accounted for ~32% of total area treated but ~45% of total volume applied. Therefore, although fungicides are the most commonly used CPP, herbicides are applied in the greatest volume (FERA, 2012) (Figure 1.2).

a) b)

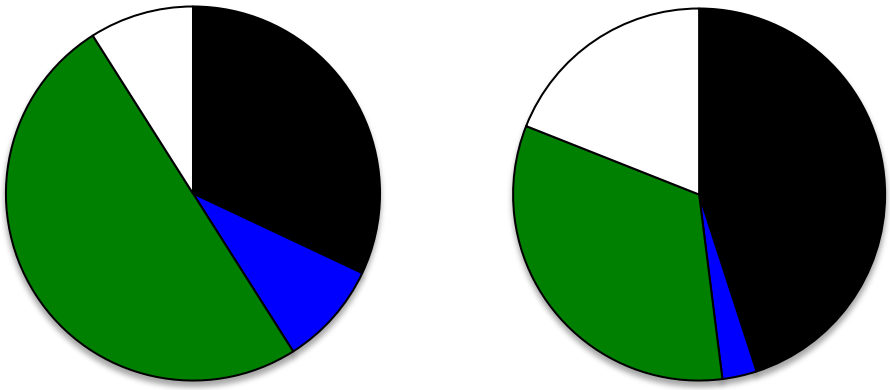


Figure 1.2: Estimated crop protection product use in the UK in 2010; (a) Total area treated (%), and; (b) Total volume applied (%). Herbicide (black); Insecticides (blue); Fungicides (green), and; 'other' CPPs (white). Taken from FERA 2012.

1.2. CROP PROTECTION PRODUCT REGULATION IN THE EU

1.2.1. Silent Spring

The publication of 'Silent Spring' (Carson, 1962) around 50 years ago marked the beginning of public awareness towards the potential adverse effects of CPPs on human health and the environment. Dichlorodiphenyltrichloroethane (DDT) was used as a case study and its impact on wildlife (e.g. eggshell thinning) and

human health (e.g. genotoxicity) was meticulously documented. The potential for CPPs to contaminate groundwater/freshwater bodies (Stoate *et al*. 2011), bioaccumulate (Coat *et al*. 2011), and impact non-target organisms such as higher vertebrates (Bernanke and Köhler, 2009), insects (Canty *et al*. 2007), and microorganisms (Li *et al*. 2010a) has since been discussed. However, CPP regulation has become considerably more stringent since the publication of 'Silent Spring', and provided CPPs are used correctly, associated adverse effects are far less common.

1.2.2. Safety testing of crop protection products in the EU

A CPP cannot be used in the EU unless it is scientifically proven that it is effective against pests without having adverse effects on human health or unacceptable effects on the environment (European Commission, 2013). An extensive dossier, which fully addresses all requirements in Commission Regulation (EU) No 544/2011 (for the active substance) and Commission Regulation (EU) No 545/2011 (for the plant protection product) must be produced for each new active ingredient (AI). The dossier must demonstrate a detailed risk assessment of the active ingredient, which includes the environmental fate data and safety testing of the AI. The Directive 2009/128/EC then sets out the rules for the sustainable use of the CPP according to Good Agricultural Practice. Further, the Organisation for Economic Co-operation and Development (OECD) provide guidance on a number of tests to evaluate a new AI.

1.2.3. Organisation for economic co-operation and development guidelines

The OECD guidelines are a collection of internationally relevant testing methods used by government, industry and independent laboratories to assess the safety of chemical products. The guidelines are divided into five sections, each of which describes a number of tests to identify specific properties of the AI or the impact of an AI on a specific system or organism. Section 1 is aimed at identifying the physical and chemical properties of the chemical, such as adsorption-desorption using a batch equilibrium method, section 2 the effects on biotic systems, such as freshwater algae and cyanobacteria growth inhibition test, section 3 degradation and accumulation, such as aerobic and anaerobic transformation in soil, section 4 health effects, such as toxicokinetics, and section 5 'other test guidelines', such as the nature of residues in food commodities.

1.3. THE FATE OF CROP PROTECTION PRODUCTS IN SOIL

Agricultural CPPs are typically sprayed directly onto the soil surface or crops. CPPs can then enter water bodies through run-off, or the atmosphere through volatilisation (U.S. EPA, 1994), however, this thesis is focused on the fate of CPPs in the soil environment. The fate of a CPP in soil can be considered in terms of; (i) transformation of the parent substance into metabolites; (ii) mineralisation to 'harmless' by-products such as CO_2, water or nitrate, and; (iii) formation of non-extractable residues (NER) (OECD Guideline 307).

1.3.1. Non-extractable residues

CPPs can either be present in the aqueous phase of soil, or be bound to soil. The latter refers to a NER, which can be either reversibly or irreversibly bound (Semple *et al*. 2004). A NER is difficult to define as the extractability of a CPP is controlled by the extraction process itself i.e. the time or intensity of extraction (Alexander, 1995) and the type of extraction procedure (Gevao *et al*. 2000). For the purpose of this thesis a NER simply refers to the proportion of a CPP and its metabolites that are not extracted following solvent extraction(s). NERs can also be referred to as bound residues in the literature. However, this term will not be used in this thesis as the difference between a bound residue and a 'free residue' is not easily defined, as many 'free-residues' will also be bound to compartments of the soil environment (Gevao *et al*. 2000).

A CPP can be bound to soil through ionic bonding, H-bonding, van der Waals forces, or covalent bonding, the latter being the most irreversible process (Gevao *et al*. 2000). The contact time between the CPP and soil is thought to be one of the most important controlling factors in NER formation, and it has been shown that a larger proportion of the CPP will be irreversibly bound to soil with a longer contact time (Xing and Pignatello, 1997). There are several biological consequences related to the soption of CPPs such as a reduction in CPP toxicity (Bowmer, 1991; Kelsey and Alexander, 1997), and a reduction in the bioavailability/bioaccessibility to microbial communities (Semple *et al*. 2004). A review by Semple *et al*. (2004) offered the following definitions of bioavailability and bioaccessibility:

"A bioavailable compound is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time. Once transfer across the membrane has occurred, storage, transformation, assimilation, or degradation can take place within the organism; however, these processes are obviously distinct from the transfer between the medium (e.g., soil) and the organism."

"A bioaccessible compound is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical. However, the chemical may be either physically removed from the organism or only bioavailable after a period of time."

The sorption of a CPP to soil has been shown to reduce CPP degradation rates due to a reduction in bioavailability/bioaccessibility (Karpouzas and Walker, 2000; Gaultier *et al*. 2008). A CPP can also be non-bioavailable/non-bioaccessible after occlusion into soil pores (Semple *et al*. 2004).

1.3.2. Factors that determine the fate of crop protection products in soil

Several researchers have investigated edaphic and climatic factors that affect the rate and route of CPP degradation (Table 1.1-1.5), as well as the genes responsible for pesticide transformation (Rodriguez-Cruz *et al*. 2010).

The rate of CPP degradation has been shown to be controlled by several abiotic factors such as photolysis and indirect photolysis, in addition to several edaphic and climatic variables, which influence biotic degradation, including pH (Table 1.1), soil organic matter content (Table 1.2), clay type (Table 1.3), water content (Table 1.4) and temperature (Table 1.5).

1.3.2.1. Abiotic factors

1.3.2.1.1. Direct photolysis

Direct photolysis refers to the absorption of actinic light (290-400 nm) by a CPP and the subsequent chemical reaction and breakdown of the CPP into fragments of lower molecular weight (Wallace *et al*. 2010). CPPs show a range of susceptibility to direct photolysis depending on their structure. Some are extensively degraded whilst others are not photolytically degraded, as they do not absorb UV light (Burrows *et al*. 2002).

2,4-dichlorophenoxyacetic acid (2,4-D) (Arkhipova *et al*. 1997) and atrazine (Beltran *et al*. 1993) have both been shown to be rapidly photodegraded in aqueous solution with more than 99% transformed within the first hour and 15 mins of treatment, respectively. Direct photolysis has been shown to be slower in soil compared to aqueous systems (Curran *et al*. 1992). For example, Curran *et al*. (1992) showed that 100% of imazapyr, imazethapyr and imazaquin and 87% and 8% of imazamthabenz and atrazine were degraded in aqueous solutions after 48 hours, respectively, however, in sand or a silty-clay loam soil, degradation was typically <10%. Reduced degradation rates in soil are likely to be due to an attenuation of light intensity below the top few millimetres of the soil surface (Benvenuti, 1995) and the presence of humic substances that absorb UV light (Beltran *et al*. 1993). For example, the addition of humic substances to an aqueous system reduced the rate of atrazine transformation (Beltran *et al*, 1993).

1.3.2.1.2. Indirect photolysis

Indirect photolysis refers to the absorption of actinic light by solutes, known as photosensitisers, and the subsequent production of reactive intermediates which can breakdown CPPs (Wallace *et al*. 2010). Dissolved organic matter (DOM) and nitrate have been highlighted as important photosensitisers due to the production of the carbonate radical, triplet state DOM, hydrogen peroxide, and the hydroxyl radical, when in direct contact with light (Brekken and Brezonick, 1998; Miller and Chin, 2002). Wallace *et al*. (2010) highlighted the importance of indirect photolysis in the photodegradation of chlorotoluron, chlorothalonil, prometryn, and propiconazole in surface waters. Photodegradation of pinoxaden, chlorotoluron, propiconazole and prometryn were all linked to the concentration of nitrate and the authors' suggested a significant role of the hydroxyl radical in the breakdown of CPPs. The specificity of photosensitisers to CPPs was also highlighted as chlorothalonil was thought to be photodegraded by the carbonate radical (Wallace *et al*. 2010). Further, nitrate-mediated hydroxyl radical degradation of atrazine has been shown to be faster than direct photolysis of atrazine (Torrents *et al*. 1997).

1.3.2.2. Biotic factors

CPP degradation can occur by growth-linked or co-metabolic catabolism. Growth-linked catabolism refers to the breakdown and use of a CPP as a C or N source. Degradation kinetics are characterised by a lag-phase whereby the appropriate enzymes required to breakdown a CPP are produced followed by an increase in the rate of degradation after the proliferation of degrader-organisms (Figure 1.3a) (Alexander, 1981). CPPs can also be co-metabolised which refers to the breakdown of a CPP by non-specific enzymes. The CPP is not used as an energy source and consequently degradation kinetics are characterised by a slow, steady rate of degradation (Figure 1.3b) (Alexander, 1981).

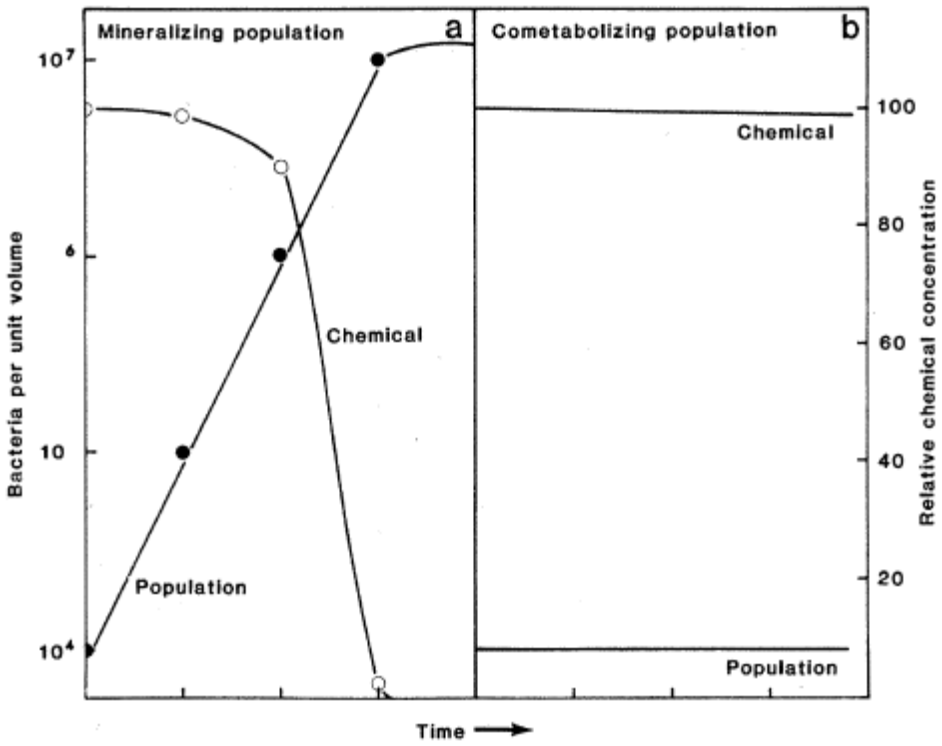


Figure 1.3: Conceptual diagram of CPP degradation kinetics and bacterial populations under (a) mineralising (growth-linked catabolism) and (b) co-metabolic degradation. (Taken from Alexander (1981)).

CPP degradation rates are also influenced by several edaphic and climatic variables that influence microbial activity, biomass and diversity (Table 1.1-1.5).

1.3.2.2.1. Effect of pH on crop protection product degradation

An increase in soil pH accelerated degradation rates of chlorpyrifos, fenamiphos, lindane and endosulfan (Table 1.1). In contrast, above a threshold pH, a further pH increase could result in reduced degradation rates, for example, Benimeli *et al*. (2007) showed that lindane degraded faster at pH7 compared to pH9.

The effect of pH on CPP degradation rates could be caused by an indirect effect on microbial community structure or activity. For example, Griffiths *et al*. (2011) comprehensively showed the effect of pH on bacterial community structure in soil across the UK. Over 1000 soil cores were taken and α diversity (sample variance within a site) was positively related to pH whereas β diversity (between sample variance in α diversity) was negatively related to pH. Highly acidic soils were dominated by few taxa and there was a clear selection for *Acidobacteria* and *Alphaproteobacteria*. pH has also been shown to affect microbial activity, for example, microbial activity in the leachates of both upper and lower layers of mor humus have been shown to be significantly greater following liming, even after five leaching events (Andersson and Nilsson, 2001).

Table 1.1: Effect of pH on crop protection product degradation

Crop protection product	Effect of pH	Reference
Chlorpyrifos	Microbial biomass and the rate of degradation increased as soil pH increased (five soils)	Singh *et a*l. 2003
Chlorpyrifos	The rate of degradation increased as pH increased up until pH6.7. A pH >6.7 did not result in a significant increase in degradation	Singh *et al*. 2006
Ethroprophos	Degradation was greatest at pH6.8 and pH8.4	Karpouzas and Walker, 2000
Fenamiphos	The rate of degradation was greatest at pH>5.7	Singh *et al*. 2006
Lindane	Degradation was greatest at pH7 compared to a pH5 and pH9	Benimeli *et al*. 2007
Endosulfan	The rate of degradation increased from pH3 to pH5, and from pH5 to pH7.5/8.5	Awasthi *et al*. 2000
Endosulfan	In aerobic systems, degradation was lowest at pH4 and pH10, and greatest at pH of 6, 7 and 8. In anaerobic systems, degradation efficiency progressively increased with an increase in pH	Kumar and Philip, 2006
Isoproturon, azoxystrobin and diflufenican	pH was significantly correlated with the $DegT_{25}$ of azoxystrobin. Isoproturon and diflufenican degradation rates were not significantly correlated with pH	Bending *et al*. 2006
Isoproturon	A strong correlation was found between isoproturon mineralisation and pH >6.5	El-Sebai *et al*. 2005

pH also impacts CPP sorption, for example, a 14-21% and 5.5% decrease in sorption has been shown per unit increase in pH for bromoxynil and diuron, respectively (Yang *et al*. 2004). This reduction in sorption was thought to be due to an increase in the deprotonation of surface functional groups at a higher pH value (Yang *et al*. 2004). Glyphosate sorption has also been studied in sandy soil and sandy loam soil receiving different additions of lime over a period of 60 and 100 years, respectively. Glyphosate sorbed more strongly to sandy soil that had received liming, which was thought to be due to an increase of reactive amorphous aluminium and iron hydrous oxides (de Jonge *et al*. 2001).

1.3.2.2.2. Effect of soil organic matter on crop protection product degradation

The term soil organic matter (SOM) collectively refers to living soil biomass, fresh and partially decomposed residues, and humus (organic matter which has been decomposed to a point of stability). 2,4-D, dicamba, ethoprophos, and metribuzin have all shown accelerated degradation rates at elevated SOM contents (Table 1.2). Similar to pH there may be a threshold where a further increase in SOM results in stronger CPP sorption, reduced bioavailability, and reduced degradation rates (Karpouzas and Walker, 2000; Gaultier *et al*. 2008).

SOM is also important in soil structure and plant growth, and as a C source for soil microbial communities (Fontaine *et al*. 2003). Consequently, microbial activity (Schnürer *et al*. 1985; Gaultier *et al*. 2008) and biomass (Schnürer *et al*. 1985; Voos and Groffman, 1997) have both been shown to be correlated with SOM.

Table 1.2: Effect of soil organic matter on crop protection product degradation

CPP	Effect of soil organic matter	Reference
2,4-D and dicamba	Degradation was studied for five different land use types. SOM and microbial biomass were positively correlated with degradation	Voos and Groffman, 1997
Imidacloprid	Sorption increased with the addition of organic amendment; imidacloprid degradation remained relatively constant	Cox *et al*. 2002
Chlorpyrifos and fenamiphos	The addition of SOM (up to 5%) to three different soil types did not impact the rate of degradation	Singh *et al*. 2006
Carboxyl and 2,4-D	Representative soils were taken from seven ecoregions. Microbial activity was greater in soil with a higher SOM content. Degradation rates were lower in soil with a higher SOM content	Gaultier *et al*. 2008
Ethoprophos	Degradation increased when SOM content was raised from 0.3% to 8.5%. Degradation was inhibited as SOM content increased >8.5%	Karpouzas and Walker, 2000
2,4-D and metribuzin	A compost amendment was applied to soil. Compound mineralisation increased with greater levels of amendment	Getenga *et al*. 2004

1.3.2.2.3. Effect of clay type on crop protection product degradation

SOM has generally been highlighted as the principal edaphic factor controlling CPP bioavailability and degradation (Gevao *et al*. 2000), however, soil clay content has also been linked with both processes (Table 1.3). The impact of clay

on CPP bioavailability is clay-specific, for example, Chen *et al*. (2009) showed the greatest sorption and lowest biodegradation of carbaryl in the presence of montmorillonite clay compared to kaolinite or goethite (Chen *et al*. 2009).

Table 1.3: Effect of clay type on crop protection product degradation

CPP	Effect of clay type	Reference
Atrazine	Cationic clays increased CPP sorption and reduced degradation	Besse-Hoggan, *et al*. 2009
2,4-D	CPP mineralisation was found to be inversely correlated to CPP sorption to clay	Hermosin *et al*. 2006
Carbaryl	Carbaryl showed the strongest sorption and lowest biodegradation to the following order of clays; montmorillonite>kaolinite>goethite	Chen *et al*. 2009

1.3.2.2.4. Effect of water content on crop protection product degradation

Accelerated degradation rates of simazine, hexazinone, 2,4-D, aldrin, dieldrin, endrin, chlordane, isoproturon, benazolin-ethyl, glyphosate, and isoproturon are commensurate with an increase in soil water content (Table 1.4). The impact of water content on CPP degradation rates has been shown to be greatest at water contents close to arid conditions. For example, an increase in water content between 4% and 10% had a more pronounced impact on simazine and hexazinone degradation rates than an increase from 10% to 18% (Garcia-Valcarcel and Tadeo, 1999).

Table 1.4: Effect of water content on crop protection product degradation

CPP	Effect of water content	Reference
Simazine and hexazinone	Degradation increased as water content increased from 4% to 10% and from 10% to 18%, with the former having a considerably larger effect	Garcia-Valcarcel and Tadeo, 1999
1,3-D	An increase in water content from 25% to 50% to 75% did not result in a faster rate of degradation	Dungan *et al*. 2001
2,4-D	Mineralisation increased considerably when moisture content was raised from 10% to 15% to 25%	Bouseba *et al*. 2009
2,4-D	2,4-D did not degrade at a soil water content <6%. Degradation proceeded above this threshold value	Cattaneo *et al*. 1997
Aldrin, dieldrin, endrin and chlordane	The rate of degradation was significantly higher at a soil water content of 19% and 33% compared to 10%	Ghardiri *et al*. 1995
Isoproturon, benazolin-ethyl and glyphosate	A linear increase in compound mineralisation was observed within the range of -20 and -0.015MPa. Above this, mineralisation was reduced	Schroll *et al*. 2006
Isoproturon	Degradation in both surface and sub-surface soil was greatest at 90% compared to 50% water holding capacity	Alletto *et al*. 2006

1.3.2.2.5. Effect of temperature on crop protection product degradation

Temperature impacts degradation rates of 1,3-D, 2,4-D, α- and β- endosulfan, lindane, isoproturon, aldrin, dieldrin, endrin, and chlordane (Table 1.5). Further, a

10°C increase in temperature has typically been shown to result in a doubling in the rate of CPP degradation (Dungan *et al*. 2001; Alletto *et al*. 2006; Arshad *et al*. 2008).

Table 1.5: Effect of soil temperature on crop protection product degradation

CPP	Effect of temperature	Reference
1,3-D	A 10°C increase in temperature doubled the rate of degradation of both isomers of 1,3-D	Dungan *et al*. 2001
2,4-D	Degradation was greater at temperatures of 20°C and 28°C compared to 10°C. There were no significant differences in degradation rates between 20°C and 28°C	Bouseba *et al*. 2009
Endosulfan	The rate of α- and β- endosulfan degradation in soil slurry almost doubled with an increase in temperature from 20°C to 30°C	Arshad *et a*l. 2008
Lindane	Degradation was greater at 30°C compared to 25°C and 35°C	Benimeli *et al*. 2007
Isoproturon	The rate of degradation doubled as temperature was increased from 10°C to 22°C in both surface and sub-surface soil	Alletto *et al*. 2006
Aldrin, dieldrin, endrin and chlordane	The rate of degradation was 3-5 times faster when incubated at 30°C compared to those under variable temperature conditions	Ghardiri *et al*. 1995

1.3. ASSESSMENT OF CROP PROTECTION PRODUCT FATE UNDER LABORATORY AND FIELD CONDITIONS

1.3.1. Regulatory laboratory studies: OECD Guideline 307 aerobic and anaerobic transformation in soil

Laboratory-based regulatory studies of CPP degradation are conducted according to OECD Guideline 307. Briefly, soil samples are treated with a ^{14}C labelled CPP and incubated in the dark under controlled temperature and moisture conditions (Figure 1.4). At appropriate time intervals, bottles are destructively sampled, and the CPP is solvent extracted and analysed for parent and metabolites using chromatography. Mineralisation of the test CPP is also quantified by trapping evolved $^{14}CO_2$ in NaOH or KOH (Fig 1.4). Finally, NERs are quantified by combustion and the mass balance is calculated. The time taken for 50% ($DegT_{50}$) or 90% ($DegT_{90}$) of the CPP to degrade can then be calculated using models that fit appropriate degradation kinetics, provided they comply with the FOrum for the Co-ordination of pesticide fate models and their USe (FOCUS) guidelines (FOCUS, 2006). The process is repeated under a range of different soil types (taxonomy, pH, SOM content) and at different temperatures to assess CPP persistence for a range of ecoregions and climatic conditions.

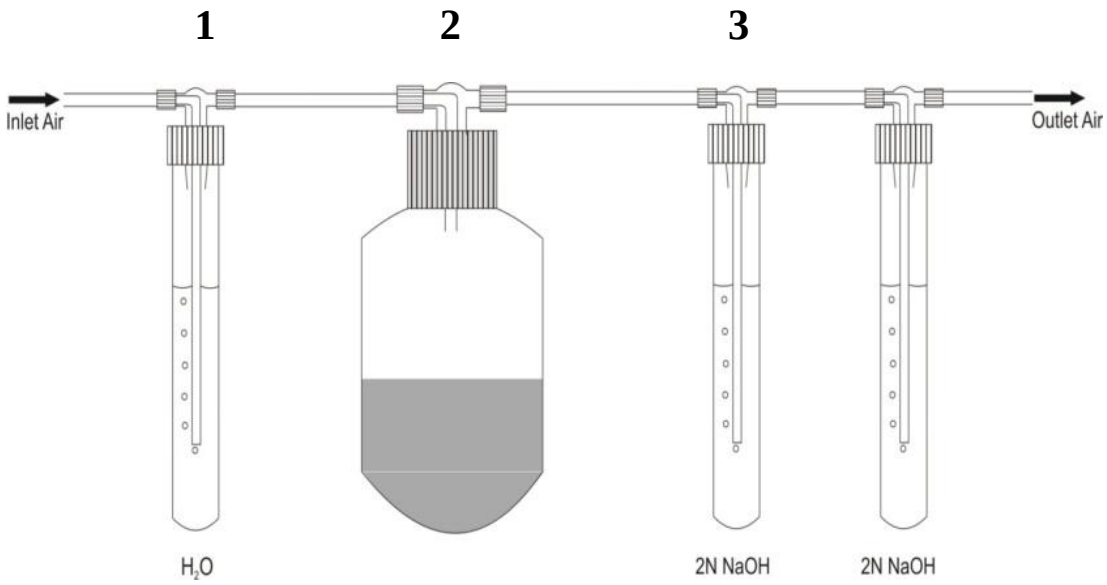


Figure 1.4: Schematic of a laboratory degradation system. 1) Air is pumped through ultra pure water where it becomes water-saturated. 2) The water-saturated air is pumped through a centrifuge vessel containing 50-200 g soil dry weight. The air acts to maintain soil moisture content and microbial activity over a test period of up to 120 days. 3) Traps are attached to the soil vessel containing NaOH or KOH, which trap $^{14}CO_2$ that is evolved by CPP mineralisation.

OECD Guideline 307 standardised the assessment of CPP degradation rates, allowing good comparison across a range of CPPs/soil types The fact that confounding environmental variables such as fluctuating temperature or water content are minimised allows good reproducibility between test systems. However, this simple laboratory system does not accurately mimic the dynamic and heterogeneous nature of an agricultural system. Firstly, soil is sieved to 2 mm, which breaks up soil structure and destroys fungal hyphae networks, which are functionally important in promoting soil aggregation (Tisdall *et al*. 2012). Water content and temperature are maintained at a constant level throughout the experiment, which differs from the diurnal shifts in temperature and dynamic rainfall events under agricultural cropping systems. Moreover, the gravimetric flow of water via soil pores

and channels, and upward flow of water by evaporation are not simulated. OECD Guideline 307 also states that the upper 15 cm of soil is used and therefore variability in the rate of CPP degradation associated with soil depth is completely omitted (Rodriguez-Cruz *et al*. 2006). Finally, tests are conducted under dark conditions and therefore any impact of phototrophs, which develop at the top millimetre of soil (Jeffery *et al*. 2009), on CPP degradation are ignored.

1.3.2. Regulatory field trials

EU Guidelines for the assessment of CPP degradation using field trials are documented by the EPA (2008) and European Food Safety Authority (EFSA, 2010). Briefly, a representative CPP formulation is applied to recently tilled soil and a timecourse of degradation is taken. At each sampling point, cores are sampled from several areas of the field site. Soil is then divided into several depths, solvent extracted and analysed for parent and metabolites, and $DegT_{50/90}$ values are calculated. Trials must be performed on several sites that are representative of the ecoregions the CPP is intended for use.

Field trials provide a realistic environment to assess the fate of CPPs in different soil types and under variable climatic conditions. CPPs are exposed to fluctuations in temperature, natural water flow, and indigenous microbial communities. However, EFSA guidance suggests the incorporation of the CPP below the soil surface or covering the surface with sand, both of which eliminate soil surface degradation processes (EFSA, 2010). Further issues with field trials include difficulty achieving an even application of the CPP to soil and CPP dissipation from

the study site by preferential flow, run-off, volatilisation or mineralisation (Oliver, pers. comm.).

1.3.3. Bridging the gap between regulatory laboratory and field tests

It has been shown that laboratory studies typically overestimate the persistence of CPPs in the field, which may be due to the absence of spatial and temporal variability in edaphic and climatic variables in laboratory systems (Beulke *et al*. 2000). For example, Beulke *et al*. (2000) reviewed 178 studies and compared the $DegT_{50}$ of CPPs from field studies with those simulated by persistence models based on laboratory data. Persistence models overestimated persistence by >1.25 fold in 44% of studies, whereas an underestimation by >1.25 fold was only found in 15% of studies. Beulke *et al*. (2005) later tested the effect of fluctuating or constant water/temperature conditions, the size of soil aggregates (<3 mm or 3-5 mm), and static vs. flow water conditions on the rate of degradation of bentazone and cyanazine in soil. CPP degradation was not significantly impacted by the different treatments and it was concluded that the assumptions underlying extrapolation of laboratory data to field conditions were acceptable under the laboratory setups that were tested (Beulke *et al*. 2005). However, this study may only serve to emphasise that the field environment cannot be reproduced in the lab.

The disparity between CPP persistence estimates in laboratory tests and field trials could also be due to factors that are present in the field but are missing from laboratory systems, such as non-UV light. It is not known if non-UV light impacts CPP degradation, however, the presence of non-UV light represents a clear

difference between laboratory systems which are kept in the dark, and field trials which can be exposed to light.

1.3.3.1. Non-UV light

UV light can directly and indirectly photodegrade CPPs (see sections 1.3.2.1.1 and 1.3.2.1.2). However, non-UV light may also impact CPP degradation by stimulating the development of phototrophs, which could directly metabolise CPPs, or indirectly impact CPP degradation by stimulating heterotrophic degrader organisms. Indeed, Thomas and Hand (2011) have already shown considerable reductions in the persistence of six CPPs in water-sediment systems containing algae that were incubated under non-UV light compared to standard dark systems. Moreover, eight green algae within the division Chlorophyta, including two *Scenedesmus* spp. and a *Chlorella* sp., and four cyanobacteria, including *Nostoc punctiforme* and *Anabaena cylindrica* have been shown to metabolise fludioxonil in pure culture (Thomas & Hand, 2012). Additional CPPs, including diclofop-methyl (Cai *et al*. 2007), fenamiphos (Caceres *et al*. 2008), and isoproturon (Mostafa & Helling, 2001) have also been shown to be transformed in pure algal cultures. The impact of phototrophs on CPP degradation rates are less well established in soil. However, inoculation of soil with *Chlorococcum* sp. or *Scenedesmus* sp. has been shown to result in an increase in the rate of α-endosulfan degradation (Sethunathan *et al*. 2004).

Phototrophs could also have an indirect effect on CPP degradation by altering the soil environment, which could in turn impact soil microbial activity or

community structure. For example, an input of C produced by photosynthesis (Yoshitake *et al*. 2010), an input of N by diazotrophic cyanobacteria (Belnap, 2002; Abed *et al*. 2010; Zhao *et al*. 2010), or an increase in pH by phototrophs taking up CO_2, which is acidic, for use in photosynthesis. To fully understand the potential of non-UV light on CPP degradation in soil, the unique community structure and functional importance of phototrophic communities that develop at the soil surface must first be introduced; a soil compartment termed the Biological Soil Crust (BSC).

1.4. THE BIOLOGICAL SOIL CRUST

The term Biological Soil Crust (BSC) refers to the upper few millimeters of soil. This area is distinct from bulk soil as it is exposed to light and other environmental factors such as wind and rain erosion. The community structure of the BSC has been shown to be different from underlying bulk soil due to the development of photosynthetic communities such as cyanobacteria, algae, mosses, and lichens (Garcia-Pichel *et al*. 2001; Redfield *et al*. 2002; Yeager *et al*. 2004; Langhans *et al*. 2009*a*; Abed *et al*. 2010; Zhang *et al*. 2011). Researchers have focused on BSCs in arid and semi-arid lands such as the Colorado plateau and Sonoran desert (Nagy *et al*. 2005) in the USA, (Garcia-Pichel *et al*. 2001; Redfield *et al*. 2002; Yeager *et al*. 2004), Gurbantunggut desert in northwest China (Zhang *et al*. 2009*a*, 2009*b* & 2011) and the Sultanate of Oman (Abed *et al*. 2010), where BSCs have been estimated to cover up to 70% of the soil surface (Pointing and Belnap, 2012). However, BSCs have also been shown to be ubiquitous in temperate soils and under agricultural crops (Veluci *et al*. 2006; Knapen *et al*. 2007; Langhans *et al*. 2009*a* & 2009*b*). The following sections describe the unique microbial communities and ecological importance of BSCs.

1.4.1. Succession of biological soil crusts

A long term study from 1956 to 1981 investigated succession at the surface of a bare sand dune in the Tengger desert, China (Li *et al*. 2002). Three stages of development were highlighted; (i) Initial colonisation of the surface within one year, particularly by Cyanobacteria such as *Microcoleus vaginatus*; (ii) Appearance of mosses after eight years, and; (iii) Dominance by algae, mosses and liverworts within 25 years. The three stages of community development have since been shown in several arid land studies such as the eastern Negev desert (Lange *et al*. 1992), Arches National Park, Utah (Belnap, 1993) and the Tengger desert, China (Li *et al*. 2010*b*).

1.4.2. Microbial community structure of biological soil crusts

1.4.2.1. Photosynthetic communities

The cyanobacterium *Microcoleus vaginatus* has been shown to be dominant, or abundant, in BSCs from geographically distinct regions, such as the Sultanate of Oman (Abed *et al*. 2010), Colorado plateau, Utah (Garcia Pichel *et al*. 2001; Redfield *et al*. 2002; Yeager *et al*. 2004), Chihuahuan desert, New Mexico (Yeager *et al*. 2004), Gurbantunggut desert, China (Zhang *et al*. 2009*a*, 2009*b* and 2011), northwestern Negev desert, Israel (Zaady *et al*. 2010), xeric Florida shrubland (Hawkes and Flechtner, 2002) and the Nevada desert (Steven *et al*. 2012). In addition, inoculation of soil with *M. vaginatus* has been shown to improve the rate of

recovery following scalping, which suggests that *M. vaginatus* are an important pioneer species of BSCs (Belnap, 1993).

There is also evidence that the stage of succession of a BSC influences cyanobacteria community structure. For example, Yeager *et al*. (2004) showed a shift from *M. vaginatus* in early successional BSCs to *Nostoc* and *Scytonema* spp. in late successional BSCs in both the Chihuahuan desert and the Colorado plateau. Indeed, this distinction between the dominant cyanobacterium is so evident that several studies have grouped BSCs into light (early successional) and dark (late successional) BSCs based on the dominance of the light green coloured *M. vaginatus* and dark green coloured *Nostoc* spp. in early- and late- successional BSCs, respectively (Garcia-Pichel *et al*. 2003; Darby *et al*. 2007; Bates and Garcia-Pichel, 2009; Soule *et al*. 2009; Bates *et al*. 2012).

Direct determination and microscopy following culturing has shown that BSCs also harbour algae, mosses and lichens. A wide range of algae have been found in BSCs, such as *Chlamydomonas ovalis*, *Chlorococcum humicola*, *Chlorella vulgaris*, and *Synechococcus parvus* (Zhang *et al*. 2011). Late-successional BSCs have also been shown to be dominated by lichen, such as *Collema tenax*, or mosses such as *Syntrichia ruralis* (Darby *et al*. 2007).

1.4.2.2. Heterotrophic communities

BSCs also harbour fungi, nematodes, archaea and heterotrophic bacteria (Nagy *et al*. 2005; Darby *et al*. 2007; Soule *et al*. 2009; Bates *et al*. 2012). The

fungal division Ascomycota and the free-living fungal genera *Alternaria* and *Acremonium* have been shown to be dominant in both early and late successional BSCs from the Colorado plateau (Bates and Garcia-Pichel, 2009). Moreover, Ascomycota have been shown to be dominant, and the order Pleosporales to be abundant, in early- and late- successional cyanobacteria dominated BSCs, and lichen-dominated BSCs of the Chihuahuan desert, Sonoran desert and the Colorado plateau (Bates *et al*. 2012).

A large study of either *M. vaginatus*-, *Nostoc* spp.- lichen- or moss-dominated BSCs of the Sonoran desert, Colorado plateau, NW Great Basin and Chihuahuan desert found archaeal community structure to be relatively consistent between crusts and locations (Soule *et al*. 2009). In addition, only six different phylotypes, all within Crenarchaea, were found across all sites (Soule *et al*. 2009). BSC research has also focussed on nematodes and has found nematode abundance to be greater at the soil surface compared to underlying bulk soil in BSCs of the Colorado plateau and Chihuahuan desert, USA (Darby *et al*. 2007). Moreover, nematode community structure has been shown to be influenced by successional stage, with a greater abundance of predators/omnivores in late compared to early successional BSCs (Darby *et al*. 2007).

The community structure of heterotrophic bacteria harboured by BSCs is less well established. Heterotrophic bacteria have been overlooked by several culture-independent studies as they have either targeted 16S rRNA of cyanobacteria (Redfield *et al*. 2002), or diazotrophic cyanobacteria by amplifying the *nifH* gene (Yeager *et al*. 2004). Further, studies that have investigated the diversity of 16S rRNA of general bacteria have found clone libraries to be dominated by sequences with close homology to cyanobacteria (Abed *et al*. 2010; Steven *et al*. 2012).

Consequently, heterotrophic bacterial community structure and diversity are not well understood. However, sequences with close homology to β-proteobacteria such as *Massilia timonae,* Actinobacteria such as *Rubrobacteriadae* sp., Chloroflexi such as *Dehalococcoides* sp., Bacteroidetes such as *Flavobacterium* sp., and *Acidobacterium* sp. such as *Holophaga* have all been detected in BSCs from the Sonoran desert, USA (Nagy *et al*. 2005).

1.4.3. The ecological importance of biological soil crusts

1.4.3.1. Nitrogen fixation

Diazotrophic cyanobacteria present at the soil surface have been shown to input a considerable amount of N at the soil surface (Belnap, 2002; Yeager *et al*. 2004; Johnson *et al*. 2005; Veluci *et al*. 2006; Wu *et al*. 2009; Abed *et al*. 2010; Zhao *et al*. 2010). For example, Belnap (2002) estimated that *M. vaginatus* and Collema dominated BSCs had the potential to fix 13 kg N_2 ha^{-1} yr^{-1}. Cyanobacteria-dominated crusts have typically been shown to fix a greater amount of N_2 than lichen or moss crusts (Belnap, 2002; Wu *et al*. 2009). Moreover, late successional BSCs characterised by diazotrophic *Nostoc* spp. typically input more N_2 compared to BSCs dominated by the non-diazotrophic cyanobacterium *M. vaginatus* (Yeager *et al*. 2004; Johnson *et al*. 2005). For example, Zhao *et al*. (2010) compared N_2 fixation rates at the soil surface at 1, 3, 7, 10, 13 and 16 years and found that; (i) N_2 fixation rates were lowest at year 1 when BSCs are typically dominated by *M. vaginatus*; (ii) N_2 fixation rates were highest in year 3 when BSCs are typically characterised by

Nostoc spp., and; (iii) N_2 fixation rates gradually reduced with time and succession from a cyanobacteria- to a moss- dominated crust.

1.4.3.2. Carbon fixation and respiration

BSC communities have also been shown to fix C through photosynthesis (Housman *et al*. 2006; Yoshitake *et al*. 2010). Similar to N_2 fixation, C fixation is higher in late- compared to early- successional BSCs of the Colorado Plateau and the Chihuahuan desert (Housman, 2006).

BSCs have also been shown to produce a considerable proportion of CO_2 through soil respiration (Castillo-Monroy *et al*. 2011). Castillo-Monroy *et al*. (2011) investigated *in-situ* soil respiration rates in areas with a surface cover of either *Stipa tenacissima* tussock, *Retama sphaerocarpa,* low, medium and high BSC coverage or bare soil. Although soil respiration did not differ between BSCs of varying cover, respiration was higher in all BSCs compared to bare soil. Further, although soil respiration was higher for *S. tenacissima* tussock compared to BSC cover, 37-42% of the estimated 240-323 C m^{-2} yr^{-1} was attributed to BSCs due to the relatively larger surface cover of BSCs compared to *S. tenacissima* (Castillo-Monroy *et al*. 2011). This is consistent with a study in the deglaciated area of Svalbard, Norway which showed that although photosynthesis and dark respiration rates of BSCs were lower than that of vascular plants and mosses, their potential net primary productivity was greater as their surface cover was >30-fold higher that that of plants (Yoshitake *et al*. 2010).

1.4.3.3. Soil structure and water infiltration

BSCs have been shown to improve soil structure and impact rates of water infiltration (Belnap and Gillette, 1997; Kidron *et al*. 2003; Zhang *et al*. 2006; Garcia-Pichel and Wojciechowski, 2009; Chamizo *et al*. 2012). Belnap and Gillette (1997) showed that BSCs undisturbed for 1, 5, 10 and 20 years were able to tolerate a greater friction threshold velocity (the velocity at which soil particles are blown away by wind) than bare sand, and that improved soil stability was commensurate with time since disturbance. Similar results have since been shown for BSCs in the Gurbantunggut desert, China (Zhang *et al*. 2006). Improved soil stability has been attributed to stacks of tightly woven filaments that are deposited during the growth of cyanobacteria, which bind sand particles together (Garcia-Pichel and Wojciechowski, 2009). *Microcoleus* spp. are thought to use this method to colonise highly eroded soil surfaces during the initial stage of BSC development. However, several fungal species have also been shown to improve soil stability by enlarging the size of soil aggregates through cross-linkage and entanglement of particles (Tisdall *et al*. 2012).

Research has since progressed from soil structure to the impact of BSCs on water infiltration and runoff. Confusingly, BSCs have been shown to both improve and reduce water infiltration rates compared to bare soil (Kidron *et al*. 2003; Kidron *et al*. 2012; Zaady *et al*. 2012; Chamizo *et al*. 2012). Contrasting results could be explained by the successional age of the crust, for example, Kidron *et al*. (2003) showed that chlorophyll *a* content was linearly and positively correlated with an increase in runoff for cyanobacteria dominated crusts, however, the inclusion of a

highly water-absorbent moss dominated crust resulted in a reduction in water run-off (Kidron *et al*. 2003). This increase in runoff in early successional BSCs and reduced runoff in late successional crusts has also been shown by Kidron *et al*. (2012), Zaady *et al*. (2012) and Chamizo *et al*. (2012).

1.4.3.4. Seed germination and plant growth

The impact of BSCs on seed germination has also been shown to be BSC-dependent. Lichen crusts have been shown to adversely impact germination time, root penetration and seedling establishment of the annual desert grasses *Bromus tectorum* L. and *Vulpia microstachys* compared to bare soil (Deines *et al*. 2007). However, a BSC composed of mixed phototrophs typically produced similar results to bare soil (Deines *et al*. 2007). The germination efficiency of seeds of four grasses; *Festuca idahoensis, Festuca ovina, Elymus wawawaiensis* and *Bromus tectorum* has also been shown to be BSC- specific; germination efficiency of seeds in short moss (*Bryum argenteum*) were generally half that of tall moss (*Tortula ruralis*) and bare soil treatments (Serpe *et al*. 2006). In contrast, the herb *Mimosa luisana* and the cactus *Myrtillocatus geometrizans* have shown improved seed germination in the presence of a BSC compared to soil without a BSC (Rivera-Aguilar *et al*. 2005).

1.4.4. The biological soil crust of temperate environments

The vast majority of BSC related research to date has been performed in arid and semi-arid lands (Garcia-Pichel *et al*. 2001; Redfield *et al*. 2002; Yeager *et al*. 2004; Langhans *et al*. 2009*a*; Abed *et al*. 2010; Zhang *et al*. 2011). The lack of knowledge regarding BSCs in temperate environments represents a significant research gap, however, the few studies that have been conducted show considerable consistency with arid land research. Firstly, Jeffery *et al*. (2007) showed the development of phototrophic communities in the top millimetre of soil, which were distinct from underlying bulk soil communities. Phototroph community structure has also been shown to be relatively similar between early- and late- successional BSCs of temperate soil of the Rhine, Germany (Langhans *et al*. 2009*a*) and arid lands (Yeager *et al*. 2004). Phototrophs were investigated directly from soil and following culture enrichment, and cyanobacteria and eukaryotic algae commonly found in arid lands were highlighted such as *Clamydomonas* sp., *Chlorella* sp., *Nostoc* sp., *Microcoleus* sp., and *Tolypothrix* sp. (Langhans *et al*. 2009*a*). Moreover, algae and cyanobacteria were dominant in early successional BSCs whereas mosses were more abundant in late successional BSCs (Langhans *et al*. 2009*a*).

The germination, emergence and survival of a range of perennial and annual plants have been shown to be adversely affected by the presence of a BSC in a temperate climate (Langhans *et al*. 2009*b*), a similar situation to results from arid lands (Serpe *et al*. 2006; Deines *et al*. 2007). However, those that survived showed improved height and biomass, which may be due to the elevated N content found at the soil surface (Langhans *et al*. 2009*b*). Potential N_2 fixation rates by diazotrophs

have also been investigated in temperate climate regions where phototrophs have been estimated to fix ~13-19 kg N_2 ha^{-1} on unfertilised land (Witty *et al*. 1979).

1.5. WHY STUDY THE AGRICULTURAL IMPORTANCE OF PHOTOTROPHS?

An investigation into both the occurrence of phototrophs in cropland soil and their impact on soil erosion highlighted the potential importance of phototrophs in agriculture (Knapen *et al*. 2007). A visual assessment of winter wheat, maize and sugar beet cropland in 62 fields, across 300 ha found that phototrophs were present in 74% of fields, and that 16% of all sites had a surface cover between 75% and 100% (Figure 1.5). Further, the rate of soil erosion was reduced by 37% and 79% in the presence of algal and moss crusts, respectively compared to bare soil (Knapen *et al*. 2007). However, although phototrophs have been shown to be present under agricultural cropping systems, their community structure and ecological importance are not well understood.

Figure 1.5: The establishment of phototrophs under a cropping system in loess-derived Belgian soil. Taken from Knapen (*et al*. 2007).

In order to effectively manage cropping systems, it is essential to understand the agricultural importance of phototrophs. For example, if phototrophs were shown to significantly reduce soil erosion under agricultural cropping systems, then a shift to a no tillage farming strategy would be appropriate for farms particularly at risk of soil erosion. Alternatively, if the presence phototroph communities impeded seed germination efficiency and establishment, soil tillage may be more appropriate.

The presence of phototrophs at the soil surface may also be an important factor for CPP fate as suggested in Section 1.3.3.1. A CPP's first point of contact to the soil environment is the soil surface. Therefore, laboratory degradation tests should aim to simulate the physical, chemical and biological properties of the soil surface. In an agricultural field the soil surface is exposed to light, which stimulates

the development of phototrophs (Knapen *et al*. 2007). Currently it is not known how non-UV light impacts CPP fate, or microbial community structure and function in temperate soil.

1.6. AIMS AND OBJECTIVES

The overall aim of the work described in this thesis was to determine if non-UV light affects the fate of CPPs and the microbial community structure and function of a pasture soil of a temperate climate. The thesis has been divided into five sections in total, with three self-contained experimental chapters (Chapters II – IV), each with their own defined aims, methods, results and discussion sections, in addition to a general introduction (Chapter I) and a general discussion (Chapter V).

Chapter II aimed to determine the effect of non-UV light on the fate of CPPs in soil. The questions considered were:

(i) Does non-UV light affect the transformation, mineralisation and formation of non-extractable residues for a range of CPPs?

(ii) What are the mechanisms responsible for non-UV light impacting CPP degradation?

Chapter III aimed to determine the effect of non-UV light on pH, nutrients, and microbial community structure of soil over a timecourse. The questions considered were:

(i) How diverse are cyanobacteria and eukaryotic phototrophs at the soil surface?

(ii) Does light influence bacterial and fungal community structure and diversity at the soil surface?

(iii) Are there successional changes in phototroph, bacterial and fungal communities at the soil surface and underlying bulk soil?

(iv) Does the establishment of soil surface communities affect chemical parameters and microbial community structure of underlying bulk soil?

Chapter IV aimed to determine the impact of non-UV light on soil surface functions using a metatranscriptomic approach. The questions considered were:

(i) How do major soil functions and functional diversity differ in the presence of soil surface communities exposed to light and dark conditions?

(ii) Are there differences in primary metabolic strategies and genes involved in the nitrogen cycle between soil exposed to light and dark conditions?

2. CHAPTER II: NON-UV LIGHT INFLUENCES THE DEGRADATION RATE OF CROP PROTECTION PRODUCTS

This Chapter has been published in *Environmental Science and Technology*, 2013, **47**, 8229-8237. The publication is displayed in Appendix I.

2.1. ABSTRACT

CPPs are subject to strict regulatory evaluation prior to approval for commercial use, including laboratory and field trials. It is important to add environmental complexity to laboratory systems, as they do not provide an accurate representation of a field environment. The following study investigated the effect of non-UV light on the rate of CPP degradation, by the inclusion of non-UV light to OECD guideline 307. The transformation of benzovindiflupyr, chlorotoluron, prometryn, cinosulfuron, imidacloprid, lufenuron, propiconazole, and fludioxonil were investigated. The time taken for 50% of benzovindiflupyr to degrade was halved from 373 to 183 days with the inclusion of light. Similarly, the time taken for 90% of chlorotoluron to degrade was halved from 79 to 35 days under light compared to dark conditions. In addition, significant reductions in extractable parent compound for prometryn (4%), imidacloprid (8%), and fludioxonil (24%) were also observed under light conditions in comparison to the dark control. However, a significantly slower rate of cinosulfuron transformation (14%) was observed under light compared to dark conditions. Non-extractable residues were significantly higher for seven of the CPPs tested under light, which may be due to enhanced transformation, and incorporation of compounds into biogenic residues. Soil

biological and chemical analyses suggest that light stimulated phototroph growth, which may directly and/or indirectly impact CPP degradation rates

2.2. INTRODUCTION

OECD guideline 307 sets out the method used to investigate aerobic and anaerobic transformation of chemicals in soil (OECD Guideline 307). The guideline states that soil should be sieved to 2 mm, kept at a constant temperature of 20°C±2°C and incubated in the dark. These conditions are not consistent with the field environment where microbial community structure and biomass are likely to differ considerably. In particular, incubation of samples in the dark prevents the development of phototrophs, which are known to be present at the soil surface of agricultural soil (Knapen *et al*. 2007). Phototrophs could have a direct role in metabolising pesticides (Thomas and Hand, 2012), or an indirect role, such as an input of C (Yoshitake *et al*. 2010) or N (Belnap, 2002), which could stimulate heterotrophic degrader taxa and consequently accelerate CPP degradation rates.

This chapter investigated the effect of non-UV light, which stimulates the development of phototrophs without photolytically degrading CPPs, on CPP degradation in soil. The Chapter aimed to answer the following questions:

(i) Does non-UV light affect the transformation, mineralisation and formation of non-extractable residues for a range of CPPs?

(ii) What are the mechanisms responsible for non-UV light impacting CPP degradation?

2.3. MATERIALS AND METHODS

2.3.1. Gartenacker soil

Gartenacker soil (silty loam) was sourced from Switzerland (CH-1896 Vouvry) in November 2009 for use in experiment 1 (section 2.3.3). Gartenacker was sourced in May 2010 for use in experiment 2 (section 2.3.5). The properties of Gartenacker are shown in Table 2.1. Soil was sieved to 2 mm and used within 3 months of collection according to OECD guideline 307.

2.3.2 Test chemicals

Studies were performed using [^{14}C] labelled compounds. The compounds used were; (i) Herbicides: chlorotoluron (specific activity (spec act) 4.570 MBq mg^{-1}), cinosulfuron (spec act 2.327 MBq mg^{-1}) and prometryn (spec act 1.136 MBq mg^{-1}); (ii) Fungicides: propiconazole (spec act 0.762 MBq mg^{-1}), fludioxonil (spec act 1.469 MBq mg^{-1}) and benzovindiflupyr (spec act 5.620 MBq mg^{-1}), and; (iii) Insecticides: lufenuron (spec act 5.132 MBq mg^{-1}) and imidacloprid (spec act 2.020 MBq mg^{-1}). Table 2.2 and Figure 2.1 show selected pesticide properties and structures, respectively.

Table 2.1: Properties of Gartenacker topsoil (20 cm depth) taken from Basel, Switzerland

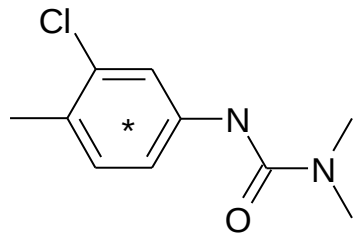
Soil	Classification	pH		% OM	CEC	Particle size analysis (%)			Moisture holding capacity (%)	
		H_2O	0.01M $CaCl_2$		meq/100g	Sand	Silt	clay	-33 kPa	-1500 kPa
Gartenacker	Silt loam	6.9	6.6	4.1	10.1	34	52	14	29.6	16.0

Table 2.2: Selected properties of crop protection products investigated

Name	Type	$DegT_{50}$ (d)	K_{ow} (logP)	Water solubility (mg l^{-1})	K_{oc} (ml g^{-1})	Mode of action
Prometryn	Methylthiotriazine herbicide	14-158	3.1	33	400	Photosynthetic electron transport inhibitor. Inhibits oxidative phosophorylation
Cinosulfuron	Sulfonylurea herbicide	20	2.0	4000	20	Inhibits biosynthesis of essential amino acids.
Chlorotoluron	Urea herbicide	30-40	2.5	74	205	Inhibitor of photosynthetic electron transport
Propiconazole	Triazole fungicide	29-70	3.7	100	1086	Steroid demethylation inhibitor
Benzovindiflupyr	Pyrazolecarboxamide fungicide	N/A*	N/A*	N/A*	N/A*	Succinate dehydrogenase inhibitor
Fludioxonil	Phenylpyrrole fungicide	140-350	4.1	1.8	75000	Inhibits protein kinase involved in regulatory step of cell division
Lufenuron	Benzoylurea insecticide	13-20	5.1	0.06	41182	Inhibits chitin synthesis
Imidacloprid	Neonicotinoid insecticide	N/A*	0.57	610	225	Antagonist to post-synaptic nicotinic receptors in central nervous system

N/A refers to not available. $DegT_{50}$ values, organic carbon water partition coefficients (K_{oc}) and modes of action are taken from Tomlin (2006).

a)



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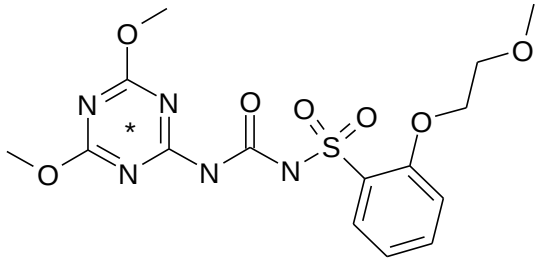

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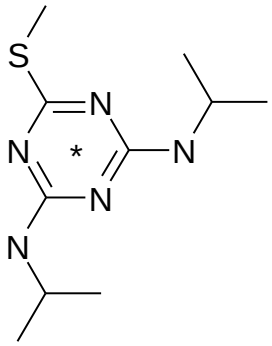

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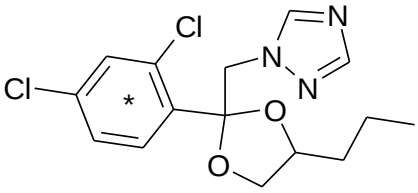

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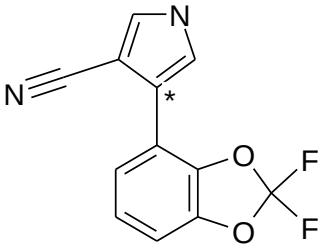

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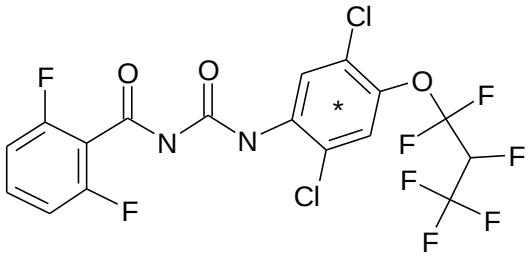

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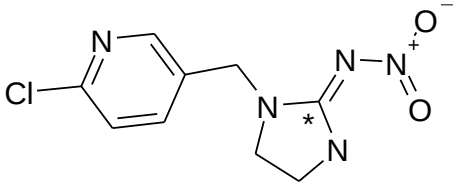

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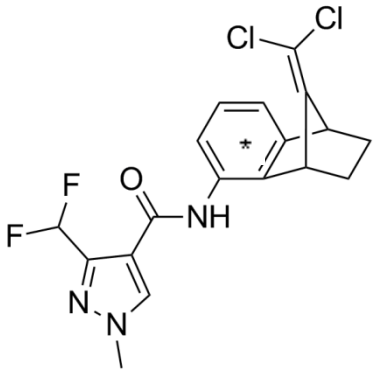


Figure 2.1: Structures of crop protection products investigated: (a) Chlorotoluron; (b) Cinosulfuron; (c) Prometryn; (d) Propiconazole; (e) Fludioxonil; (f) Lufenuron; (g) Imidacloprid, and; (h) Benzovindiflupyr. The position of radiolabelling is indicated by a (*).

2.3.3. Experiment 1: The effect of non-UV light on the degradation of benzovindiflupyr and chlorotoluron

2.3.3.1. Light and temperature readings

A Bentham instruments photomultiplier DH-3 (DM150 double monochromator) and a Bentham cone diffuser sn9941 probe were used to record light levels from a Sanyo Gallenkamp Environmental Chamber illuminated with Philips Master fluorescent lights (>360nm) TLD 36W/840 and natural light during Spring and Summer (Figure 2.2). The light simulated treatment was setup in a Sanyo environmental cabinet at 20°C±2°C using a 16 hour light:8 hour dark cycle. The dark simulated treatment was setup in a controlled temperature (CT) room at 20°C±2°C. Temperature readings were taken using a Comark EvolutionN1001 thermometer to ensure that light and dark treatments were not incubated under considerably different temperatures. Probes were placed in mock vessels containing 100 g dry weight equivalent (dwe) Gartenacker soil approximately 1 cm above the soil surface, and temperature was recorded over 7 days. The effect of crowding on vessel temperature was investigated by setting up mock vessels such that one was surrounded by eleven vessels (crowded) and the other was not surrounded by any vessels (isolated) (Table 2.3).

2.3.3.2. Test System

Approximately 100 g dry weight equivalent (dwe) of Gartenacker soil at 35% (pF=2 or -30 kPa) moisture content was transferred to 250 ml centrifuge vessels and pre-incubated for 7 d under aerobic conditions prior to benzovindiflupyr application. Soil was pre-incubated for 22 d for the chlorotoluron study to allow the development of phototrophs prior to application. Subsequent work on chlorotoluron applied after only 7 d incubation gave similar results (Table 2.4). Soil moisture content was monitored weekly (by weight) and maintained at 35% by the addition of sterile ultra pure water (U.P. water).

2.3.3.3. Application and sampling of crop protection products

Test compounds were dissolved in acetonitrile and applied drop-wise using a micro-pipette, onto the soil surface. Following application of the test compound, vessels were mixed for 10 min on a roller to achieve a homogenous distribution of the CPP. CPPs were applied at, or close to field application rates to monitor transformation at environmentally relevant concentrations. Application rates were 0.8 µg g^{-1} and 0.1 µg g^{-1} for chlorotoluron and benzovindiflupyr, respectively, which are equivalent to field application rates of 600 g ha^{-1} for chlorotoluron and 75 g ha^{-1} for benzovindiflupyr. Application rates are based on the OECD calculation, which assumes an incorporation depth of 5 cm and a bulk density of 1.5 g cm^{-3} (OECD Guideline 307).

Systems were destructively sampled in triplicate at 0 days after treatment (DAT) and compound specific sampling points were taken thereafter; chlorotoluron (3, 7, 14, 28, and 61 DAT) and benzovindiflupyr (22, 59, 90, and 120 DAT). A time course of transformation was taken during experiment 1 with the aim of providing sufficient data to enable the calculation of robust rates of transformation.

At each sampling point, CPPs were extracted with solvents. Extraction solvents were; (i) Chlorotoluron: 2 x 100 ml acetonitrile:U.P. water (80:20 v/v), and; (ii) Benzovindiflupyr: 2 x 100ml acetonitrile:0.1 M aqueous ammonium acetate (80:20 v/v), followed by 100 ml acetonitrile:U.P. water (pH3 adjusted using formic acid) (80:20 v/v). After each solvent addition, samples were shaken at 300 rpm for 1 hr, centrifuged at 2500 rpm for 10 min; each extract decanted and pooled with successive extractions (Thomas & Hand, 2011). Soil was left to dry before being ground to a fine powder using a mortar and pestle, and subjected to 2 x 20 min cycles of accelerated solvent extraction (ASE) using acetonitrile:0.3% acetic acid (70:30 v/v) at 100°C and 1500 psi. ASE extractions were not performed on 0 DAT extractions or for chlorotoluron at sampling points 3 and 7 DAT.

2.3.3.4. Analysis of crop protection products

The total ^{14}C-activity recovered in primary extracts, ASE extracts, and $^{14}CO_2$ traps were quantified by Liquid Scintillation Counting (LSC) using a Packard Tri-Carb (3100TR) Liquid Scintillation Counter (Perkin Elmer, Boston, MA). The ^{14}C-activity remaining in soil was termed non-extractable residues (NER) and was quantified by sample oxidation using a Packard Model 307 combustor. The total ^{14}C-

activity recovered in each fraction was calculated as a percentage of total applied, and summed to give the mass balance in each vessel.

The primary extracts were analysed using High Performance Liquid Chromatography (HPLC) to determine the percentage of parent compound remaining in the sample. It was assumed that ASE extracts contained the same percentage of parent compound as main extracts. Aliquots were concentrated prior to analysis under nitrogen gas using a Turbovap II (Caliper Life Sciences).

HPLC was performed using an Agilent HP1200 HPLC system (Agilent Technologies, UK Limited) connected to a Packard model 4 β-RAM radiodetector (IN/US systems). Reversed phase gradient elution was used for both compounds. Benzovindiflupyr was run on a Luna C18 column (250 mm x 4.0 mm, 5 µm particle size), starting at 95% U.P. water (0.1% formic acid): 5% acetonitrile, progressing to 95% acetonitrile: 5% U.P. water (0.1% formic acid) over 25 mins using a linear gradient. Chlorotoluron was run on a Luna C18 column (150 mm x 4.60 mm, 5 µm particle size), starting at 95% U.P. water (0.1% acetic acid): 5% acetonitrile, progressing to 100% acetonitrile over 20 mins using a linear gradient (Thomas & Hand, 2011).

The levels of parent compound, expressed as a percentage of applied radioactivity, recovered in the extracts were plotted using Simple First Order Kinetics (SFO) for benzovindiflupyr under light and dark conditions and chlorotoluron under light. A biphasic plot (Double First-Order Kinetics; DFOP) was more appropriate for chlorotoluron under dark conditions. The modeling program KinGUI v1.1, (conforms to the requirements of FOCUS kinetics) was used to estimate the time it takes for 50% and 90% of the compound to degrade ($DegT_{50}$/$DegT_{90}$) (FOCUS, 2006.

2.3.4. Biological and chemical properties of Gartenacker soil during benzovindiflupyr and chlorotoluron degradation

Soil was sub-sampled from test vessels during the timecourse of degradation of benzovindiflupyr and chlorotoluron, and used to assess pH, and bacterial, fungal and archaeal copy numbers.

2.3.4.1. Chlorophyll a

Chlorophyll *a* was extracted using a modified version of Ritchie (2006). In full, the absorbance of solvent extract (1 ml) from the CPP extraction method was measured at 664 nm and 750 nm. Absorbance at 750 nm was used to record absorbance by background particles, and was subtracted from absorbance at 664 nm. The solution was then acidified by adding 200 µl of 3M HCl to convert chlorophyll *a* to pheophytin. Absorbance was measured at 665 nm and 750 nm after 90 secs of acidification. Absorbance readings at 750 nm were deducted from absorbance at 665 nm, and chlorophyll *a* was calculated according to the formulas given in Hansson (1988).

2.3.4.2. pH

Soil (2 g) was shaken with 5 ml U.P water for 15 mins at 200 rpm before measuring pH using a BASIC pH meter (Denver Instrument Company, Norfolk, UK) with a Russell electrode (Fisher Scientific, Leicestershire, UK) (Emmett *et al*. 2008).

2.3.4.3. DNA extraction and qPCR amplification of ribosomal RNA markers to assess bacterial, archaeal and fungal copy number

DNA was extracted using a FastDNA Spin Kit (Qbiogene, Loughborough, UK) soil kit according to the manufacturer's handbook. All centrifuging was performed at 14, 000 x *g*. In full, 978 µl sodium phosphate buffer and 122 µl of MT buffer was added to 500 mg soil. Samples were placed in a Hybaid RiboLyser Homogenizer (Fisher Scientific UK Ltd., Leicestershire, UK) at maximum velocity for 3 mins and centrifuged for 30 secs before transferring the supernatant to a 2 ml eppendorf tube. PPS reagent (250 µl) was added, samples were inverted 10-times, centrifuged for 5 mins, and the supernatant transferred to a 15 ml falcon tube. Binding matrix suspension (1 ml) was added and tubes were inverted for 2 mins and allowed to settle for 3 mins, with the upper 500 µl discarded. The binding matrix was re-suspended and 600 µl was transferred to a SPIN^{TM} filter and centrifuged for 1 min. The catch tube was emptied and the process was repeated. 500 µl of SEWS-M was added, centrifuged for 1 min and the supernatant discarded. Tubes were centrifuged for 2 mins to dry the matrix. The filter was transferred to a clean catch tube and air-dried for 5 mins. DNA was eluted by adding 30 µl of DES and

centrifuging for 1 min. The quality and quantity of DNA in extracts was analysed using a nanodrop ND-1000 spectrophotometer (Labtech International Ltd., Sussex, UK). Sample quality was also checked by gel electrophoresis using a 1% agarose gel in 0.5% tris acetate EDTA.

Bacterial copy number was assessed by qPCR targeting 16S rRNA genes using primers BA519f (CAGCMGCCGCGGTAANWC) and BA907R (CCGTCAATTCMTTTRAGTT) (Lane, 1991). Archaeal 16S rRNA genes were amplified using primers A364aF (CGGGGYGCSCAGGCGCGAA) and A934b (GTGCTCCCCCGCCAATTCCT) (Burgraff *et al*. 1997; Grosskopf *et al*. 1998), and for analysis of fungi, qPCR targeted the ITS region using primers 5.8S (CGCTGCGTTCTTCATCG) and ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993; Fierer *et al*. 2005) (Sigma-Aldrich Company Ltd., Dorset, UK).)

qPCR was performed using 12.5 µL Sybr Green Jumpstart Taq Ready-Mix (Sigma-Aldrich Company Ltd., Dorset, UK), 1 µL of 10 ng μl^{-1} DNA, and either 0.125 µl, 0.15 µl or 0.10 µl of 50 µM forward and reverse primers for bacteria, archaea and fungi, respectively. Additionally, 3 µl (bacteria) or 4 µl (archaea/fungi) of 25 mM $MgCl_2$ was added. Bovine serum albumin (20 µg μl^{-1}) was also added to bacterial (0.25 µl) and fungal (0.4 µl) reactions (Roche Diagnostics Ltd., Sussex, UK). All reactions were made up to 25 µl with DNase/RNase free-water (QIAGEN Ltd., Sussex) and run on a CFX Connect™ real time PCR detection system (BIO-RAD Laboratories Ltd,. Hertfordshire, UK). For bacteria, denaturation was performed at 96°C for 10 mins, followed by 40 cycles of 96°C for 30 secs, 52°C for 30 secs, and 72°C for 1 min. For archaea, denaturation was performed at 94°C for 6 mins, followed by 45 cycles of 94°C for 35 secs, 66°C for 30 secs, 72°C for 45 secs

and 86.5°C for 10 secs. A melt curve was constructed for bacterial and archaeal reactions from 75.0 to 95°C, with 0.2°C increase per cycle. For fungi, denaturation was performed at 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs, 53°C for 30 secs, 72°C for 30 secs. A melt curve was constructed from 60°C - 95°C, with an increase of 0.2°C every 6 secs.

2.3.5. Experiment 2: Compound screen; The effect of non-UV light on the degradation of six crop protection products with a range of physico-chemical properties

2.3.5.1. Test System, application and sampling of crop protection products

Unless specified, the test system, application and sampling were the same as described previously in section 2.3.3.2. Cinosulfuron, fludioxonil, imidacloprid, lufenuron, prometryn, and propiconazole were applied at environmentally relevant rates of 0.13 μg g^{-1}, 0.27 μg g^{-1}, 0.27 μg g^{-1}, 0.13 μg g^{-1}, 2 μg g^{-1}, and 0.67 μg g^{-1}, respectively which are equivalent to field application rates of 100 g ha^{-1} for cinosulfuron and lufenuron, 200 g ha^{-1} for fludioxonil and imidacloprid, 1500 g ha^{-1} prometryn, and 500 g ha^{-1} propiconazole. Application rates are based on the OECD calculation, which assumes an incorporation depth of 5 cm and a bulk density of 1.5 g cm^{-3} (OECD Guideline 307).

The time of sampling was compound specific and at a single timepoint; lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole

(42 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Quadruplicate samples were set up and extractions/HPLC was performed on a single centrifuge vessel from both light and dark treatments approximately at the $DegT_{50}$ of the CPP. CPP degradation under light and dark conditions compared, and provided significant degradation had occurred, extractions were taken from the remaining three vessels. A sampling point was also taken at 0 DAT for all compounds to ensure CPP application rates were appropriate.

2.3.5.2. Analysis of crop protection products

Extraction solvents were compound specific; (i) Cinosulfuron, propiconazole and imidacloprid: 3 x 100 ml acetonitrile : U.P. water (80:20 v/v); (ii) Prometryn: 2 x 100 ml acetone : U.P. water (80:20 v/v), and; (iii) Lufenuron: 2 x 100 ml acetone : U.P. water (80:20 v/v), followed by 100 ml acetone. Unless specified, the proportion of parent compound remaining in samples was determined using the same method as described in Section 2.3.3.4 (Thomas and Hand, 2011).

Reversed phase gradient elution was used for all compounds, however, the column, gradients and mobile phases used differed between CPPs; (i) Fludioxonil, prometryn, and propiconazole were run on a Luna C18 column (150 mm x 4.60 mm, 5 µm particle size) starting at 95% U.P. water (0.1% acetic acid) : 5% acetonitrile for 5 mins, progressing to 100% acetonitrile over 20 mins using a linear gradient; (ii) Imidacloprid was run on ACE 5 C18 column (250 mm x 4.00 mm, 5 µm particle size), starting at 95% 0.02 M H_3PO_4: 5% acetonitrile progressing to 35% acetonitrile over 30 mins using a linear gradient, followed by 99% acetonitrile for 5 mins, and;

(iii) Lufenuron was run on a Luna C18 column (250 mm x 4.6 mm, 5 µm particle size), starting at 75% 0.02 M H_3PO_4: 5% acetonitrile for 11 mins, progressing to 95% acetonitrile over 10 mins using a linear gradient. Cinosulfuron was run on the same column as lufenuron, but using a gradient starting at 95% U.P. water (0.125% trifluoroacetic): 5% acetonitrile, progressing to 50% acetonitrile over 42 mins using a linear gradient, followed by 95% acetonitrile for 8 mins (Thomas and Hand, 2011). Fludioxonil mineralisation values under dark conditions are taken from only two samples as one sample failed to show considerable mineralisation.

A metabolite of lufenuron was volatile. Therefore, extracts could not be concentrated prior to HPLC analysis as the sample would not be representative of the original extract. Lufenuron extracts were analysed by direct injection into a SoFiE stop-flow controller attached to a HPLC, which has a lower limit of detection than a standard HPLC system.

2.3.6. Statistical analysis

Parametric tests were performed on non-transformed data where possible. If assumptions were not met, data was log transformed, or a non-parametric test was performed instead. A mixture of two-way ANOVA with Tukey test (with treatment, time and treatment*time as factors), correlation analysis, Kruskall-Wallis, and t-tests were performed on data. Errors are all ±1 standard error (S.E). All analyses were performed using Minitab version 15 and Figures were plotted using Sigmaplot v. 12.0.

2.4. RESULTS

2.4.1. Light spectra and temperature

The majority of light was at a wavelength >380 nm i.e. non-UV light (visible light) (Figure 2.2). The light intensity was far higher under natural light compared to laboratory test systems (Figure 2.2). It is important that UV light was not present in laboratory test systems as this can both adversely affect microorganisms (Lin *et al*. 1997), and photodegrade CPPs (Beltran *et al*. 1993; Arkhipova *et al*. 1997).

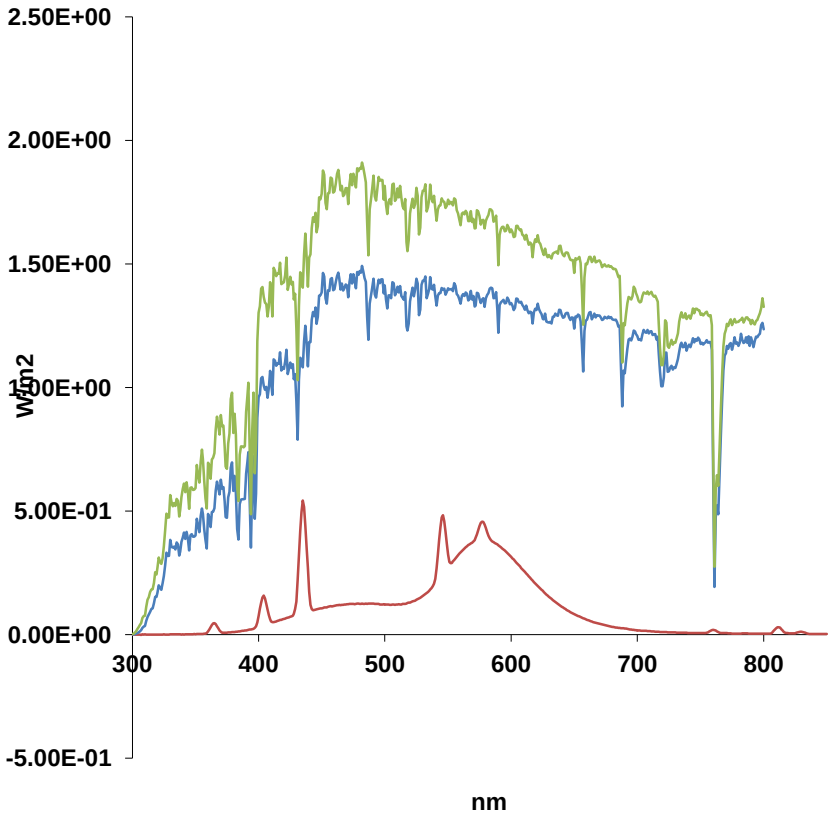


Figure 2.2: Light spectra taken from a Sanyo environmental chamber (red) and outside during Spring (blue) and Summer (green) (280-800nm).

Bottle crowding resulted in an increase in temperature of approximately 1°C in both the Sanyo cabinet and CT room (Table 2.3). The temperature in the Sanyo cabinet was approximately 1°C higher compared to the CT room, which may be due to a production of heat by lights in the Sanyo cabinet (Table 2.3).

Table 2.3: Mean, median and range of temperatures (°C) in a controlled temperature (CT) room and Sanyo environmental chamber over 7 days

Condition	Range (°C)	Mean (°C)	Median (°C)
Sanyo crowded	19.8-20.7	21.03	21.4
Sanyo isolated	19-19.7	20.09	20.1
CT crowded	19.9-21.7	20.01	20.0
CT room isolated	19.3-21.0	19.25	19.2

2.4.2. Experiment 1: Degradation of benzovindiflupyr and chlorotoluron under light and dark conditions

Benzovindiflupyr mass balances were between 86% and 100% for all sampling times with the exception of 120 DAT under light conditions, which had a mass balance of 83%. Light treatment ($p \leq 0.001$) and sampling time ($p \leq 0.001$) both had a significant effect on the proportion of extractable benzovindiflupyr (Figure 2.3). Benzovindiflupyr transformation was more rapid under light conditions with parent levels declining from 95.4%±S.E.0.9% at 0 DAT to 58.8%±S.E.1.1% in the light and 70.6%±S.E. 1.4% in the dark at 120 DAT (Figure 2.3). The $DegT_{50}$ of benzovindiflupyr was approximately halved under light from 373 d in the dark to 183 d in the light. The $DegT_{90}$ value was 608 d under light conditions and >1000 d in the

dark treatment (chi-squared error value was <15 for $DegT_{50/90}$, which suggests an acceptable fit for degradation kinetics). The impact of a slightly elevated temperature under light compared to dark conditions on the $DegT_{50/90}$ of benzovindiflupyr was adjusted according to Culleres (2007), however, it did not impact $DegT_{50/90}$ results.

Light also had an impact on the proportion of benzovindiflupyr NERs (Figure 2.3). NERs increased over the timecourse of benzovindiflupyr degradation and were significantly higher under light compared to dark conditions at 90 DAT ($p \leq 0.05$) and 120 DAT ($p \leq 0.001$). For example, at 120 DAT, NERs accounted for 15.0%±S.E.0.2% and 9.5%±S.E.0.2% of total radioactivity under light and dark conditions, respectively. Benzovindiflupyr mineralisation was minimal at <2% under both light and dark conditions at 120 DAT (Figure 2.3).

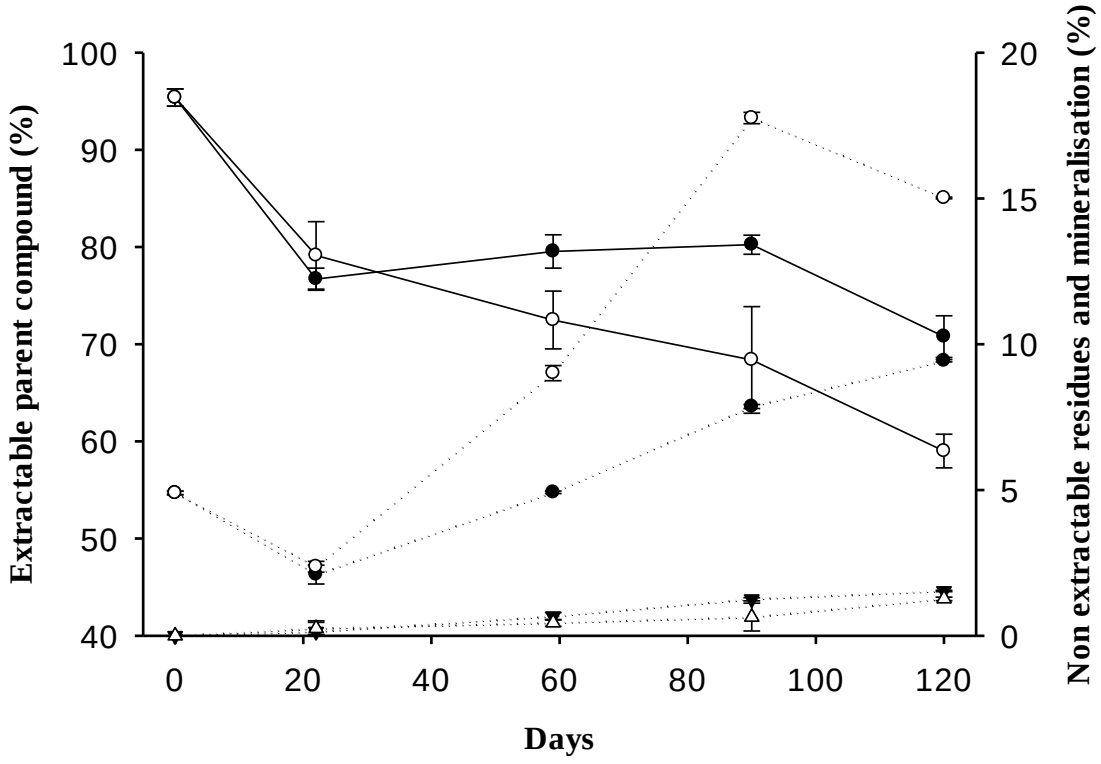


Figure 2.3: Mass balance for the fungicide benzovindiflupyr under light (open symbols) and dark (closed symbols) conditions. The partitioned radioactivity is shown for extractable parent compound (–●–), mineralisation (···▲···) and non extractable residues (···●···). Error bars are ±1 S.E.

Mass balances for chlorotoluron were between 94% and 102% for all sampling points. Light treatment ($p \leq 0.001$) and sampling time ($p \leq 0.001$) also had a significant effect on the proportion of extractable chlorotoluron (Figure 2.4). Moreover, a significant interaction was observed between sampling time and light treatment ($p \leq 0.001$). Chlorotoluron transformation was more rapid under light conditions, with parent levels declining from 97.3%±S.E. 3.9% at 0 DAT to 11.6%±S.E. 1.0% in the light and 26.5%±S.E. 0.6% in the dark at 28 DAT ($p \leq 0.001$) (Figure 2.4). Chlorotoluron $DegT_{50}$ values were approximately similar at 10 d and 15 d under light and dark conditions, respectively. However, chlorotoluron $DegT_{90}$ value was approximately halved from 79 d in the dark to 35 d under light (chi-squared error value was <15 for $DegT_{50/90}$, which suggests an acceptable fit for degradation kinetics). The impact of a slightly elevated temperature under light compared to dark conditions on the $DegT_{50/90}$ of chlorotoluron was adjusted according to Culleres (2007), however, it did not impact $DegT_{50/90}$ results.

Chlorotoluron NERs increased during the timecourse of degradation. At 61 DAT, NERs accounted for 55.7% ±S.E 3.2% and 39.2% ±S.E 1.7% of total radioactivity under light and dark conditions, respectively ($p < 0.05$) (Figure 2.4). Chlorotoluron mineralisation also increased over the timecourse of degradation with 12.4%±S.E. 1.3% and 16.1%±S.E. 0.2% at 61 DAT under light and dark conditions, respectively. Mineralisation was significantly higher under dark conditions at 3 DAT ($p \leq 0.001$) and 28 DAT ($p \leq 0.05$) (Figure 2.4).

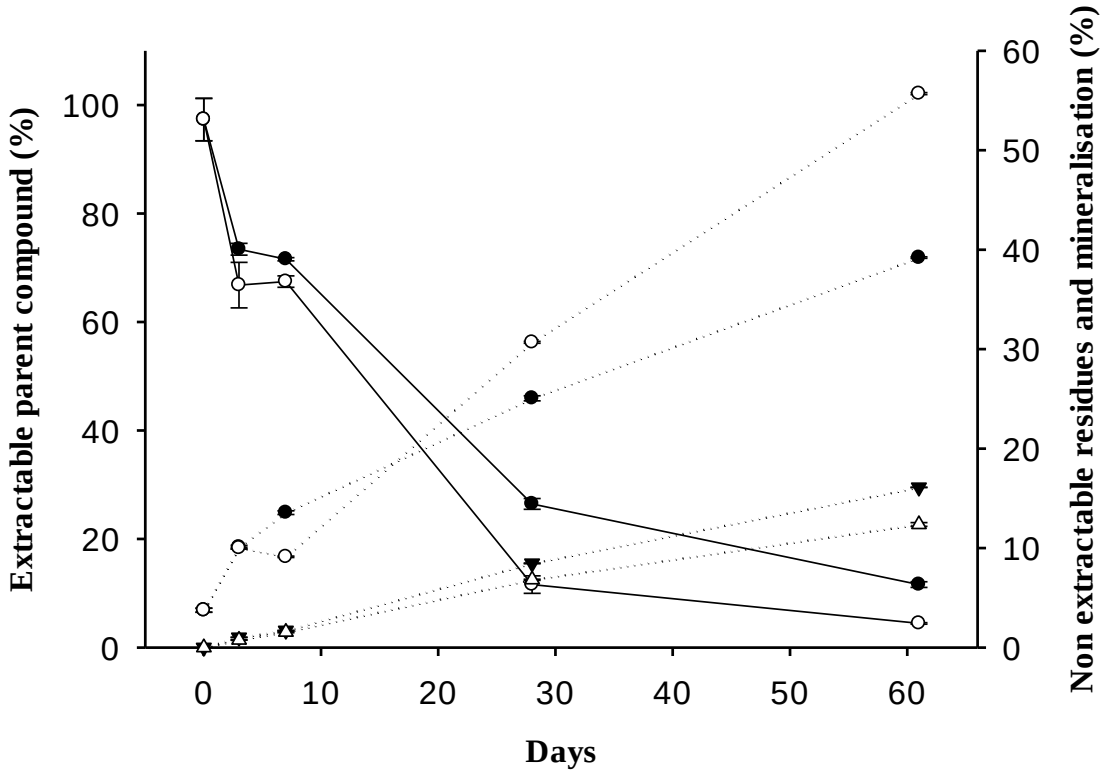


Figure 2.4: Mass balance for the herbicide chlorotoluron under light (open symbols) and dark (closed symbols) conditions. The partitioned radioactivity is shown for extractable parent compound (–●–), mineralisation (···▲···) and non-extractable residues (···●···). Error bars are ±1 S.E.

Table 2.4 shows that the duration of pre-incubation time prior to chlorotoluron application did not impact chlorotoluron degradation rates under light or dark conditions, for example, there was approximately 14.9% and 13.1% less chlorotoluron remaining under light compared to dark conditions after 7 and 22 days pre-incubation, respectively.

Table 2.4: Comparison of the effect of incubation time on extractable chlorotoluron in Gartenacker soil after incubation under light and dark conditions

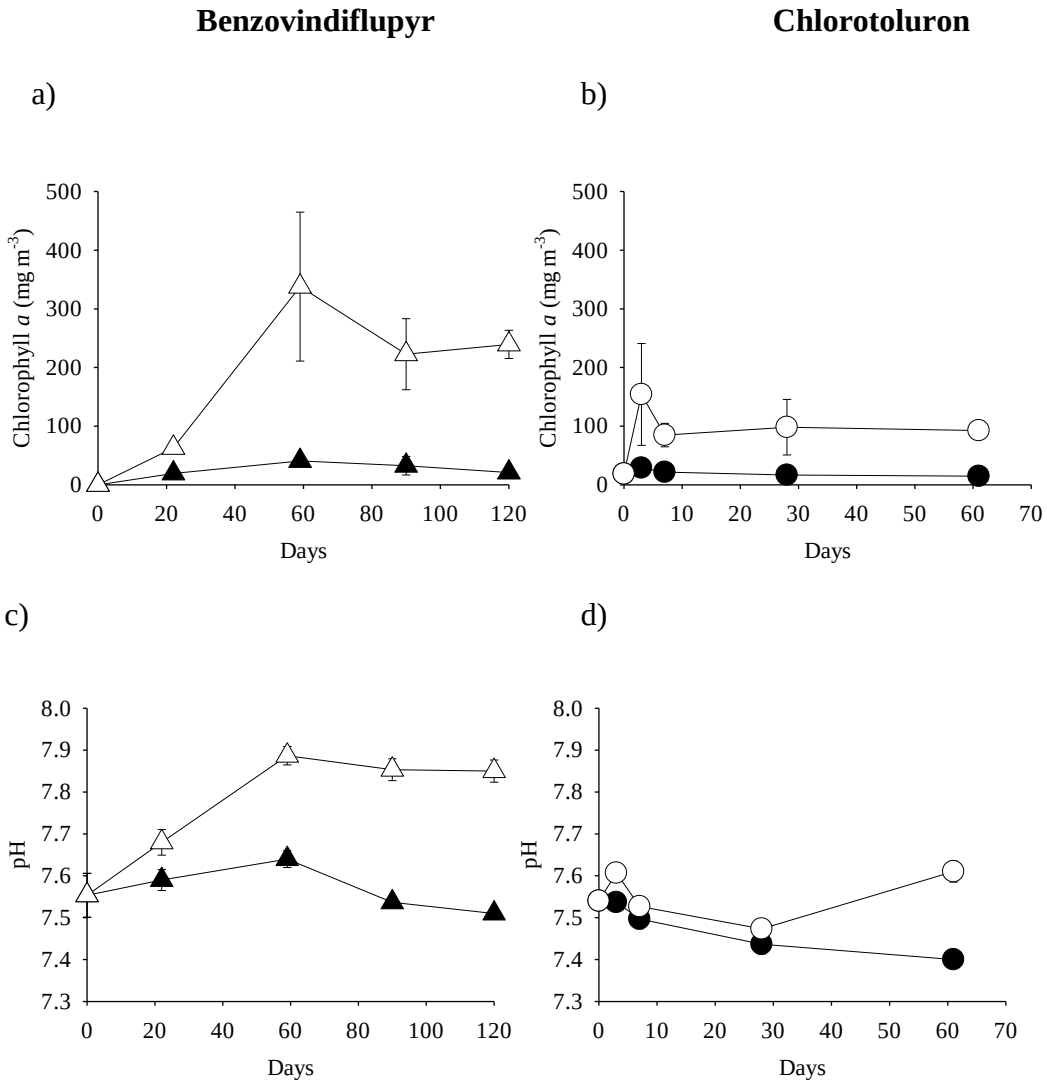
Pre-incubation time (days)	Extractable parent compound (%)*	
	Light	Dark
7	11.6±S.E. 1.62	26.5±S.E. 1.0
22	10.0±S.E. 1.02	23.1±S.E. 0.29

*** Samples with a pre-incubation time of 7 and 22 days were destructively sampled after 24 and 28 days, respectively.**

2.4.3. Chlorophyll *a*, pH and bacterial, fungal and archaeal copy numbers across a time course of benzovindiflupyr and chlorotoluron degradation under light and dark conditions

Light had a significant effect on chlorophyll *a* abundance ($p \leq 0.001$) (Figures 2.5a-2.5b), and both light ($p \leq 0.001$) and time ($p \leq 0.01$) significantly impacted soil pH during the degradation of benzovindiflupyr and chlorotoluron (Figures 2.5c-2.5d). Light treatment did not have a significant impact on bacterial copy number during benzovindiflupyr degradation, for example, at day 120, there were 1.26×10^8 ±S.E 1.26×10^7 and 2.90×10^8 ±S.E 2.15×10^7 copies under light and dark conditions, respectively (Figure 2.5e). Similarly, copies of bacterial 16S rRNA genes did not differ significantly between light (3.8×10^8 ±S.E 1.05×10^8) and dark (1.43×10^8 ±S.E 7.02×10^7) conditions 61 days after chlorotoluron application (Figure 2.5f). Archaeal 16S rRNA copy numbers were relatively similar under light and dark conditions during benzovindiflupyr degradation, for example, at 120 DAT there were 2.0×10^6 ±S.E 3.60×10^5 and 3.94×10^6 ±S.E 8.02×10^5 under light and dark conditions (Figure 2.5g). Similarly, there were no apparent differences in archaeal

copy number during chlorotoluron degradation, for example, at 61 DAT there were 5.72 x 10^6 ±S.E 1.01 x 10^6 and 6.39 x 10^6 ±S.E 7.21 x 10^5 under light and dark conditions, respectively (Figure 2.5h). Again, light had no considerable impact on fungal abundance, with 8.71 x 10^6 ±S.E 3.48 x 10^5 and 1.58 x 10^7 ±S.E and 3.00 x 10^6 copies of the ITS region under light and dark conditions, respectively during benzovindiflupyr degradation, (Figure 2.5i) and 3.87 x 10^7 ±S.E 1.14 x 10^7 and 2.53 x 10^7 ±S.E 4.33 x 10^6 copies of the ITS region under light and dark (Figure 2.5j).



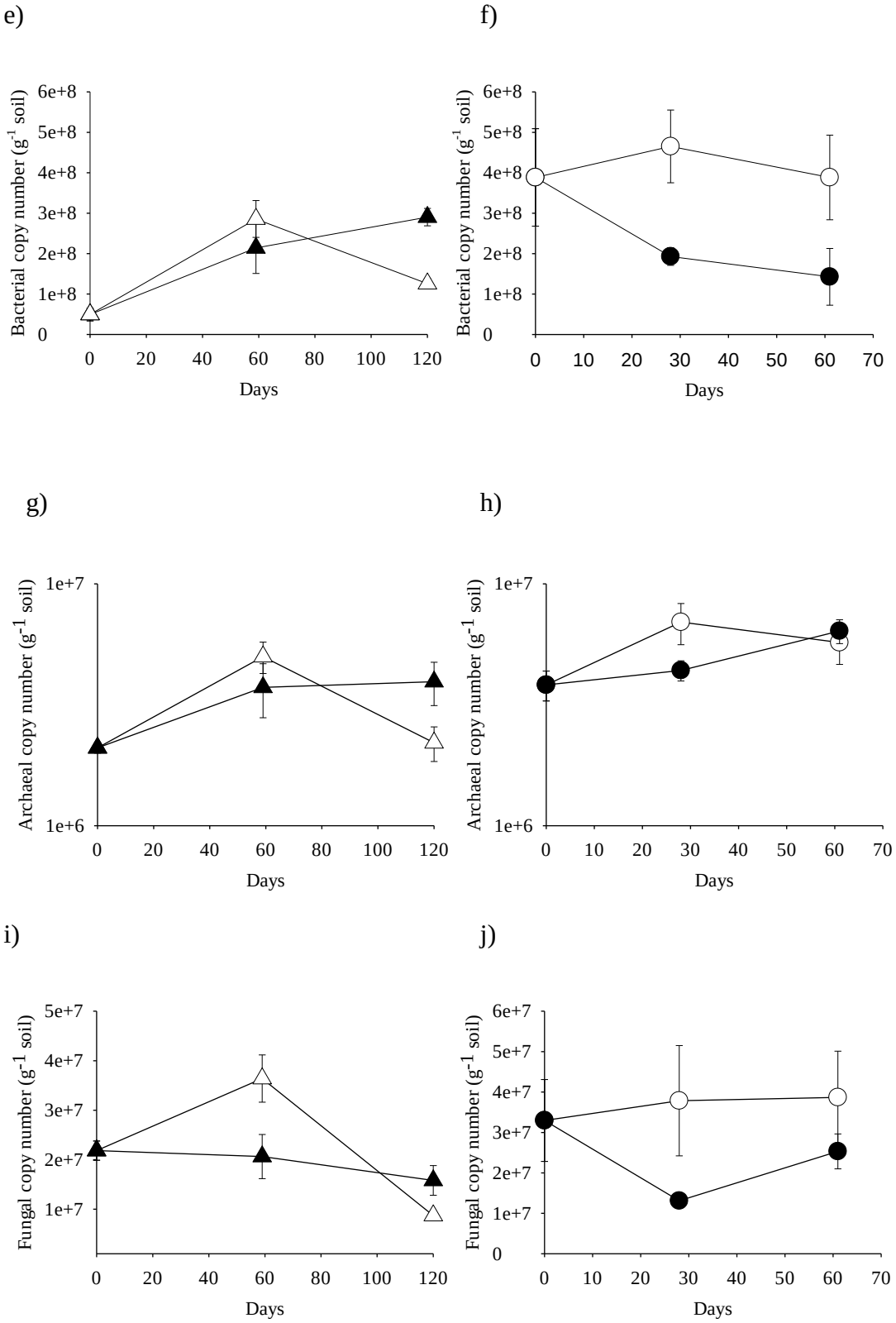


Figure 2.5: Soil chemical and biological properties during a timecourse of benzovindiflupyr (▲) and chlorotoluron (●) degradation under light (open symbols) and dark (closed symbols) conditions; (a – b) Chlorophyll *a*; (c – d) pH; (e – f) Bacterial copy number (16S rRNA); (g – h) Archaeal copy number (16S rRNA), and; (i – j) Fungal copy number (ITS region). Error bars are ±1 S.E

2.4.4. Experiment 2: The compound screen

2.4.4.1. Growth of phototrophs

Chlorophyll *a* analysis showed significant development of phototrophs under light compared to dark conditions for lufenuron, prometryn, propiconazole fludioxonil, and imidacloprid ($p \leq 0.05$) (Table 2.5). In contrast, there was no significant difference in chlorophyll *a* abundance under light and dark conditions during the degradation of cinosulfuron (Table 2.5).

Table 2.5: Chlorophyll *a* (mg m^{-3}) values for Gartenacker soil applied with a crop protection product and incubated under light and dark conditions

Compound	Sampling time (DAT)	Light	Dark	Probability
Lufenuron	25	75.5±0.2	6.9±0.8	$p \leq 0.05$
Prometryn	32	165.4±0.3	21.8±0.2	$p \leq 0.05$
Cinosulfuron	34	24.2±1.0	14.5±1.0	NS
Propiconazole	43	64.2±0.4	8.6±1.2	$p \leq 0.05$
Fludioxonil	69	133.7±0.6	3.2 ±1.0	$p \leq 0.05$
Imidacloprid	102	268.3±0.1	7.8±0.9	$p \leq 0.01$

2.4.4.2. Degradation of a variety of crop protection products under light and dark conditions

The compound screen showed the effect of light on the transformation of CPPs to be compound specific (Figure 2.6). Mass balances were between 91% and 96% for all compounds and treatments. Extractable parent compound was significantly lower under light relative to dark conditions for prometryn (6.4%±S.E 0.4% and 10.3±S.E 0.5%; $p \leq 0.01$), fludioxonil (41.6%±S.E 1.3% and 65.7%±S.E 0.4%; $p \leq 0.01$), and imidacloprid (29.8%±S.E 1.1% and 48.1%±S.E 1.5%; $p \leq 0.001$). Cinosulfuron behaved atypically, with an increase in compound persistence under light conditions, with 42.2%±S.E 2.3% and 28.4%±S.E 0.7% under light and dark conditions, respectively ($p \leq 0.05$). There were no significant differences in the rate of transformation between treatments for propiconazole and lufenuron (Figure 2.6).

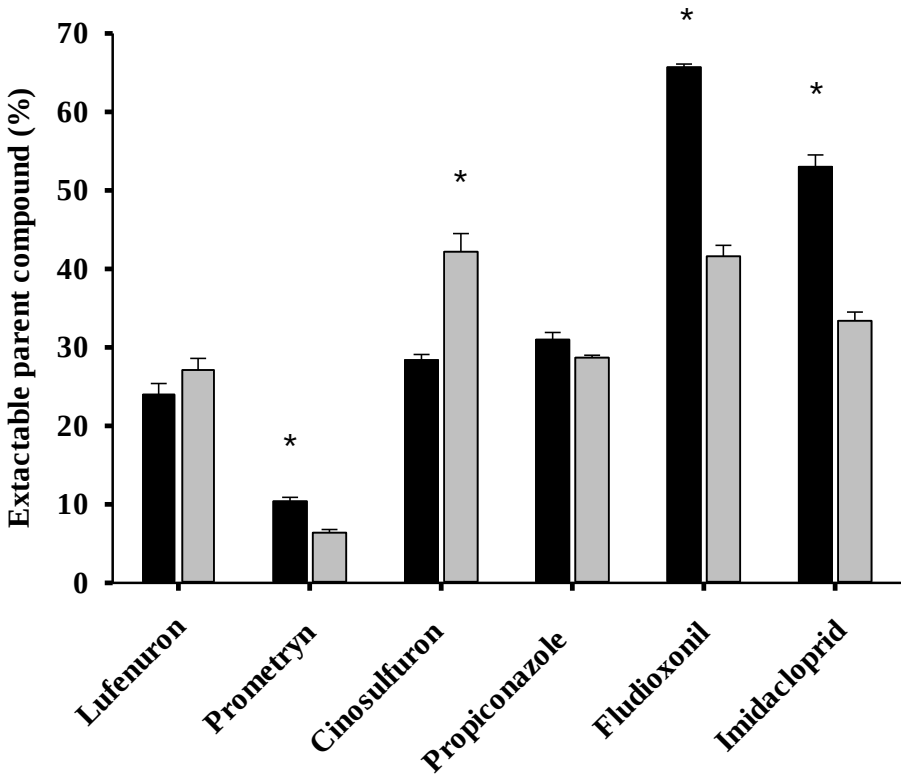


Figure 2.6: Extractable parent compound for a variety of crop protection products under dark (black) and light (grey) conditions. Sampling times were; lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole (43 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Significant differences are indicated by an asterisk (*) ($p \leq 0.01$). Error bars are ±1 S.E.

NER (Figure 2.7) and mineralisation (Figure 2.8) values from the compound screen were typically consistent with those from the $DegT_{50}$ degradation study (Figures 2.3 and 2.4). NERs were significantly higher under light for prometryn ($p \leq 0.05$), fludioxonil ($p \leq 0.01$), propiconazole ($p \leq 0.001$), lufenuron ($p \leq 0.05$), and imidacloprid ($p \leq 0.01$) (Figure 2.7). However, no significant difference was observed for cinosulfuron (Figure 2.7). Particularly large differences in NER were observed for imidacloprid and fludioxonil, with an approximate 3.5-fold increase under light (Figure 2.7). Mineralisation was significantly higher under dark conditions for

prometryn (p≤0.01), cinosulfuron (p≤0.01), lufenuron (p≤0.001) and propiconazole (p≤0.05) (Figure 2.8). In contrast, a significantly greater proportion of fludioxonil mineralised under light compared to dark conditions (p≤0.01) (Figure 2.8). Imidacloprid mineralisation did not differ significantly between light and dark conditions (Figure 2.8).

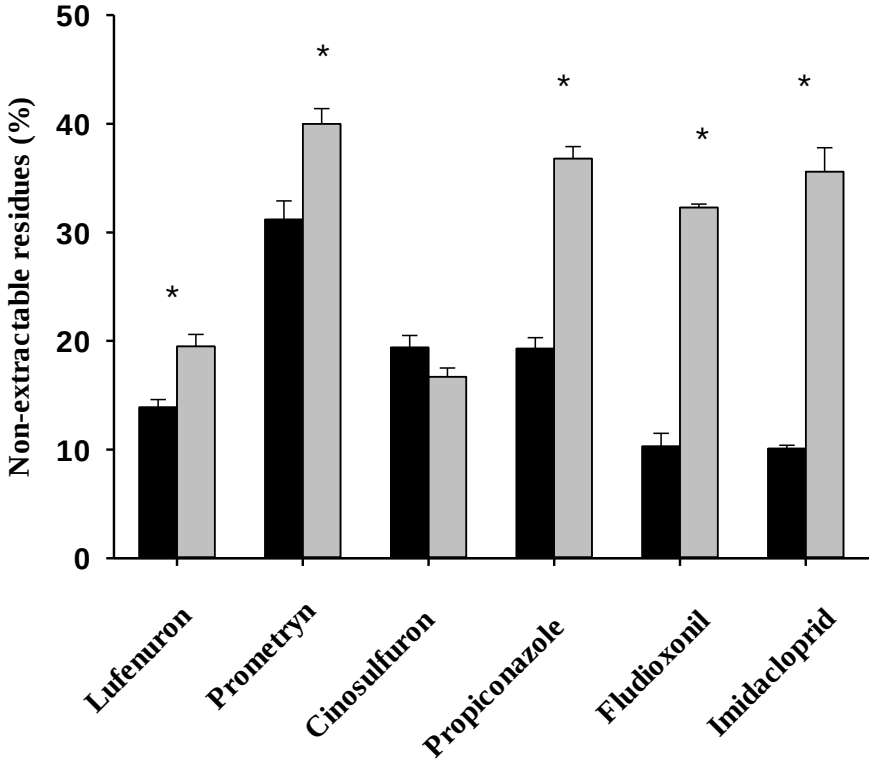


Figure 2.7: Non-extractable residues for a variety of crop protection products under dark (black) and light (grey) conditions. Sampling times were; lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole (43 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Significant differences are indicated by an asterisk (*) (p≤0.01). Error bars are ±1 S.E.

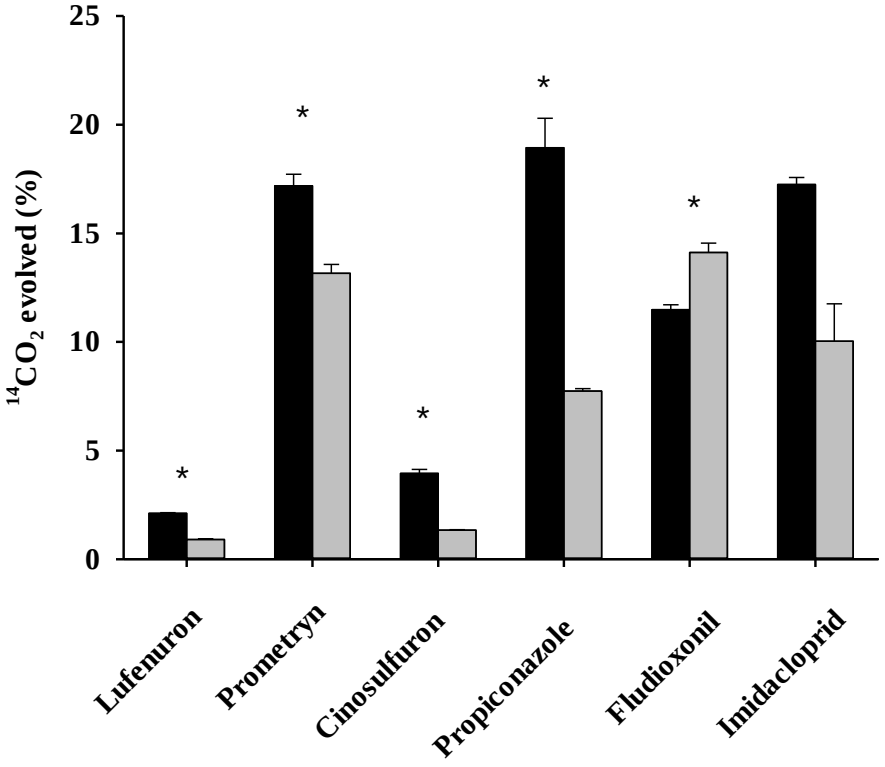


Figure 2.8: Mineralisation values for a variety of crop protection products under dark (black) and light (grey) conditions. Sampling times were; lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole (43 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Significant differences are indicated by an asterisk (*) ($p \leq 0.01$). Error bars are ±1 S.E.

2.4.5. Correlations between physico-chemical properties and the difference in degradation rates between light and dark conditions

There were no significant correlations between the difference in degradation between light and dark conditions and either chlorophyll *a* content, $DegT_{50}$ values, K_{oc} or water solubility (Figure 2.9).

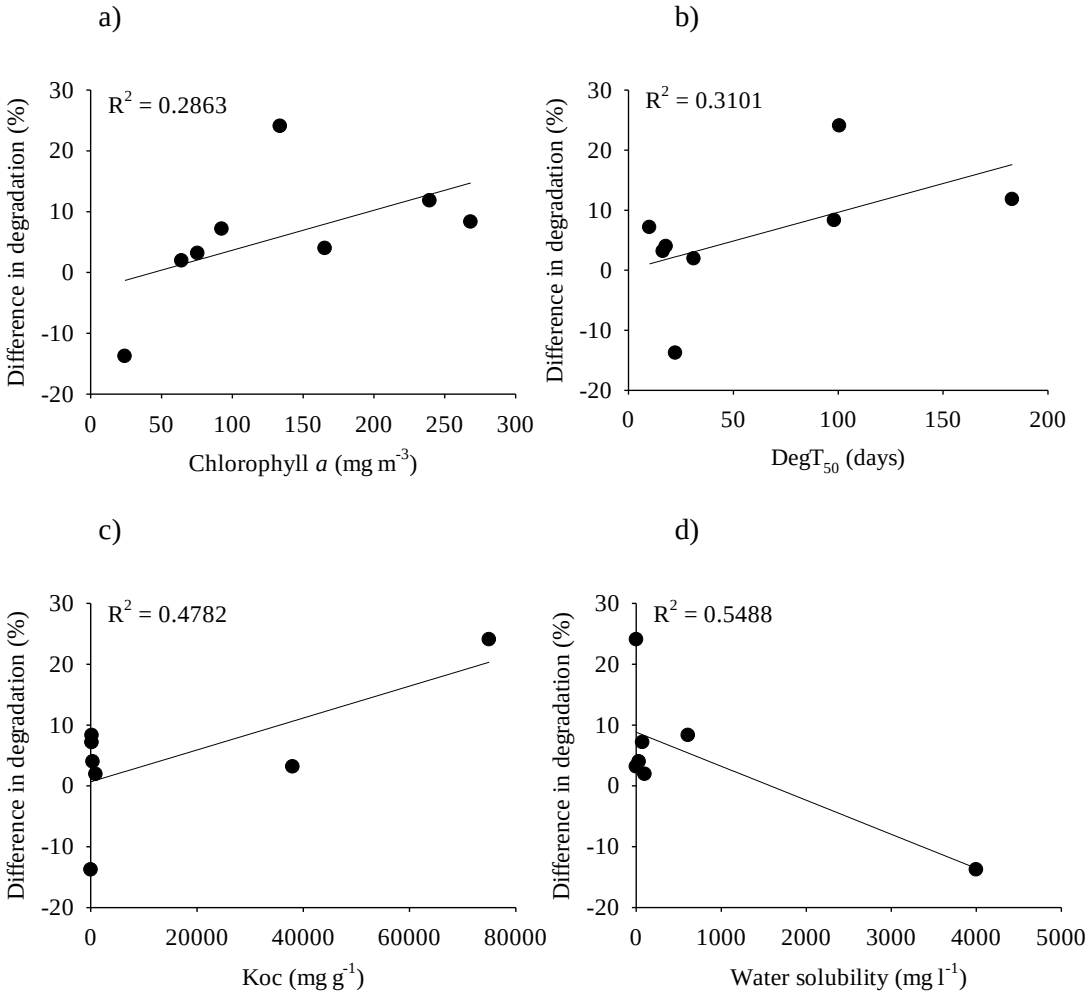


Figure 2.9: Correlations between the difference in extractable parent compound of benzovindiflupyr, chlorotoluron, prometryn, cinosulfuron, imidacloprid, fludioxonil, lufenuron, and propiconazole when applied to soil and incubated under light and dark conditions and several crop protection product properties: (a) Chlorophyll *a*; (b) $DegT_{50}$ (days); (c) Koc (mg g^{-1}), and; (d) Water solubility (mg l^{-1}).

2.5. DISCUSSION

The inclusion of non-UV light to OECD guideline 307 had a compound specific effect on the rate of CPP degradation. Light was found to significantly increase the rate of transformation for five out of the eight CPPs tested, and reduced the rate of degradation of one CPP. Light treatment also resulted in an increase in the rate of NER formation for seven CPPs. A variety of mechanisms could be responsible for the 'light effect', including the proliferation of phototroph communities, and pH shifts.

The transformation rates of chlorotoluron, benzovindiflupyr, prometryn, imidacloprid, and fludioxonil significantly increased under light relative to dark conditions (Figures 2.3, 2.4 and 2.6). For example, the inclusion of light resulted in an approximate halving of the $DegT_{50/90}$ of benzovindiflupyr (Figure 2.3), a halving of the $DegT_{90}$ of chlorotoluron (Figure 2.4), and significantly lower extractable parent compound for prometryn (4%), imidacloprid (18%), and fludioxonil (24%) under light conditions compared to in the dark (Figure 2.6). A similar impact of light has also been observed in water-sediment systems containing algae or macrophytes, for example, Thomas and Hand (2011) showed considerable reductions in the persistence of chlorotoluron, fludioxonil, prometryn, pinoxeden metabolite (diketone), and propiconazole under light compared to dark controls. The study showed a 4-fold, 5-fold, 7-fold and 20-fold enhancement of transformation for chlorotoluron, prometryn, propiconazole, and fludioxonil.

In this Chapter, light clearly influenced the rate of CPP transformation for six out of eight CPPs tested but the mechanisms responsible may be compound specific. Firstly, phototrophs proliferated under light conditions for all CPPs tested, with the exception of cinosulfuron (Figures 2.5a-2.5b & Table 2.5). A direct effect of

phototrophs on CPP degradation is supported by several studies indicating the potential for algae and cyanobacteria to degrade CPPs in axenic cultures (Sethunathan *et al*. 2004; Cai *et al*. 2007; Caćeres *et al*. 2008; Mostafa and Helling, 2011; Thomas and Hand, 2011 & 2012). Phototrophs could also indirectly affect CPP degradation through a shift in soil pH. Indeed, a significant increase in pH under light compared to dark conditions during the degradation of benzovindiflupyr and chlorotoluron (Figures 2.5c-2.5d) would have been caused by the uptake of CO_2, which is acidic, by phototrophs during photosynthesis (Thomas and Hand, 2010). Both light (Jeffery *et al*. 2007 and 2009) and pH (Griffiths *et al*. 2011) have previously been shown to influence microbial community composition in soil, which could in turn impact the rate of CPP transformation in the current system. The fact that no correlation was found between the difference in CPP transformation between light and dark treatments and chlorophyll *a* abundance (Figure 2.9a), suggests that potential shifts in heterotroph communities caused by the impact of phototrophs on soil pH could also be important (Figures 2.5c-2.5d).

An additional impact of light on soil communities may be driven by an input of C by photosynthesis, which could potentially increase viable biomass or microbial activity, and consequently the rate of CPP transformation. If true, this mechanism would be analogous to 'the rhizosphere effect', which has been documented for several organic compounds (Shaw and Burns, 2003). For example, Marchland *et al*. (2002) observed that under 4-week old maize seedlings, 61% of atrazine had mineralized compared to only 48% in non-planted soil (Marchland *et al*. 2002). However, our results do not support this theory as there were no significant differences in bacterial, fungal or archaeal copy numbers between light and dark

treatments during a time course of benzovindiflupyr and chlorotoluron degradation (Figures 2.5e-2.5j).

A lag phase is often observed in the degradation of growth-linked catabolised compounds to allow microorganisms' time to induce specific degradative enzyme systems to catabolise the compound (Alexander, 1981). In the current system, there may also be a lag phase to allow the development of phototrophs. At the end of the lag phase, phototrophs are able to directly or indirectly impact the rate of CPP transformation. Indeed, this is supported by the timecourse of chlorotoluron degradation where the $DegT_{90}$ was halved under light compared to dark conditions, but no impact was observed on the $DegT_{50}$ (Figure 2.4).

The physico-chemical properties or mode of action of the CPPs may also play a role, however, the difference in CPP transformation between light and dark treatments was not correlated with the environmental persistence ($DegT_{50}$) or bioavailability (K_{oc} value or water solubility) of the CPPs tested (Figures 2.9b-2.9d). Further, the mode of action of the CPPs tested did not have as dramatic effect as expected. For example, prometryn and chlorotoluron both inhibit the electron transport system in photosytem II (Tomlin, 2006). Therefore, an application of prometryn or chlorotoluron to soil may inhibit the growth of photosynthetic organisms, thereby reducing any contribution of phototrophs to CPP transformation. However, both CPPs were found to have significantly faster rates of transformation under light conditions.

It is also important not to overlook potential abiotic differences between light and dark treatments such as indirect photolysis. Indirect photolysis refers to the absorbance of light by photosensitisers to form reactive intermediates such as hydroxyl radicals, which react with CPPs (Wallace *et al*. 2010). In soil, nitrate and

humic acids could be considered to be the most likely photosensitisers. UV is thought to be primarily responsible for the photosensitisation of humic acids (Zepp *et al*. 1985), and nitrate does not absorb light at <350 nm (Bravo *et al*. 2009), and therefore neither would photosensitise in a non-UV light system (see Figure 2.2 for the spectrum and intensity of light). Therefore, indirect photolysis is unlikely to be responsible for the 'light effect', however, unknown photosensitisers cannot be eliminated from contributing to the effect. Overall, the 'light effect' and the mechanisms responsible for the effect appear to be CPP specific. For example, phototrophs proliferated for all CPPs that degraded faster under light, however, accelerated degradation rates were not observed for all CPPs that showed phototroph proliferation. Further, the contribution of heterotrophs to the 'light effect' could also be CPP specific.

The inclusion of light in laboratory degradation test systems also has the potential to improve the accuracy of CPP persistence estimates. OECD guideline 307 states that degradation studies can only be conducted for a maximum of 120 d, as after this time, microbial biomass and activity are reduced considerably. The $DegT_{50}$ of highly persistent compounds are therefore estimated using regression analysis. However, the rate of CPP degradation may change from first-order kinetics following 120 d, and thus $DegT_{50}$ estimations by regression may not be accurate. The addition of C to the system via phototrophs may act to maintain microbial biomass and activity for a longer duration. Hence, studies could be carried out for longer than 120 d, potentially increasing the accuracy of $DegT_{50}$ values for persistent CPPs, or other xenobiotics. In addition, the presence of phototrophs in agricultural soil surfaces may affect CPP mobility, particularly for less polar CPPs such as lufenuron and fludioxonil (Table 2.2). These compounds may be more likely to sorb to soil

phototroph communities rather than dissolve in the aqueous phase of soil, potentially accelerating rates of degradation through an extended exposure to phototrophs, heterotrophs and photolysis at the soil surface.

Although CPP transformation was typically faster in the light, mineralisation was considerably greater under dark conditions for all CPPs tested with the exception of imidacloprid and fludioxonil (Figures 2.3, 2.4 and 2.7). Phototrophs may be utilising $^{14}CO_2$ for photosynthesis, reducing the overall amount collected in NaOH traps. Therefore, mineralisation results may not be reliable under light conditions. Fludioxonil behaved atypically by being the only compound tested which showed higher rates of mineralisation under light conditions. Transformation under light may be increased to such an extent as to offset any uptake of $^{14}CO_2$ by phototrophs. This is supported by fludioxonil showing the greatest difference in transformation between light and dark conditions compared to all other CPPs (Figures 2.3, 2.4 and 2.7). Moreover, fludioxonil has been shown to be degraded by eight green algae and four cyanobacteria in pure culture (Thomas and Hand, 2012).

NER formation was greater under light for all CPPs with the exception of cinosulfuron (Figure 2.7). The increase in NER could be due to a variety of reasons, for instance: (i) a change in the chemical properties of soil incubated under light increasing the sorption of the CPP to soil, (ii) a greater rate of CPP degradation and incorporation into biogenic residues by photosynthetic or non-photosynthetic organisms under light, or (iii) the assimilation of $^{14}CO_2$ into the biomass of phototrophs. The third explanation was tested by accounting for the potential uptake of the additional $^{14}CO_2$ found in dark systems by phototrophs in light systems. It was found that NER were still higher under light conditions and significantly higher for fludioxonil ($p \leq 0.01$), imidacloprid ($p \leq 0.01$), lufenuron ($p \leq 0.05$), and

benzovindiflupyr ($p \leq 0.001$). Therefore, it is likely that an increase in CPP degradation and incorporation into biomass and/or an increase in sorption are responsible for greater NERs under light conditions. NERs are not typically viewed as bioavailable, rather they are relatively stable, and slowly degraded over an extended period (Gevao *et al*. 2000). Therefore, an increase in NERs under light conditions could translate to greater CPP sorption under agricultural cropping systems following the development of soil surface phototrophs.

The inclusion of non-UV light to standard laboratory studies increased the rate of CPP transformation for five CPPs, and NER formation for seven out of the eight CPPs tested. This effect may have been driven by the presence of soil phototrophs, which could directly degrade CPPs or indirectly impact heterotroph community composition and/or alter soil chemical properties such as pH. Phototrophs represent the first point of contact for CPPs applied to the soil surface, and therefore the results have important implications for CPP legislation. It is important to further investigate the mechanisms responsible, test if the 'light effect' is soil-specific and if natural light also has a similar effect, and continue to alter additional variables, with the ultimate aim of bridging the gap between laboratory studies and field-applications, and improving the risk assessment of CPPs.

3. CHAPTER III: NON-UV LIGHT STRUCTURES PHOTOTROPH, BACTERIAL AND FUNGAL COMMUNITIES AT THE SOIL SURFACE

This Chapter has been published in *PLoS ONE*, 2013, **8** (7), e69048. The publication is displayed in Appendix II.

3.1. ABSTRACT

The upper few millimeters of soil harbour photosynthetic microbial communities that are structurally distinct from those of underlying bulk soil due to the presence of light. Previous studies in arid zones have demonstrated the functional importance of these communities in reducing soil erosion, and enhancing carbon and nitrogen fixation. Despite being widely distributed, comparative understanding of the biodiversity of the soil surface and underlying soil is lacking, particularly in temperate zones. This chapter investigated the establishment of soil surface communities on pasture soil in microcosms exposed to light or dark conditions, focusing on changes in phototroph, bacterial and fungal communities at the soil surface (0-3 mm) and bulk soil (3-12 mm) using ribosomal marker gene analyses. Microbial community structure changed with time and structurally similar phototrophic communities were found at the soil surface and in bulk soil in the light exposed microcosms suggesting that light can influence phototroph community structure even in the underlying bulk soil. 454 pyrosequencing showed a significant selection for diazotrophic cyanobacteria such as *Nostoc punctiforme* and *Anabaena* spp., in addition to the green alga *Scenedesmus obliquus*. The soil surface also

harboured distinct heterotrophic bacterial and fungal communities in the presence of light, in particular, the selection for the phylum Firmicutes. However, these light driven changes in bacterial community structure did not extend to the underlying soil suggesting a discrete zone of influence, analogous to the rhizosphere.

3.2. INTRODUCTION

Chapter II showed that non-UV light impacted degradation rates for a range of CPPs. This chapter investigated the effect of non-UV light on the biological and chemical properties of soil. Arid land studies have shown the soil surface to harbour distinct photosynthetic communities compared to the underlying bulk soil due to the development of phototrophs such as cyanobacteria, algae, mosses, and lichens (Garcia-Pichel *et al*. 2001; Redfield *et al*. 2002; Yeager *et al*. 2004; Langhans *et al*. 2009*a*; Abed *et al*. 2010; Zhang *et al*. 2011). Phototroph communities have also been shown to be widespread in temperate soils and under agricultural crops (Veluci *et al*. 2006; Knapen *et al*. 2007; Langhans *et al*. 2009*a*), however, little is known about their community structure and ecological significance.

In arid environments, a succession from cyanobacteria dominated to lichen- and moss- dominated crusts have been shown (Lange *et al*. 1992; Belnap, 1993; Li *et al*. 2002*b*). Further, a succession within cyanobacteria dominated crusts has also been noted from *Microcoleus vaginatus* to *Nostoc* spp./*Tolypothrix* spp. (Yeager *et al*. 2004). However, our understanding of the community structure remains very limited, not least because the majority of studies investigating phototroph diversity in soil have used culture dependent methods which are prone to bias (Hawkes and Flechtner, 2002; Li *et al*. 2002*b*; Langhans *et al*. 2009*a*; Li *et al*. 2010; Zhang *et al*.

2011), or molecular methods that target 16S rRNA of bacteria, which ignore the diversity of eukaryotic phototrophs (Garcia-Pichel *et al*. 2001; Redfield *et al*. 2002; Nagy *et al*. 2005; Abed *et al*. 2010; Zaady *et al*. 2010; Steven *et al*. 2012). Further, molecular microbial community analysis of bacterial diversity of BSCs has shown a dominance by cyanobacteria (Garcia-Pichel *et al*. 2001; Redfield *et al*. 2002; Abed *et al*. 2010; Zaady *et al*. 2010) e.g. Abed *et al*. (2010) found that 77-81% of clones from BSCs of Oman had close homology to cyanobacteria. Consequently, it remains unclear whether BSCs also harbour distinct heterotrophic bacterial populations in addition to phototrophic communities. Likewise, although fungi have been shown to provide key ecosystem services of BSCs such as structural cohesion provided by hyphal entanglement (Tisdall *et al*. 2012), little is known regarding the fungal community structure of BSCs (Bates and Garcia-Pichel, 2009; Bates *et al*. 2012).

This Chapter investigated shifts in phototroph, bacterial and fungal community structure between the soil surface and bulk soil of a pasture soil from a temperate climate throughout the development of phototroph communities at the soil surface. Universal phototroph primers designed to amplify any plastid-containing organisms, 454 pyrosequencing of PCR amplicons, and soil pH and nutrient measurements were taken with the aim of answering the following questions:

(i) How diverse are cyanobacteria and eukaryotic phototrophs at the soil surface?

(ii) Does light influence bacterial and fungal community structure and diversity at the soil surface?

(iii) Are there successional changes in phototroph, bacterial and fungal communities at the soil surface and underlying bulk soil?

(iv) Does the establishment of soil surface communities affect chemical parameters and microbial community structure of underlying bulk soil?

3.3. MATERIALS AND METHODS

3.3.1. Soil

Soil was sourced from the same area and treated using the same method as described in Section 2.3.1. Soil was sampled in October, 2010.

3.3.2. Test System and sampling the soil surface

To follow development of soil surface communities (Figure 3.1), a modified design was used from Jeffery *et al*. (2009) with dimensions of 20 cm x 15.5 cm x 1.8 cm. Trays were filled with 600 g of Gartenacker soil (35% water content) and soil was flattened to minimise soil surface heterogeneity. Trays were covered with either: (i) DS 226 light filter (0% transmission of wavelengths < 380 nm), or (ii) an opaque filter (Lee Filters, Andover, UK). In order to study the impact of light on microbial community development, soil was incubated in a controlled constant environment chamber on a 16h:8h light:dark cycle at 200 µmol s^{-1} m^{-1} (Philips Master fluorescent lights (>360 nm) TLD 36W/840) at a constant temperature of 20°C ± 2°C. This allowed the development of soil surface communities to be investigated under controlled conditions by removing confounding climatic variables. Trays were setup

in triplicate using a randomised design; moisture content was checked weekly by weight and maintained by watering from above using a pipette.

Triplicate trays were destructively sampled at 0, 20, 40, and 80 days. This sampling strategy aimed to follow the development of early-successional phototroph communities based on previous work, which showed development of phototrophs under cropping systems between 50 and 80 days following tillage (Knapen *et al*. 2007). At each sampling point, a stainless steel sheet was run under the soil surface at a measured depth of 3 mm to separate the soil surface (upper 3 mm) from the underlying bulk soil (4-12 mm). Surface and bulk soil samples were frozen at -20°C in polyethylene zip bags for 48 h before freeze-drying for 72 h. Freeze-dried soil was homogenised using a mortar and pestle and stored at -20°C.

3.3.3. Soil chemical properties

Soil pH was measured by adding 25 ml water to 10 g freeze-dried soil and shaking at 200 rpm for 15 mins prior to pH measurement using an Accumet AR50 electrode (VWR, Leicestershire, UK). Extractable Mg and K were measured by adding 50 ml 1M NH_4NO_3 solution to 10 g freeze-dried soil and shaking at 200 rpm for 30 mins. The solution was filtered prior to analysis using an ULTIMA 2 Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) (HORIBA Jobin Yvon, Middlesex, UK). Extractable nitrate (NO_3) was measured by adding 50 ml saturated $CaSO_4$ to 20 g freeze-dried soil and shaking at 200 rpm for 30 mins. The solution was filtered prior to colorimetric analysis using a FIAstar 5000 flow injection analyser (FOSS UK Ltd, Warrington, UK). Extractable P was measured by

adding 100 ml 0.5M $NaHCO_3$ solution (pH 8.5) to 5 g freeze-dried soil and shaking at 200 rpm for 30 mins. The solution was filtered prior to analysis by ICP-AES (Faithful, 2002).

3.3.4. Characterisation of soil surface communities

3.3.4.1. Chlorophyll a

Chlorophyll *a* was quantified using the method described in section 2.3.4.1

3.3.4.2. Most probable number of algae

At day 80, the number of algal cells at the soil surface under light and dark conditions was estimated using MPN. Fresh soil was homogenized and 1 g was transferred aseptically to 10 ml sterile Bold's Basal Media (BBM) (Bold, 1949). Bold's Basal Media was produced by adding the following stock solutions to 940 ml d. H_2O: 10 ml $NaNO_3$ (25 g l^{-1}), 10 ml $CaCl_2.2H_2O$ (2.5 g l^{-1}), 10 ml $MgSO_4.7H_2O$ (7.5 g l^{-1}), 10 ml K_2HPO_4 (7.5 g l^{-1}), 10 ml KH_2PO_4 (17.5 g l^{-1}), 10 ml NaCl (2.5 g l^{-1}), 1 ml EDTA (50 g l^{-1}) and KOH (31 g l^{-1}), 1 ml $FeSO_4.7H_2O$ (4.98 g l^{-1}) with 1 ml H_2SO_4, 1 ml H_3BO_3 (11.42 g l^{-1}), and 1 ml micronutrients solution ($ZnSO_4.7H_2O$ (8.82 g l^{-1}) $MnCl_2.4H_2O$ (1.44 g l^{-1}), MoO_3 (0.71 g l^{-1}), $CuSO_4.5H_2O$ (1.57 g l^{-1}), and $Co(NO_3)_2.6H_2O$ (0.49 g l^{-1})). Serial dilutions were performed at 2, 4, 5, 6, 8, 10, 15, 20, and 25-fold dilutions and 5 replicates of 1 ml aliquots were transferred to a

microtitre plate, covered with cling film and incubated for 21 days under a 16h:8h light:dark cycle at 200 µmol s^{-1}. Algal growth was recorded by a colour change of BBM from clear to green. Algal abundance was estimated using a MPN calculator according to Blodgett (2006).

3.3.4.3. Microbial community structure at the soil surface

3.3.4.3.1 DNA extraction, PCR amplification of ribosomal RNA markers and terminal restriction fragment length polymorphism (TRFLP) to assess phototroph, fungal and bacterial community structure

DNA was extracted from surface and bulk soil samples after 0, 20, 40 and 80 days incubation under light and dark conditions according to Section 2.3.4.3. The diversity of phototrophs was analysed by PCR targeting 23S rRNA genes of plastids using primers p23SrV_f1 and p23SrV_R1-HEX (Table 3.1), which produced a product approximately 410 bp in length (Sherwood and Presting, 2007). Samples were run on a GeneAmp 9700 thermocycler under the following conditions: initial denaturation at 94°C for 2 mins, followed by 35 cycles of 94°C for 20 secs, an annealing step at 55°C for 30 secs and extension at 72°C for 30 secs, before a final extension period at 72°C for 10 mins (Sherwood and Presting, 2007).

Bacterial 16S rRNA genes were amplified using primers 63f and 1087r-VIC (Table 3.1) giving a 1 kb product (Hauben *et al*. 1997; Marchesi *et al*. 1998), and for

analysis of fungi, PCR targeted the ITS region using primers ITS1f-PET and ITS4r (White *et al*. 1990; Gardes and Bruns, 1993) (Table 3.1). Samples were run under the following conditions: initial denaturation at 95°C for 3 mins, followed by 30 cycles of 95°C for 30 secs, 55°C for 1 min and 72°C for 1 min, before a final extension period at 72°C for 10 mins.

PCR was performed using 47 µl MegaMix (Microzone Ltd, Haywards Heath, UK), 1 µl of DNA (10 ng μl^{-1}) and 1 µl of either 5 µM (bacteria/phototrophs) or 25 µM (fungi) forward and reverse primers (Table 3.1). Samples were run on a GeneAmp 9700 thermocycler (Applied Biosystems, Warrington, UK).

PCR products were purified using a QIAquick PCR purification kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Restriction digests were performed at 37°C for 4 hrs followed by 95°C for 15 mins. Digests of 23S rRNA gene fragments of phototrophs used 500 ng PCR product, 2 µl 10X buffer, 0.5 µl 5U *Dde*I (New England Biolabs, Hitchin, UK), made up to 20 µl with UltraPure DNase/RNase-free distilled water (Invitrogen, Paisley, UK). *Dde*I was used based on clone libraries using the Restriction Enzyme Mapping Application (REMA, http://bioperl.macaulay.ac.uk). Digests of 16S rRNA gene fragments of bacteria and ITS fragments of fungi used 500 ng and 400 ng of PCR product, respectively, and 2 µl 10X buffer, 0.25 µl 5U of either *Msp*I or *Hha*I (New England Biolabs, Hitchin, UK), made up to 20 µl with sterilised distilled water. *Msp*I and *Hha*I were used as they have previously been shown to provide good differentiation between bacterial and fungal taxa (Hilton *et al*. 2013). Restriction digests using *Hha*I also contained 0.2 µl (10 mg ml^{-1}) bovine serum albumin (New England Biolabs, Hitchin, UK).

All samples were cleaned using sephadex spin columns and LIZ1200 standard was added prior to electrophoresis using an ABI PRISM 3130xl genetic analyser (Applied Biosystems, Warrington, UK). GeneMarker (Softgenetics, USA) was used to quantify peak height of terminal restriction fragments (TRFs) and values were transformed to relative abundance to standardise data. A constant percentage threshold was selected according to Sait *et al*. (2003) to minimise a correlation between total peak height and number of TRFs.

Table 3.1: Primer pairs used to investigate bacterial, fungal and phototroph community structure in Gartenacker soil incubated under light and dark conditions

Taxa	Gene	Primer and label	Fragment size (bp)	Restriction enzyme
Phototrophs	23S rRNA plastid	**p23SrV_R1-HEX** (5′TCAGCCTGTTATCCC TAGAG 3′) **p23SrV_f1** (5′GGACAGAAAGACCCT ATGAA 3’)	410	*Dde*I
Bacteria	16S rRNA	**63f** (5′AGGCCTAACACATGCAA GTC3’) **1087r-VIC** (5′CTCGTTGCGGGACTTACC CC3′)	1000	*Hha*I, *Msp*I
Fungi	ITS	**ITS1f-PET** (5′CTTGGTCATTTAGAGGAAG TAA3’) **ITS4r** (5′TCCTCCGCTTATTGATAT GC3’)	750	*Hha*I, *Msp*I

3.3.4.3.2. 454 amplicon pyrosequencing to determine diversity of phototrophs, fungi and bacteria

Phototroph, bacterial and fungal PCR amplicons from the soil surface incubated under light and dark conditions for 80 days were pyrosequenced by Research and Testing Laboratory (Lubbock, TX, USA) (RTL) using a Roche 454 FLX instrument and Titanium reagents. Bacterial Tag-encoded pyrosequencing was performed as described previously by Dowd *et al*. (2008). Fungal and phototroph pyrosequencing were performed according to the same protocol using the primer pairs shown in Table 3.1. Pyrosequencing gave a total of 67, 658 reads for bacteria, 22, 672 for fungi, and 77, 470 for phototrophs across six samples.

3.3.4.3.3. Processing of 454 sequence data

Sequences were processed using QIIME v. 1.4.0 (Caporaso *et al*. 2010*a*) by selecting sequences with an average quality score >25, containing no ambiguous bases or homopolymers longer than six base pairs, without any primer mismatches, and a sequence length between 250-430 bp (bacteria), 250-390 bp (fungi) and 330-410 bp (phototrophs). Sequences were also denoised using Denoiser (Reeder and Knight, 2010). Following denoising, methods of data processing differed for bacteria, fungi and phototrophs. Bacterial operational taxonomic units (OTUs) were picked at a 97% similarity threshold using UCLUST (Edgar, 2010) and

representative sequences were picked using the most abundant method before PyNAST aligning (Caporaso *et al*. 2010*b*) with the 16S rRNA greengenes database aligned at 97% (DeSantis *et al*. 2006). Chimeras were identified using ChimeraSlayer (Haas *et al*. 2011) and taxonomy was assigned using the RDP classifier (Wang *et al*. 2007). Processing of fungi and phototrophs used UCHIME (Edgar *et al*. 2011) for *de novo* chimera identification. Taxonomy was assigned using the RDP classifier for fungi (Wang *et al*. 2007) and BLAST (Altschul *et al*. 1990) for phototrophs. Figures showing sequence abundance data were created using MEGAN 4 (Huson *et al*. 2011). Full details of the number of sequences removed at each processing step are shown in Tables 3.2-3.4.

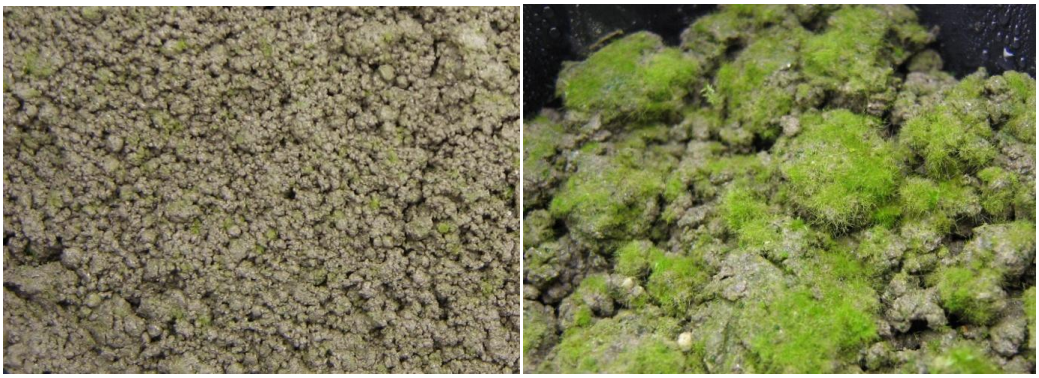
3.3.5. Statistical analysis

Parametric tests on non-transformed data were performed where possible. If assumptions were not met, data was transformed. One-way ANOVA was performed on chlorophyll *a*, pH and soil nutrient data, and t-tests were performed on MPN for algae and phototroph abundance data. All analyses were performed using Minitab version 15. TRF data was analysed using GeneMarker and statistically analysed using non-metric multidimensional analysis using PRIMER6 (Plymouth, UK), ANOSIM and SIMPER. Pyrosequencing data was rarefied at 3, 317 reads for phototrophs, 6, 322 for bacteria and 964 for fungi. QIIME v.1.4.0 was used for ANOVAs that compared taxonomy abundance data, t-tests that compared α diversity, Chao1 as a mark-release-recapture assessment of α diversity (Chao, 1984) and Observed Species as an assessment of the number of unique OTUs.

3.4. RESULTS

Phototroph development was visible at the soil surface under light conditions compared to in the dark (Figure 3.1).

a) b)



c)

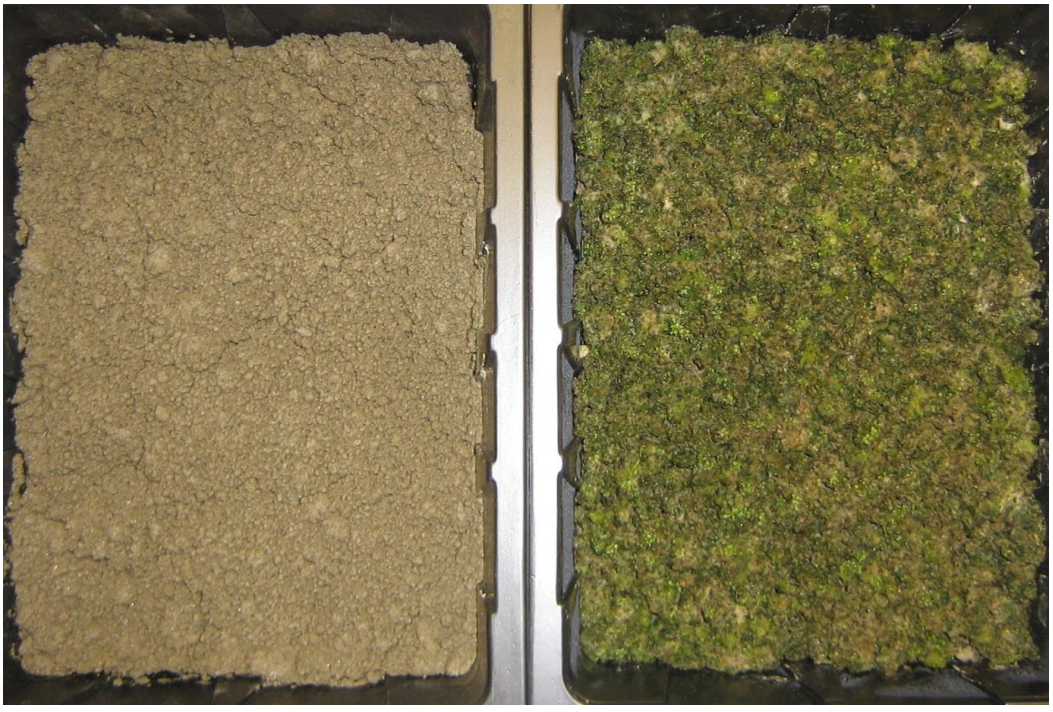


Figure 3.1: Development of phototrophs at the surface of a pasture soil; (a) 9 days incubation under light conditions; (b) 14 days incubation under light conditions, and; (c) Comparison of dark and light incubated soil after 40 days incubation.

3.4.1 Soil pH and nutrients

Light had a significant effect on extractable NO_3, pH and Mg ($p \leq 0.001$) at all sampling points (Figures 3.2a-3.2c). At day 80, pH ($p \leq 0.01$) was higher and extractable NO_3 ($p \leq 0.01$) and Mg ($p \leq 0.01$) were lower under light compared to dark incubated samples, however, there was no effect of depth (Figures 3.2a-3.2c). Depth influenced extractable K content with the soil surface having significantly higher extractable K than underlying bulk soil after 80 days incubation under light conditions ($p \leq 0.01$) (Figure 3.2d). Light did not influence extractable P, however, P was significantly higher at the soil surface compared to underlying bulk soil after 80 days incubation under light conditions ($p \leq 0.01$) (Figure 3.2e).

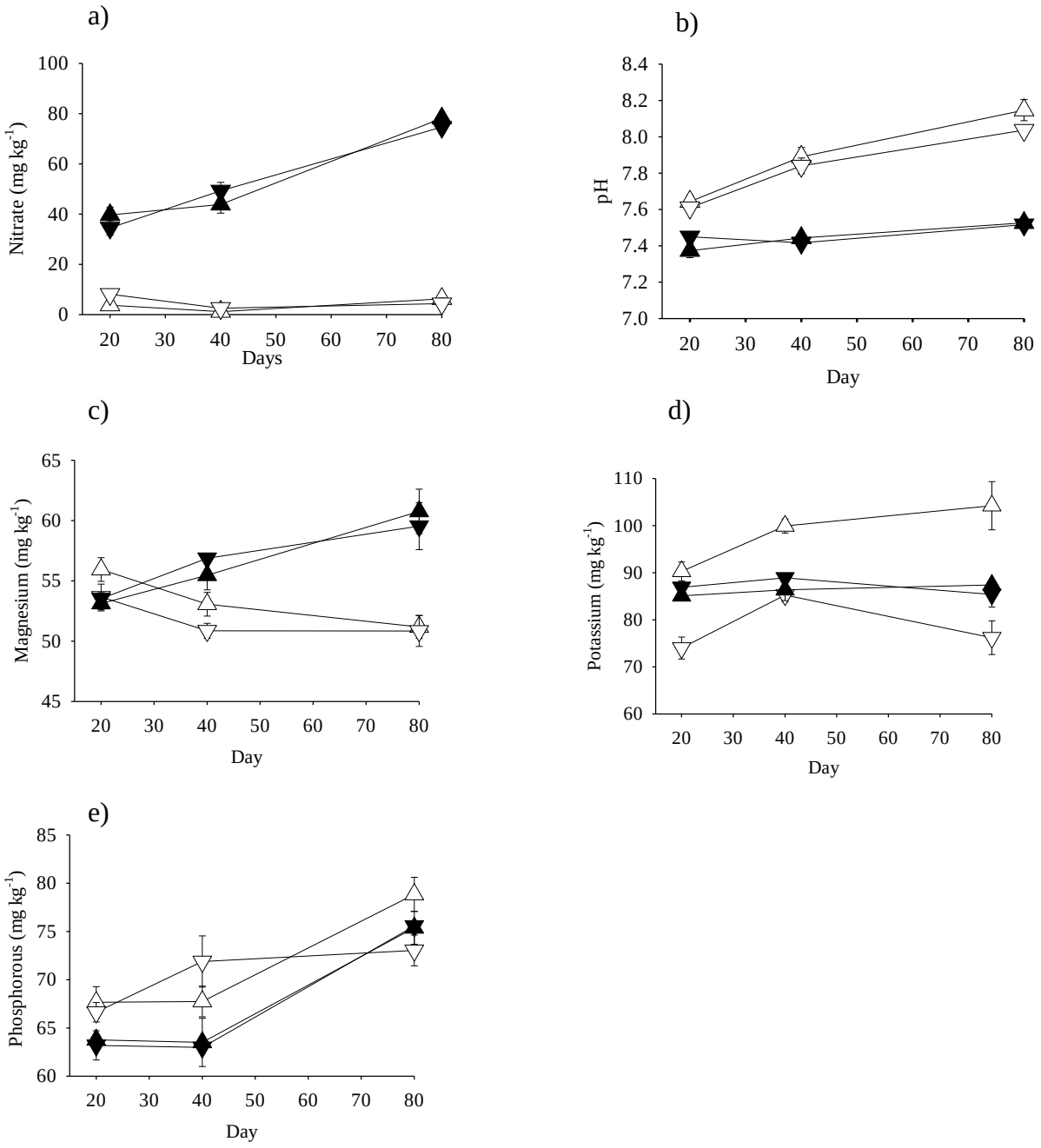


Figure 3.2: Timecourse of (a) extractable nitrate; (b) pH; (c) extractable magnesium; (d) extractable potassium, and; (e) extractable phosphorous at the surface (▲) and bulk (▼) of Gartenacker soil incubated under light (open symbols) and dark (closed symbols) conditions. Error bars are ±1S.E.

3.4.2. Biological characterisation of surface and bulk soil communities

3.4.2.1. Most probable number for algae and the growth of phototrophs

MPN assessment of algal abundance estimated a >60-fold higher algal population in the soil surface incubated under light compared to dark conditions for 80 days ($p \leq 0.01$). In addition, light ($p \leq 0.001$) and depth ($p \leq 0.001$) had a significant effect on chlorophyll *a* (Figure 3.3). Chlorophyll *a* was significantly higher at the soil surface under light at day 20, 40 and 80 ($p \leq 0.001$). Chlorophyll *a* was not detected in bulk soil under light or under dark conditions (Figure 3.3).

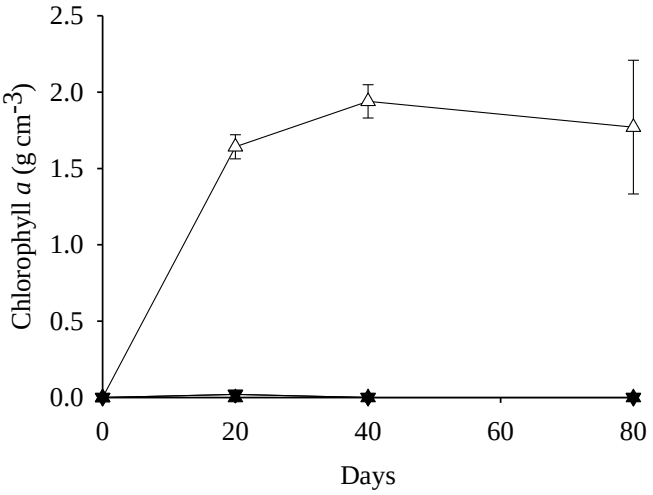


Figure 3.3: Timecourse of chlorophyll *a* development at the surface (▲) and bulk (▼) of Gartenacker soil incubated under light (open symbols) and dark (closed symbols) conditions. Error bars are ±1S.E.

3.4.2.2. TRFLP analysis of phototroph, bacterial and fungal community structure at the soil surface and underlying bulk soil under light and dark conditions

Phototroph community structure was significantly different at the soil surface ($p \leq 0.01$) and in bulk soil ($p \leq 0.05$) under light conditions compared to dark incubated soil (Figures 3.4a-3.4c). There were no significant differences in phototroph community structure between the soil surface and bulk soil incubated under light. NMDS analysis of TRFLP data showed two distinct clusters of samples: Grp I and Grp II (Figure 3.4a). Dark incubated samples were present in both Grp I and Grp II (Figures 3.4a-3.4b), however, all light incubated samples clustered within Grp II (Figure 3.4c), which suggests that phototroph community structure was more variable under dark compared to light conditions (Figures 3.4a-3.4c).

The soil surface incubated under light conditions had significantly different heterotrophic bacterial and fungal communities compared to bulk soil incubated under light and dark incubated samples ($p \leq 0.01$) (Figures 3.4d-3.4e). There was no significant difference in heterotrophic bacterial and fungal community structure between bulk soil incubated under light and dark conditions (Figures 3.4d and 3.4e). At day 80, the soil surface harboured distinct bacterial communities under light conditions (Figure 3.4d).

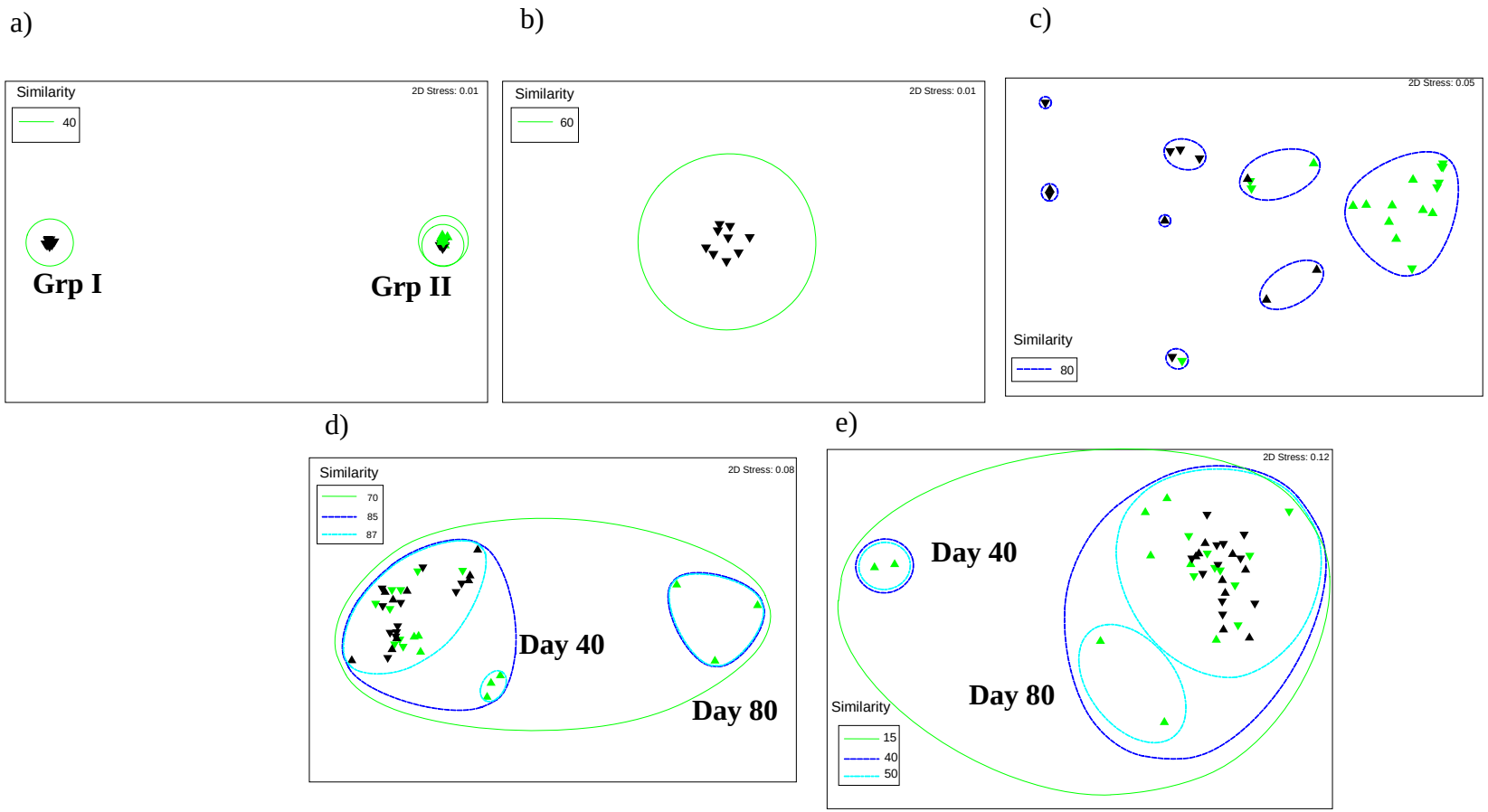


Figure 3.4: Ordination plots derived from non-metric multidimensional scaling analysis of Bray-Curtis distances of phototrophs, bacterial and fungal communities for surface (▲) and bulk (▼) of Gartenacker soil under light (green) and dark (black) conditions; (a) phototrophs all samples; (b) phototrophs close up of Grp I samples; (c) phototrophs close up of Grp II samples; (d) bacteria all samples, and; (e) fungi all samples.

3.4.3. Microbial community structure and taxonomic diversity at the soil surface and in bulk soil after 80 days of incubation

3.4.3.1. Processing pyrosequencing data

A total of 39, 267 (50.7%), 17, 892 (26.4%) and 8, 095 (35.7%) reads were discarded when processing phototroph (Table 3.2), bacterial (Table 3.3) and fungal (Table 3.4) 454-pyrosequencing data. A high proportion of phototroph sequence data (50.7%) was discarded, however, 10.4% of sequences were discarded as they shared close homology with heterotrophic bacteria (Table 3.2)

Table 3.2: Number of reads discarded during the processing of phototroph 454-pyrosequencing data

	Raw data	Split libraries	Chimera removal	phototrophs*
No. seqs	77 470	50 962	46 281	38 203
Minimum No. seqs	18 558	5 292	4 814	3 317
Maximum No. seqs	16 223	11 365	10 256	10 188
Mean No seqs	12 912	8 494	7 714	6 367

*** The phototroph processing step refers to the removal of all representative sequences that shared close homology with heterotrophic bacteria**

Table 3.3: Number of reads discarded during the processing of bacterial 454-pyrosequencing data

	Raw data	Split libraries	Chimera removal
No. seqs	67 658	54 767	49 766
Minimum No. seqs	8 536	6 853	6 322
Maximum No. seqs	13 552	11 059	9 834
Mean No seqs	11 276	9 127	8 294

Table 3.4: Number of reads discarded during the processing of fungal 454-pyrosequencing data

	Raw data	Split libraries	Chimera removal
No. seqs	22 672	15 141	14 577
Minimum No. seqs	1 574	1 012	964
Maximum No. Seqs	5 469	3 761	3 647
Mean No seqs	3 779	2 524	2 430

3.4.3.2. Phototroph community structure

Pyrosequencing revealed a total of 533 phototrophic OTUs across all samples with an average length of 351 bp, and an average of 71.7 reads assigned to each OTU, out of a total of 38, 203 processed reads. Chao1 and Observed species both show α diversity to be significantly higher at the soil surface incubated in the dark compared to light conditions ($p \leq 0.001$) (Figures 3.5a & 3.5b). Moreover, there were an estimated 246 unique phototroph OTUs under dark conditions compared to only 80 under light conditions (Figure 3.5b). Figs 3.5a and 3.5b both show that diversity plateaus under light as sampling depth increased, however, under dark conditions a plateau was not observed. NMDS analysis of phototroph community structure showed a greater grouping of samples under light compared to dark conditions, which suggests that phototroph community structure was less variable under light conditions (Figure 3.6).

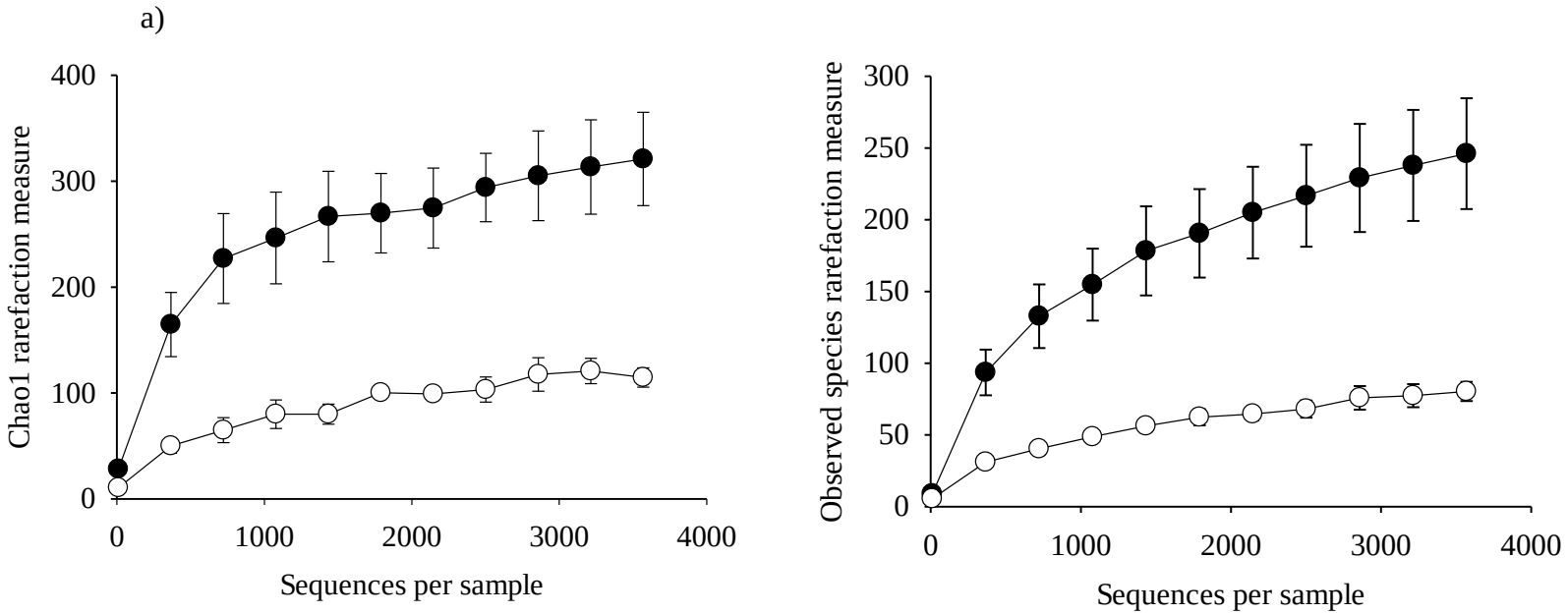


Figure 3.5: α diversity estimates Chao1 (a) and Observed Species and (b) and non-metric dimensional scaling of community structure similarity (23S rRNA genes of plastids) of pasture soil after 80 days incubation under light (open symbols) and dark (closed symbols) conditions. OTU clustering was performed at the 97% similarity threshold using UCLUST. Error bars are ±1 S.E.

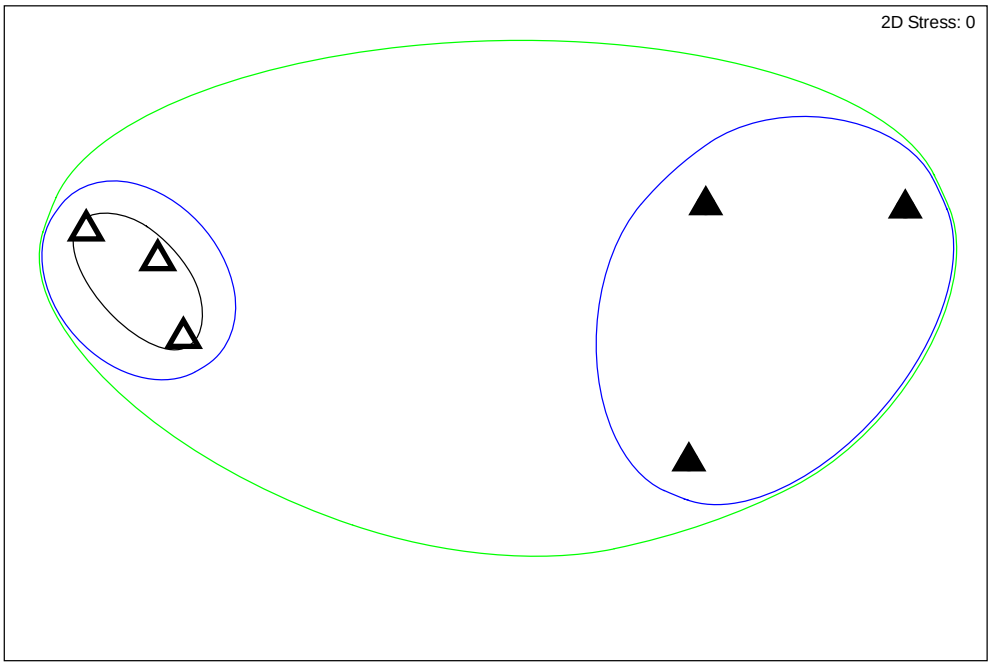


Figure 3.6: Ordination plots derived from non-metric multidimensional scaling analysis of Bray-Curtis distances of community structure similarity for phototrophs (23S rRNA genes of plastids) at the soil surface of Gartenacker soil after 80 days incubation under light (open symbols) or dark (closed symbols) conditions. OTU clustering was performed at the 97% similarity threshold using UCLUST. Non-metric multidimensional scaling shows clustering based on the similarity of microbial community structure between treatments: 20% (green), 25% (blue) and 80% (black).

A wide range of cyanobacteria and eukaryotic phototrophs were detected, including green, red and brown algae, cryptomonads, diatoms, mosses, and angiosperms (Figure 3.7). Relative composition analysis showed that cyanobacteria, rather than eukaryotic phototrophs, dominated under both treatments, with a relatively greater number of reads assigned to cyanobacteria under light compared to dark conditions ($p<0.01$) (Table 3.5). Further, the relative composition of cyanobacteria differed between light treatments e.g. 65.1%±SE 0.96% and 12.6%±S.E 2.17% of reads had close homology to *N. punctiforme* PCC 73102 under light and dark conditions, respectively ($p\leq0.001$), 11.6%±S.E 2.02% and 2.4%±S.E

0.11% of reads had close homology to *Anabaena variabilis* ATCC 29413 under light and dark, respectively ($p \leq 0.01$), and 2.5%±S.E 0.26% and 1.0%±S.E 0.29% of reads had close homology to *A. cylindrica* PCC 7122 under light and dark, respectively ($p \leq 0.05$) (Figure 3.7). There were no clearly dominant taxa under dark conditions, rather, seven taxa had a relative read abundance between 6% and 15%, which ranked as follows: *Cyanothece* sp. > *N. punctiforme* > *Thermosynechococcus elongatus* > *Cryptomonas paramecium* > *Ricinus communis* > *Gloeobacter violaceus* > *Scenedesmus obliquus* (Figure 3.7).

Relative composition analysis showed that a greater proportion of reads were assigned to eukaryotic phototrophs under dark compared to light conditions ($p < 0.001$), in particular, cryptomonads, red algae, brown algae, mosses and angiosperms ($p < 0.05$) (Table 3.5). In contrast, relative composition analysis showed 6.2%±S.E 1.25% and 14.8%±S.E 1.88% of reads were assigned to *Scenedesmus obliquus* under dark and light conditions, respectively ($p \leq 0.05$). Relative composition analysis also showed a greater number of reads assigned to the green algae *Chlorella variabilis* ($p \leq 0.05$) and *Chlorogonium elongatum* ($p \leq 0.05$), brown alga *Ectocarpus siliculosus* ($p \leq 0.001$), moss *Syntrichia ruralis* ($p \leq 0.05$), angiosperm *Jacobaea vulgaris* ($p \leq 0.001$), diatom *Phaeodactylum tricornutum* ($p \leq 0.05$), and cryptomonads *Rhodomonas salina* ($p \leq 0.001$) and *Cryptomonas curvata* ($p \leq 0.01$) under dark compared to light conditions.

Table 3.5: Relative read abundance of sequences with close homology to cyanobacteria and eukaryotic phototrophs from the soil surface of a pasture soil after incubation under light or dark conditions (± 1 S.E)

Taxonomy	Dark (%)	Light (%)
Cyanobacteria	63.8± 3.4	82.7± 2.0**
Eukaryotes	36.2± 3.4	17.3±2.0**
- Green algae	12.8± 1.8	15.8± 1.9
- Red algae	1.1± 0.04	0.01± 0.0*
- Brown algae	0.67± 0.1	0.03± 0.03**
- Diatoms	1.1± 0.2	0.93± 0.2
- Cryptomonads	10.6± 3.9	0.24± 0.2**
- Mosses	1.1± 0.2	0.1± 0.03**
- Angiosperms	9.5± 3.2	0.11± 0.1*

Significant differences between light and dark treatments are indicated by a * ($p<0.05$) and ** ($p<0.01$)

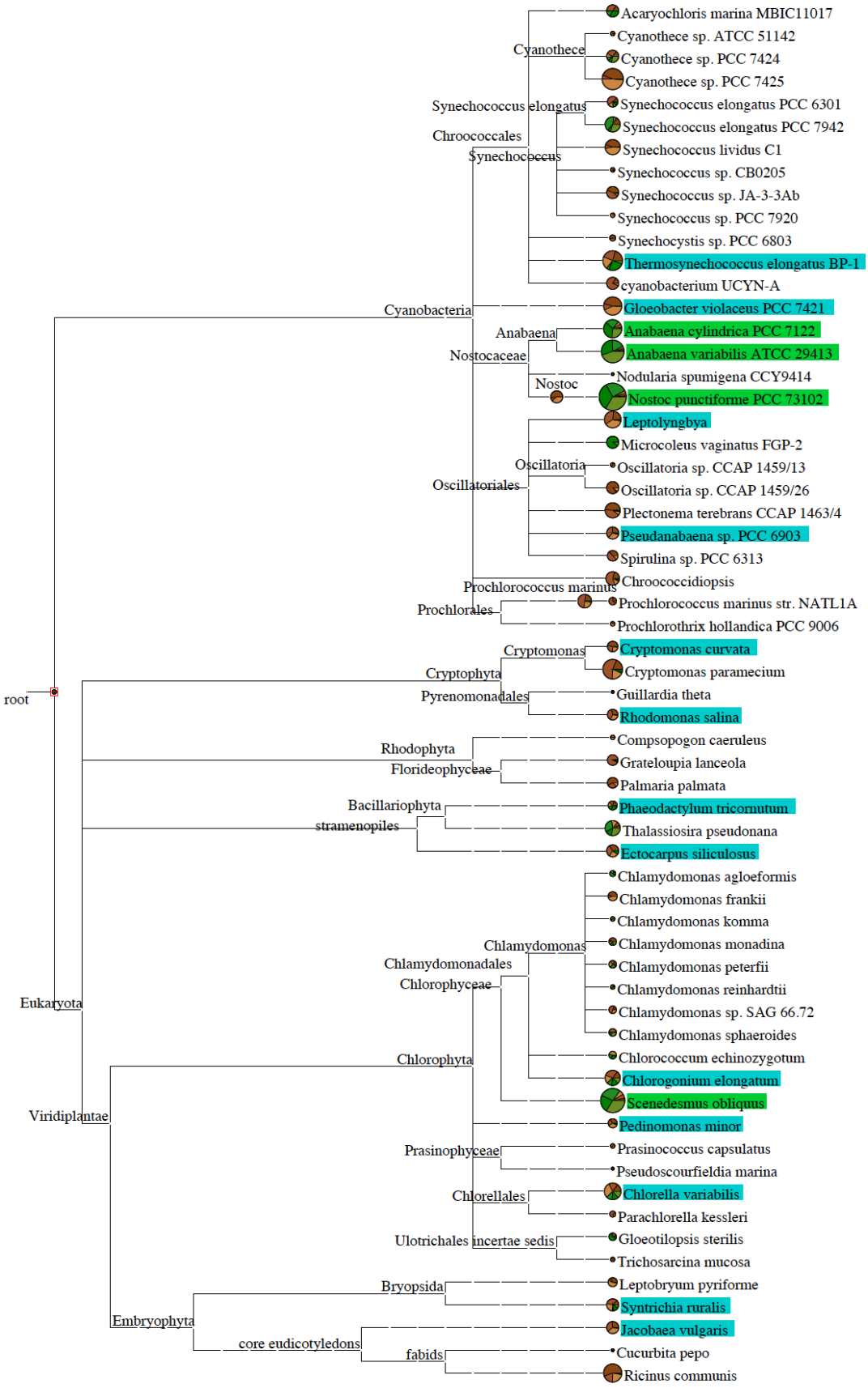

Acaryochloris marina MBIC11017
Cyanothece sp. ATCC 51142
Cyanothece
Cyanothece sp. PCC 7424
Cyanothece sp. PCC 7425
Synechococcus elongatus PCC 6301
Synechococcus elongatus
Synechococcus elongatus PCC 7942
Chroococcales
Synechococcus lividus C1
Synechococcus
Synechococcus sp. CB0205
Synechococcus sp. JA-3-3Ab
Synechococcus sp. PCC 7920
Synechocystis sp. PCC 6803
Thermosynechococcus elongatus BP-1
cyanobacterium UCYN-A
Cyanobacteria
Gloeobacter violaceus PCC 7421
Anabaena
Anabaena cylindrica PCC 7122
Anabaena variabilis ATCC 29413
Nostocaceae
Nodularia spumigena CCY9414
Nostoc
Nostoc punctiforme PCC 73102
Leptolyngbya
Microcoleus vaginatus FGP-2
Oscillatoria
Oscillatoria sp. CCAP 1459/13
Oscillatoriales
Oscillatoria sp. CCAP 1459/26
Plectonema terebrans CCAP 1463/4
Pseudanabaena sp. PCC 6903
Spirulina sp. PCC 6313
Chroococcidiopsis
Prochlorococcus marinus
Prochlorococcus marinus str. NATL1A
Prochlorales
Prochlorothrix hollandica PCC 9006
Cryptomonas
Cryptomonas curvata
Cryptophyta
Cryptomonas paramecium
Pyrenomonadales
Guillardia theta
root
Rhodomonas salina
Compsopogon caeruleus
Rhodophyta
Florideophyceae
Grateloupia lanceola
Palmaria palmata
Bacillariophyta
Phaeodactylum tricornutum
stramenopiles
Thalassiosira pseudonana
Ectocarpus siliculosus
Chlamydomonas agloeformis
Chlamydomonas frankii
Chlamydomonas komma
Chlamydomonas
Chlamydomonas monadina
Chlamydomonadales
Chlamydomonas peterfii
Chlorophyceae
Chlamydomonas reinhardtii
Chlamydomonas sp. SAG 66.72
Eukaryota
Chlamydomonas sphaeroides
Chlorococcum echinozygotum
Chlorophyta
Chlorogonium elongatum
Scenedesmus obliquus
Viridiplantae
Pedinomonas minor
Prasinococcus capsulatus
Prasinophyceae
Pseudoscourfieldia marina
Chlorellales
Chlorella variabilis
Parachlorella kessleri
Ulotrichales incertae sedis
Gloeotilopsis sterilis
Trichosarcina mucosa
Bryopsida
Leptobryum pyriforme
Syntrichia ruralis
Embryophyta
Jacobaea vulgaris
core eudicotyledons
Cucurbita pepo
fabids
Ricinus communis

Figure 3.7: Diversity and relative abundance of phototrophs at the soil surface after 80 days incubation under light or dark conditions. Data is presented as an OTU table created in QIIME at the 97% similarity threshold (uclust). The number of reads assigned to each taxa is shown at the end of each node. Pie charts show the proportion of reads assigned to each sample incubated under light (green) and dark (brown) conditions with shades representative of triplicate samples. Taxonomic assignments with only one read were removed. Significant differences in sequence abundance between light and dark samples are highlighted in green when abundance is significantly higher under light conditions and in blue when abundance is significantly higher under dark conditions ($p<0.05$).

3.4.3.3. Bacterial community structure

Analysis of pyrosequencing data for bacteria (49, 766 reads) clustered read data into 6, 517 bacterial OTUs with an average read length of 340 bp and an average of 7.6 reads assigned to each OTU. Chao1 and Observed Species were significantly higher at the soil surface under dark compared to light conditions ($p \leq 0.001$) (Figures 3.8a & 3.8b). In contrast to phototrophs, NMDS analysis of bacterial community structure showed a greater grouping of dark compared to light incubated samples, which suggests that bacterial community structure was more variable at the soil surface under light conditions (Figure 3.9).

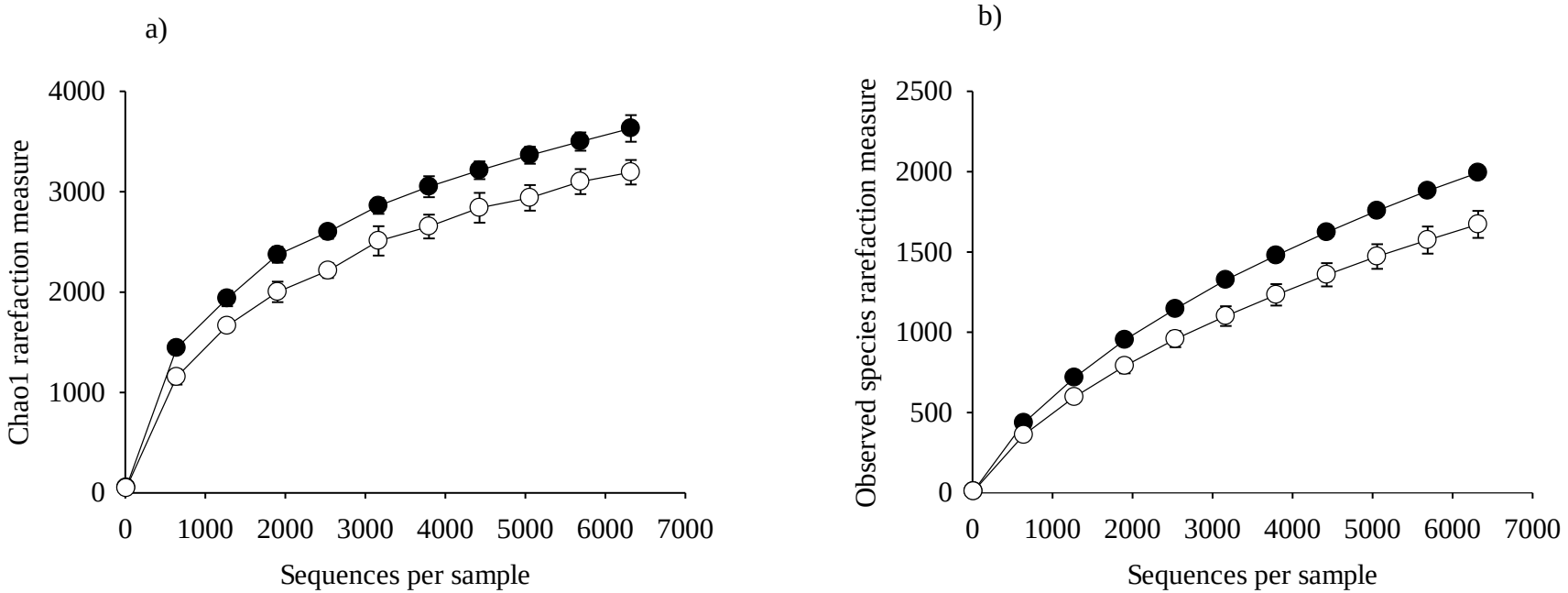


Figure 3.8: α diversity estimates Chao1 (a) and Observed Species (b) bacteria (16S rRNA) at the soil surface of Gartenacker soil after 80 days incubation under light (open symbols) and dark (closed symbols) conditions. OTU clustering was performed at the 97% similarity threshold using uclust. Error bars are ± 1 S.E.

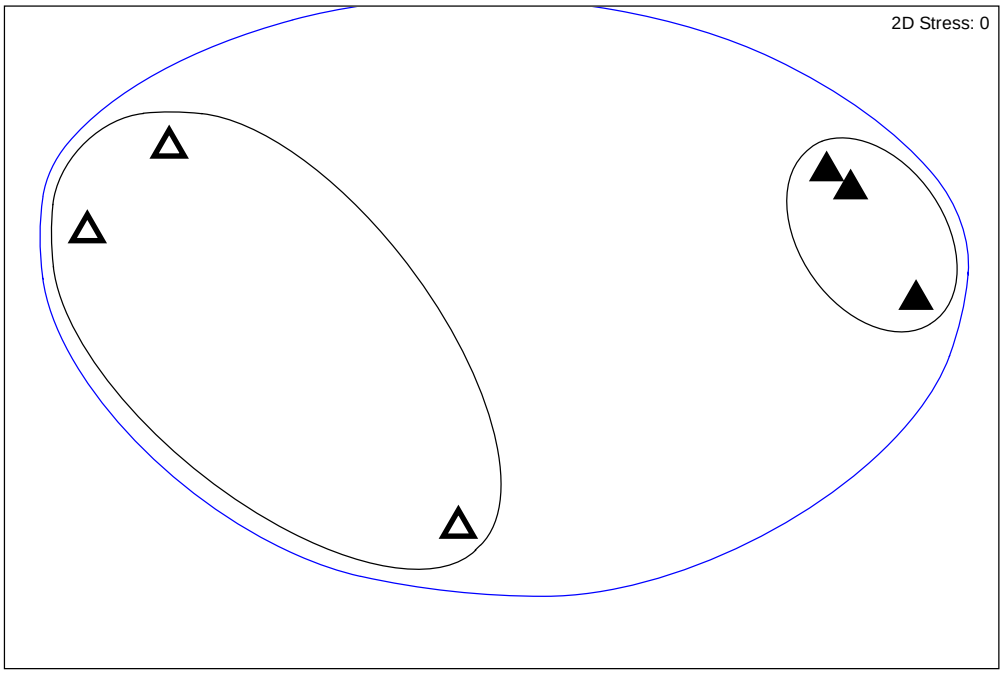
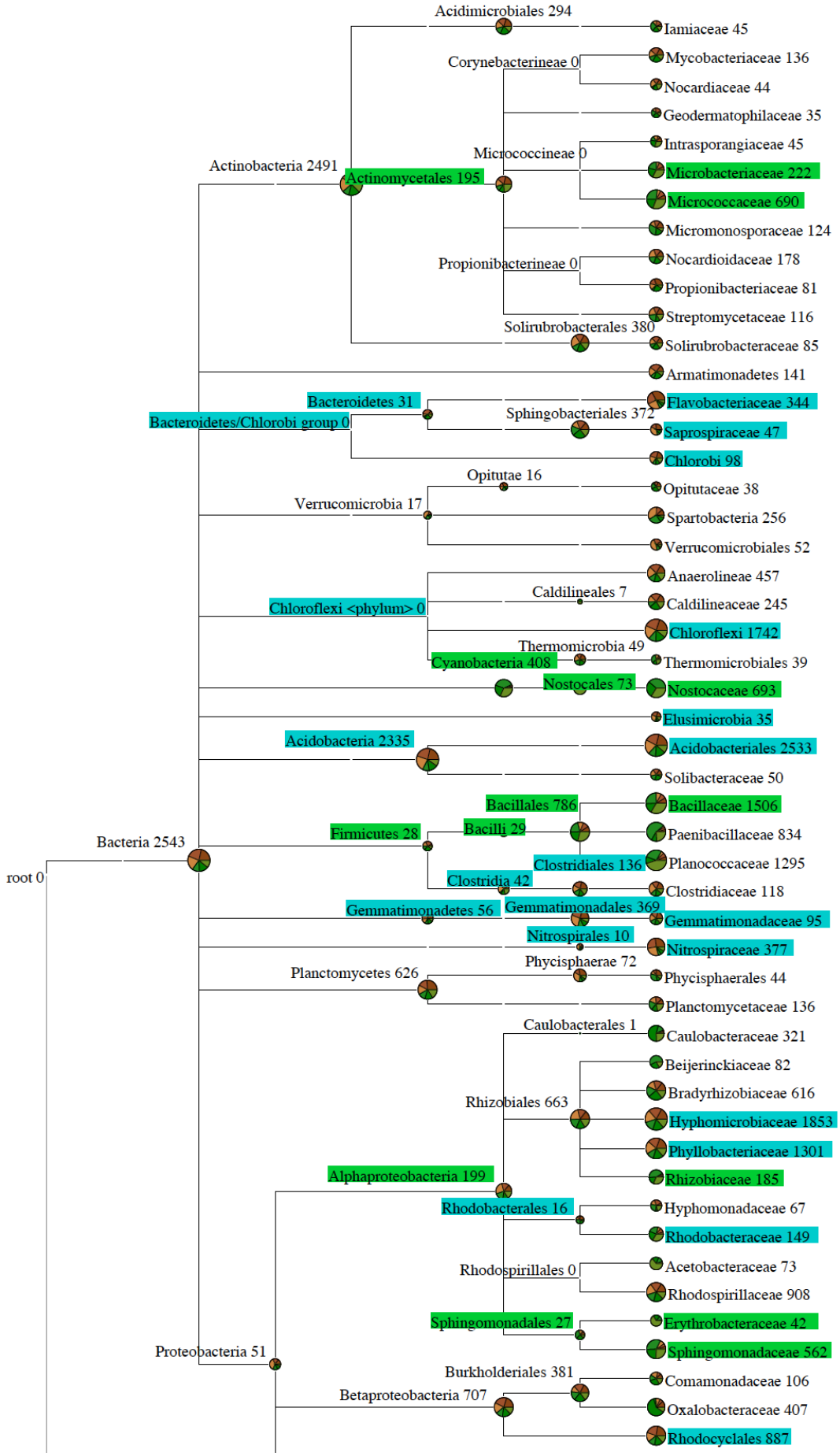


Figure 3.9 Ordination plots derived from non-metric multidimensional scaling analysis of Bray-Curtis distances of community structure similarity for bacteria (16S rRNA) at the soil surface of Garteacker soil after 80 days incubation under light (open symbols) or dark (closed symbols) conditions. OTU clustering was performed at the 97% similarity. Non-metric multidimensional scaling shows clustering based on the similarity of microbial community structure between treatments: 40% (blue) and 55% (black).

At the phylum level, relative composition analysis showed that Proteobacteria dominated the soil surface with 35.1%±S.E 0.21% and 36.4%±S.E 2.66% of reads assigned under dark and light conditions, respectively (Figure 3.10). The relative composition of samples showed that 19.3%±S.E 4.39% and 5.9%±S.E 0.18% of reads had close homology to the phylum Firmicutes under light and dark conditions, respectively ($p \leq 0.05$), and 5.9%±S.E 1.21% and 2.0%±S.E 0.03% of reads were assigned to the family Bacillaceae under light and dark conditions, respectively ($p \leq 0.05$) (Figure 3.10). Moreover, relative composition analysis showed that more reads were assigned to the class α-Proteobacteria ($p \leq 0.05$), the order

Sphingomonadales ($p \leq 0.001$) and the families Sphingomonadaceae ($p \leq 0.01$) and Rhizobiaceae ($p \leq 0.05$) under light compared to dark conditions (Figure 3.10).

Relative composition analysis also showed that 5.4%±SE 0.14% and 3.0%±S.E 0.04% of reads had close homology to δ-Proteobacteria under dark and light conditions, respectively ($p \leq 0.01$), and 2.5%±S.E 0.02% and 1.4%±S.E 0.3% of reads had close homology to Syntrophobacteraceae under dark and light conditions, respectively ($p \leq 0.05$) (Figure 3.10).


Acidimicrobiales 294
Iamiaceae 45
Corynebacterineae 0
Mycobacteriaceae 136
Nocardiaceae 44
Geodermatophilaceae 35
Micrococcineae 0
Intrasporangiaceae 45
Actinobacteria 2491
Actinomycetales 195
Microbacteriaceae 222
Micrococcaceae 690
Micromonosporaceae 124
Propionibacterineae 0
Nocardioidaceae 178
Propionibacteriaceae 81
Streptomycetaceae 116
Solirubrobacterales 380
Solirubrobacteraceae 85
Armatimonadetes 141
Bacteroidetes 31
Flavobacteriaceae 344
Sphingobacteriales 372
Bacteroidetes/Chlorobi group 0
Saprospiraceae 47
Chlorobi 98
Opitutae 16
Opitutaceae 38
Verrucomicrobia 17
Spartobacteria 256
Verrucomicrobiales 52
Anaerolineae 457
Caldilineales 7
Chloroflexi <phylum> 0
Caldilineaceae 245
Chloroflexi 1742
Thermomicrobia 49
Cyanobacteria 408
Thermomicrobiales 39
Nostocales 73
Nostocaceae 693
Elusimicrobia 35
Acidobacteria 2335
Acidobacteriales 2533
Solibacteraceae 50
Bacillales 786
Bacillaceae 1506
Firmicutes 28
Bacilli 29
Paenibacillaceae 834
Bacteria 2543
Clostridiales 136
Planococcaceae 1295
Clostridia 42
Clostridiaceae 118
root 0
Gemmatimonadetes 56
Gemmatimonadales 369
Gemmatimonadaceae 95
Nitrospirales 10
Nitrospiraceae 377
Phycisphaerae 72
Planctomycetes 626
Phycisphaerales 44
Planctomycetaceae 136
Caulobacterales 1
Caulobacteraceae 321
Beijerinckiaceae 82
Rhizobiales 663
Bradyrhizobiaceae 616
Hyphomicrobiaceae 1853
Phyllobacteriaceae 1301
Alphaproteobacteria 199
Rhizobiaceae 185
Rhodobacterales 16
Hyphomonadaceae 67
Rhodobacteraceae 149
Rhodospirillales 0
Acetobacteraceae 73
Rhodospirillaceae 908
Sphingomonadales 27
Erythrobacteraceae 42
Proteobacteria 51
Sphingomonadaceae 562
Burkholderiales 381
Comamonadaceae 106
Betaproteobacteria 707
Oxalobacteraceae 407
Rhodocyclales 887

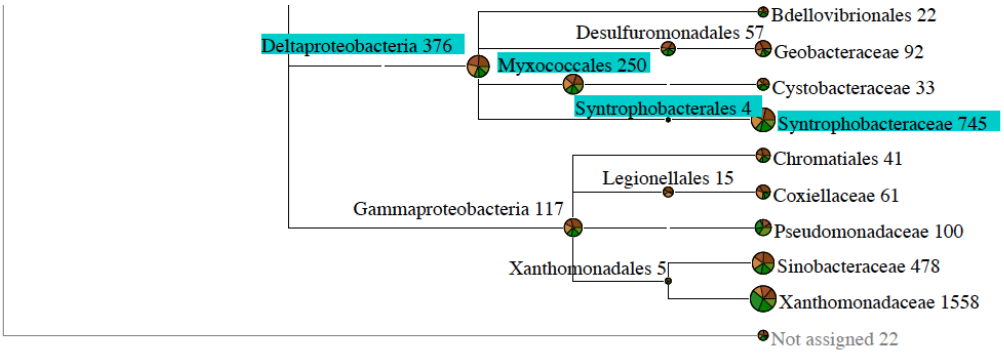


Figure 3.10: The diversity and relative abundance of bacteria at the soil surface of Gartenacker soil after 80 days incubation under light or dark conditions. Data is presented as an OTU table created in QIIME at the 97% similarity threshold (uclust). The OTU table is presented at the taxonomic Level of Family. The number of reads assigned to each Family is shown at the end of each node. Pie charts show the proportion of reads assigned to each sample incubated under light (green) and dark (brown) conditions with shades representing triplicate samples. Taxonomic assignments accounting for <0.5% total sequence abundance were removed. Significant differences in sequence abundance between light and dark samples are highlighted in green when abundance is significantly higher under light conditions and in blue when abundance is significantly higher under dark conditions ($p<0.05$).

3.4.3.4. Fungal community structure

Pyrosequencing (14, 577 reads) revealed 472 fungal OTUs with an average length of 316 bp and an average of 30.9 reads assigned to each OTU. However, Observed Species showed a significantly higher number of unique OTUs under dark compared to light conditions ($p \leq 0.001$) (Figures 3.11a-3.11b). NMDS analysis of fungal community structure showed a poor grouping of light incubated samples under light conditions; one sample shared a greater similarity to dark incubated

rather than light incubated samples, which suggests that fungal community structure was more variable under light compared to dark conditions (Figure 3.12).

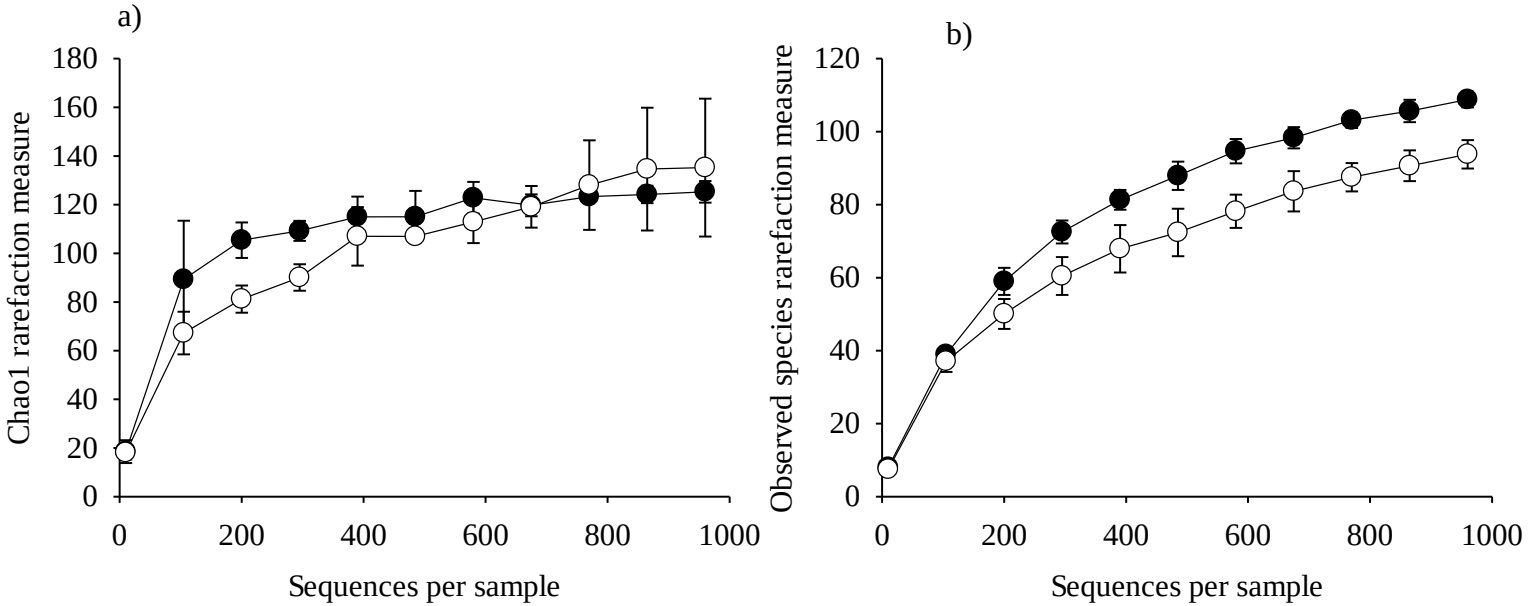


Figure 3.11: α diversity estimates Chao1 (a) and Observed Species (b) for fungi (ITS region) at the soil surface after 80 days incubation under light (open symbols) or dark (closed symbols) conditions. OTU clustering was performed at the 97% similarity threshold using uclust. Error bars are ±1 S.E.

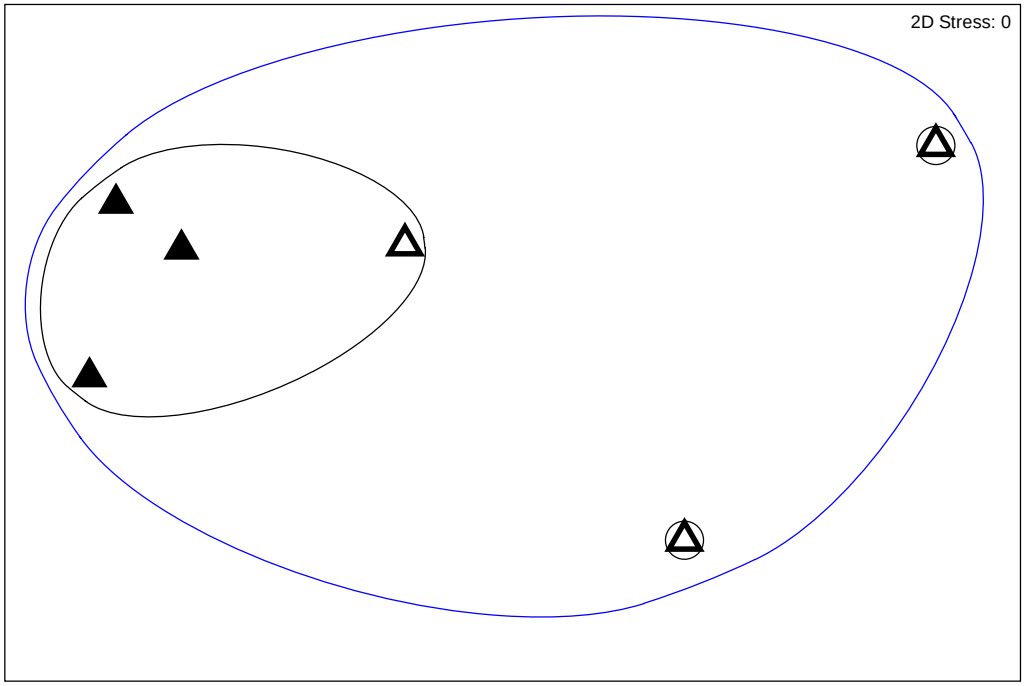


Figure 3.12: Ordination plots derived from non-metric dimensional scaling analysis of Bray-Curtis similarity for fungi (ITS region) at the soil surface of Gartenacker soil after 80 days incubation under light (open) or dark (closed) conditions. OTU clustering was performed at the 97% similarity threshold using uclust. Non-metric multidimensional scaling shows clustering based on the similarity of microbial community structure between treatments: 55% (blue) and 70% (black).

Relative composition analysis showed Ascomycota to be the dominant division of fungi at the soil surface with 57.9%±S.E 5.96% and 62.4%±S.E 2.79% of reads showing close homology under light and dark conditions, respectively (Figure 3.13). The presence of light produced few shifts in fungal community structure, however, relative composition analysis showed that 2.3%±S.E 0.09% and 4.3%±S.E 0.53% of reads were assigned to Hypocreales under dark and light conditions, respectively ($p \leq 0.05$) (Figure 3.13). Relative composition analysis also showed a relatively greater number of reads assigned to both Sordariomycetes incertae sedis and Clavicipitaceae under dark compared to light conditions ($p \leq 0.05$) (Figure 3.13).

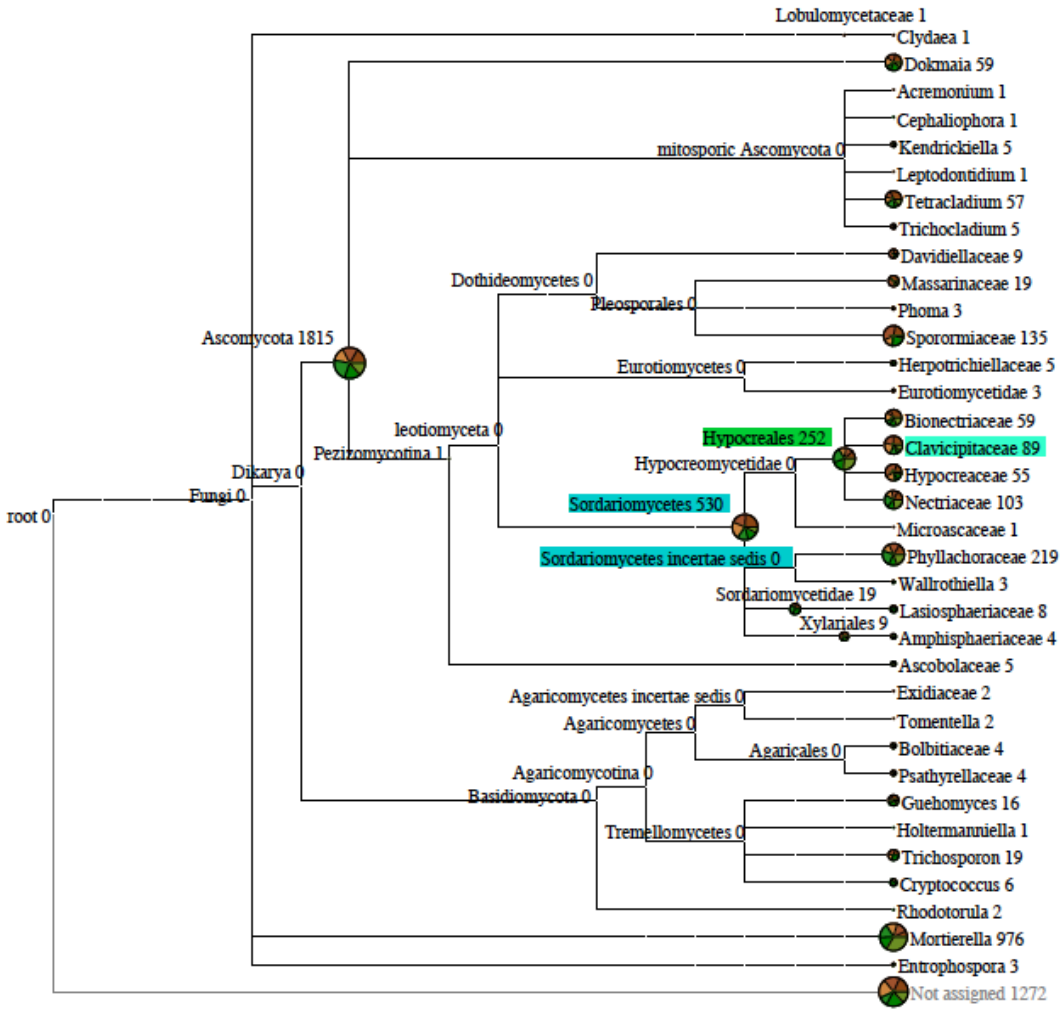


Figure 3.13: The diversity and relative abundance of fungi at the soil surface of Gartenacker soil after 80 days incubation under light or dark conditions. Data is presented as an OTU table created in QIIME at the 97% similarity threshold (uclust). The number of reads assigned to each taxa is shown at the end of each node. Pie charts show the proportion of reads assigned to each sample incubated under light (green) and dark (brown) conditions with shades representing triplicate samples. Significant differences in sequence abundance between light and dark samples are highlighted in green when abundance is significantly higher under light conditions and in blue when abundance is significantly higher under dark conditions ($p<0.05$).

3.5 DISCUSSION

Light had a significant effect on phototroph community structure, soil nutrients and pH, and this effect extended to the underlying bulk soil. Light also had a significant, time-dependent impact on heterotrophic bacterial and fungal community structure which was restricted to the soil surface. Soil surface communities are typically defined by the presence of photosynthetic communities in the top 1-3 mm of soil (Jeffery *et al*. 2007), however, this Chapter shows changes in phototroph community structure at a depth greater than 3 mm, and the presence of distinct heterotrophic microbial communities at the soil surface in the presence of light.

Chlorophyll *a* analysis was used as a broad-scale assessment of phototroph biomass development, and it indicated both the development of phototrophs at the soil surface after 20 days and the restriction of phototrophs to the soil surface under light conditions (Figure 3.3). The presence of light also significantly increased soil pH and reduced extractable NO_3 and extractable Mg at both the soil surface and underlying bulk soil under light compared to dark conditions (Figures 3.2a-3.2c). Therefore, although phototrophs appeared to be restricted to the soil surface, the influence of light extended to bulk soil (Figure 3.2a-3.2c and 3.3).

TRFLP analysis of phototrophs was used as a fine-scale assessment of community structure, and it showed development of distinct communities at the soil surface and bulk soil under light compared to dark incubated soil (Figures 3.4a-3.4c). In contrast to chlorophyll *a* data, TRFLP analysis showed no difference in phototroph community structure between the soil surface and underlying bulk soil under light conditions (Figures 3.4a-3.4c). Therefore, fine-scale molecular analysis

has shown a new depth of influence of light on phototroph community structure that previous broad-scale assessments have missed (Jeffery *et al*. 2007). It has previously been shown that approximately 0.3% of light is transmitted beyond the top 3 mm of soils with the highest transmittance of light (Benvenuti, 1995). Therefore, these shifts in phototroph community structure in bulk soil may be driven by attenuated light penetrating small cracks present at the soil surface. Alternatively, penetration of filamentous cyanobacteria into underlying soil may be a consequence of primary production at the soil surface under light conditions. These hypotheses require further testing, particularly in cracking clay soils where light penetration through soil cracks could result in significant shifts in phototroph community structure at even greater depths.

Distinct bacterial and fungal communities developed at the soil surface under light conditions compared to bulk soil, and dark incubated soil (Figures 3.4d-3.4e). Although chlorophyll *a* data showed the growth of phototrophs after only 20 days (Figure 3.3), shifts in bacterial and fungal communities were only evident after 40 days (Figures 3.4d-3.4e). This time lag may be controlled by the time taken for light to indirectly affect soil pH and/or nutrient availability. The influence of light on bacterial and fungal communities was restricted to the soil surface which suggests they are either directly responding to light which is attenuated at lower depths, and/or indirectly responding to nutrients that are only altered at the soil surface, presumably as a result of the growth of phototrophs, such as extractable P or extractable K. Alternatively, heterotrophic bacterial and fungal communities may have an indirect response to availability of C fixed by phototrophs at the soil surface.

Light may also exert an additional indirect effect on community structure by elevating temperature and therefore accelerating the frequency of drying-rewetting

cycles at the soil surface. It has previously been shown that drying-rewetting regimes can influence bacterial composition (Fierer *et al*. 2003; Placella *et al*. 2012) and fungal PLFA (Gordon *et al*. 2008). Placella *et al*. (2012) showed significant declines in the relative abundances of Actinobacteria and Acidobacteria, significant increases in the relative abundances of β- and γ- proteobacteria, and specific α-proteobacteria such as Sphingomonadales, and a bell-shaped response for Bacilli after soil re-wetting (Placella *et al*. 2012). Relative composition analysis showed a similar effect of light on Bacilli and Sphingomonadales in the current study, which could be a consequence of more pronounced wet-dry cycles under light compared to dark conditions (Figure 3.10). However, it is important to note that Placella *et al*. (2012) investigated shifts in active communities over a short time-period (72-hour) after total soil water content was increased by ~30% (Placella *et al*. 2012). In contrast, weekly monitoring of soil moisture content in the current study showed water content did not differ by >1% between light and dark incubated samples.

Studies of the soil surface have typically focused on how bacterial and fungal communities differ based on geographical location, desert type, or aridity level; a direct impact of light on heterotrophic communities, however, has not been reported previously (Abed *et al*. 2010; Zaady *et al*. 2010; Bates *et al*. 2012). Figure 3.4 shows community shifts between 40 and 80 days following a simulated tillage event, which adds to studies conducted under agricultural cropping systems, which have shown phototroph development between 50 and 80 days after tillage (Knapen *et al*. 2007).

Phototroph diversity has been investigated using cultivation-dependent techniques (Hawkes and Flechtner, 2002; Li *et al*. 2002; Langhans *et al*. 2009*a*; Li *et al*. 2010*b*; Zhang *et al*. 2011) or molecular analysis targeting bacterial diversity in arid lands (Garcia-Pichel *et al*. 2001; Redfield *et al*. 2002; Nagy *et al*. 2005; Abed *et*

al. 2010; Zaady *et al*. 2010; Steven *et al*. 2012), however, Figure 3.7 reveals the diversity of both cyanobacteria and eukaryotic phototrophs at the soil surface of a temperate soil using 454 pyrosequencing. Specific cyanobacterial taxa were selected for by light, namely *N. punctiforme, A. cylindrica* and *A. variabilis* (Figure 3.7). The fact that relative composition analysis showed that significantly more reads were assigned to cryptomonads, red algae, brown algae, mosses, and angiosperms in the dark reflects that these proliferated less than cyanobacteria in the light but are nonetheless present in the seed bank of phototrophs (Figure 3.7; Table 3.5). The dominant cyanobacteria of BSCs has been shown to be influenced by several factors, including the type of BSC (Redfield *et al*. 2002), successional stage (Yeager *et al*. 2004), underlying soil substrata (Garcia-Pichel *et al*. 2002), and level of aridity (Zaady *et al*. 2010). The selection for the diazotrophic cyanobacterium *N. punctiforme* at the surface of temperate soil in the current study is consistent with results documented in mature, or late-successional BSCs from arid lands (Belnap, 2002; Yeager *et al*. 2004; Johnson *et al*. 2005; Zhao *et al*, 2010; Liu *et al*. 2012) (Figure 3.7). This suggests that diazotrophic cyanobacteria may also be important ecosystem engineers in temperate environments, in addition to arid zones (Belnap, 2002; Yeager *et al*. 2004, Johnson *et al*. 2005; Li *et al*. 2010*b*; Zhao *et al*. 2010; *et al*. 2011*a* and 2011*b*). However, the contribution of surface communities to N_2 fixation in temperate soils or agricultural systems remains to be elucidated. Such data could be beneficial for informing agricultural management decisions, for example, the realization that diazotrophs were able to fix an agriculturally significant proportion of N_2 could influence decisions relating to soil tillage and the amount, frequency and timing of N fertiliser application under cropping systems.

454 pyrosequencing revealed that light also selected for heterotrophic bacteria at the soil surface (Figure 3.10). In contrast to the desert soils studied to date (Garcia-Pichel, 2001; Redfield *et al*. 2002; Abed *et al*. 2010; Zaady *et al*. 2010), few bacterial sequences (<4%) had close homology to cyanobacteria, allowing shifts in heterotrophic bacteria to be assessed (Figure 3.10). The comparative reduction in bacterial diversity under light conditions was not due to a selection for cyanobacteria (Figures 3.9a-3.9b) as α diversity was still significantly lower under light conditions ($p \leq 0.01$) after the removal of photosynthetic bacterial OTUs from analysis. The differences in diversity may be due to an input of C through photosynthesis and or N by N_2 fixation, which could indirectly select for specific heterotrophic bacteria. This is analogous to the 'rhizosphere effect.' The rhizosphere is the area of soil under the influence of roots. Studies have shown that the rhizosphere can select for particular microbial communities and that this selection is plant-specific (Morgan *et al*. 2005). A similar effect may be occurring at the soil surface under light conditions. Moreover, taken with TRFLP results which show that the impact of light on bacterial community structure is restricted to the upper 3 mm of the soil surface (Figure 3.4d), a new research area of microbial influence may be emerging.

TRFLP and 454 pyrosequencing revealed that light also significantly impacted fungal community structure at the soil surface (Figures 3.4e and 3.11). The relatively few shifts in fungal communities could be due to the development stage of phototroph communities. BSCs typically undergo a succession from cyanobacteria- to lichen- to moss- dominated crusts in arid zones (Li *et al*. 2002). In the present study, the soil surface was dominated by cyanobacteria (Table 3.5). However, if the surface was left to develop to a lichen dominated community, more significant shifts in fungal community structure may be evident as lichen symbioses develop.

However, parallels can still be drawn between soil surface fungal communities of temperate and arid lands, for example, relative composition analysis showed that Ascomycota were the dominant fungi in the present study in addition to surveys in the Colorado plateau, Chihuahuan desert and Sonoran deserts, USA (Bates & Garcia-Pichel, 2009; Bates *et al*. 2012).

In conclusion, the application of fine-scale molecular analysis gave new insights into soil surface community structure. Phototroph community structure was different in bulk soil in the presence of light compared to dark incubated soil, which has not previously been detected. The soil surface was also shown to harbour distinct heterotrophic bacterial and fungal communities. Future work should focus on the ecological significance of both phototrophic and heterotrophic communities, particularly in temperate zones, including their functional importance in agro-ecosystems.

4. CHAPTER IV: NON-UV LIGHT IMPACTS ACTIVELY TRANSCRIBING ORGANISMS AND FUNCTION AT THE SOIL SURFACE

4.1. ABSTRACT

The soil surface harbours photosynthetic communities that are distinct from those of underlying bulk soil. These soil surface communities are important in reducing soil erosion, improving water infiltration and enhancing carbon and nitrogen fixation. Despite their wide distribution, comparative understanding of soil surface functions and the organisms responsible for these are lacking. A metatranscriptomic approach was used to identify differences in general soil surface functions, key genes in the N cycle, and actively transcribing eukarya, bacteria and archaea in the top three millimeters of a pasture soil incubated under light and dark conditions. Under light, actively transcribing phototrophic eukaryotes were selected for at the soil surface, including Chlorophyceae, Coniferopsida and diazotrophic Nostocaceae cyanobacteria. In addition, light selected heterotrophic eukarya, namely Saccharomycetes. Relative transcript analyses suggested that autotrophic and heterotrophic processes were dominating in light and dark incubated samples, respectively. Together, these results suggest that autotrophy and actively transcribing eukaryotes are more important in soil exposed to light. Analysis of predicted protein coding regions demonstrated reduced functional alpha and beta diversity under light compared to dark conditions. However, the use of defined functions produced contrasting results, which is thought to be due to superior annotation of functions

selected for by light, including photosynthesis. This highlights an important bias introduced by current functional gene databases.

4.2. INTRODUCTION

Light is a key parameter with important impacts on soil metabolism. It was shown that light impacted the rate of CPP degradation (Chapter II), and that it changed soil chemical properties and both phototrophic and heterotrophic community composition and diversity at the soil surface (Chapter III). In this Chapter, a metatranscriptomic approach was used to investigate whether light also impacts soil surface processes and actively transcribing organisms. Metatranscriptomic analysis represents the current state of the art in the molecular assessment of the functional diversity of a complex community. It attempts to investigate the total diversity and transcript abundances of all functions in mixed community RNA.

Metatranscriptomic analysis has only recently become possible with the advent of new sequencing technology, and as such, relatively few studies have implemented the technique, particularly in the soil environment (Bailly *et al*. 2007; Urich *et al*. 2008; Stewart *et al*. 2011*b*; Damon *et al*. 2012; de Menezes *et al*. 2012; Tveit *et al*. 2012). The approach is useful as it can be used to assess known and unknown functions simultaneously, such as genes involved in the degradation of organic compounds, stress responses and biogeochemical cycles. An investigation into biogeochemical cycles is particularly useful in soil surface studies where N_2 fixation and ammonia oxidation are known to occur (Yeager *et al*. 2004; Johnson *et al*. 2005).

The technique has been split into two fields, the first aims to investigate "who is there?" and "what are they doing?" using rRNA and mRNA, respectively. Urich *et al*. (2008) pioneered the simultaneous assessment of soil microbial community structure and function in soil, and it has since been used to investigate the organisms and gene pool of microbiota driving soil organic C transformations in peatlands (Tveit *et al*. 2012). The drawback of this approach is the dominance of rRNA in samples and therefore an underrepresented assessment of community function using mRNA. The second approach attempts to overcome this drawback and focus on the question "What are they doing?" by selectively enriching for mRNA of eukarya or bacteria by enriching for polyA-tailed mRNA (Bailly *et al.* 2007; Damon *et al*. 2012) or depleting rRNA (Stewart *et al*. 2011b; de Menezes *et al*. 2012), respectively. This Chapter used the latter approach for an in-depth investigation of how light impacts functional diversity and actively transcribing organisms at the soil surface, with the aim of answering the following questions:

(i) How do major soil functions and functional diversity differ in the presence of soil surface communities exposed to light and dark conditions?

(ii) Are there differences in primary metabolic strategies and genes involved in the nitrogen cycle between soil exposed to light and dark conditions?

4.3. MATERIALS AND METHODS

4.3.1. Soil

Soil was sourced during October 2010, from the same area and treated using the same method as described in section 2.3.1.

4.3.2. Test system and sampling

A core system was used which only exposed the soil surface to light. The sides of the cores were opaque, therefore restricting light and subsequently phototroph development to the soil surface. The system was designed to mimic the design described in Chapter II (Section 2.3.3.2) in that ~33% of the total soil volume was exposed to light. Cores had a radius of 5.9 cm and a total soil depth of 9 mm, and it was assumed that light only penetrated the top 3 mm of soil (Benevenuti, 1995). Cores consisted of two parts, the bottom part being 0.6 cm in height and the top part being 2 cm. Soil (66 g dwe) was added to each core to a depth of 9 mm and the surface was flattened to minimise surface heterogeneity. Light and dark conditions were simulated using filters as described in section 3.3.2. In order to track moisture content throughout the study, each core was setup on an individual stainless steel sheet and samples were weighed throughout the experiment. Moisture content was maintained at 35% by the addition of U.P H_2O drop-wise to the soil surface. Samples were taken at 0, 3, 5, 10, 14, and 30 days. At sampling points, a stainless steel metal sheet was run between the two cores, accurately separating the top 3 mm

of soil from the underlying 6 mm of bulk soil (see Appendix III for design and sampling). Surface and bulk soil samples were homogenised separately by mixing, and sub samples were frozen at -80°C for RNA extractions. The remaining surface and bulk soil was stored at -20°C prior to chlorophyll *a* extraction.

4.3.3. Chlorophyll *a*

Chlorophyll *a* was determined according to the method described in section 2.3.4.1.

4.3.4. RNA extraction from soil and enrichment of mRNA

Nucleic acids (DNA/RNA) were extracted from triplicate soil surface samples after 21 days incubation under light and dark conditions using a modified version of the procedure of Griffiths *et al*. (2000). Only certified DNase- and RNase-free plasticware were used throughout the extraction method. In full, 500 mg soil (wet weight) was added to lysing matrix E 2 ml tubes containing 1.4 mm ceramic spheres, 0.1 mm silica spheres and one 4 mm glass bead (MP Biomedicals, Cambridge, UK). 500 µl 6% hexadecyltrimethylammonium bromide (CTAB) extraction buffer (12% CTAB/0.7 M NaCl:240 mM potassium phosphate buffer pH 8.0 (1:1 v/v)) and 500 µl phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0) was added and samples were mixed by vortex before bead beating for 30 secs at 5.5 m/s using a Hybaid Ribolyser (Fisher Scientific UK Ltd., Leicestershire, UK), followed by centrifugation at 16, 000 x *g* for 5 mins at 4°C. The aqueous layer was

transferred to a new 2 ml centrifuge taking care to avoid the protein phase. Phenol was removed by adding an equal volume (ca. 700 µl) of chloroform-isoamyl alcohol (25:24:1, pH 8.0) (Sigma-Aldrich company Ltd., Dorset, UK) before mixing by vortex, and centrifugation at 16, 000 x *g* for 5 mins at 4°C. Total nucleic acids were precipitated from the extracted aqueous layer with 2 volumes (ca. 800 µl) of 30% (wt/vol) polyethylene glycol 6, 000 in 1.6 M NaCl for 2 h at room temperature, followed by centrifugation at 18, 000 x *g* for 10 mins at 4°C. Pelleted nucleic acids were washed in 0.5 ml ice-cold 70% ethanol (v/v), followed by centrifugation at 18, 000 x *g* for 10 mins at 4°C. The pellet was air-dried at room temperature and then re-suspended in 100 µl DNase/RNase free H_2O.

DNA was removed from samples by the addition of 37.5 µl DNase/RNase free H_2O, 10 µl RDD buffer and 2.5 µl DNase (QIAGEN Ltd., Sussex, UK) to 50 µl of DNA/RNA extract. Pooled RNA samples (2x) were then purified using RNeasy MinElute Cleanup Kit (QIAGEN Ltd., Sussex, UK) according to the manufacturer's protocol and eluted using 30 µl DNase/RNase free H_2O.

The rRNA was depleted from samples by Terminator^{TM} 5'-Phosphate-Dependent Exonuclease (Cambio Ltd., Cambridge, UK) which digests RNA that has a 5' -monophosphate end (prokaryotic 16S and 23S rRNA and eukaryotic 18S and 28S rRNA), but does not digest RNA that has a 5' -triphosphate, 5' -cap (most eukaryotic mRNAs) or 5' -hydroxyl group (primary prokaryotic RNA transcripts. The digestion was performed by the addition of 5.5 µl DNase/RNase free H_2O, 0.5 µl Protector RNase Inhibitor (Roche Diagnostics Ltd., Sussex, UK), 2 µl Terminator 10X reaction buffer A, 1 µl Terminator Exonuclease (1 U) to 11 µl RNA and incubating for 30 mins at 42°C using a thermocycler. The reaction was terminated by the addition of 1 µl 100 mM EDTA, pH 8.0 (Life Technologies Ltd., Paisley, UK),

and samples were purified using RNeasy MinElute Cleanup Kit (QIAGEN Ltd., Sussex, UK) according to the manufacturer's protocol, and eluted by the addition of 30 µl DNase/RNase free H_2O.

An Agilent 2100 bioanalyzer (Agilent Technologies Ltd., Berkshire, UK) and RNA 6000 Nanokit (Agilent Technologies Ltd., UK) were used to assess the depletion of rRNA from samples according to the manufacturer's protocol. RNA samples were incubated for 2 mins at 72°C using a GeneAmp 9700 thermocycler to denature RNA secondary structure. The bioanalyzer electrodes were decontaminated with RNaseZAP® (Invitrogen, UK) for 1 min and washed with DNase/RNase free H_2O (Life Technologies Ltd., Paisley, UK) for 2 mins prior to analysing denatured RNA. RNA samples were run pre- and post- treatment with Terminator™ 5'-Phosphate-Dependent Exonuclease to assess the efficiency of rRNA removal. The quantity of RNA and the percentage 16S rRNA and 23S rRNA as a proportion of total RNA was calculated using 2100 Expert Software B.02.08 (Agilent, free software).

4.3.5. Reverse transcription and qPCR amplification of the 16S rRNA bacterial marker

Reverse transcription-PCR (RT-PCR) was performed on soil RNA extracted from triplicate soil surface samples after incubation under light and dark conditions for 21 days. Firstly, 8 µl DNase/RNase free H_2O, 1 µl random hexamer primer (Roche Diagnostics Ltd., Sussex, UK) (1:50 dilution) and 1 µl 10 ng RNA μl^{-1} was incubated at 75°C for 5 mins to allow primers to anneal. First strand synthesis was

performed by adding 1 µl 10 mM dNTPs, 4 µl M-MLV 5x reaction buffer (Invitrogen, UK), 2 µl 0.1M dithiothreitol (Invitrogen, UK), 0.4 µl RNAse-inhibitor (40U μl^{-1}) (Roche Diagnostics Ltd., Sussex, UK), 0.8 µl M-MLV reverse transcriptase (200U μl^{-1}) (Invitrogen, UK) and 1.8 µl DNase/RNase free H_2O followed by incubation at 37°C for 1 hour. qPCR amplification of bacterial 16S rRNA was performed as described in section 2.3.4.3.

4.3.6. cDNA library preparation for 454 pyrosequencing of soil metatranscriptome

A cDNA library was prepared for RNA extracted from triplicate soil surface samples after 21 days incubation under light and dark conditions using a GS FLX Rapid Library Preparation Kit (Roche Diagnostics Ltd., Sussex, UK) according to the manufacturer's protocol (carried out by Micropathology Ltd,. University of Warwick Research Park, Coventry, UK). Unless otherwise stated, all buffers and reagents were present in the GS FLX Rapid Library Preparation Kit. Preparation of a cDNA library consisted of nine steps; (i) Fragmentation of RNA; (ii) Double-stranded cDNA synthesis; (iii) Fragment end repair; (iv) AMPure bead preparation; (v) Adaptor ligation; (vi) Small fragment removal; (vii) Library quantitation; (viii) cDNA library quality assessment, and; (ix) Preparation of working aliquots.

A sample volume ≤ 19 µl and a sample concentration ≥ 200 ng RNA was required for cDNA library preparation. Of the 19 µl sample, 1 µl was removed to compare to the prepared fragmented RNA. To fragment RNA, 2 µl RNA fragmentation solution (9 ml 0.1M $ZnCl_2$ and 1 ml 1M Tris-HCl pH 7.0) was added

to the remaining 18 µl sample and incubated at 70°C for 30 secs, before being placed on ice. Following this, 2 µl 0.5M EDTA (pH 8.0) and 28 µl Tris HCl (pH 7.5) and 80 µl RNAClean reagent, containing Solid Phase Reversible Immobilisation (SPRI) beads (Agencourt, High Wycombe, UK) was added and samples were incubated at room temperature for 10 mins to allow binding of RNA to SPRI beads. Samples were placed on a Magnetic Particle Concentrator (MPC) (Agencourt, High Wycombe, UK), supernatant was removed, and beads were washed three times in 200 µl 70% ethanol, before air-drying at room temperature for 3 mins. To re-suspend RNA from SPRI beads, 19 µl 10mM Tris HCl (pH 7.5) were added before vortexing, and pelleting beads using the MPC. The supernatant containing RNA was transfered to a new tube and analysed on an RNA 6000 Nano Chip using an Agilent 2000 Bioanalyzer to confirm RNA fragmentation.

For double stranded cDNA synthesis, a cDNA Synthesis System Kit was used, according to the manufacturer's protocol (Roche Diagnostics Ltd., Sussex, UK). In full, 4 µl 400 µM Roche Primer 'random' was added to fragmented RNA and incubated at 70°C for 10 mins to denature RNA. Then, first strand cDNA synthesis progressed by the addition of 8 µl 5X RT-buffer AMV, 4 µl 0.1M DTT, 4 µl 10mM dNTPs, 1 µl Protector RNase inhibitor (25 U μl^{-1}), and 2 µl AMV RT (25 U μl^{-1}), followed by incubation at 25°C for 10 mins, and then 42°C for 60 mins. Second strand cDNA synthesis progressed by the addition of 30 µl 5X 2^{nd} strand synthesis buffer, 72 µl DNAse/RNAse free water, 1.5 µl 10mM dNTPs, and 6.5 µl 2^{nd} strand enzyme. Samples were then incubated at 16°C for 5 mins, and the reaction was stopped by the addition of 17 µl 0.2M EDTA (pH 8.0).

For double-stranded cDNA purification, 300 µl AMPure beads (Agencourt, High Wycombe, UK) were added to cDNA. Samples were incubated at room

temperature for 5 mins to promote the binding of amplicons to SPRI beads. Beads were pelleted using the MPC and washed three times by the addition of 800 µl 70% ethanol, before air-drying at room temperature for 3 mins. Then, 16 µl 10 mM Tris-HCl (pH 7.5) was added and the sample was mixed using a vortex, before placing on a MPC and transferring the supernatant containing double-stranded cDNA.

For fragment end repair, 9 µl End repair mix was added to cDNA (2.5 µl 10X PNK buffer, 2.5 µl ATP, 1 µl dNTP, 1 µl T4 polymerase, 1 µl PNK, 1 µl Taq polymerase (Roche Diagnostics Ltd., Sussex, UK). Samples were mixed using a vortex and incubated at 25°C for 20 mins, followed by 72°C for 20 mins. Adapters were then ligated to cDNA by the addition of 1 µl RL adapter and 1 µl RL Ligase, followed by incubation at 15°C for 10 mins.

The removal of small fragments (<100 bp) required the use of AMPure beads. These were prepared by first separating AMPure beads from 125 µl AMPure bead solution using the MPC before the addition of 73 µl TE Buffer, and 500 µl Sizing Solution. To remove small fragments from samples, the cDNA with ligated adapters was added to the AMPure bead preparation and incubated at room temperature for 5 mins. The supernatant was discarded and 100 µl TE Buffer and 500 µl Sizing Solution was added, and samples were incubated at room temperature for 5 mins. The previous step was repeated with the supernatant being discarded each time. AMPure beads were washed twice using 1 ml 70% ethanol, and the pellet was left to air-dry for 2 mins at room temperature. Finally, 53 µl TE Buffer was added and 50 µl of the supernatant was transferred to a new tube for library quantitation.

A standard curve consisting of eight samples ranging from 1.46×10^8 to 2.50×10^9 molecules μl^{-1} was generated for use in library quantitation by diluting RL

Standard with TE Buffer. The relative fluorescence units of standards and samples were analysed using the TBS 380 Fluorometer. At this point, the cDNA library required a concentration ≥1.46 x 10^8 molecules μl^{-1}. The quality of cDNA was then assessed using an Agilent Bioanalyzer High Sensitivity DNA chip, which gave an average fragment length between 600 bp and 1200 bp, and a lower size cut-off of <10% below 500 bp. A working stock of cDNA was prepared by diluting samples to 1 x 10^7 molecules μl^{-1} using TE Buffer. Samples were stored at -20°C.

4.3.7. Emulsion-based clonal PCR amplification (emPCR) and sequencing

Unless stated, all reagents used were included in the GS FLX Titanium MV emPCR Kit (Lib-L). The manufacturer's protocol was followed for the GS Junior Titanium Series of Rapid, Paired End, SeqCap, Unidirectional fusion primer, and cDNA Rapid Libraries (Lib-L) (carried out by Micropathology Ltd,. University of Warwick Research Park, Coventry, UK).

EmPCR was composed of seven steps; (i) Preparation of reagents and emulsion oil; (ii) DNA library capture; (iii) Emulsification; (iv) Amplification; (v) Bead recovery; (vi) DNA library bead enrichment, and; (vii) Seq primer annealing. Reagents and emulsion oil were prepared according to the manufacturer's protocol. Capture of sample cDNA onto beads was prepared by first denaturing cDNA by incubating at 95°C for 2 mins. The volume of cDNA to be added to beads was calculated according to the following equation:

$$\mu L\ of\ DNA\ library\ per\ tube = \frac{Desired\ molecules\ per\ bead\ \times\ 10\ million\ beads}{Library\ concentration\ (molecules\ per\ \mu L)}$$

In this instance, 10^7 molecules of each sample were added in order to produce bead enrichment between 5% and 20%. Then, emulsification was performed by adding 1.2 ml of Live Amp Mix, transferring to a Turrax stirring tube and mixing in the Ultra Turrax Tube Drive (IKA, Manchester, UK) at 2000 rpm for 5 mins. For amplification, 100 µl was added into eight strip-cap tubes, and amplified according to the following reaction; 94°C for 4 mins, followed by 50 cycles of 94°C for 30 secs, 58°C for 4.5 mins and 68°C for 30 secs.

To recover DNA-bound beads, the emulsion was aspirated from the strip cap tubes (according to the manufacturer's protocol) such that beads were trapped in a 50 ml falcon tube and emulsion oil was collected separately. Beads were then washed in 35 ml isopropanol, centrifuged at 9, 000 x g for 5 mins and the supernatant discarded. This was repeated twice and beads were then washed with 35 ml ethanol, followed by 35 ml Enhancing Buffer.

DNA library bead enrichment was first performed by the addition of 1 ml Melt Solution to DNA-bound beads. Samples were incubated at room temperature for 2 mins and the supernatant discarded. Then, 1 ml Annealing Buffer was added, mixed by vortex, and the supernatant discarded. A further 45 µl Annealing Buffer and 25 µl Enrich Primer were added before placing in a heat block at 65°C for 5 mins and cooling on ice for 2 mins. Then, 1 ml Enhancing Buffer was added to DNA-bound beads, mixed by vortex and the supernatant discarded. This was repeated twice before beads were re-suspended in 1 ml Enhancing Buffer. 80 µl of washed Ennrichment Beads were then added and the sample was rotated on a LabQuake (Fisher Scientific UK Ltd., Leicestershire, UK) for 5 mins before a MPC

was used to pellet beads and discard the supernatant. Beads were washed 10 times by adding 2 ml Enhancing Buffer, before vortexing and using a MPC to pellet beads. Then, 700 µl Melt Solution was added and the supernatant discarded after beads formed a pellet using the MPC. This step was repeated and 1 ml Annealing Buffer was added. The supernatant was discarded and 100 µl Annealing Buffer was added to re-suspended the beads.

Seq primer annealing was performed by first adding 25 µl Seq Primer and placing in a heat block at 65°C for 5 mins before cooling on ice for 2 mins. Then, 1 ml Annealing Buffer was added, and the supernatant discarded. This step was repeated twice before a final addition of 1 ml Annealing Buffer. The amount of enriched beads was evaluated using a GS Junior Bead Counter, and compared to the optimal value of 500, 000 enriched beads. Enriched beads were then immediately used in the sequencing reaction.

A total of three sequencing runs were performed at Micropathology Ltd. (Coventry, UK) using a GS Junior System. Light samples 1 and 2, light sample 3 and dark sample 1, and dark samples 2 and 3 were grouped, respectively across three sequencing runs. To test consistency between sequencing runs, light sample 2 was also loaded onto sequencing runs 2 and 3 at a concentration of 4% of the total cDNA to be run. Sequencing produced a total of 325, 995 reads with 127, 382 attributed to sequencing run 1, 103, 511 to run 2, and 95, 102 to run 3. Of these sequences, a total of 12, 953 and 10, 410 reads were attributed to the light sample 2 control from sequencing runs 1 and 2, respectively.

4.3.8. Bioinformatic analysis

MACQIIME v 1.6 was used to process and quality control sequence data (Caporaso *et al*. 2010*a*). Firstly, fasta files were de-multiplexed and split based on sample identifiers, and light sample 2 control reads were removed from runs 2 and 3. All sequence files (except controls) were concatenated and quality controlled based on the following parameters: no ambiguous bases, quality score of <0.2% (min. phred quality score = 28), sequence length between 120 bp and 1000 bp. Taxonomy was assigned using the RDP classifier (Wang *et al*. 2007) and the SILVA 108 rRNA database, which contained both small subunit (16S/18S, SSU) and large subunit (23S/28S, LSU) sequence data for bacteria, eukarya and archaea (Quast *et al*. 2013). If the RDP classifier could not assign the transcript to any of the three major phyla, the transcript was labelled as a putative mRNA read. The effect of the confidence level used during taxonomy assignment on the number of reads classified as putative mRNA was tested by assigning taxonomy at several confidence levels; 5% increments from 80% - 100% and 10% increments from 10% - 80%.

Exact duplicate sequences were removed (Gomez-Alvarez *et al*. 2009) from putative mRNA sequences. Functional and taxonomic annotation was performed using the Meta-Genomic Rapid Annotations using Subsystems Technology (MG-RAST) server (Meyer *et al*. 2008). Protein coding regions were first predicted using FragGeneScan (Rho *et al*. 2010) and then clustered at 90% using uclust. For functional binning, the Subsystems database and default parameters were used (max. *e*-value cut-off 1e – 5, min % identity cut-off 60% and min. alignment length cut-off 15). Functions were classified as primary or secondary based on >1% or <1% of transcripts being assigned to the function for both treatments, respectively.

Taxonomic binning was performed using the MG-RAST best-hit classification against the RefSeq protein database and default parameters.

Putative mRNA sequences were searched for similarity to genes encoding for specific functions using custom databases from UniProtKB/Swiss-Prot and UniProtKB/TrEMBL (http://www.uniprot.org), specifically, *nifH*, *amoA*, *nirS*, *nirK*, *napA*, *narG*, *norB*, *nosZ*, genes (tBLASTn: *e*-value 1e – 10). Unique identifiers were extracted for sequence hits against the above mentioned databases, and taxonomic binning was performed by a BLASTx (*e*-value 1e – 10) search against the RefSeq database. Then, taxonomical and functional annotations were binned using MEGAN (parameters: min. bit score 30, min. support 1, top percent 10; 50 best BLAST hits) (Huson *et al*. 2011).

4.3.9. Statistical analysis

ANOVA was used to assess Chlorophyll *a* abundance between treatments with time, light, depth, and time*light*depth as factors. Chi-squared and functional alpha diversity measures implemented in QIIME v1.4.0 were used to test for differences between controls on each of the three sequencing runs. Meta-transcriptomic data was rarefied at 4, 450 and 17, 866 reads per sample for functional and taxonomic annotations, respectively. Taxonomic and functional annotations were statistically analysed using STAtistical analysis of Metagenomic Profiles (STAMP) v 2.0.0 release candidate 5 (Parks and Beiko, 2010) (parameters: remove unclassified reads). QIIME was used for ANOVAs to compare relative transcript abundances for predicted protein coding regions (CRs), and t-tests to compare functional alpha

diversity. Chao1 was used as a mark-release-recapture assessment of functional alpha diversity (Chao, 1984). Functional beta diversity was assessed by Non-metric MultiDimensional Scaling analysis (NMDS) (Bray-Curtis distance) using PRIMER6 (Plymouth, UK).

4.4. Results

4.4.1. Growth of phototrophs

Light, time and depth all had a significant effect on Chlorophyll *a* abundance, and there was a significant interaction between all three variables ($p \leq 0.001$) (Figure 4.1). At day 30, chlorophyll *a* was significantly more abundant at the soil surface under light conditions compared to all other treatments ($p \leq 0.001$). There were no significant differences in chlorophyll *a* abundance between bulk soil under light conditions and dark-incubated samples (Figure 4.1).

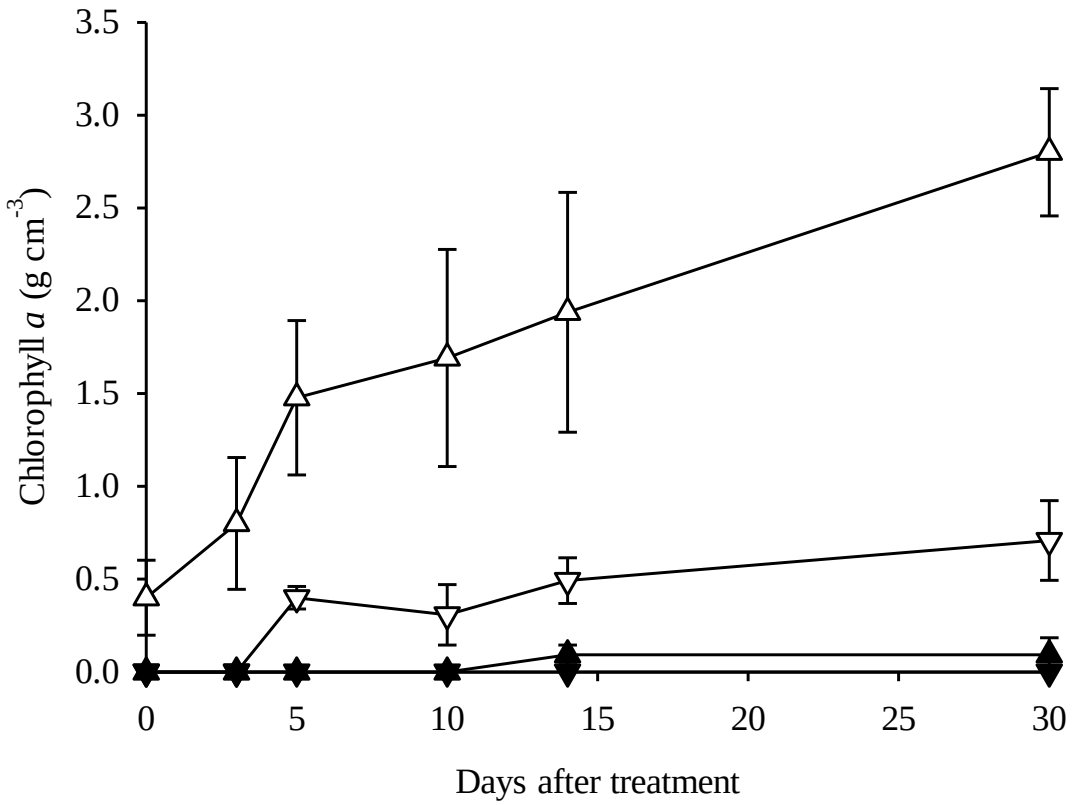


Figure 4.1: Timecourse of chlorophyll *a* development at the surface (▲) and bulk (▼) of Gartenacker soil incubated under light (open symbols) and dark (closed symbols) conditions. Error bars are ±1 S.E.

4.4.2. The abundance of active bacterial communities at the soil surface

After 21 days incubation, there were significantly more copies of bacterial 16S rDNA under light compared to dark conditions at 3.36×10^9 ±S.E 6.90×10^8 copies g^{-1} soil and 3.07×10^8 ±S.E 5.04×10^7 copies g^{-1} soil, respectively ($p<0.01$).

4.4.3. Terminator 5’ phosphate dependent exonuclease digestion of total RNA and ribosomal RNA

RNA yields were significantly higher under light (210±S.E. 26) compared to dark (71±S.E. 11) conditions ($p<0.05$) (Table 4.1). Following digestion with the Terminator 5’ phosphate dependent exonuclease, 59.4%±S.E. 2.4% and 42.4%±S.E. 18.4% of rRNA was depleted from total RNA extracted from the soil surface under light and dark conditions, respectively. Total RNA was reduced by 88.8% ± S.E.2.8% and 85.3% ± S.E.4.1% under light and dark conditions, respectively (Table 4.1).

Table 4.1: Relative quantity of 16S rRNA and 23S rRNA, total RNA extracted from samples and the impact of Terminator™ 5'-Phosphate-Dependent Exonuclease on removal of rRNA and total RNA

Sample	Pre-Terminator				Post-Terminator				Impact of Terminator	
	16S rRNA (% area)	23S rRNA (% area)	rRNA (% area)	RNA (ng/µl)	16S rRNA (% area)	23S rRNA (% area)	rRNA (% area)	RNA (ng/µl)	Reduction rRNA (%)	Reduction RNA (%)
Light 1	16.0	10.3	26.3	220	3.2	6.2	9.4	27	64.3	87.7
Light 2	16.0	9.8	25.8	162	5.6	5.4	11	25	57.4	84.6
Light 3	16.7	8.4	25.1	249	4.1	6.8	10.9	15	56.6	94.0
Dark 1	19.4	12.5	31.9	85	6.2	13.3	19.5	8	38.9*	90.6
Dark 2	16.9	11.0	27.9	50	10.3	14.1	24.4	6	12.5*	88.0
Dark 3	19.5	13.2	32.7	79	3.3	4.6	7.9	18	75.8	77.2

*** The comparative reduction in rRNA was lower for these samples compared to all other samples. The quantity of total RNA in these samples was very low. This resulted in the automatic noise filterer used by the Bioanalyzer software ignoring genuine mRNA from traces following digestion with the 5' Terminator. Consequently, the proportion of rRNA that remained in samples after digestion was over -estimated**

4.4.4. Bioinformatic processing of sequence data

The number of transcripts assigned to samples following sequencing ranged from 24, 793 (Dark 2) to 83, 579 (Light 2) (Table 4.2). A total of 50, 183 (16.6%) reads were removed during quality control. The proportion of transcripts removed after failing quality control parameters were ranked as: quality score <0.2% error, 24, 616 reads (49.1%); duplicate reads, 13, 764 reads (27.4%); ambiguous bases, 9, 351 reads (18.6%); read length <120 bp, 2, 452 reads (4.9%) (Table 4.2). A total of 252, 448 transcripts passed all quality control checks.

Table 4.2: Transcript removal following QIIME quality control

Sample	No. transcripts	No. transcripts after quality control (% reduction)
Light 1	43 903	35 019 (20.2%)
Light 2	83 479	66 915 (19.8%)
Light 3	47 229	40 947(13.3%)
Dark 1	43 329	37 750 (12.9%)
Dark 2	24 793	20 881 (15.8%)
Dark 3	59 899	50 937 (14.9%)
Total	**302 632**	**252 448 (16.6%)**

Transcripts were assigned to the SILVA 108 rRNA database using the RDP classifier. If the RDP classifier could not assign the transcript to one of the three major phyla (at a given confidence level), the transcript was labelled as putative mRNA. An increase in the confidence level used to assign taxonomy to transcripts resulted in an increase in the number of putative mRNA reads (Table 4.3). For example, using a confidence level of 80% or 100% to assign taxonomy resulted in 165, 461 (65.5%) and 182, 476 (72.3%) transcripts being assigned as putative mRNA, respectively (Table 4.3). The default confidence level of 80% used by QIIME was used to classify sequences as putative mRNA.

Table 4.3: Number of putative mRNA reads assigned under different confidence levels using the RDP classifier and SILVA108 database

Confidence Level (%)	No. of putative mRNA reads	Putative mRNA reads (%)
10	878	0.4
20	6 615	2.6
30	12 141	4.8
40	24 608	9.7
50	55 577	22.0
60	98 404	39.0
70	139 080	55.1
80	165 461	65.5
85	173 181	68.6
90	177 951	70.5
95	180 414	71.5
100	182 476	72.3

When separating mRNA from rRNA reads it is important to maintain a confidence level high enough to avoid protein coding sequences with close homology to rRNA being selected as putative mRNA reads. To do this, a variety of confidence levels were tested and the lowest confidence level at which there was only a small shift in the proportion of assignable mRNA was selected (80%) i.e. between confidence levels of 90% and 80%, only 5% fewer reads were assigned as mRNA, however, between confidence levels of 80% and 70%, 10.4% fewer reads were assigned as mRNA, marking a considerable drop-off.

4.4.5. Consistency across sequencing runs

A comparison of functional alpha diversity values (Figure 4.2) was used to assess consistency between the standards loaded onto three pyrosequencing runs. Functional alpha diversity values were similar across runs for both transcripts clustered at 90% similarity level, and for functionally annotated reads (Figure 4.2).

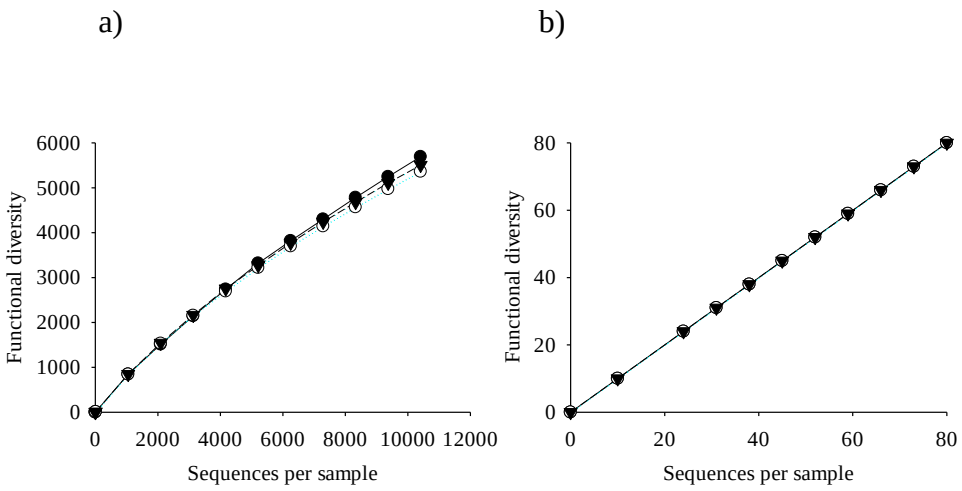


Figure 4.2: Functional alpha diversity assessed by Oberved Species for the standard runs across 454-pyrosequencing; runs 1 (●) 2 (○) and 3 (▼). Functional diversity was calculated using QIIME for: (a) transcripts clustered at 90% similarity, and; (b) transcripts annotated in MG-RAST using the Subsystems database and default settings.

4.4.6. Predicted protein coding regions and functional categories at the soil surface under light and dark conditions

Approximately 75% and 70% of putative mRNA reads were assigned to predicted protein CRs under light and dark conditions, respectively (Table 4.4). The

samples incubated under light had approximately 10, 000 more transcripts on average assigned to predicted protein CRs and as putative mRNA, compared to samples incubated in the dark. Only ~27% (12, 438) and ~35% (12, 632) of putative mRNA reads could be assigned to defined functional categories under light and dark conditions, respectively (Table 4.4).

Table 4.4: Number of predicted protein coding regions (protein CRs) and functional categories described for mRNA reads from the surface of Gartenacker soil incubated under light and dark conditions for 21 days

Sample	Putative mRNA reads	Transcripts assigned to predicted protein CRs	Predicted protein CRs	Functional categories	Transcripts assigned to functional categories
Light 1	35 019	26 692 (76.2%)	16 366	2 368	11 250 (32.1%)
Light 2	66 915	50 421 (75.4%)	25 917	3 588	15 908 (23.8%)
Light 3	40 947	30 552 (74.6%)	16 874	1 935	10 157 (24.8%)
Average	**47 627± 9795**	**35 888± 7351 (75.4%± 0.5%)**	**19 719± 3102**	**2630± 495**	**12 438± 1763 (26.9%± 2.6%)**
Dark 1	37 750	26 495 (70.2%)	20 815	2 648	13 150 (34.8%)
Dark 2	20 881	14 729 (70.5%)	12 101	1 600	7 991 (38.3%)
Dark 3	50 937	35 410 (69.5%)	25 501	3 292	16 755 (32.9%)
Average	**36 523± 8698**	**25 545± 5989 (70.1%± 0.3%)**	**19 472± 3926**	**2513± 493**	**12 632± 2543 (35.3%± 1.6%)**

4.4.7. Functional and taxonomic annotation of transcripts from the soil surface incubated under light and dark conditions

Transcripts from light and dark conditions were assigned to an average of 274±S.E 73 and 203±S.E 19 distinct functions, respectively (Table 4.5). There was a considerable range in values between samples, most notably highlighted between samples 2 and 3 in the light, which assigned transcripts to 155 and 406 functions, respectively. Taxonomically, transcripts were assigned to an average of 129± S.E 12 and 119±S.E 6 families under light and dark conditions respectively (Table 4.5). There were no significant differences between the average number of functions or actively transcribing taxonomic families at the soil surface under light and dark conditions.

Table 4.5: The number of annotated functions and taxonomic families at the soil surface under light and dark conditions

	Light			Dark		
	1	2	3	1	2	3
No. functions	262	155	406	222	165	221
Average		**274± S.E 73**			**203± S.E 19**	
No. families	106	143	138	130	117	110
Average		**119± S.E 6**			**129± S.E 12**	

Functional and taxonomic annotations were rarefied at 4450 and 17866 reads, respectively.

4.4.7.1. Actively transcribing organisms at the soil surface under light and dark conditions

4.4.7.1.1 Dominant organisms actively involved in soil surface functions across all samples

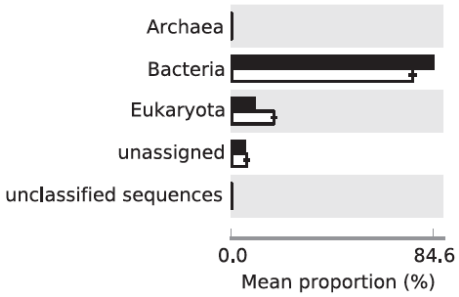
Out of the 107, 196 transcripts that were taxonomically binned, 80.2% were assigned to Bacteria, 13.7% to Eukarya, and 0.07% to Archaea. The remaining transcripts were unassigned (6.0%) or derived from unclassified sequences (0.03%) (Figure 4.3a).

At the phylum level, the majority of transcripts were assigned to Firmicutes, (29.6%), Proteobacteria (24.3%) and Actinobacteria (14.2%). Streptophyta (Plantae) (4.8%) and Ascomycota (3.0%) were the phototroph and fungal phyla with the greatest proportion of transcripts assigned to them, respectively. A considerable proportion of transcripts were also assigned to Cyanobacteria (3.5%) (Figure 4.3b).

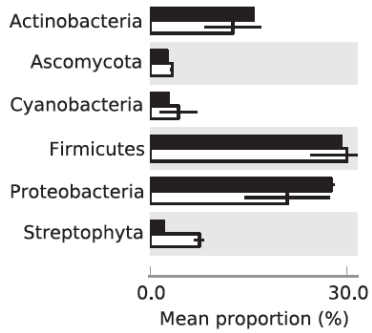
At the class level, transcripts were assigned to five primary taxa, which were ranked in the following order of relative abundance: Clostridia (18.7%) > Actinobacteria (14.2%) > Bacilli (10.5%) > Gammaproteobacteria (10.0%) > Alphaproteobacteria (5.2%) (Figure 4.3c). At the family level, 48.8% of transcripts were assigned to 11 taxa, namely Bacillaceae, Brucellaceae, Burkholderiaceae, Clostridiaceae, Corynebacteriaceae, Enterobacteriaceae, Heliobacteriaceae, Kineosporiaceae, Lactobacillaceae, Streptomycetaceae, and unclassified derived

from Chroococcales (Figure 4.3d). The remaining 51.2% (54, 884 reads) of transcripts were assigned to 374 families.

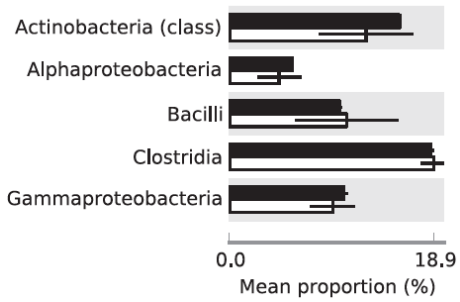
a)



b)



c)



d)

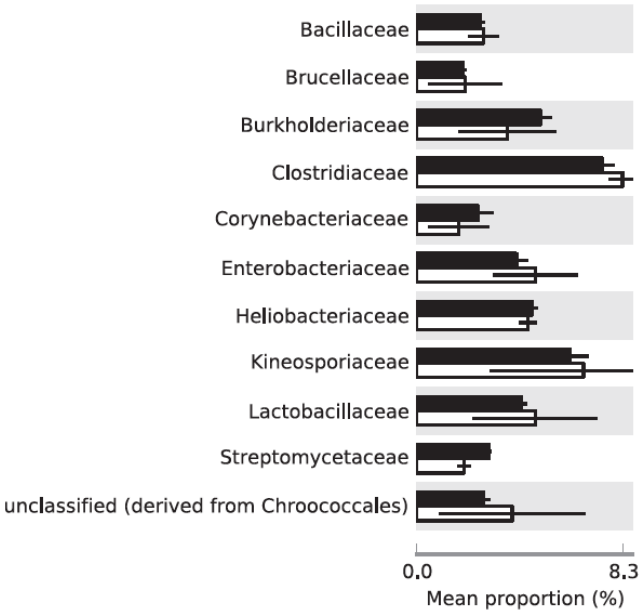


Figure 4.3: The dominant actively transcribing organisms (≥3% reads assigned) at the soil surface after 21 days incubation under light (open bar) and dark (closed bar) conditions. The relative abundances of transcripts taxonomically assigned using the RefSeq protein database at default setting are shown at taxonomic levels of: (a) domain; (b) phylum; (c) class, and (d) family. Corrected p-values and confidence intervals were created using STAMP. Error bars are ±1 S.E.

4.4.7.1.2. Community shifts in actively transcribing organisms at the soil surface under light and dark conditions

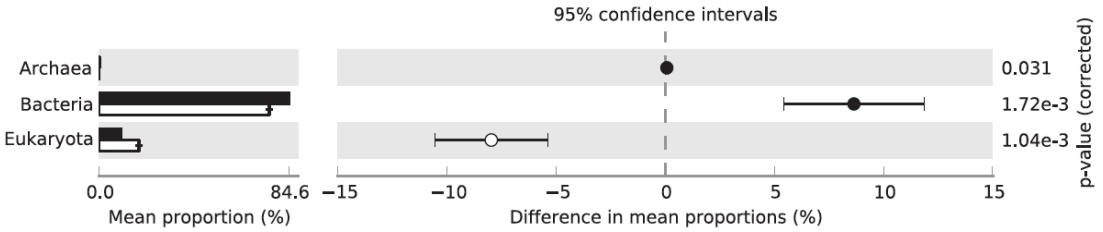
At the domain level, a significantly greater proportion of transcripts were assigned to bacterial and archaeal domains under dark conditions ($p<0.05$), and Eukarya under light conditions ($p<0.001$) (Figure 4.4a). At the phylum level, relative composition analysis showed that significantly more transcripts were assigned to Streptophyta, Ascomycota, Chlorophyta (green alga) and Cnidaria (medusozoa) in the light compared to dark conditions ($p>0.05$) (Figure 4.4b). In contrast, the bacterial phyla Acidobacteria, Aquificae, and Planctomycetes had significantly greater relative transcript abundances under dark compared to light conditions ($p>0.05$) (Figure 4.4b).

Relative composition analysis revealed a number of eukaryotic classes to have a greater relative transcript abundance under light compared to dark conditions, such as Anthozoa (Cnidarian) and Saccharomycetes (Ascomycota), as well as classes of phototrophs; Bryopsida (Bryophytes), Chlorophyceae (green alga), Coniferopsida (conifers), Liliopsida (plantae), and unclassified (derived from Streptophyta) ($p<0.05$) (Figure 4.4c). In contrast, bacterial classes such as Aquificae (Class), Deinococci, Erysipelotrichi, Planctomycetia, and unclassified (derived from Acidobacteria (Class), and the archaeal class Thermococci had a greater relative transcript abundance under dark compared to light conditions ($p<0.05$) (Figure 4.4c).

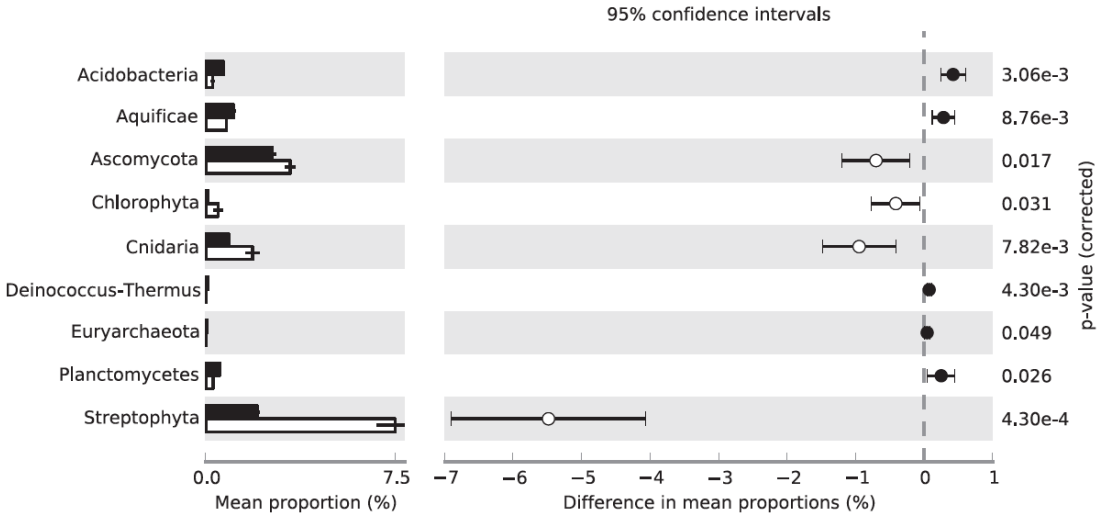
Finally, transcripts were assigned to different families under light and dark conditions, for example, a significantly greater proportion of transcripts were

assigned to Protists (Perkinsidae), Cyanobacteria (Nostocaceae), Cnidaria (Edwardsiidae) and Plantae (Orchidaceae, Pinaceae and Poaceae) under light compared to dark conditions ($p<0.05$) (Figure 4.4d). The majority of families with a significantly greater relative transcript abundance under dark compared to light conditions were bacterial, for example, Actinomycetaceae, Bifidobacteriaceae, Comamonadaceae, Deinococcaceae, and Hydrogenothermaceae, however, the eukaryotic family Caligidae also had a greater relative transcript abundance in the dark ($p<0.05$) (Figure 4.4d).

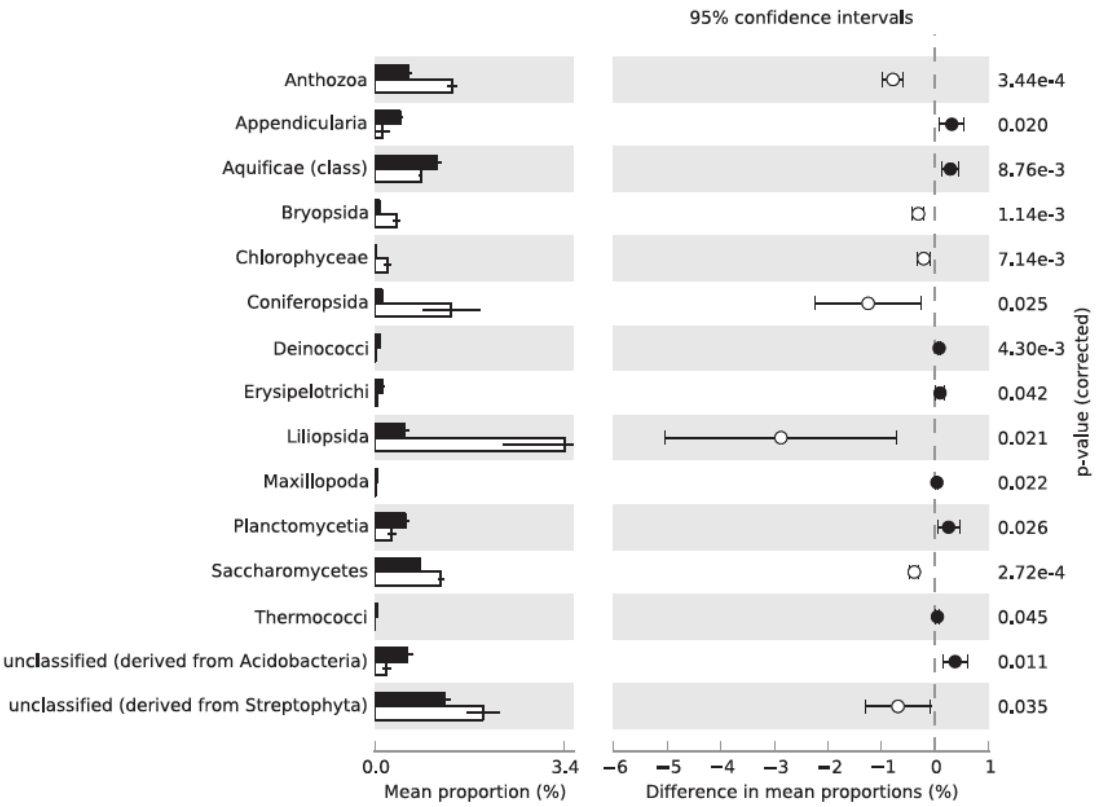
a)



b)



c)


95% confidence intervals
Anthozoa
Appendicularia
Aquificae (class)
Bryopsida
Chlorophyceae
Coniferopsida
Deinococci
Erysipelotrichi
Liliopsida
Maxillopoda
Planctomycetia
Saccharomycetes
Thermococci
unclassified (derived from Acidobacteria)
unclassified (derived from Streptophyta)
3.44e-4
0.020
8.76e-3
1.14e-3
7.14e-3
0.025
4.30e-3
0.042
0.021
0.022
0.026
2.72e-4
0.045
0.011
0.035
p-value (corrected)
0.0
3.4
Mean proportion (%)
−6
−5
−4
−3
−2
−1
0
1
Difference in mean proportions (%)

d)

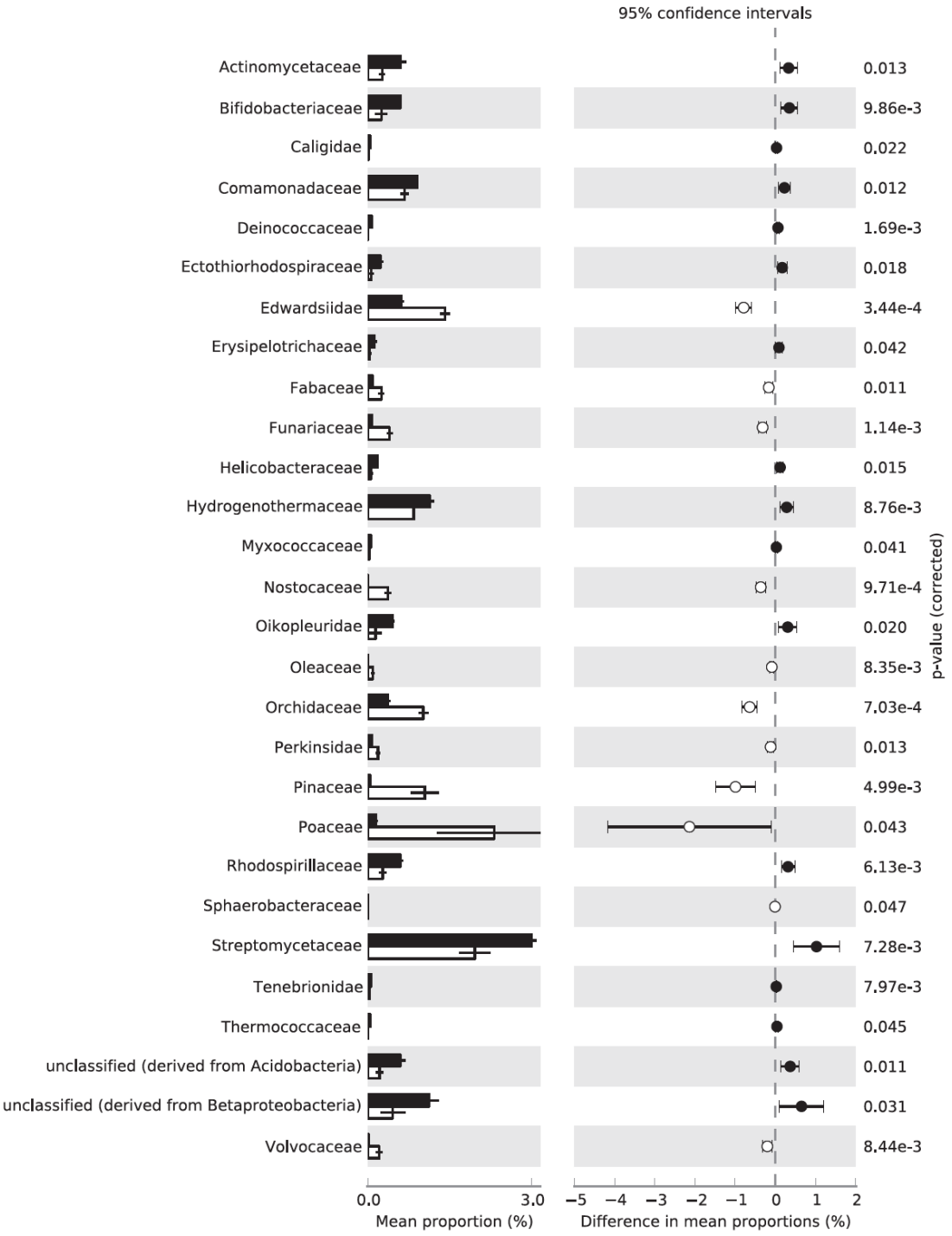


Figure 4.4: Community shifts in actively transcribing organisms at the soil surface after 21 days incubation under light (open bar) and dark (closed bar) conditions. The relative abundances of transcripts assigned using the RefSeq protein database at default setting are shown at the taxonomic level of; (a) domain; (b) phylum; (c) class, and; (d) family. Corrected p-values and confidence intervals were created using STAMP. Error bars are ±1 S.E.

4.4.7.2. Functional annotation of transcripts from the soil surface across all samples

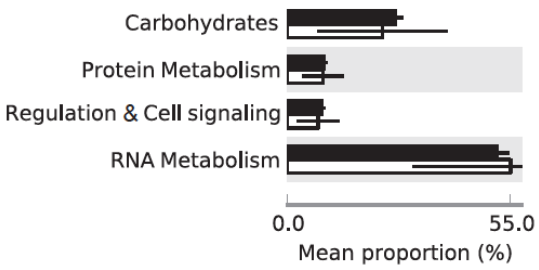
Functional annotation has been presented as a series of Levels specified by the Subsystems database utilised by MG-RAST, namely Level 1, Level 2, Level 3, and Function Level. Level 1 provides the broadest description of functional classification, and this becomes more specific down the classification Levels. The final level, termed Function, can provide an enzyme commission number to highlight the specific reaction the enzyme catalyses.

4.4.7.2.1. Primary and secondary functions at the soil surface

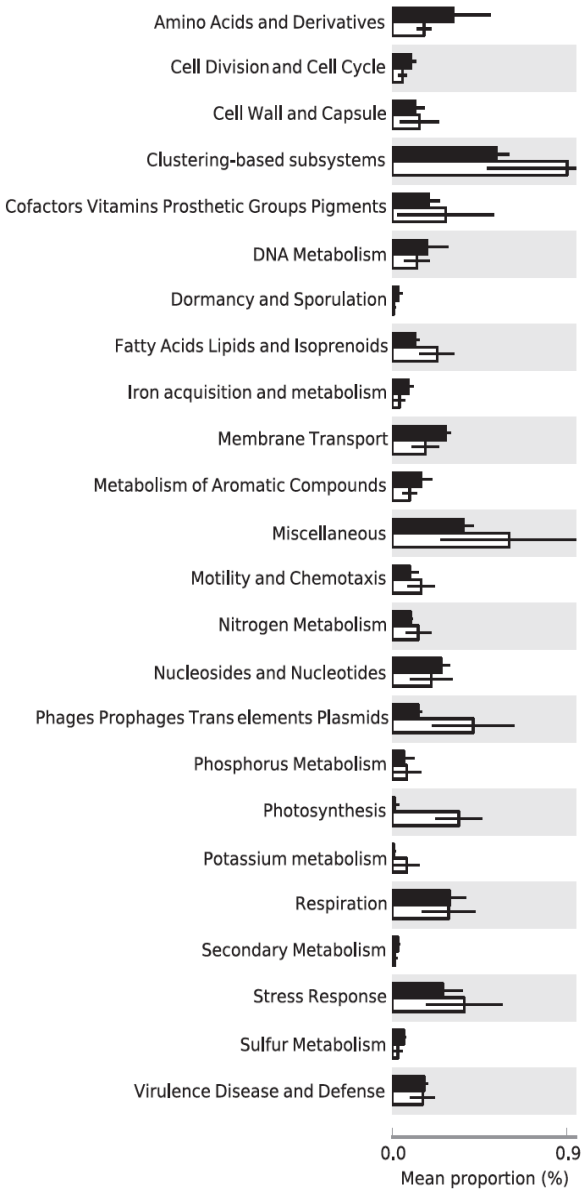
Approximately 95.6% (71, 902 reads) of all transcripts were assigned to four primary MG-RAST Level 1 functions, (>1% of transcripts), the relative abundance of which were ranked in the following order: RNA metabolism (53.4%) > carbohydrates (25.9%) > protein metabolism (8.9%) > regulation and cell signalling (8.2%) (Figure 4.5a). The remaining 4.4% (3, 309 reads) transcripts were assigned to 22 secondary functions (<1% of transcripts), including respiration, secondary metabolism, cell division and cycling, iron acquisition and metabolism, metabolism of aromatic compounds, photosynthesis, membrane transport, and sulphur, potassium, phosphorous and nitrogen metabolism (Figure 4.5b).

A similar trend was observed at the MG-RAST Function Level with 77.3% (58 183 reads) of transcripts assigned to five primary functions, and the remaining 22.7% (17, 072 reads) of transcripts being assigned to 829 secondary functions. The five primary functions were ranked in the following order: histone acetyl transferase ELP3 family (26.7%, RNA metabolism) > retron-type reverse transcriptase (26.3%, RNA metabolism) > Ribokinase (E.C.2.7.1.15 (8.2%, carbohydrates) > [NiFe] hydrogenase metallocentre assembly protein HypC (8.1%, protein metabolism) > heptaprenyl disphosphate synthase component I (E.C.2.5.1.30 (8.0%, regulation and cell signalling) (Figure 4.5c).

a)



b)


Amino Acids and Derivatives
Cell Division and Cell Cycle
Cell Wall and Capsule
Clustering-based subsystems
Cofactors Vitamins Prosthetic Groups Pigments
DNA Metabolism
Dormancy and Sporulation
Fatty Acids Lipids and Isoprenoids
Iron acquisition and metabolism
Membrane Transport
Metabolism of Aromatic Compounds
Miscellaneous
Motility and Chemotaxis
Nitrogen Metabolism
Nucleosides and Nucleotides
Phages Prophages Trans elements Plasmids
Phosphorus Metabolism
Photosynthesis
Potassium metabolism
Respiration
Secondary Metabolism
Stress Response
Sulfur Metabolism
Virulence Disease and Defense
0.0
0.9
Mean proportion (%)

c)

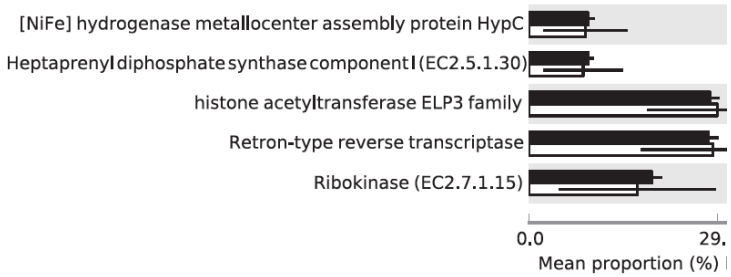


Figure 4.5: Soil surface functions after 21 days incubation under light (open bar) and dark (closed bar) conditions. The relative abundance of transcripts assigned using MG-RAST Subsystems database at default settings are shown for: (a) MG-RAST Level 1 primary functions; (b) MG-RAST Level 1 secondary functions, and (c) primary functions at MG-RAST Function Level. Error bars are ±1 S.E.

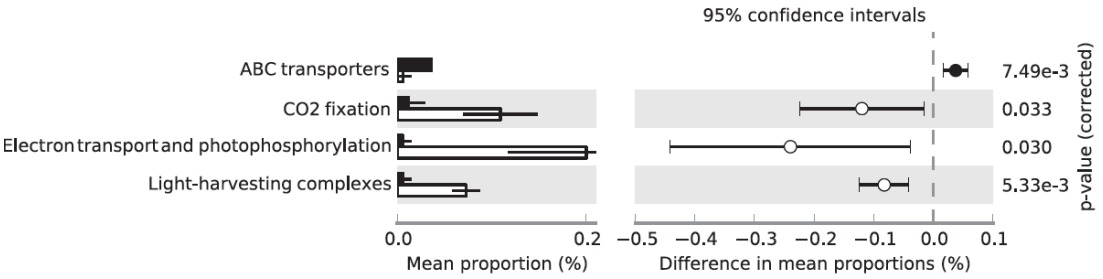
4.4.7.2.2. Functional differences at the soil surface under light and dark conditions

At MG-RAST Level 1, relative composition analysis revealed a significantly greater abundance of transcripts assigned to photosynthesis at the soil surface in the presence of light compared to in the dark ($p<0.05$). Several processes within the major function of photosynthesis were also shown to have significantly greater transcript abundances; MG-RAST Level 2 (CO_2 fixation, electron transport and phosphorylation, and light harvesting complexes), MG-RAST Level 3 (Calvin-Benson cycle, photosystem I and phycobilisome), and at MG-RAST Function Level (phycobilisome rod linker polypeptide (phyocyanin associated) under light compared to dark treatments ($p<0.05$) (Figures 4.6a-4.6c).

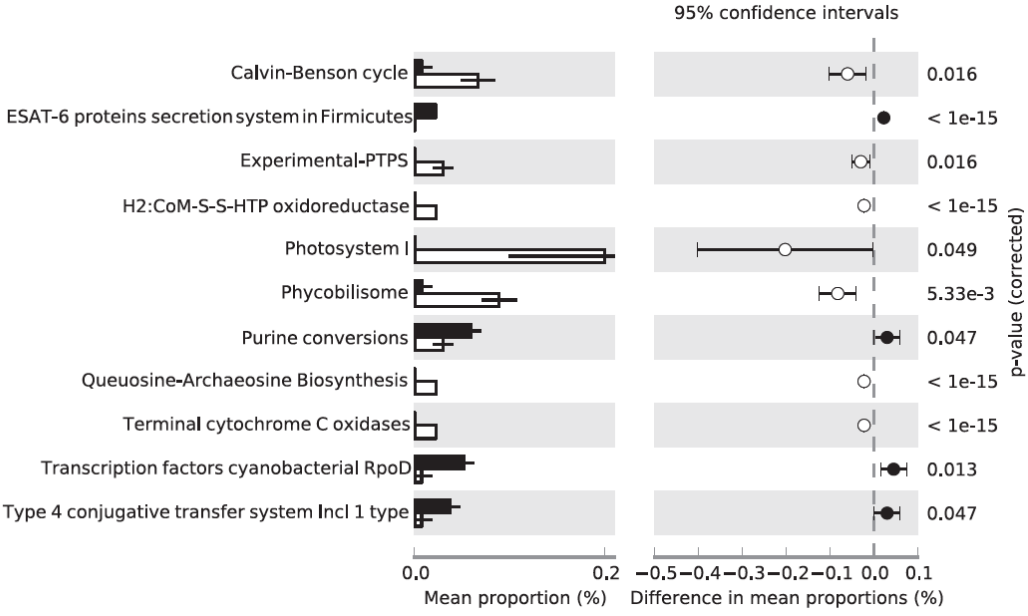
Processes classified as miscellaneous also had significantly greater relative transcript abundance in the light, such as experimental-PTPS ($p<0.05$), which are involved in folate and biopterin biosynthesis (EC 3.5.4.16), hydroxylation of phenylalanine to generate tyrosine (EC 1.14.16.1) and queuosine biosynthesis (Figure 4.6b). Lastly, queuosine-archaeosine biosynthesis, which are involved in RNA processing and modification (RNA metabolism) and terminal cytochrome C oxidase, which are involved in the electron accepting reactions of respiration had significantly greater relative transcript abundances in the light compared to dark conditions ($p<0.05$) (Figure 4.6b).

Under dark conditions, relative transcript abundance was greater for ABC transporters compared to light conditions ($p<0.05$) (Figure 4.6a). At MG-RAST Level 3, relative transcript abundance was higher for ESAT-6 protein secretion system in Firmicutes, which are involved in protein translocation across cytoplasmic membranes during membrane transport, transcription factors cyanobacterial RpoD-like sigma factors, which are involved in transcription during RNA metabolism, type 4 conjugative transfer system 1 type, which are involved in protein and nucleoprotein secretion system in membrane transport, and purine conversion under dark compared to light conditions ($p<0.05$) (Figure 4.6b). At MG-RAST Function Level, ammonium transporters (involved in nitrogen metabolism) and RNA polymerase sigma factor RpoD (involved in RNA metabolism) had a greater relative transcript abundance under dark compared to light conditions ($p<0.05$) (Figure 4.6c).

a)



b)



c)

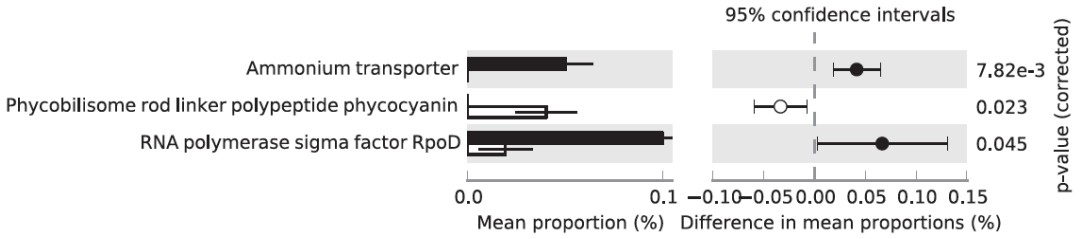


Figure 4.6: Relative transcript abundances at the soil surface after 21 days incubation under light (open bar) and dark (closed bar) conditions. The relative abundance of transcripts using MG-RAST Subsystems database at default settings are shown for: (a) MG-RAST Level 2; (b) MG-RAST Level 3, and; (c) MG-RAST Function Level.

Corrected p-values and confidence intervals were created using STAMP. Error bars are ±1 S.E.

4.4.7.3. Differences in predicted protein CRs at the soil surface under light and dark conditions

Out of 37, 387 predicted protein CRs, 576 and 448 had significantly greater relative transcript abundances under light and dark conditions, respectively ($p<0.05$). Out of these predicted protein CRs, only six had >1% of all transcripts assigned to that function under either light or dark conditions (Table 4.6).

Table 4.6: Relative transcript abundances of major predicted protein CRs at the soil surface under light and dark treatments

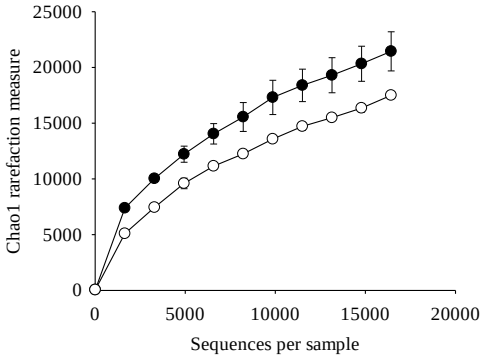
Function	Transcripts assigned to protein CRs (%)		p-value
	Light	Dark	
Protein CR 1	1.64	6.26e-05	0.01
Protein CR 2	1.67	3.08e-05	0.02
Protein CR 3	1.46	0.64	0.01
Protein CR 4	0.94	1.90	0.0007
Protein CR 5	3.11	0.29	0.006
Protein CR 6	2.05	8.46e-06	0.011

4.4.7.4. Functional and taxonomic diversity of soil surface communities under light and dark conditions

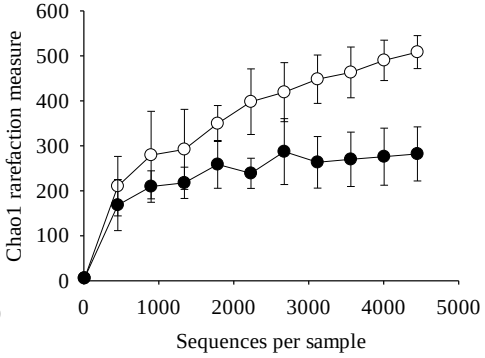
4.4.7.4.1. Alpha diversity patterns

The Chao1 diversity value of predicted protein CRs at the soil surface was significantly greater under dark compared to light conditions, with values of 21, 445±S.E 1, 757 and 17, 500±S.E 224, respectively ($p<0.05$) (Figure 4.7a). In contrast, the Chao1 diversity of assigned functions was significantly higher at the soil surface under light (508±S.E 37) compared to dark (282±S.E 60) conditions ($p<0.001$) (Figure 4.7b). These differences were primarily due to photosynthesis (Figure 4.7c), stress response (Figure 4.7d) and motility and chemotaxis (Figure 4.7e), which all showed elevated functional alpha diversity under light compared to dark conditions. The functional diversity of core functions (present in ≥50% of samples) differed little in their Chao1 diversity values under light and dark conditions, with scores of 92±S.E 26 and 73±S.E 15, respectively at a maximum sampling depth of 3, 799 sequences (Figure 4.7f). However, satellite functions (present in <50% of samples) had proportionally greater Chao1 diversity scores of 1, 207±S.E 195 and 537±S.E 223 at a maximum sampling depth of 115 sequences under light and dark conditions, respectively and differed considerably between treatments (Figure 4.7g). Lastly, the Chao1 values of actively transcribing organisms were almost identical under light and dark conditions at 262±S.E 18 and 245±S.E 11 under light and dark conditions, respectively (Figure 4.7h).

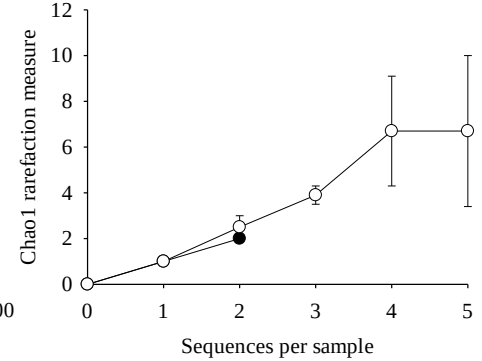
a) Predicted protein CRs



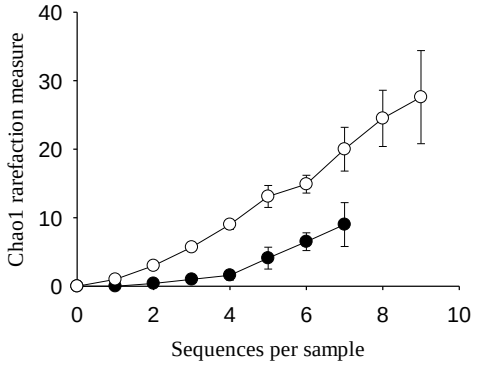
b) Assigned functions



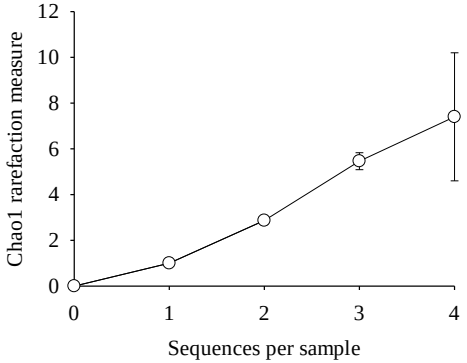
c) Photosynthesis



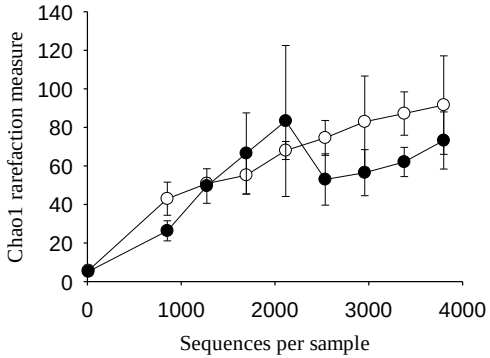
d) Stress response



e) Motility and chemotaxis



f) Core functions



g) Satellite functions h) Taxonomy

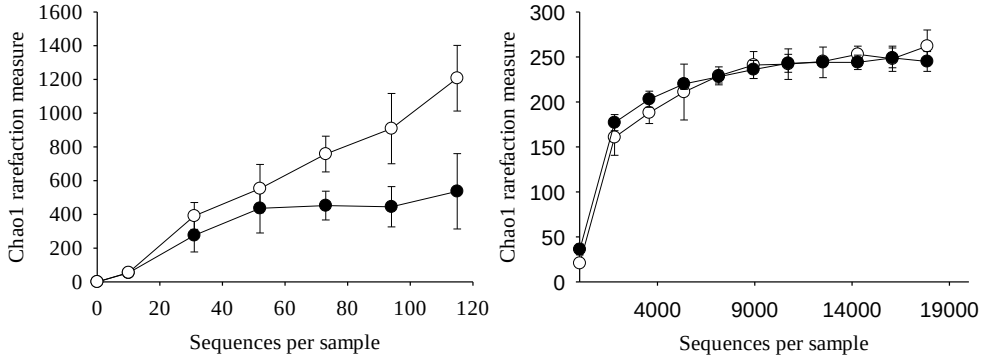
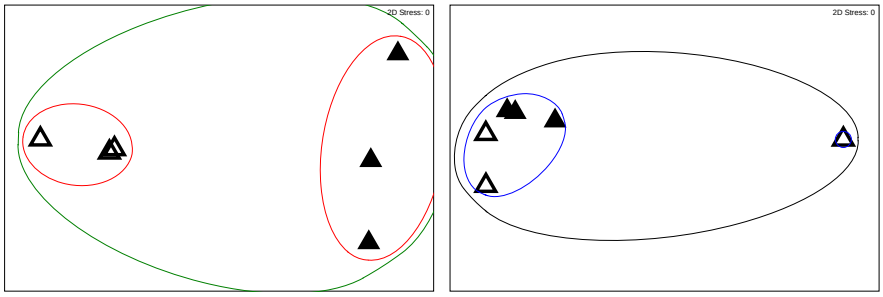


Figure 4.7: Chao1 functional alpha diversity at the soil surface after incubation under light (open symbols) and dark (closed symbols) conditions for 21 days. Chao1 diversity values were calculated for a number of data types: (a) Predicted protein CRs; (b) Functions assigned using Subsystems database in MG-RAST; (c) photosynthesis; (d) stress response; (e) motility and chemotaxis; (f) Core functions (present in ≥50% of samples) assigned using Subsystems database in MG-RAST; (g) Satellite functions (present in <50% of samples) assigned using Subsystems database in MG-RAST, and; (h) Actively transcribing organisms using RefSeq protein database in MG-RAST.

4.4.7.4.2. Beta diversity patterns

NMDS plots show that triplicate samples of predicted protein CRs were more closely clustered under light compared to dark conditions (Figure 4.8a). In contrast, after taxonomical and functional assignment using MG-RAST, NMDS plots show that triplicate samples are more closely clustered under dark compared to light conditions (Figures 4.8b-4.8c).

a) b)



c)

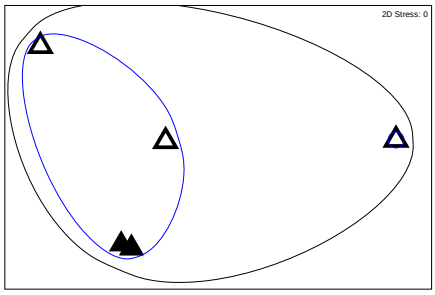


Figure 4.8: Ordination plots derived from non-metric multidimensional scaling analysis of Bray-Curtis distances of functions and actively transcribing organisms at the soil surface after incubation under light (open symbols) and dark (closed symbols) conditions. Beta diversity values were calculated for a number of data types: (a) Predicted protein coding regions; (b) Functions assigned using Subsystems database in MG-RAST, and; (c) Taxonomic annotation of transcripts using the RefSeq protein database in MG-RAST. Clustering is highlighted at a number of similarity levels: 20% (green), 35% (red), 45% (black) and 70% (blue).

4.4.7.5. Relative transcript abundances of key nitrogen cycle genes at the soil surface under light and dark conditions

The relative transcript abundances of *nifH*, *amoA*, *nirS*, *nirK*, *narG*, *napA*, *nosZ* and *norB* genes were assessed at the soil surface under light and dark conditions (Table 4.7). There were no significant differences between transcript abundances of genes involved in the soil N cycle under light and dark conditions. Very few reads were assigned to each of the genes, for example, the maximum number of reads assigned to a gene involved in the soil N cycle was the *amoA* gene in dark sample 3, which had five reads assigned (Table 4.7). Several genes received no hits such as *nirS* and *norB*, and others only received one, such as *nifH* and *nosZ*.

Table 4.7: Transcript abundances assigned to key steps in the soil nitrogen cycle

Function	**Form**	**Gene**	**Light**			**Dark**		
			1	**2**	**3**	**1**	**2**	**3**
Nitrogen fixation	N_2	*nifH*	0	0	1	0	0	0
Ammonia oxidation	NH_4^+	*amoA*	1	1	0	1	1	5
Nitrite reduction	NO_2^-	*nirK*	1	1	1	0	0	0
		nirS	0	0	0	0	0	0
Nitrate reduction	NO_3^-	*narG*	1	0	0	1	0	0
		napA	3	2	1	3	0	1
Nitrous oxide reduction	N_2O	*nosZ*	1	0	0	0	0	0
Nitric oxide reduction	NO	*norB*	0	0	0	0	0	0
No. reads			**35019**	**66915**	**40947**	**37750**	**20881**	**50937**

4.5. DISCUSSION

Phototrophs were significantly more abundant at the soil surface under light compared to dark conditions ($p<0.001$) (Figure 4.1). Light was found to select for actively transcribing phototrophic eukaryotes at the soil surface, such as Chlorophyceae and Coniferopsida, in addition to heterotrophic eukaryotes such as Saccharomycetes (Figure 4.4c) and diazotrophic cyanobacteria such as Nostocaceae (Figure 4.4d). The fact that light selected for photosynthesis and that the relative transcript abundances of functions associated with heterotrophs were greater in the dark suggests different primary metabolic strategies between the two treatments (Figure 4.6). Very few transcripts were assigned to key genes involved in the nitrogen cycle, and therefore any potential differences between treatments could not be detected (Table 4.6). Although annotated functions and predicted protein CRs produced contrasting results, it is thought that the selection pressure exerted by light caused a reduction in functional alpha and beta diversity compared to in the dark; as shown by the analysis predicted protein CRs (Figure 4.7 and 4.8). Therefore, use of predicted protein CRs are thought to overcome database bias introduced during the analysis of annotated functions.

4.5.1. Actively transcribing organisms

Relative composition analysis showed important differences in actively transcribing organisms between light and dark systems. Both light and dark systems were dominated by actively transcribing bacteria, which is consistent with other soil

metatranscriptomes (Urich *et al*. 2008; de Menezes *et al*. 2012, Tveit *et al*. 2012) (Figure 4.3a). However, it is important to note that taxonomic and functional binning has been shown to be problematic due to the unequal representation of bacterial reference genomes/transcriptomes compared to those of eukarya or archaea (Urich *et al*. 2008). The fact that the majority of transcripts in our study were not taxonomically binned further highlights the uncertainty of interpreting our system as bacterially dominated.

Although bacterial transcripts dominate soil metatranscriptomes in the literature, the proportion of reads assigned to the domain differ considerably, which could be due to differences in intrinsic soil characteristics. For example, transcripts from a soil nearby an Irish timber facility were overwhelmingly assigned to bacteria (95%) (de Menezes *et al*. 2012) whereas bacterial transcripts comprised only ~70% of total reads in peatland soil (Urich *et al*. 2012), with boreal soil (Urich *et al*. 2008) and the temperate pasture soil used in the present study lying halfway with ~80% of transcripts assigned as bacterial (Figure 4.4a). To some extent this was also evident for major bacterial phyla, with our system showing similarity (Figure 4.4b) to that of boreal soil with ~24% of transcripts assigned to Proteobactera (Urich *et a*l. 2008) compared to ~40% in peatland soil and soil in close proximity to a timber facility (de Menezes *et al*. 2012; Tviet *et al*. 2012). The difference in the proportion of transcripts assigned to bacteria were generally made up by the proportion of transcripts assigned to eukarya, with the relative read abundance assigned to archaea remaining relatively constant at ~1% in all studies (Urich *et al*. 2008; de Menezes *et al*. 2012, Tveit *et al*. 2012) (Figure 4.3a).

Tveit *et al*. (2012) suggested that bacterial energy channels were more important than the fungal energy channel in peatland soil after showing that a greater

proportion of rRNA reads were assigned to bacteria compared to fungi. By applying the same rationale to taxonomically assigned mRNA reads, the eukaroytic energy channel may be more important in light compared to dark incubated surface soil in the current study (Figure 4.4a). Further, elevated RNA yields and bacterial copy numbers under light compared to dark incubated soil suggests elevated microbial and bacterial activity, respectively. In contrast, relative composition analysis suggests that the bacterial and archaeal energy channels were significantly more important under dark compared to light conditions (Figure 4.4a). Plants such as Streptophyta and fungi, particularly within the division Ascomycota, were shown to be the dominant actively transcribing eukaryotic taxa under both treatments (Figure 4.4b). This was consistent with other studies that showed fungi were among the dominant eukaryotic organisms (Urich *et al*. 2008; de Menezes *et al*. 2012) but contrasts with peatland soil where protists were the dominant eukarya (Tveit *et al.* 2008).

At the Class level, light selected for actively transcribing phototrophs such as Plantae (Lilopsida and Coniferopsida), Bryophytes (Bryopsida), green algae (Chlorophyceae), Anthozoa (Cnidarian), Protists (Perkinsida), in addition to heterotrophs such as Fungi (Saccharomycetes) and Cnidaria (Anthozoa) (Figure 4.4c). The selection of light for heterotrophs could be due to the development of phototroph-heterotroph symbioses, for example, lichen symbiosis. Further study should focus on how climate, soil type or environmental/geographic distance influences soil function and the proportion of actively transcribing eukarya and archaea, as these factors will have important implications for managing soil energy channels, and ultimately ecosystem services.

4.5.2. Soil surface functions

As expected, relative transcript abundance of photosynthesis, a light driven process, was significantly greater under light compared to dark conditions (Figure 4.6). This expected difference served as an important control as it assigned greater confidence to other differences detected in the transcriptional profiles of light and dark incubated soil surfaces. Of the 26 major functions assigned to the soil surface, only four were classified as primary functions (>1% of reads), namely carbohydrates, protein metabolism, regulation and cell signalling and RNA metabolism, and these did not differ statistically between light and dark incubated soil (Figure 4.5a). Both light and dark systems were dominated by reads assigned to RNA metabolism (~ 53%), which differed markedly with other soil metatranscriptomes, which showed protein metabolism to be the dominant function (Urich *et al*. 2008; de Menezes *et al*. 2012). For example, an assessment of the impact of phenanthrene on soil function found ~25% of reads assigned to protein metabolism, and only ~7% assigned to RNA metabolism (de Menezes *et al*. 2012). Further, transcripts assigned to regulation and cell signalling were typically ~1% of total reads, however, in our system ~8% of transcripts were assigned to this major function (Urich *et al*. 2008; de Menezes *et al*. 2012) (Figure 4.5a).

By applying our primary function classification system to published metatranscriptomes, a far broader spread of primary functions was found (Urich *et al*. 2008; de Menezes *et al*. 2012). For example, there would be 22 primary functions in the system used by de Menezes *et al*. (2012), compared to only four in our system (Figure 4.5a). The fact that soil in our system and de Menezes' system were both sieved and lab incubated under similar controlled conditions suggests that soil

primary functions may be heavily influenced by soil type and sampling location, even after incubation.

Although light and dark incubated soil showed similar primary functions, there is evidence to suggest differences in microbial metabolism pathways between the two systems. For example, relative composition analysis revealed a significantly greater abundance of transcripts assigned to photosynthesis in light systems which suggests a selection for autotrophy (Figure 4.6), whereas the significantly greater transcript abundance assigned to ABC transporters (Figure 4.6a) in the dark suggests that heterotrophy was the dominant form of microbial metabolism. ABC transporters are often used for the transport of substrates across membranes for use in growth and division (Higgins, 2001), and therefore a significantly greater relative transcript abundance of ABC transporters under dark compared to light conditions provides support for microbial metabolism in dark incubated soil being heterotrophic. In addition, significantly greater relative transcript abundances assigned to several membrane transporters, such as ESAT-6 protein secretion system in Firmicutes and type 4 conjugative transfer system 1 type, as well as the conversion of organic purine also supports this hypothesis (Figure 4.6b).

Although there were differences in microbial metabolism between light and dark incubated systems, no differences in relative transcript abundances were found for genes involved in the N cycle (Table 4.6). The fact that actively transcribing diazotrophic organisms, such as Nostocaceae (Figure 4.4d) were selected for in the presence of light suggests that N_2 fixation was greater at the soil surface under light compared to dark conditions. Indeed, desert soil research has shown N_2 fixation and ammonia oxidation to be greater in the top few millimetres of soil compared to the underlying bulk soil when harboring diazotrophic cyanobacteria such as *Nostoc* spp.

(Yeager *et al*. 2004; Johnson *et al*. 2005). In the current study, an accurate comparison could not be made between treatments as very few transcripts were assigned to genes involved in nitrogen fixation, ammonia oxidation, and the reduction of nitrate, nitrite, nitric oxide and nitrous oxide (Table 4.6). Therefore, an absence of selection for nitrogen fixation may be a product of sequencing depth, and perhaps future soil metatranscriptomic work would benefit from prioritising the read numbers offered by other sequencing platforms, rather than the greater read length offered by 454-pyrosequencing, which has been used in published works to date (Urich *et al*. 2008; de Menezes *et al*. 2012; Stewart *et al*. 2011b; Tveit *et al*. 2012). The requirement for greater sequencing depth was also supported by a lack of plateau shown for the functional alpha diversity of predicted protein CRs (Figure 4.7a).

Metatranscriptomic research is currently limited by the lack of functional and taxonomical annotation. Indeed, only 75, 211 out of 252, 449 (~29%) transcripts were functionally binned (Table 4.4). Large differences between light and dark treatments could therefore be missed simply due to a lack of annotation. To overcome this issue, the 184, 299 (~73%) transcripts assigned to predicted protein CRs were investigated. This revealed far greater functional differences between soil surface communities incubated under light and dark conditions, compared to data based on annotated functions (Table 4.6). The use of predicted protein CRs therefore highlighted the drawbacks of current metatranscriptomic research, as well as its future potential

4.5.3. Functional alpha and beta diversity

The study of functional alpha diversity has become increasingly common for microbial ecologists who have attempted to link it to both taxonomic and phylogenetic diversity (Fierer *et al*. 2012) and primary production (Raes *et al*. 2011). For example, Fierer *et al*. (2012) recently showed a significant positive correlation between taxonomic/phylogenetic diversity and functional alpha diversity and suggested that, in some cases, functional gene categories could be predicted from taxonomic/phylogenetic diversity.

We have shown a doubling of functional alpha diversity at the soil surface under light compared to dark conditions (Figure 4.7b), which is due in part to a greater diversity of photosynthesis, stress response and motility and chemotaxis related functions in the light (Figure 4.7c-4.7e). In contrast, the alpha diversity of actively transcribing organisms was almost identical between light and dark incubated soil (Figure 4.7h). Functional alpha diversity patterns between annotated functions and predicted protein CRs yielded contrasting results, and therefore these results require careful interpretation (Figure 4.7a-4.7b). Indeed, current database bias could be responsible for the appearance of functional alpha diversity being significantly higher under light compared to dark conditions. For example, this result could simply be a consequence of a selection for well annotated functions in the light, such as photosynthesis (Figures 4.6 and 4.7c).

The use of predicted protein CRs was used to overcome the inherent bias of current databases. Analysis of predicted protein CRs showed a significant reduction in functional alpha diversity under light compared to dark conditions, which suggests

that light exerted a selection pressure for specific functions (Figure 4.7a). Similarly, lower functional beta diversity under light compared to dark conditions also suggests that light selected for specific functions at the soil surface (Figure 4.8). The fact that known functions and predicted protein CRs produced contrasting results suggests that taxonomic and ecosystem predictions based on functional diversity should be made carefully. However, the use of metagenomic and metatransciptomic work has huge potential and future work will no doubt shine light on some of the most fundamental microbial ecology questions, such as why microorganisms live where they do and how they will respond to environmental perturbation.

In conclusion, light selected for actively transcribing photosynthetic and heterotrophic eukarya, as well as diazotrophic cyanobacteria. Similar differences in genes involved in the N cycle were not detected between treatments, however, this could be due to insufficient sequencing depth. Differences in microbial metabolic strategies were also shown, with light and dark conditions being dominated by autotrophy and heterotrophy, respectively. Finally, assessment of functional alpha and beta diversity using annotated functions and predicted protein CRs produced contrasting results, however, the selection pressure of light reduced both functional alpha and beta diversity compared to dark systems.

5. CHAPTER V: GENERAL DISCUSSION

5.1. GENERAL OVERVIEW OF FINDINGS

This work has shown that non-UV light impacts CPP degradation, and microbial community structure and function in an aerobic soil environment. This has a range of implications for CPP regulatory studies and our understanding of C and N dynamics in agricultural systems. In all experiments, phototroph growth was stimulated by the presence of light, but was not detected under dark conditions. This suggests that phototrophs play an important role in the impact of light on the soil environment.

Light was found to significantly impact degradation rates for six out of eight CPPs tested. The $DegT_{50}$ and $DegT_{90}$ of the fungicide benzovindiflupyr and the herbicide chlorotoluron were halved under light compared to dark conditions, respectively. Further, prometryn, imidacloprid and fludioxonil all showed significantly faster degradation rates under light compared to dark conditions. NERs were also shown to increase under light compared to dark conditions for seven CPPs. In contrast, cinosulfuron degraded significantly slower under light compared to dark conditions, and there were no differences in the formation of NERs. The fact that there was a significant growth of phototroph communities during the degradation of all CPPs tested (except cinosulfuron) suggests that phototrophs could directly or indirectly impact CPP degradation.

Direct degradation of CPPs by phototrophs was not tested in this project, however, several CPPs had previously been shown to be degraded by phototrophs (Cai *et al*. 2007; Caćeres *et al*. 2008; Mostafa and Helling; 2011; Thomas and Hand,

2012). Several indirect impacts of light on chemical and biological soil properties were shown, for example, significant shifts in soil pH, extractable nitrate and magnesium, phototroph, bacterial and fungal community structure, actively transcribing taxa, and soil function were all detected at the soil surface incubated under light compared to dark conditions. The fact that bacterial, fungal and archaeal copy number did not differ between light and dark incubated systems suggested that light did not impact microbial abundance during CPP degradation.

The impact of light on chlorotoluron degradation was further tested in Chapters III and IV using open incubation systems that limited the development of phototrophs to the surface (Appendix IV). Chlorotoluron was first applied to the soil surface following the development of soil surface communities over 85 days under light and dark conditions. The presence of light did not impact chlorotoluron degradation rates (Appendix IV; Table 1). However, only a single sampling point was taken to assess chlorotoluron degradation, and therefore a potential effect of light on degradation rates at earlier sampling points may have been missed. In addition, by confining phototroph development to the soil surface only ~17% of the total soil volume was exposed to light compared to ~33% in the closed system used in Chapter II. To remove these effects, the final chlorotoluron degradation experiment was designed such that ~33% of the total soil volume was exposed to light, and a timecourse of chlorotoluron degradation was plotted over six sampling points (Appendix IV; Figure 1). Again, the degradation rate of chlorotoluron was not impacted by light with $DegT_{50}$ values of 8 and 9 days under light and dark conditions, respectively (Appendix IV; Figure 1).

The fact that chlorotoluron degraded at similar rates under light and dark conditions in the two open systems used, but showed an accelerated rate of

degradation under light compared to dark conditions in the closed system suggests that the effect of light could be due to an artefact of the test system or variation in microbial communities at different sampling times. In particular, potential reductions in gaseous exchange in the closed system compared to the open system could be responsible for the effect of light. For example, in the closed system there could be a build up of oxygen fixed by phototrophs under light but not dark conditions. The elevated aerobic environment in the light could be responsible for accelerated degradation rates under light conditions by increasing the activity or biomass of important degrader organisms.

Alternatively, a lack of an effect of light on chlorotoluron degradation in open systems could be an artefact of accelerated degradation rates shown in these systems. Chapter II showed that light did not impact the $DegT_{50}$ but halved the $DegT_{90}$ under light compared to dark conditions. This suggested the importance of a lag phase whereby phototroph communities develop and subsequently impact chlorotoluron degradation rates. It may be that this important lag phase was missed in the open systems as chlorotoluron showed elevated rates of degradation, for example, ~97% had degraded after 30 days compared to ~88% after 61 days in the previous system.

In terms of soil biological properties, light was shown to have a significant and time-dependent impact on bacterial community structure at the soil surface. Overall, a reduction in alpha diversity showed that light selected for both phototrophs and heterotrophic bacteria at the soil surface compared to dark conditions. More specifically, light selected for diazotrophic cyanobacteria *Nostoc punctiforme*, *Anabaena variabilis* and *Anabaena cylindrica*, in addition to several heterotrophic bacteria, particularly within the family Bacillaceae. Further,

metatranscriptomic research showed that light also selected for actively transcribing diazotrophs (Nostocales). However, very few reads were assigned to genes involved in the N cycle and therefore relative transcript abundances of these genes did not differ between soil surfaces incubated under light and dark conditions.

Metatranscriptome analysis showed a clear selection for actively transcribing phototrophic eukaryotes at the soil surface, such as Chlorophyceae and Coniferopsida and the diazotrophic cyanobacterium Nostocaceae, in addition to heterotrophic eukarya such as Saccharomycetes. Relative transcript analyses suggested a preference for autotrophy and heterotrophy in light and dark incubated samples, respectively. Together, these results suggest that autotrophy and the eukaryotic energy channel are more important in soil exposed to light. Finally, the fact that metatranscriptomic work (RNA) showed a clearer selection for fungi than amplicon work (DNA) suggests that light has a more pronounced influence on active fungal communities.

5.2. BROAD IMPLICATIONS OF WORK AND FUTURE QUESTIONS

The following section describes the implications of this work for the wider research environment, in terms of CPP regulatory studies, the fate of CPPs in the environment, and agricultural management practices.

5.2.1. Laboratory-based crop protection product regulatory studies

Laboratory-based CPP regulatory studies are conducted in the dark, which prevents the development of phototrophs. Although the majority of the soil environment is not exposed to light (Benvenuti, 1995), a CPP's first point of contact with the soil environment is the soil surface. This compartment has been shown to have distinct physical, chemical and biological properties as a result of direct and indirect effects of light (Belnap and Gillette, 1997; Chapters II-IV). Chapter II also showed that light could significantly impact CPP degradation rates and the formation of NERs in an aerobic soil environment, which provides support for the inclusion of light in CPP regulatory studies. The fact that light also impacts soil properties provides further support for the inclusion of light in regulatory studies that assess CPP degradation in a soil environment. However, the lack of a 'light effect' shown in two additional experiments (Appendix IV; Table 1 and Figure 1) showed that the effect was not reproducible under different test systems. Additional work is required to test the effect of light on CPP degradation and its reproducibility. It may be prudent to consider the inclusion of light to OECD guideline 307 alongside studies that are conducted under dark conditions to more effectively simulate the different fractions of the soil environment.

In a broader sense, this work has important implications for the relevance of current laboratory-based regulatory studies to an agricultural environment, particularly OECD guideline 307. Beulke *et al.* (2000) found that laboratory tests and simulation models overestimated and underestimated actual pesticide persistence in the field by a factor of >1.25 in 44% and 17% of studies, respectively. Modification of OECD guideline 307 to consider a greater range of environmental parameters that

are currently omitted from tests could reduce the frequency and magnitude of the overestimation of CPP persistence in the field. For the five CPPs that showed accelerated degradation rates in the light, CPP persistence was overestimated by a factor of 1.53 on average (Table 5.1). Therefore, inclusion of light to OECD tests could contribute to reducing the disparity between $DegT_{50}$ values under laboratory and field conditions.

Table 5.1: Estimated differences in the persistence of crop protection products between light and dark conditions

CPP	$DegT_{50}$ light	$DegT_{50}$ dark	Difference in $DegT_{50}$ estimation under dark compared to light conditions
Prometryn	18.9	20.4	>1.08
Cinosulfuron	29.4	23.7	<1.24
Lufenuron	17.1	16.4	<1.04
Fludioxonil	59.1	100.6	>1.70
Imidacloprid	150.4	199.2	>1.32
Propiconazole	51.6	49.3	<1.04
Benzovindiflupyr	183	373	>2.03
Chlorotoluron	10	15	>1.50

Benzovindiflupyr and chlorotoluron $DegT_{50}$ values were taken from the time course studies in Chapter II. The $DegT_{50}$ of the remaining CPPs were estimated by calculating daily degradation rates based on degradation by single first order kinetics.

Soil temperature (Ghardiri *et al*. 1995; Dungan *et al*. 2001; Alletto *et al*. 2006; Benimeli *et al*. 2007; Arshad *et a*l. 2008; Bouseba *et al*. 2009) and moisture

content (Ghardiri *et al*. 1995; Cattaneo *et al*. 1997; Garcia-Valcarcel & Tadeo, 1999; Alletto *et al*. 2006; Schroll *et al*. 2006; Bouseba *et al*. 2009) have previously been shown to impact CPP degradation rates. However, the impact of fluctuating temperature and/or moisture contents that occur diurnally in agricultural environments are not well established. Both are important factors determining the frequency and magnitude of wet-dry cycles, which impact both fungal communities (Gordon *et al*. 2008) and active bacterial communities (Placella *et al*. 2012). However, preliminary work has shown that the $DegT_{50}$ of cyanazine and bentazone were similar under constant and fluctuating temperature/moisture conditions, and under static water and flowing water conditions (Beulke *et al*. 2005). Further work is required to confirm the effect of fluctuating temperature/moisture conditions using additional CPPs and soils.

Sieving has also been shown to impact fungal communities (Petersen and Klug, 1994) and CPP persistence (Beulke *et al*. 2005; Bending and Rodríguez-Cruz, 2007). Beulke *et al*. (2005) showed that the $DegT_{50}$ of bentazone was lowered from ~58 days in soil sieved to 5 mm compared to ~38 days in soil sieved to 3 mm. Further, isoproturon degradation rates have been shown to be slower in intact cores compared to sieved soil (Bending and Rodríguez-Cruz, 2007). These results suggest that sieving soil prior to conducting degradation studies could underestimate the persistence of some CPPs in an agricultural environment.

More general points relate to soil type and depth of sample collection. OECD 307 states that degradation rates must be determined in at least four soil types that the CPP is likely to be applied to. However, extrapolation of degradation results from reference soils to rare soil types such as highly acidic soils may not be accurate. Soil pH has been shown to impact CPP degradation rates, with faster rates typically

shown as pH nears neutral (Awasthi *et al*. 2000; Kumar and Philip, 2006; Singh *et a*l. 2006; Benimeli *et al*. 2007). Therefore, CPPs are likely to show slower rates of degradation in highly acidic soils, particularly for ionisable CPPs such as dicamba, metsulfuron-methyl, fluazifop-P, metribuzin, 2,4-D, flupyrsulfuron-methyl, fluroxypyr, terbutryn, pirimicarb, and fenpropimorph (Kah *et al*. 2007). This is particularly complex for CPPs that contain acid groups as an increase in pH could alter polarity, and therefore water solubility/bioavailability (U.S EPA, 1994).

Finally, OECD guideline 307 recommends that soil sampled from the A horizon or upper 20 cm is used. However, CPPs are known to leach to greater depths than 20 cm, particularly in cracking clay soils where a large volume of water can flow through macropores (Harris *et al*. 1994). Indeed, isoproturon, bentazone and mecoprop have all shown reduced degradation rates in subsoil (50-60 cm) compared to topsoil (0-15 cm) (Rodríguez-Cruz *et al*. 2006), with further research showing isoproturon and bentazone degradation rates to decline progressively in soil sampled at five depths between 0 and 80 cm (Bending and Rodríguez-Cruz, 2007). In contrast, Karpouzas *et al*. (2001) have shown greater rates of carbofuran degradation in subsoil compared to topsoil.

Overall, laboratory-based CPP regulatory studies are designed to mimic the agricultural environment the CPP will be exposed to. However, it is unlikely that a laboratory microcosm could effectively mimic the dynamic and heterogeneous agricultural environment. Therefore, increasingly sophisticated models are implemented to improve the assessment of CPP fate (Sweeney pers. comm). However, standardised changes to regulatory studies or higher tier testing could also improve the environmental realism of studies where appropriate, for example, using

packed soil cores sampled from the field, the inclusion of light, fluctuating temperature, continuous water-flow, and using soil sampled at a variety of depths.

5.2.2. The environmental fate of crop protection products in an agricultural environment

The impact of light on pesticide degradation and NER formation (Chapter II) could also have important implications for the fate of CPPs under agricultural cropping systems. Similar to laboratory-based regulatory studies, field-based regulatory guidelines remove phototrophs by soil tillage prior to CPP application (Oliver pers. comm.). Knapen *et al*. (2007) showed that phototrophs developed under the majority of 52 Winter wheat, maize and sugar beet fields spanning 300 ha of agricultural land in Belgium. Therefore, the removal of these phototroph communities prior to conducting an assessment of CPP fate may not be relevant to agricultural systems. It is important to note that the timing of application is also important as phototrophs may not have developed prior to pre-emergence applications.

Chapter II showed a significant increase in NER formation for seven out of eight CPPs tested under light compared to dark conditions. This could be due to faster rates of degradation and incorporation into biomass, or stronger sorption of parent material under light conditions. The latter could have important implications for an agricultural environment as it could translate to a greater retention of CPPs at the soil surface. Greater sorption of CPPs at the soil surface could result in faster degradation rates for CPPs that are susceptible to photolysis, or actively degraded by

phototrophs, such as the fungicide fludioxonil (Thomas and Hand, 2012). Conversely, stronger sorption could result in a reduction in bioavailability and consequently reduced CPP degradation rates. These effects are likely to be CPP specific and require further testing.

Phototroph communities could also impact CPP leaching rates and run-off into adjacent water bodies. Arid land research has shown improved water infiltration rates and reduced run-off in moss-dominated BSCs compared to soil without a BSC, which is thought to be due to high water absorption by mosses (Kidron *et al*. 2003; Zhang *et al*. 2009; Chamizo *et al*. 2012). In contrast, early successional crusts dominated by cyanobacteria have typically been shown to form a seal at the soil surface and reduce water infiltration rates (Chamizo *et al*. 2012; Kidron *et al*. 2012; Zaady *et al*. 2012). Provided similar trends are observed under agricultural cropping systems, the presence of cyanobacterial or moss dominated communities could result in elevated or reduced rates of CPP run-off, respectively. This is of particular relevance to highly water soluble CPPs commonly found in drinking water such as metaldehyde, carbetamide, 2,4-D, mecoprop-P and MCPA (Pesticides in the UK, 2011).

The effect of phototrophs on CPP degradation, sorption and run-off in an agricultural system has not been directly tested, however, the effect of conservation tillage on pesticide fate in relation to conventional tillage has been extensively studied. Conservation tillage covers a variety of practices, however, they are all characterised by crop residues covering a greater proportion of the soil surface than under conventional tillage. Although not directly relevant, these studies compare agricultural management techniques that are likely to result in the development of a range of soil surface communities, from conventional tillage where phototroph

communities may be removed by tillage annually, to conservation tillage where phototroph communities may be present. A detailed review of the impact of soil tillage on CPP degradation, dissipation and transfer concluded that effects were highly variable and can be contradictory (Alleto *et al*. 2010). Conservation tillage typically resulted in a build up of organic matter at the soil surface, which increased retention and decreased CPP bioavailability and pesticide degradation rates (Alleto *et al*. 2010). However, it is important to note that climatic and edaphic conditions were typically shown to be of greater importance to pesticide fate than the tillage practice. Future studies could directly test the impact of phototrophs at the soil surface on the degradation, sorption, transfer, and run-off of CPPs.

5.2.3. Nitrogen and carbon input in agriculture

Under light conditions, the soil surface selected for the diazotrophic cyanobacteria *N. punctiforme*, *A. variablis* and *A. cylindrica* (Chapter III), and actively transcribing diazotrophs (Nostocales) (Chapter IV) compared to dark conditions. Consequently, N_2 fixation could be higher at the soil surface when diazotroph communities are present, which could have important implications for the nutrient status of agricultural land in the presence or absence of these communities.

Arid land research has shown that BSCs dominated by Nostocales fix ecologically significant amounts of N_2 (Belnap, 2002; Yeager *et al*. 2004; Johnson *et al*. 2005; Zhao *et al*. 2010; Stewart *et al*. 2011*a*; Liu *et al*. 2012). Phototroph communities have also been shown to fix ~13-19 kg N ha^{-1} on unfertilised land under a temperate climate (Witty *et al*. 1979). Witty *et al*. (1979) showed that phototroph communities could substantially contribute to soil fertility, but could not fix

agriculturally significant amounts of N_2. Witty *et al*. (1979) also showed that an application of 48 kg N ha^{-1} resulted in a ~20% algal and cyanobacterial surface cover and a N_2 fixation potential of ~25-28 kg ha^{-1}, however, an application of 196 kg N ha^{-1} suppressed N_2 fixation and reduced algal and cyanobacterial surface cover to ~1%. Witty (1979) furthered this work by investigating the impact of liquid or dry sand inoculation of diazotrophic cyanobacteria *Nostoc ellipsosporum*, *A. cylindrica* and *N. punctiforme* on N_2 fixation and winter wheat yield. Similarly, all inoculants could fix ecologically significant, but not agriculturally relevant amounts of N_2.

Witty provided an excellent introduction to the potential for diazotrophs to fix N_2 in temperate field environments. However, maximum N_2 fixation potential of soil surface diazotroph communities was not fully tested as surface cover only reached a maximum of ~20%, and desiccation by a lack of crop cover for substantial times across the period of sampling would have considerably reduced N_2 fixation rates (Witty *et al*. 1979). Indeed, Austin *et al*. (2004) showed that N_2 inputs from BSCs were highly sensitive to pulsed rain events.

Relative composition analysis showed a significantly greater abundance of transcripts assigned to photosynthesis under light compared to dark conditions, in addition to a selection for a range of actively transcribing phototrophs (Chapter IV). This suggests that there would be a greater input of labile C at the soil surface under light compared to dark conditions. The presence of light significantly increased bacterial abundance at the soil surface, which could impact a range of downstream microbial processes. Indeed, arid land research has shown that BSCs have a greater net primary production potential than vascular plants (Yoshitake *et al*. 2010). It is important to quantify the input of C as well as N by phototrophs under agricultural

cropping systems as these communities could improve soil nutrient status and accelerate rates of C turnover.

5.3. FUTURE DIRECTION OF STUDY

This work highlights a range of areas for future research on the impact of light on CPP fate in laboratory and field systems, the development of phototrophs under agricultural cropping systems, and the impact of agricultural management on phototrophs and soil surface community function. Broad questions for future research include:

5.3.1. Is the effect of light on crop protection product degradation influenced by edaphic properties and climatic variables?

It is important to investigate if the impact of light on CPP degradation is generic to a range of soil types, or if it is specific to certain edaphic or climatic variables. Edaphic properties such as pH, organic matter and clay content, and climatic variables such as temperature and water content (Tables 1.1-1.5) have all been shown to influence CPP degradation rates, and they could also impact the effect of light on CPP degradation. It is also important to fully test the mechanisms responsible for the effect of light on CPP degradation by separating the direct effects of phototrophs, such as phototroph degradation of CPPs, from the indirect effects of phototrophs on the physical, chemical and biological properties of soil.

5.3.2. Which factors influence the development of phototrophic communities under agricultural cropping systems?

Phototrophs have been shown to develop under agricultural cropping systems 80 days following soil tillage (Knapen *et al*. 2007). It is important to determine the relative importance of factors that influence phototroph development in an agricultural environment. For example, is the development of phototrophs primarily determined by edaphic factors such as pH, nutrients, organic matter content, or soil texture and structure, or are climatic factors equally important, such as rainfall, wet-dry cycles, temperature, light intensity and hours of sunlight. Further, it is important to determine if edaphic properties, climatic variables or crop-type influence the composition of soil surface communities.

5.3.3. Does non-UV light influence crop protection product degradation and mobility in a field environment?

Non-UV light was shown to impact the rate of degradation for six out of eight CPPs tested. It is important to test if these results are repeated in a field environment, and under cropping systems. It is also important to test if the presence of soil surface communities impacts CPP mobility, and if this changes with the succession of phototroph communities.

5.3.4. How do soil management practices impact C and N fixation under agricultural cropping systems?

Research in arid and temperate environments has shown that soil surface communities are able to fix an ecologically significant proportion of N_2 (Witty *et a*l. 1979; Witty, 1979; Belnap, 2002; Yeager *et al*. 2004; Johnson *et al*. 2005; Zhao *et al*. 2010; Stewart *et al*. 2011*a*; Liu *et al*. 2012). Further testing is required to assess if these communities can fix agriculturally significant amounts of N_2 or C. Further research could assess how soil management practices such as tillage, fertiliser and pesticide application and crop rotation impact C and N_2 fixation. A direct quantification of N_2 fixation using the stable isotope ^{15}N is also missing from the literature, as studies to date have indirectly assessed N_2 fixation using the acetylene reduction assay. A carefully designed stable isotope probing experiment could determine which organisms are responsible for C and N_2 fixation and how these energy sources filter down through the microbial food-web (Radejewski *et al*. 2000)

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APPENDIX I

NON-UV LIGHT INFLUENCES THE DEGRADATION RATE OF CROP PROTECTION PRODUCTS

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Non-UV Light Influences the Degradation Rate of Crop Protection Products

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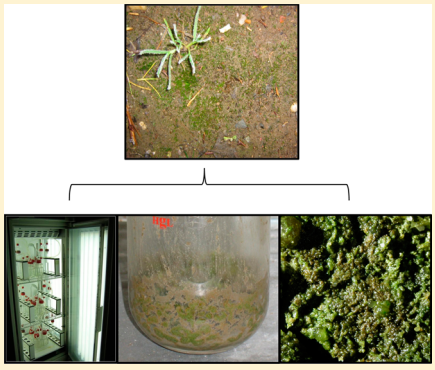
Supporting Information

ABSTRACT: Crop protection products (CPPs) are subject to strict regulatory evaluation, including laboratory and field trials, prior to approval for commercial use. Laboratory tests lack environmental realism, while field trials are difficult to control. Addition of environmental complexity to laboratory systems is therefore desirable to mimic a field environment more effectively. We investigated the effect of non-UV light on the degradation of eight CPPs (chlorotoluron, prometryn, cinosulfuron, imidacloprid, lufenuron, propiconazole, fludioxonil, and benzovindiflupyr) by addition of non-UV light to standard OECD 307 guidelines. Time taken for 50% degradation of benzovindiflupyr was halved from 373 to 183 days with the inclusion of light. Similarly, time taken for 90% degradation of chlorotoluron decreased from 79 to 35 days under light conditions. Significant reductions in extractable parent compound occurred under light conditions for prometryn (4%), imidacloprid (8%), and fludioxonil (24%) compared to dark controls. However, a significantly slower rate of cinosulfuron (14%) transformation was observed under light compared to dark conditions. Under light conditions, nonextractable residues were significantly higher for seven of the CPPs. Soil biological and chemical analyses suggest that light stimulates phototroph growth, which may directly and/or indirectly impact CPP degradation rates. The results of this study strongly suggest that light is an important parameter affecting CPP degradation, and inclusion of light into regulatory studies may enhance their environmental realism.



INTRODUCTION

Crop protection products (CPPs) have been an essential factor in improving crop productivity and food security; however, their inherent ability to reduce crop pests can potentially result in adverse environmental effects.[1] CPPs are therefore subject to a strict regulatory evaluation. The Organisation for Economic Co-operation and Development (OECD) provides an in depth description of the holistic processes involved in the risk assessment of CPPs, including, physicochemical properties, health effects, degradation and accumulation, and ecological risk assessment. An integral part of CPP risk assessment involves evaluating their toxicity,[2] toxicokinetics, and carcinogenicity[3] to humans and other nontarget organisms. Results are evaluated alongside investigations into the rate and route of compound transformation[4] to predict the concentration of CPPs in the environment and their overall risk.

The most important process in the environmental fate of CPPs is generally considered to be microbial degradation.[5] Prior to regulatory approval, the rate of degradation of CPPs and other organic chemicals in soil is determined using the OECD 307 test.[4] The test provides good reproducibility between samples, and allows the transformation, mineralization, and compartmentalization of the residual chemical to be determined with high precision. However, conditions may not be environmentally realistic; for example, in lab test systems temperature and moisture are kept constant, soil is sieved to 2 mm and homogenized, and soil is incubated in the dark.[4] Beulke et al. reviewed 178 studies and found that laboratory tests and simulation models overestimated actual pesticide persistence in the field by a factor of >1.25 in 44% of studies.[6] However, an underestimation by a factor of >1.25 was only found in 17% of studies.[6] It is likely that such discrepancies may be due to poor reproduction of environmental conditions in laboratory tests, and it is therefore essential to try and bridge the gap between laboratory and field studies. Improvement of the predictions of the persistence of CPPs should be possible by progressively adding complexity to laboratory systems in order to better mimic the natural environment.

A CPPs first point of contact with the soil environment is the soil surface. Therefore, laboratory degradation tests should aim to simulate the physical, chemical, and biological properties of the soil surface. In an agricultural field the soil surface is exposed to light; however, laboratory degradation tests are incubated in the dark.[4] The presence of light has been shown to drive the formation of a biological soil crust (BSC) composed

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Table 1. Selected Properties of the Crop Protection Products Investigated in This Study

name	type	$DegT_{50}$ (d)	k_{ow} (log P)	water solubility (mg/L)	K_{oc} (mL/g)	mode of action
prometryn	methylthiotriazine herbicide	14−158	3.1	33	400	photosynthetic electron transport inhibitor; inhibits oxidative phosophorylation
cinosulfuron	sulfonylurea herbicide	20	2.0	4000	20	inhibits biosynthesis of essential amino acids
chlorotoluron	urea herbicide	30−40	2.5	74	205	inhibitor of photosynthetic electron transport
propiconazole	triazole fungicide	29−70	3.7	100	1086	steroid demethylation inhibitor
benzovindiflupyr	triazole fungicide	N/A[a]	N/A[a]	N/A[a]	N/A[a]	succinate dehydrogenase inhibitor
fludioxonil	phenylpyrrole fungicide	140−350	4.1	1.8	75000	inhibits protein kinase involved in the regulatory step of cell division
lufenuron	benzoylurea insecticide	13−20	5.1	0.06	41182	inhibits chitin synthesis
imidacloprid	neonicotinoid insecticide	N/A[a]	0.57	610	225	antagonist to postsynaptic nicotinic receptors in the central nervous system

[a]N/A refers to information not available. Taken from ref 18.

of cyanobacteria, heterotrophic bacteria, lichens, algae, mosses, fungi, and archaea.[7] BSCs in arid and semiarid environments have been shown to have considerable functional importance in soil formation,[8] erosion protection,[9] and biological N_2 fixation.[10] Few studies have investigated the functional importance of BSCs in an agricultural environment. However, BSCs have been shown to develop under winter wheat, sugar beet, and maize cropping systems, even in tilled systems.[9] Further, evidence suggests that microbial community structure within the soil surface (upper 1 mm of soil) is distinct from that found at greater soil depths after only 4−6 months.[11] There is currently a trend toward zero and reduced tillage in agricultural environments, which is likely to enhance the development of BSCs in temperate cropping systems.[12]

The implication of the BSC for the fate of CPPs and other organic chemicals remains to be established. However, several studies have indicated the potential for algae to transform CPPs, although these studies typically focus on pure algal cultures. For example, diclofop-methyl,[13] fenamiphos,[14] and isoproturon,[15] have all been found to be degraded by pure algal cultures in growth media. Moreover, Thomas and Hand have shown considerable reductions in the persistence of several CPPs in water-sediment systems containing algae that are incubated under non-UV light compared to systems in the dark.[16] Sethunathan et al. also showed that the inoculation of soil with the alga *Chlorococcum* sp. or *Scenedesmus* sp. resulted in increased degradation rates of the insecticide α-endosulfan.[17]

In this study, we investigated the effect of non-UV light on the transformation, formation of nonextractable residues and mineralization of eight CPPs in soil by the inclusion of non-UV light to standard laboratory OECD 307 degradation studies. High performance liquid chromatography, chlorophyll *a* analysis, pH measurements and qPCR analysis of bacterial, fungal and archaeal copy numbers were assessed with the aim of answering the following questions: (i) Does non-UV light effect the rate of CPP degradation? (ii) Is the effect of non-UV light on CPP degradation the same across a range of fungicides, herbicides, and insecticides? (iii) What are the mechanisms responsible for non-UV light impacting CPP degradation?

MATERIALS AND METHODS

Soil. The top 15 cm of Gartenacker soil (silty loam) was sourced from Switzerland (CH-1896 Vouvry) in November 2009 (time course study) and May 2010 (compound screen study) [soil properties are shown in Table 1 of the Supporting Information (SI)]. Soil was sieved to 2 mm and used within 3 months of collection according to OECD guideline 307.[4]

Test Chemicals. Studies were performed using ^{14}C-labeled compounds (≥99% purity) (Greensboro, NC). The compounds used were (i) herbicides, chlorotoluron [specific activity (spec act.) 4.570 MBq/mg)], cinosulfuron (spec act. 2.327 MBq/mg) and prometryn (spec act. 1.136 MBq/mg); (ii) fungicides, propiconazole (spec act. 0.762 MBq/mg), fludioxonil (spec act. 1.469 MBq/mg), and benzovindiflupyr (spec act. 5.620 MBq/mg); and (iii) insecticides, lufenuron (spec act 5.132 MBq/mg) and imidacloprid (spec act 2.020 MBq/mg) (Table 1, and Figure 1 of the SI). These CPPs were selected as they represent a mixture of fungicides, herbicides and insecticides with a broad range of environmental persistency and physicochemical properties (Table 1)

Time Course Study of the Effect of Non-UV Light on the Degradation of Benzovindiflupyr and Chlorotoluron. *Test System.* Approximately 100 g dry weight equivalent (dwe) of Gartenacker soil at 35% moisture content was transferred to 250 mL centrifuge vessels and preincubated for 7 d under aerobic conditions prior to benzovindiflupyr application. Soil was preincubated for 22 d for the chlorotoluron study to allow the development of phototrophs prior to application. However, subsequent work on chlorotoluron applied after only 7 d incubation gave similar results (Table 2 of the SI).

A conventional flow-through test system based on OECD guideline 307 was used.[4] Vessels were incubated at a constant temperature of 20 ± 0.2 °C, under dark conditions, with a constant flow of moistened air. Exhaust gases were passed through a 2 M NaOH trap to capture any $^{14}CO_2$ evolved through compound mineralization (a schematic is shown in Figure 2 of the SI). The light treatment was incubated under identical conditions; however, vessels were illuminated with Philips Master fluorescent lights (>360 nm) TLD 36W/840 in a Sanyo Gallenkamp environmental chamber on a 16 h light:8 h dark cycle to reflect maximal diurnal light cycles during summer. UV light was omitted to minimize the adverse effects on microorganisms and to eliminate compound photolysis (the intensity and spectrum of light used in the test system is shown in Figure 3 of the SI). Soil moisture content was monitored weekly (by weight) and maintained at 35% by the addition of sterile ultrapure water (UP water).

Application and Sampling. Test compounds were dissolved in acetonitrile and applied dropwise using a micropipet, onto the soil surface. Following application of the test compound,

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vessels were mixed for 10 min on a roller to achieve a homogeneous distribution of the CPP. CPPs were applied at or close to field application rates to monitor transformation at environmentally relevant concentrations.[18] Application rates were 0.8 and 0.1 μg/g for chlorotoluron and benzovindiflupyr, respectively, which is equivalent to field application rates of 600 g/ha for chlorotoluron and 75 g/ha for benzovindiflupyr, assuming a distribution depth of 5 cm. Triplicate vessels were destructively sampled at 0 days after treatment (DAT), and compound-specific sampling points were taken thereafter (chlorotoluron, 3, 7, 14, 28, and 61 DAT; benzovindiflupyr, 22, 59, 90, and 120 DAT). A time course of transformation was taken during experiment 1 with the aim of providing sufficient data to enable the calculation of robust rates of transformation.

At each sampling point, CPPs were extracted with solvents. Extraction solvents were as follows: (i) chlorotoluron, 2 × 100 mL acetonitrile:UP water (80:20 v/v); (ii) benzovindiflupyr, 2 × 100 mL acetonitrile:0.1 M aqueous ammonium acetate (80:20 v/v), followed by 100 mL acetonitrile:UP water (pH 3) (80:20 v/v). After each solvent addition, samples were shaken at 300 rpm for 1 h and centrifuged at 2500 rpm for 10 min, with each extract decanted and pooled with successive extractions. Soil was left to dry before being ground to a fine powder using a mortar and pestle and subjected to 2 × 20 min cycles of accelerated solvent extraction (ASE) using acetonitrile:0.3% acetic acid (70:30 v/v) at 100 °C and 1500 psi. ASE extractions were not performed on 0 DAT extractions or for chlorotoluron at sampling points 3 and 7 DAT.[16]

Analysis. The total ^{14}C-activity recovered in primary extracts, ASE extracts, and $^{14}CO_2$ traps was quantified by liquid scintillation counting (LSC) using a Packard Tri-Carb (3100TR) liquid scintillation counter (Perkin-Elmer, Boston, MA). The ^{14}C-activity that remained in soil was termed nonextractable residues (NERs) and was quantified by sample oxidation using a Packard Model 307 combustor. The total ^{14}C-activity recovered in each fraction was calculated as a percentage of total applied and summed to give the mass balance in each vessel.

The primary extracts were analyzed using high performance liquid chromatography (HPLC) to determine the percentage of parent compound remaining in the sample. A precautionary approach was taken for ASE extracts by assuming the extract contained the same percentage of parent compound as the main extracts. Aliquots were concentrated prior to analysis under nitrogen gas using a Turbovap II (Caliper Life Sciences).

HPLC was performed using an Agilent HP1200 HPLC system (Agilent Technologies, UK Ltd.) connected to a Packard model 4 β-RAM radiodetector (IN/US systems). Reversed-phase gradient elution was used for both compounds. Benzovindiflupyr was run on a Luna C18 column (250 mm × 4.0 mm, 5 μm particle size), starting at 95% UP water (0.1% formic acid):5% acetonitrile, progressing to 95% acetonitrile:5% U.P. water (0.1% formic acid) over 25 min using a linear gradient. Chlorotoluron was run on a Luna C18 column (150 mm × 4.60 mm, 5 μm particle size), starting at 95% UP water (0.1% acetic acid):5% acetonitrile, progressing to 100% acetonitrile over 20 min using a linear gradient.[16]

The levels of parent compound, expressed as a percentage of applied radioactivity, recovered in the extracts were plotted using simple first-order kinetics (SFO) for both benzovindiflupyr light treatments and chlorotoluron under light. However, a biphasic plot (double first-order kinetics; DFOP) was more appropriate for chlorotoluron under standard dark conditions. The modeling program KinGUI v1.1 (conforms to the requirements of FOCUS kinetics) was used to estimate the time it takes for 50% and 90% of the compound to degrade ($DegT_{50}$/$DegT_{90}$).

Biological and Chemical Properties of Gartenacker Soil during Benzovindiflupyr and Chlorotoluron Degradation. Soil was subsampled from test vessels during the time course of degradation of benzovindiflupyr and chlorotoluron and used to assess chlorophyll *a*, pH, and bacterial, fungal, and archaeal copy numbers.

Chlorophyll a. The absorbance of solvent extract (1 mL) was measured using an Agilent UV−visible scanning spectrophotometer at 664 and 750 nm before acidifying with 3 M HCl for 90 s and remeasuring at 665 and 750 nm.[19] Chlorophyll *a* values were calculated from the formulas given in Hansson [20] (SI, eqs 1 and 2).

pH. Soil (2 g) was shaken with 5 mL of UP water for 15 min at 200 rpm before measuring pH using a BASIC pH meter (Denver Instrument Co., Norfolk, UK) with a Russell electrode (Fisher Scientific, Leicestershire, UK).[21]

DNA Extraction and qPCR Amplification of rRNA Markers To Assess Bacterial, Archaeal, and Fungal Copy Number. DNA was extracted using a FastDNA Spin Kit (Qbiogene, Loughborough, UK) according to the manufacturer's handbook. The quantity and quality of DNA in extracts was analyzed using a nanodrop ND-1000 spectrophotometer (Labtech International Ltd., Sussex, UK) and by agarose gel electrophoresis, respectively.

Bacterial copy number was assessed by qPCR targeting 16S rRNA genes using primers BA519f and BA907R.[22] Archaeal 16S rRNA genes were amplified using primers A364aF and A934b,[23,24] and for analysis of fungi, qPCR targeted the ITS region using primers 5.8S and ITS1F[25,26] (Sigma-Aldrich Co. Ltd., Dorset, UK). Details of all primer pairs (Table 3 of the SI), reagents, and qPCR cycles are given in the SI.

Compound Screen: The Effect of Non-UV Light on the Degradation of Six CPPs with a Range of Physicochemical Properties. *Test System, Application, and Sampling.* Unless specified, the test system and application method were the same as described above in the time course experiment. Cinosulfuron, fludioxonil, imidacloprid, lufenuron, prometryn, and propiconazole were applied at environmentally relevant rates of 0.13, 0.27, 0.27, 0.13, 2, and 0.67 μg/g, respectively, which are equivalent to field application rates of 100 g/ha for cinosulfuron, 200 g/ha for fludioxonil and imidacloprid, 100 g/ha lufenuron, 1500 g/ha prometryn, and 500 g/ha propiconazole, assuming a distribution depth of 5 cm.[18] The time of sampling was compound specific and at a single time point: lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole (43 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Quadruplicate samples were set up and extractions/HPLC were performed on a single centrifuge vessel from both light and dark treatments approximately at the $DegT_{50}$ of the CPP. CPP degradation under light and dark conditions was compared, and provided that significant degradation had occurred, extractions were taken from the remaining three vessels. A sampling point was also taken at 0 DAT for all compounds. A comparison was made between light and dark conditions at a single sampling point with the aim of assessing the impact of light on a range of CPPs. The extraction solvents and HPLC methods used are described in ref 16 and fully detailed in the SI.

Statistical Analysis. Provided the assumptions related to ANOVA were met, parametric tests were performed on nontransformed data. If assumptions were not met, data was log transformed, or a nonparametric test was performed instead. A mixture of two-way ANOVA with Tukey test (with treatment, time, and treatment × time as factors), correlation analysis, Kruskall–Wallis, and *t* tests were performed on data. Errors are all ±1 standard error (SE). All analyses were performed using Minitab version 15, and figures were plotted using Sigmaplot v. 12.0.

RESULTS

Time Course of Degradation of Benzovindiflupyr and Chlorotoluron under Light and Dark Conditions. Benzovindiflupyr mass balances were between 86% and 100% for all sampling times with the exception of 120 DAT under light conditions, which had a mass balance of 83%. Light treatment ($p \leq 0.001$) and sampling time ($p \leq 0.001$) both had a significant effect on the proportion of extractable benzovindiflupyr (Figure 1). Benzovindiflupyr transformation was more rapid under light conditions with 58.8% ± 1.1% parent remaining in the light and 70.6% ± 1.4% in the dark at 120 DAT (Figure 1). The $DegT_{50}$ of benzovindiflupyr was approximately halved under light from 373 to 183 d. The $DegT_{90}$ value was 608 d under light conditions and >1000 d in the dark treatment (the χ^2 error value was <15 for $DegT_{50/90}$).

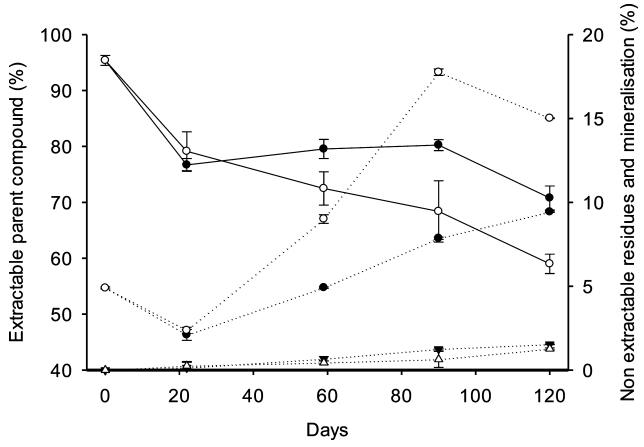

Figure 1. Mass balance for benzovindiflupyr under light (open symbols) and dark (closed symbols) conditions. The partitioned radioactivity is shown for extractable parent compound (–●–), mineralization (···▲···), and nonextractable residues (···●···). Error bars are ±1 SE.

Light was found to affect the proportion of benzovindiflupyr NERs (Figure 1). NER increased over the time course of benzovindiflupyr degradation and values were significantly higher under light compared to dark conditions at 90 DAT ($p \leq 0.05$) and 120 DAT ($p \leq 0.001$). For example, at 120 DAT, NER accounted for 15.0% ± 0.2% and 9.5% ± 0.2% of total radioactivity under light and dark conditions, respectively. Benzovindiflupyr mineralization was minimal at <2% under both light and dark conditions at 120 DAT (Figure 1).

Chlorotoluron mass balances were between 94% and 102% for all sampling points. Light treatment ($p \leq 0.001$) and sampling time ($p \leq 0.001$) also had a significant effect on the proportion of extractable chlorotoluron (Figure 2). Moreover, a significant interaction was observed between sampling time and light treatment ($p \leq 0.001$). Chlorotoluron transformation was more rapid under light conditions with 11.6% ± 1.0% parent remaining in the light and 26.5% ± 0.6% in the dark at 28 DAT ($p \leq 0.001$) (Figure 2). Chlorotoluron $DegT_{50}$ values were 10 and 15 d under light and dark conditions, respectively. The chlorotoluron $DegT_{90}$ value was approximately halved from 79 d in the dark to 35 d under light (χ^2 error value was <15 for $DegT_{50/90}$).

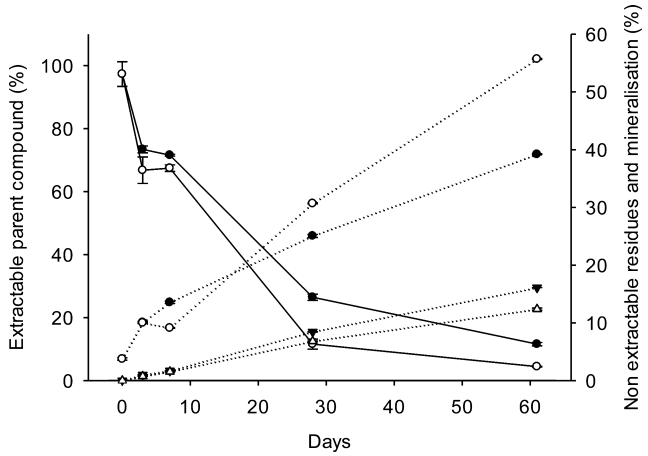

Figure 2. Mass balance for the herbicide chlorotoluron under light (open symbols) and dark (closed symbols) conditions. The partitioned radioactivity is shown for extractable parent compound (–●–), mineralization (···△···), and nonextractable residues (···●···). Error bars are ±1 SE.

Chlorotoluron NER increased during the time course of degradation and were significantly higher under light conditions at 61 DAT with 55.7% ± 3.2% and 39.2% ± 1.7% ($p < 0.05$) of total radioactivity under light and dark conditions, respectively. Chlorotoluron mineralization increased over the time course of degradation with 12.4% ± 1.3% and 16.1% ± 0.2% at 61 DAT under light and dark conditions, respectively. Mineralization was significantly higher under dark conditions at 3 DAT ($p \leq 0.001$) and 28 DAT ($p \leq 0.05$) Figure 2).

Chlorophyll *a*, pH, and Bacterial, Fungal and Archaeal Copy Numbers across a Time Course of Benzovindiflupyr and Chlorotoluron Degradation under Light and Dark Conditions. Light had a significant effect on chlorophyll *a* abundance ($p \leq 0.001$) (Figure 3a,d), and both light ($p \leq 0.001$) and time ($p \leq 0.01$) significantly impacted soil pH during the degradation of benzovindiflupyr and chlorotoluron (Figure 3b,e). Light treatment did not have a significant impact on bacterial copy number during benzovindiflupyr degradation; for example, at day 120, there were $(1.26 \times 10^8) \pm (1.26 \times 10^7)$ and $(2.90 \times 10^8) \pm (2.15 \times 10^7)$ copies under light and dark conditions, respectively ($p = 0.35$) (Figure 3c). Similarly, copies of bacterial 16S rRNA genes did not differ significantly between light $[(3.8 \times 10^8) \pm (1.05 \times 10^8)]$ and dark $[(1.43 \times 10^8) \pm (7.02 \times 10^7)]$ conditions 61 days after chlorotoluron application ($p = 0.71$) (Figure 3f). Archaeal copy numbers were not significantly different between light and dark conditions for either benzovindiflupyr ($p = 0.08$) or chlorotoluron ($p = 0.62$). Similarly, fungal copy number was not significantly different between light and dark conditions for either benzovindiflupyr ($p = 0.06$) or chlorotoluron ($p = 0.31$) (full details are shown in Figure 4 of the SI).

Compound Screen: The Degradation of a Variety of CPPs under Light and Dark Conditions. The compound

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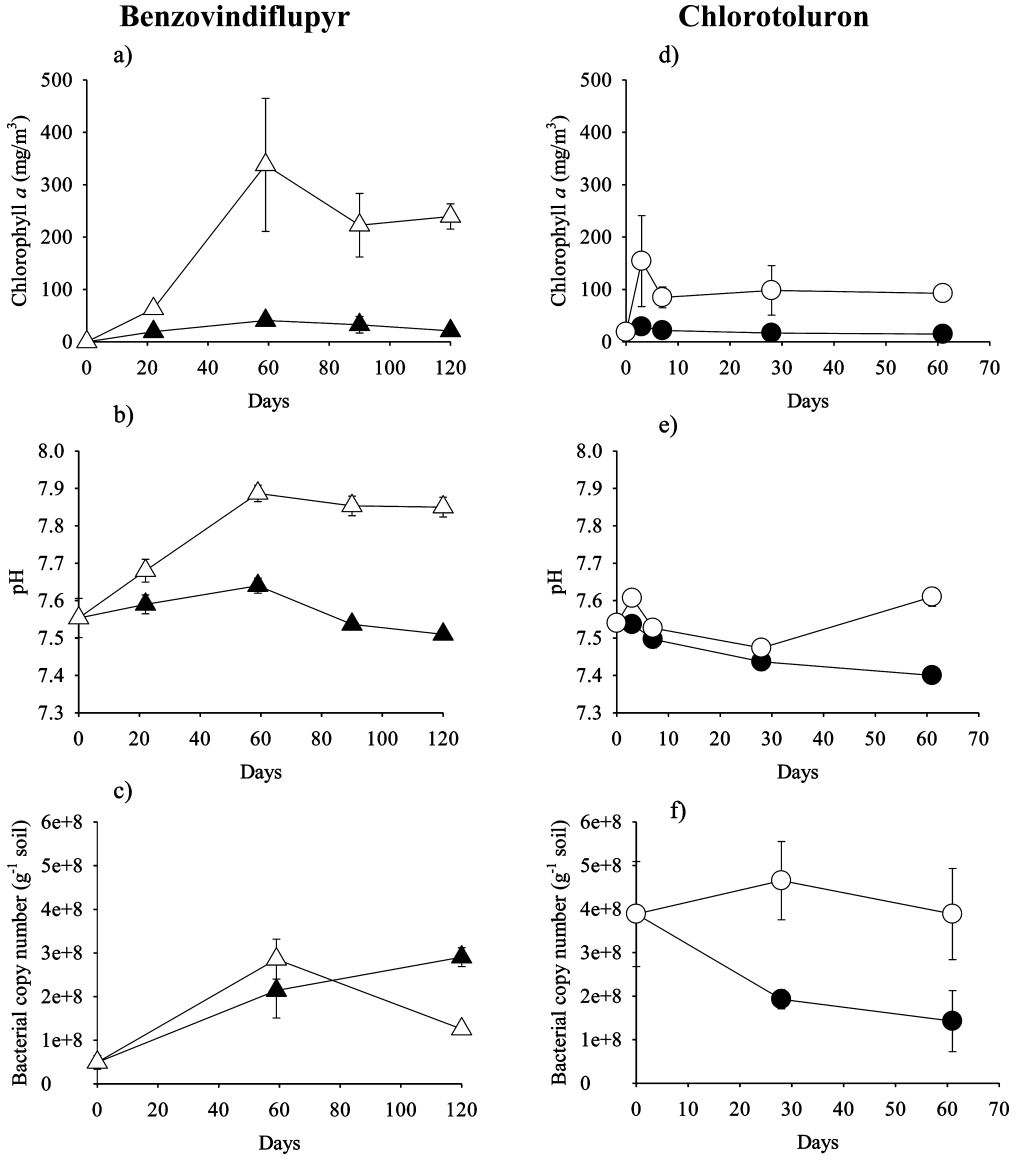

Figure 3. Chlorophyll *a*, pH, and bacterial (16S rRNA gene) copy number for soil incubated under light (open symbols) and dark (closed symbols) conditions during a time course of benzovindiflupyr (triangles) and chlorotoluron (circles) degradation. Error bars are ±1 SE.

screen showed the effect of light on the transformation of CPPs to be compound specific (Figure 4). Extractable parent compound was significantly lower under light relative to dark conditions for prometryn (6.4% ± 0.4% and 10.3 ± 0.5%; $p \leq 0.01$), fludioxonil (41.6% ± 1.3% and 65.7% ± 0.4%; $p \leq 0.01$), and imidacloprid (29.8% ± 1.1% and 48.1% ± 1.5%; $p \leq 0.001$). Cinosulfuron behaved atypically, with an increase in compound persistence under light conditions, with 42.2% ± 2.3% and 28.4% ± 0.7% under light and dark conditions, respectively ($p \leq 0.05$). There were no significant differences in the rate of transformation between light and dark treatments for propiconazole and lufenuron.

NER and mineralization results from the compound screen (Figure 5) were typically consistent with those from the $DegT_{50}$ degradation study (Figures 1 and 2). NERs were significantly higher under light for prometryn ($p \leq 0.05$), fludioxonil ($p \leq 0.01$), propiconazole ($p \leq 0.001$), lufenuron ($p \leq 0.05$), and imidacloprid ($p \leq 0.01$). However, no significant difference was observed for cinosulfuron. Particularly large differences in NER were observed for imidacloprid and fludioxonil, with an approximate 3.5-fold increase under light. Mineralization was significantly higher under dark conditions for prometryn ($p \leq 0.01$), cinosulfuron ($p \leq 0.01$), lufenuron ($p \leq 0.001$), and propiconazole ($p \leq 0.05$) (Figure 5). In contrast, a significantly greater proportion of fludioxonil mineralized under light compared to dark conditions ($p \leq 0.01$) (Figure 5). Imidacloprid mineralization did not differ significantly between light and dark conditions (Figure 5) (full details of compound screen results are shown in Table 4 of the SI).

■ DISCUSSION

The inclusion of non-UV light to the OECD guideline 307 had a compound specific effect on the rate of CPP degradation. Light was found to significantly increase the rate of transformation for five out of the eight CPPs tested and reduced the rate of degradation of one CPP. Light treatment also resulted in an increase in the rate of NER formation for seven CPPs. A variety of mechanisms could be responsible for the "light effect", including the proliferation of phototroph communities

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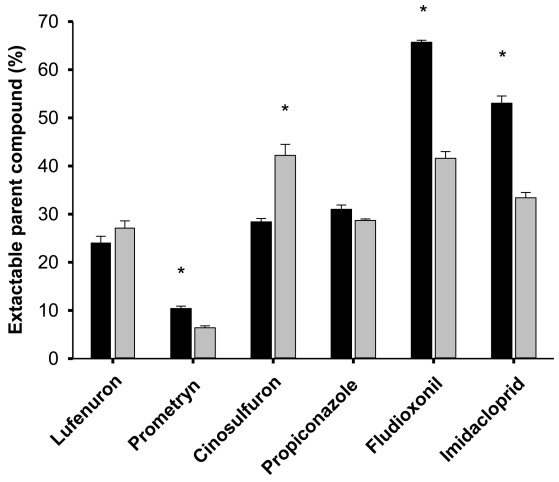

Figure 4. Extractable parent compound for a variety of crop protection products under light (gray) and dark conditions (black). Sampling times were as follows: lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole (43 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Significant differences are indicated by an asterisk (*) ($p \leq 0.01$). Error bars are ±1 SE.

and pH shifts. This is the first study to report that non-UV light affects the rate of CPP degradation in soil.

The transformation rates of chlorotoluron, benzovindiflupyr, prometryn, imidacloprid, and fludioxonil significantly increased under light relative to dark conditions (Figures 1, 2, and 4). For example, the inclusion of light resulted in an approximate halving of the $DegT_{50/90}$ of benzovindiflupyr (Figure 1), a halving of the $DegT_{90}$ of chlorotoluron (Figure 2), and significantly lower extractable parent compound for prometryn (4%), imidacloprid (18%), and fludioxonil (24%) under light conditions compared to in the dark (Figure 4). A similar impact of light has also been observed in water−sediment systems containing algae or macrophytes; for example, Thomas and Hand showed considerable reductions in the persistence of chlorotoluron, fludioxonil, prometryn, pinoxeden metabolite (diketone), and propiconazole under light compared to dark controls.[16] The study showed a 4-, 5-, 7-, and 20-fold enhancement of transformation for chlorotoluron, prometryn, propiconazole, and fludioxonil.

In this study, light clearly influenced the rate of CPP transformation for six out of eight CPPs tested, but the mechanisms responsible may be compound specific. First, phototrophs proliferated under light conditions for all CPPs tested, with the exception of cinosulfuron (Figure 3a,b, and Table 5 of the SI). A direct effect of phototrophs on CPP degradation is supported by several studies indicating the potential for algae and cyanobacteria to degrade CPPs in axenic cultures.[13−17] Phototrophs could also indirectly affect CPP degradation through a shift in soil pH. Indeed, a significant increase in pH under light compared to dark conditions during the degradation of benzovindiflupyr and chlorotoluron (Figure 3c,d) would have been caused by the uptake of CO_2, which is acidic, by phototrophs during photosynthesis.[16] Both light[11] and pH[27] have previously been shown to influence microbial community composition in soil, which could in turn impact the rate of CPP transformation in the current system. For the CPPs tested, the pH increase is not thought to impact CPP dissociation as the soil pH is not within 2 pH units of the pK_a values of the CPPs tested. The fact that no correlation was found between the difference in CPP transformation between light and dark treatments and chlorophyll *a* abundance (Figure 5a of the SI) suggests that potential shifts in heterotroph communities caused by the impact of phototrophs on soil pH could be important (Figure 3c,d). The mechanisms responsible for enhanced degradation rates of cinosulfuron in the dark are unknown; however, it could be due to light preferentially selecting against organisms that are directly or indirectly important in cinosulfuron degradation.

An additional impact of light on soil communities may be driven by an input of C by photosynthesis, which could potentially increase viable biomass or microbial activity and consequently the rate of CPP transformation. If true, this mechanism would be analogous to "the rhizosphere effect", which has been documented for several organic compounds.[28] For example, Marchland et al. observed that under 4-week-old maize seedlings, 61% of atrazine had mineralized compared to only 48% in nonplanted soil.[29] However, our results do not support this theory, as there were no significant differences in bacterial, fungal, or archaeal copy numbers between light and dark treatments during a time course of benzovindiflupyr and chlorotoluron degradation (Figure 3, and Figure 4 of the SI).

A lag phase is often observed in the degradation of growth-linked catabolized compounds to allow microorganisms time to induce specific degradative enzyme systems to catabolize the compound.[30] In the current system, there may also be a lag phase to allow the development of phototrophs. At the end of the lag phase, phototrophs are able to directly or indirectly impact the rate of CPP transformation. Indeed, this is supported by the time course of chlorotoluron degradation, where the $DegT_{90}$ was halved under light compared to dark conditions, but no impact was observed on the $DegT_{50}$ (Figure 2).

The physicochemical properties or mode of action of the CPPs may also play a role; however, the difference in CPP transformation between light and dark treatments was not correlated with the environmental persistence ($DegT_{50}$) or bioavailability (K_{oc} value or water solubility) of the CPPs tested (Figure 5b−d of the SI). Further, the mode of action of the CPPs tested did not have as dramatic effect as expected. For example, prometryn and chlorotoluron both inhibit the electron transport system in photosytem II.[18] Therefore, an application of prometryn or chlorotoluron to soil may inhibit the growth of photosynthetic organisms, thereby reducing any contribution of phototrophs to CPP transformation. However, both CPPs were found to have significantly faster rates of transformation under light conditions.

It is also important not to overlook potential abiotic differences between light and dark treatments, such as indirect photolysis. Indirect photolysis refers to the absorbance of light by photosensitizers to form reactive intermediates such as hydroxyl radicals, which react with CPPs.[31] In soil, nitrate and humic acids could be considered to be the most likely photosensitizers. UV is thought to be primarily responsible for the photosensitization of humic acids,[32] and nitrate does not absorb light at >350 nm,[33] and therefore, neither would photosensitize in a non-UV light system (see Figure 3 of the SI for the spectrum and intensity of light). Therefore, indirect photolysis is unlikely to be major factor responsible for the "light effect"; however, unknown photosensitizers cannot be eliminated from contributing to the effect. Overall, the "light effect" and the mechanisms responsible for the effect appear to be CPP specific. For example, phototrophs proliferated for all

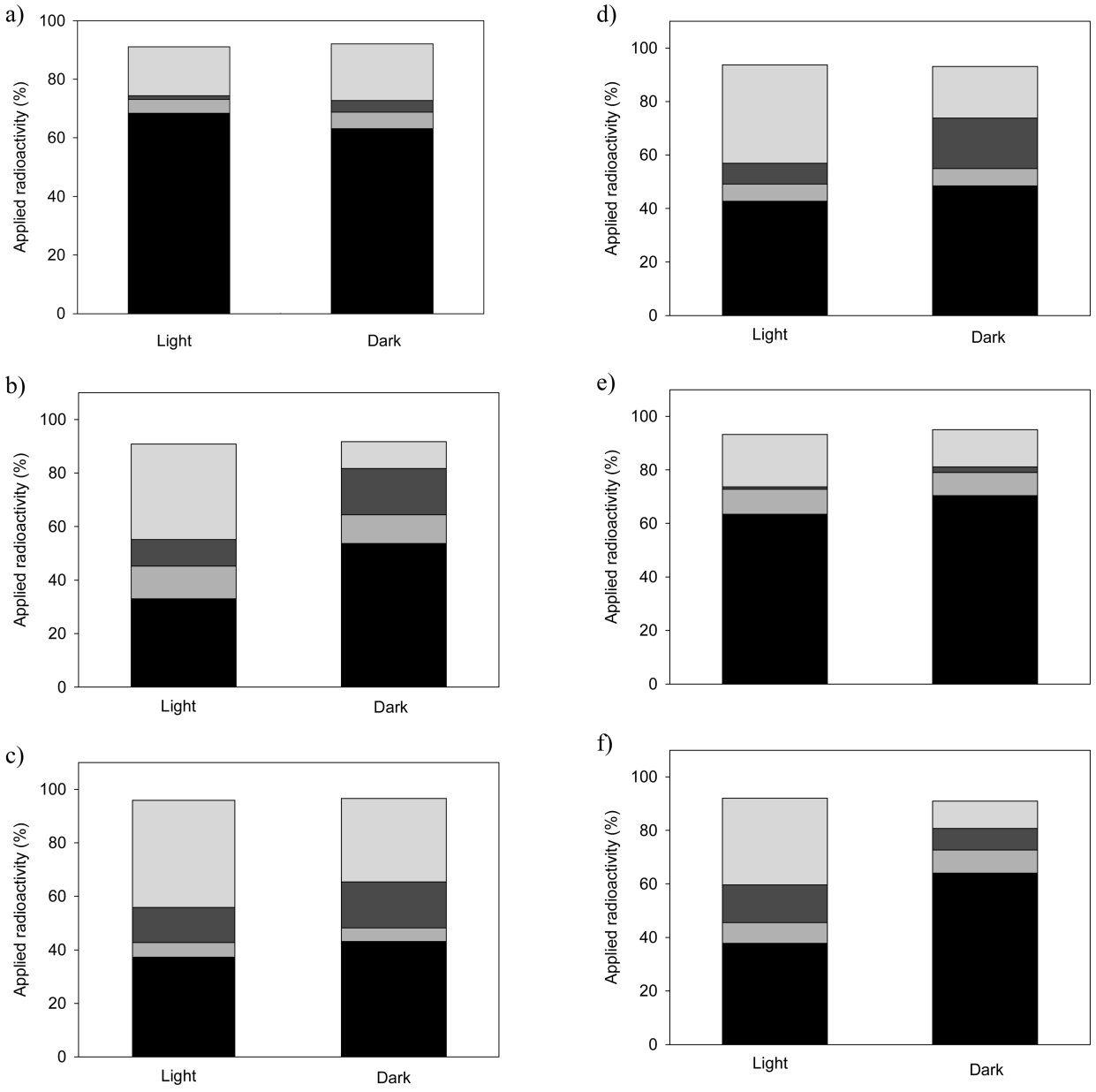

Figure 5. Recovery of partitioned radioactivity: (a) cinosulfuron (34 DAT), (b) imidacloprid (102 DAT), (c) prometryn (32 DAT), (d) propiconazole (43 DAT), (e) lufenuron (25 DAT), and (f) fludioxonil (69 DAT); main extract (black), ASE extract (light gray), mineralization (dark gray), and nonextractable residues (white).

CPPs that degraded faster under light; however, accelerated degradation rates were not observed for all CPPs that showed phototroph proliferation. Further, the contribution of heterotrophs to the "light effect" could also be CPP specific.

The use of light in laboratory degradation test systems has the potential to improve the accuracy of estimated CPP persistence in degradation studies. OECD guideline 307 states that degradation studies can only be conducted for a maximum of 120 d, as after this time, microbial biomass and activity are reduced considerably. The $DegT_{50}$ of highly persistent compounds are therefore estimated using regression analysis. However, the rate of CPP degradation may change from first-order kinetics following 120 d, and thus $DegT_{50}$ estimations by regression may not be accurate. The addition of C to the system via phototrophs may act to maintain microbial biomass and activity for a longer duration. Hence, studies could be carried out for longer than 120 d, potentially increasing the accuracy of $DegT_{50}$ values for persistent CPPs or other xenobiotics. In addition, the presence of phototrophs in an agricultural soil crust may affect CPP mobility, particularly for less polar CPPs such as lufenuron and fludioxonil (Table 1). These compounds may be more likely to sorb to the BSC rather than dissolve in the aqueous phase of soil, potentially accelerating rates of degradation through an extended exposure to phototrophs, heterotrophs, and photolysis at the soil surface.

Although CPP transformation rates were typically faster in the light, mineralization was considerably greater under dark conditions for all CPPs tested with the exception of imidacloprid and fludioxonil (Figures 1, 2, and 5). Phototrophs may be utilizing $^{14}CO_2$ for photosynthesis, reducing the overall amount collected in NaOH traps. Therefore, mineralization results may not be reliable under light conditions. Fludioxonil

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behaved atypically by being the only compound tested that showed higher rates of mineralization under light conditions. Transformation under light may be increased to such an extent as to offset any uptake of $^{14}CO_2$ by phototrophs. This is supported by fludioxonil showing the greatest difference in transformation between light and dark conditions compared to all other CPPs (Figure 4). Moreover, fludioxonil has been shown to be degraded by eight green algae and four cyanobacteria in pure culture.[34]

NER formation was greater under light for all CPPs with the exception of cinosulfuron. The increase in NER could be due to a variety of reasons, for instance (i) a change in the chemical properties of soil incubated under light increasing the sorption of the CPP to soil, (ii) a greater rate of CPP degradation and incorporation into biogenic residues by photosynthetic or nonphotosynthetic organisms under light, or (iii) the assimilation of $^{14}CO_2$ into the biomass of phototrophs. The third explanation was tested by accounting for the potential uptake of the additional $^{14}CO_2$ found in dark systems by phototrophs in light systems. It was found that NER were still higher under light conditions and significantly higher for fludioxonil ($p \leq 0.01$), imidacloprid ($p \leq 0.01$), lufenuron ($p \leq 0.05$), and benzovindiflupyr ($p \leq 0.001$). Therefore, it is likely that an increase in CPP degradation and incorporation into biomass and/or an increase in sorption are responsible for greater NERs under light conditions. NERs are not typically viewed as bioavailable, as particular forms of NER do not pose a short- or long-term threat to ecology, rather they are relatively stable and slowly degraded over an extended period.[35] Therefore, an increase in NERs under light conditions could translate to greater CPP sorption under agricultural cropping systems when a soil crust has developed.

This is the first study to investigate the effect of non-UV light on the degradation of CPPs. The inclusion of non-UV light to standard laboratory studies increased the rate of CPP transformation for five CPPs and NER formation for seven out of the eight CPPs tested. This effect may have been driven by the presence of soil phototrophs, which could directly degrade CPPs or indirectly impact heterotroph community composition and/or alter soil chemical properties such as pH. Phototrophs represent the first point of contact for CPPs applied to the soil surface, and therefore, the results have important implications for pesticide legislation. It is important to further investigate the mechanisms responsible, test if the "light effect" is soil-specific and if natural light also has a similar effect, and continue to alter additional variables, with the ultimate aim of bridging the gap between laboratory studies and field applications and improving the risk assessment of CPPs.

■ ASSOCIATED CONTENT

Supporting Information

Tables 1−5, Figures 1−5, and eqs 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org/

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Notes

The authors declare no competing financial interest.

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APPENDIX II

LIGHT STRUCTURES PHOTOTROPH, BACTERIAL AND FUNGAL COMMUNITIES AT THE SOIL SURFACE

Environmental Science & Technology

Article

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Non-UV Light Influences the Degradation Rate of Crop Protection Products

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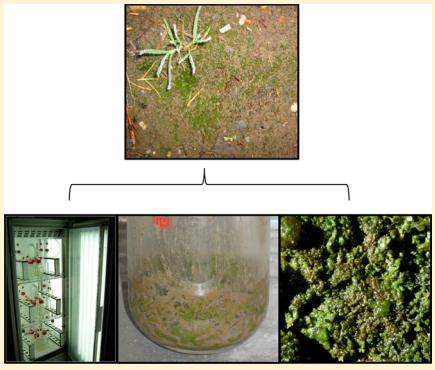
Supporting Information

ABSTRACT: Crop protection products (CPPs) are subject to strict regulatory evaluation, including laboratory and field trials, prior to approval for commercial use. Laboratory tests lack environmental realism, while field trials are difficult to control. Addition of environmental complexity to laboratory systems is therefore desirable to mimic a field environment more effectively. We investigated the effect of non-UV light on the degradation of eight CPPs (chlorotoluron, prometryn, cinosulfuron, imidacloprid, lufenuron, propiconazole, fludioxonil, and benzovindiflupyr) by addition of non-UV light to standard OECD 307 guidelines. Time taken for 50% degradation of benzovindiflupyr was halved from 373 to 183 days with the inclusion of light. Similarly, time taken for 90% degradation of chlorotoluron decreased from 79 to 35 days under light conditions. Significant reductions in extractable parent compound occurred under light conditions for prometryn (4%), imidacloprid (8%), and fludioxonil (24%) compared to dark controls. However, a significantly slower rate of cinosulfuron (14%) transformation was observed under light compared to dark conditions. Under light conditions, nonextractable residues were significantly higher for seven of the CPPs. Soil biological and chemical analyses suggest that light stimulates phototroph growth, which may directly and/or indirectly impact CPP degradation rates. The results of this study strongly suggest that light is an important parameter affecting CPP degradation, and inclusion of light into regulatory studies may enhance their environmental realism.



INTRODUCTION

Crop protection products (CPPs) have been an essential factor in improving crop productivity and food security; however, their inherent ability to reduce crop pests can potentially result in adverse environmental effects.[1] CPPs are therefore subject to a strict regulatory evaluation. The Organisation for Economic Co-operation and Development (OECD) provides an in depth description of the holistic processes involved in the risk assessment of CPPs, including, physicochemical properties, health effects, degradation and accumulation, and ecological risk assessment. An integral part of CPP risk assessment involves evaluating their toxicity,[2] toxicokinetics, and carcinogenicity[3] to humans and other nontarget organisms. Results are evaluated alongside investigations into the rate and route of compound transformation[4] to predict the concentration of CPPs in the environment and their overall risk.

The most important process in the environmental fate of CPPs is generally considered to be microbial degradation.[5] Prior to regulatory approval, the rate of degradation of CPPs and other organic chemicals in soil is determined using the OECD 307 test.[4] The test provides good reproducibility between samples, and allows the transformation, mineralization, and compartmentalization of the residual chemical to be determined with high precision. However, conditions may not be environmentally realistic; for example, in lab test systems temperature and moisture are kept constant, soil is sieved to 2 mm and homogenized, and soil is incubated in the dark.[4] Beulke et al. reviewed 178 studies and found that laboratory tests and simulation models overestimated actual pesticide persistence in the field by a factor of >1.25 in 44% of studies.[6] However, an underestimation by a factor of >1.25 was only found in 17% of studies.[6] It is likely that such discrepancies may be due to poor reproduction of environmental conditions in laboratory tests, and it is therefore essential to try and bridge the gap between laboratory and field studies. Improvement of the predictions of the persistence of CPPs should be possible by progressively adding complexity to laboratory systems in order to better mimic the natural environment.

A CPPs first point of contact with the soil environment is the soil surface. Therefore, laboratory degradation tests should aim to simulate the physical, chemical, and biological properties of the soil surface. In an agricultural field the soil surface is exposed to light; however, laboratory degradation tests are incubated in the dark.[4] The presence of light has been shown to drive the formation of a biological soil crust (BSC) composed

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Table 1. Selected Properties of the Crop Protection Products Investigated in This Study

name	type	$DegT_{50}$ (d)	k_{ow} (log P)	water solubility (mg/L)	K_{oc} (mL/g)	mode of action
prometryn	methylthiotriazine herbicide	14−158	3.1	33	400	photosynthetic electron transport inhibitor; inhibits oxidative phosophorylation
cinosulfuron	sulfonylurea herbicide	20	2.0	4000	20	inhibits biosynthesis of essential amino acids
chlorotoluron	urea herbicide	30−40	2.5	74	205	inhibitor of photosynthetic electron transport
propiconazole	triazole fungicide	29−70	3.7	100	1086	steroid demethylation inhibitor
benzovindiflupyr	triazole fungicide	N/A[a]	N/A[a]	N/A[a]	N/A[a]	succinate dehydrogenase inhibitor
fludioxonil	phenylpyrrole fungicide	140−350	4.1	1.8	75000	inhibits protein kinase involved in the regulatory step of cell division
lufenuron	benzoylurea insecticide	13−20	5.1	0.06	41182	inhibits chitin synthesis
imidacloprid	neonicotinoid insecticide	N/A[a]	0.57	610	225	antagonist to postsynaptic nicotinic receptors in the central nervous system

[a]N/A refers to information not available. Taken from ref 18.

of cyanobacteria, heterotrophic bacteria, lichens, algae, mosses, fungi, and archaea.[7] BSCs in arid and semiarid environments have been shown to have considerable functional importance in soil formation,[8] erosion protection,[9] and biological N_2 fixation.[10] Few studies have investigated the functional importance of BSCs in an agricultural environment. However, BSCs have been shown to develop under winter wheat, sugar beet, and maize cropping systems, even in tilled systems.[9] Further, evidence suggests that microbial community structure within the soil surface (upper 1 mm of soil) is distinct from that found at greater soil depths after only 4−6 months.[11] There is currently a trend toward zero and reduced tillage in agricultural environments, which is likely to enhance the development of BSCs in temperate cropping systems.[12]

The implication of the BSC for the fate of CPPs and other organic chemicals remains to be established. However, several studies have indicated the potential for algae to transform CPPs, although these studies typically focus on pure algal cultures. For example, diclofop-methyl,[13] fenamiphos,[14] and isoproturon,[15] have all been found to be degraded by pure algal cultures in growth media. Moreover, Thomas and Hand have shown considerable reductions in the persistence of several CPPs in water-sediment systems containing algae that are incubated under non-UV light compared to systems in the dark.[16] Sethunathan et al. also showed that the inoculation of soil with the alga *Chlorococcum* sp. or *Scenedesmus* sp. resulted in increased degradation rates of the insecticide α-endosulfan.[17]

In this study, we investigated the effect of non-UV light on the transformation, formation of nonextractable residues and mineralization of eight CPPs in soil by the inclusion of non-UV light to standard laboratory OECD 307 degradation studies. High performance liquid chromatography, chlorophyll *a* analysis, pH measurements and qPCR analysis of bacterial, fungal and archaeal copy numbers were assessed with the aim of answering the following questions: (i) Does non-UV light effect the rate of CPP degradation? (ii) Is the effect of non-UV light on CPP degradation the same across a range of fungicides, herbicides, and insecticides? (iii) What are the mechanisms responsible for non-UV light impacting CPP degradation?

MATERIALS AND METHODS

Soil. The top 15 cm of Gartenacker soil (silty loam) was sourced from Switzerland (CH-1896 Vouvry) in November 2009 (time course study) and May 2010 (compound screen study) [soil properties are shown in Table 1 of the Supporting Information (SI)]. Soil was sieved to 2 mm and used within 3 months of collection according to OECD guideline 307.[4]

Test Chemicals. Studies were performed using ^{14}C-labeled compounds (≥99% purity) (Greensboro, NC). The compounds used were (i) herbicides, chlorotoluron [specific activity (spec act.) 4.570 MBq/mg)], cinosulfuron (spec act. 2.327 MBq/mg) and prometryn (spec act. 1.136 MBq/mg); (ii) fungicides, propiconazole (spec act. 0.762 MBq/mg), fludioxonil (spec act. 1.469 MBq/mg), and benzovindiflupyr (spec act. 5.620 MBq/mg); and (iii) insecticides, lufenuron (spec act 5.132 MBq/mg) and imidacloprid (spec act 2.020 MBq/mg) (Table 1, and Figure 1 of the SI). These CPPs were selected as they represent a mixture of fungicides, herbicides and insecticides with a broad range of environmental persistency and physicochemical properties (Table 1)

Time Course Study of the Effect of Non-UV Light on the Degradation of Benzovindiflupyr and Chlorotoluron. *Test System.* Approximately 100 g dry weight equivalent (dwe) of Gartenacker soil at 35% moisture content was transferred to 250 mL centrifuge vessels and preincubated for 7 d under aerobic conditions prior to benzovindiflupyr application. Soil was preincubated for 22 d for the chlorotoluron study to allow the development of phototrophs prior to application. However, subsequent work on chlorotoluron applied after only 7 d incubation gave similar results (Table 2 of the SI).

A conventional flow-through test system based on OECD guideline 307 was used.[4] Vessels were incubated at a constant temperature of 20 ± 0.2 °C, under dark conditions, with a constant flow of moistened air. Exhaust gases were passed through a 2 M NaOH trap to capture any $^{14}CO_2$ evolved through compound mineralization (a schematic is shown in Figure 2 of the SI). The light treatment was incubated under identical conditions; however, vessels were illuminated with Philips Master fluorescent lights (>360 nm) TLD 36W/840 in a Sanyo Gallenkamp environmental chamber on a 16 h light:8 h dark cycle to reflect maximal diurnal light cycles during summer. UV light was omitted to minimize the adverse effects on microorganisms and to eliminate compound photolysis (the intensity and spectrum of light used in the test system is shown in Figure 3 of the SI). Soil moisture content was monitored weekly (by weight) and maintained at 35% by the addition of sterile ultrapure water (UP water).

Application and Sampling. Test compounds were dissolved in acetonitrile and applied dropwise using a micropipet, onto the soil surface. Following application of the test compound,

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vessels were mixed for 10 min on a roller to achieve a homogeneous distribution of the CPP. CPPs were applied at or close to field application rates to monitor transformation at environmentally relevant concentrations.[18] Application rates were 0.8 and 0.1 μg/g for chlorotoluron and benzovindiflupyr, respectively, which is equivalent to field application rates of 600 g/ha for chlorotoluron and 75 g/ha for benzovindiflupyr, assuming a distribution depth of 5 cm. Triplicate vessels were destructively sampled at 0 days after treatment (DAT), and compound-specific sampling points were taken thereafter (chlorotoluron, 3, 7, 14, 28, and 61 DAT; benzovindiflupyr, 22, 59, 90, and 120 DAT). A time course of transformation was taken during experiment 1 with the aim of providing sufficient data to enable the calculation of robust rates of transformation.

At each sampling point, CPPs were extracted with solvents. Extraction solvents were as follows: (i) chlorotoluron, 2 × 100 mL acetonitrile:UP water (80:20 v/v); (ii) benzovindiflupyr, 2 × 100 mL acetonitrile:0.1 M aqueous ammonium acetate (80:20 v/v), followed by 100 mL acetonitrile:UP water (pH 3) (80:20 v/v). After each solvent addition, samples were shaken at 300 rpm for 1 h and centrifuged at 2500 rpm for 10 min, with each extract decanted and pooled with successive extractions. Soil was left to dry before being ground to a fine powder using a mortar and pestle and subjected to 2 × 20 min cycles of accelerated solvent extraction (ASE) using acetonitrile:0.3% acetic acid (70:30 v/v) at 100 °C and 1500 psi. ASE extractions were not performed on 0 DAT extractions or for chlorotoluron at sampling points 3 and 7 DAT.[16]

Analysis. The total ^{14}C-activity recovered in primary extracts, ASE extracts, and $^{14}CO_2$ traps was quantified by liquid scintillation counting (LSC) using a Packard Tri-Carb (3100TR) liquid scintillation counter (Perkin-Elmer, Boston, MA). The ^{14}C-activity that remained in soil was termed nonextractable residues (NERs) and was quantified by sample oxidation using a Packard Model 307 combustor. The total ^{14}C-activity recovered in each fraction was calculated as a percentage of total applied and summed to give the mass balance in each vessel.

The primary extracts were analyzed using high performance liquid chromatography (HPLC) to determine the percentage of parent compound remaining in the sample. A precautionary approach was taken for ASE extracts by assuming the extract contained the same percentage of parent compound as the main extracts. Aliquots were concentrated prior to analysis under nitrogen gas using a Turbovap II (Caliper Life Sciences).

HPLC was performed using an Agilent HP1200 HPLC system (Agilent Technologies, UK Ltd.) connected to a Packard model 4 β-RAM radiodetector (IN/US systems). Reversed-phase gradient elution was used for both compounds. Benzovindiflupyr was run on a Luna C18 column (250 mm × 4.0 mm, 5 μm particle size), starting at 95% UP water (0.1% formic acid):5% acetonitrile, progressing to 95% acetonitrile:5% U.P. water (0.1% formic acid) over 25 min using a linear gradient. Chlorotoluron was run on a Luna C18 column (150 mm × 4.60 mm, 5 μm particle size), starting at 95% UP water (0.1% acetic acid):5% acetonitrile, progressing to 100% acetonitrile over 20 min using a linear gradient.[16]

The levels of parent compound, expressed as a percentage of applied radioactivity, recovered in the extracts were plotted using simple first-order kinetics (SFO) for both benzovindiflupyr light treatments and chlorotoluron under light. However, a biphasic plot (double first-order kinetics; DFOP) was more appropriate for chlorotoluron under standard dark conditions. The modeling program KinGUI v1.1 (conforms to the requirements of FOCUS kinetics) was used to estimate the time it takes for 50% and 90% of the compound to degrade ($DegT_{50}$/$DegT_{90}$).

Biological and Chemical Properties of Gartenacker Soil during Benzovindiflupyr and Chlorotoluron Degradation. Soil was subsampled from test vessels during the time course of degradation of benzovindiflupyr and chlorotoluron and used to assess chlorophyll *a*, pH, and bacterial, fungal, and archaeal copy numbers.

Chlorophyll a. The absorbance of solvent extract (1 mL) was measured using an Agilent UV−visible scanning spectrophotometer at 664 and 750 nm before acidifying with 3 M HCl for 90 s and remeasuring at 665 and 750 nm.[19] Chlorophyll *a* values were calculated from the formulas given in Hansson [20] (SI, eqs 1 and 2).

pH. Soil (2 g) was shaken with 5 mL of UP water for 15 min at 200 rpm before measuring pH using a BASIC pH meter (Denver Instrument Co., Norfolk, UK) with a Russell electrode (Fisher Scientific, Leicestershire, UK).[21]

DNA Extraction and qPCR Amplification of rRNA Markers To Assess Bacterial, Archaeal, and Fungal Copy Number. DNA was extracted using a FastDNA Spin Kit (Qbiogene, Loughborough, UK) according to the manufacturer's handbook. The quantity and quality of DNA in extracts was analyzed using a nanodrop ND-1000 spectrophotometer (Labtech International Ltd., Sussex, UK) and by agarose gel electrophoresis, respectively.

Bacterial copy number was assessed by qPCR targeting 16S rRNA genes using primers BA519f and BA907R.[22] Archaeal 16S rRNA genes were amplified using primers A364aF and A934b,[23,24] and for analysis of fungi, qPCR targeted the ITS region using primers 5.8S and ITS1F[25,26] (Sigma-Aldrich Co. Ltd., Dorset, UK). Details of all primer pairs (Table 3 of the SI), reagents, and qPCR cycles are given in the SI.

Compound Screen: The Effect of Non-UV Light on the Degradation of Six CPPs with a Range of Physicochemical Properties. *Test System, Application, and Sampling.* Unless specified, the test system and application method were the same as described above in the time course experiment. Cinosulfuron, fludioxonil, imidacloprid, lufenuron, prometryn, and propiconazole were applied at environmentally relevant rates of 0.13, 0.27, 0.27, 0.13, 2, and 0.67 μg/g, respectively, which are equivalent to field application rates of 100 g/ha for cinosulfuron, 200 g/ha for fludioxonil and imidacloprid, 100 g/ha lufenuron, 1500 g/ha prometryn, and 500 g/ha propiconazole, assuming a distribution depth of 5 cm.[18] The time of sampling was compound specific and at a single time point: lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole (43 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Quadruplicate samples were set up and extractions/HPLC were performed on a single centrifuge vessel from both light and dark treatments approximately at the $DegT_{50}$ of the CPP. CPP degradation under light and dark conditions was compared, and provided that significant degradation had occurred, extractions were taken from the remaining three vessels. A sampling point was also taken at 0 DAT for all compounds. A comparison was made between light and dark conditions at a single sampling point with the aim of assessing the impact of light on a range of CPPs. The extraction solvents and HPLC methods used are described in ref 16 and fully detailed in the SI.

Statistical Analysis. Provided the assumptions related to ANOVA were met, parametric tests were performed on nontransformed data. If assumptions were not met, data was log transformed, or a nonparametric test was performed instead. A mixture of two-way ANOVA with Tukey test (with treatment, time, and treatment × time as factors), correlation analysis, Kruskall–Wallis, and *t* tests were performed on data. Errors are all ±1 standard error (SE). All analyses were performed using Minitab version 15, and figures were plotted using Sigmaplot v. 12.0.

RESULTS

Time Course of Degradation of Benzovindiflupyr and Chlorotoluron under Light and Dark Conditions. Benzovindiflupyr mass balances were between 86% and 100% for all sampling times with the exception of 120 DAT under light conditions, which had a mass balance of 83%. Light treatment ($p \leq 0.001$) and sampling time ($p \leq 0.001$) both had a significant effect on the proportion of extractable benzovindiflupyr (Figure 1). Benzovindiflupyr transformation was more rapid under light conditions with 58.8% ± 1.1% parent remaining in the light and 70.6% ± 1.4% in the dark at 120 DAT (Figure 1). The $DegT_{50}$ of benzovindiflupyr was approximately halved under light from 373 to 183 d. The $DegT_{90}$ value was 608 d under light conditions and >1000 d in the dark treatment (the χ^2 error value was <15 for $DegT_{50/90}$).

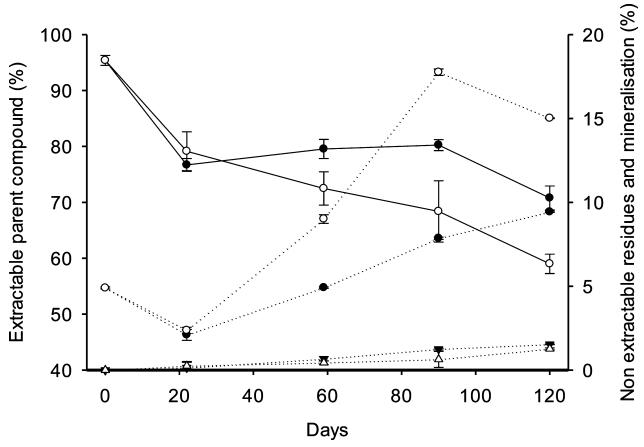

Figure 1. Mass balance for benzovindiflupyr under light (open symbols) and dark (closed symbols) conditions. The partitioned radioactivity is shown for extractable parent compound (–●–), mineralization (···▲···), and nonextractable residues (···●···). Error bars are ±1 SE.

Light was found to affect the proportion of benzovindiflupyr NERs (Figure 1). NER increased over the time course of benzovindiflupyr degradation and values were significantly higher under light compared to dark conditions at 90 DAT ($p \leq 0.05$) and 120 DAT ($p \leq 0.001$). For example, at 120 DAT, NER accounted for 15.0% ± 0.2% and 9.5% ± 0.2% of total radioactivity under light and dark conditions, respectively. Benzovindiflupyr mineralization was minimal at <2% under both light and dark conditions at 120 DAT (Figure 1).

Chlorotoluron mass balances were between 94% and 102% for all sampling points. Light treatment ($p \leq 0.001$) and sampling time ($p \leq 0.001$) also had a significant effect on the proportion of extractable chlorotoluron (Figure 2). Moreover, a significant interaction was observed between sampling time and light treatment ($p \leq 0.001$). Chlorotoluron transformation was more rapid under light conditions with 11.6% ± 1.0% parent remaining in the light and 26.5% ± 0.6% in the dark at 28 DAT ($p \leq 0.001$) (Figure 2). Chlorotoluron $DegT_{50}$ values were 10 and 15 d under light and dark conditions, respectively. The chlorotoluron $DegT_{90}$ value was approximately halved from 79 d in the dark to 35 d under light (χ^2 error value was <15 for $DegT_{50/90}$).

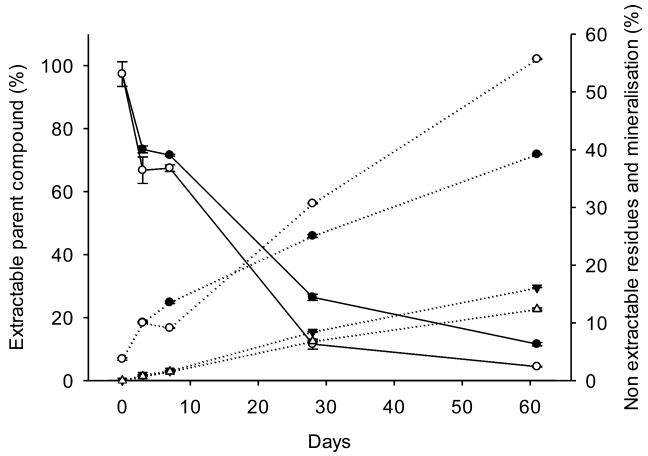

Figure 2. Mass balance for the herbicide chlorotoluron under light (open symbols) and dark (closed symbols) conditions. The partitioned radioactivity is shown for extractable parent compound (–●–), mineralization (···△···), and nonextractable residues (···●···). Error bars are ±1 SE.

Chlorotoluron NER increased during the time course of degradation and were significantly higher under light conditions at 61 DAT with 55.7% ± 3.2% and 39.2% ± 1.7% ($p < 0.05$) of total radioactivity under light and dark conditions, respectively. Chlorotoluron mineralization increased over the time course of degradation with 12.4% ± 1.3% and 16.1% ± 0.2% at 61 DAT under light and dark conditions, respectively. Mineralization was significantly higher under dark conditions at 3 DAT ($p \leq 0.001$) and 28 DAT ($p \leq 0.05$) Figure 2).

Chlorophyll *a*, pH, and Bacterial, Fungal and Archaeal Copy Numbers across a Time Course of Benzovindiflupyr and Chlorotoluron Degradation under Light and Dark Conditions. Light had a significant effect on chlorophyll *a* abundance ($p \leq 0.001$) (Figure 3a,d), and both light ($p \leq 0.001$) and time ($p \leq 0.01$) significantly impacted soil pH during the degradation of benzovindiflupyr and chlorotoluron (Figure 3b,e). Light treatment did not have a significant impact on bacterial copy number during benzovindiflupyr degradation; for example, at day 120, there were $(1.26 \times 10^8) \pm (1.26 \times 10^7)$ and $(2.90 \times 10^8) \pm (2.15 \times 10^7)$ copies under light and dark conditions, respectively ($p = 0.35$) (Figure 3c). Similarly, copies of bacterial 16S rRNA genes did not differ significantly between light $[(3.8 \times 10^8) \pm (1.05 \times 10^8)]$ and dark $[(1.43 \times 10^8) \pm (7.02 \times 10^7)]$ conditions 61 days after chlorotoluron application ($p = 0.71$) (Figure 3f). Archaeal copy numbers were not significantly different between light and dark conditions for either benzovindiflupyr ($p = 0.08$) or chlorotoluron ($p = 0.62$). Similarly, fungal copy number was not significantly different between light and dark conditions for either benzovindiflupyr ($p = 0.06$) or chlorotoluron ($p = 0.31$) (full details are shown in Figure 4 of the SI).

Compound Screen: The Degradation of a Variety of CPPs under Light and Dark Conditions. The compound

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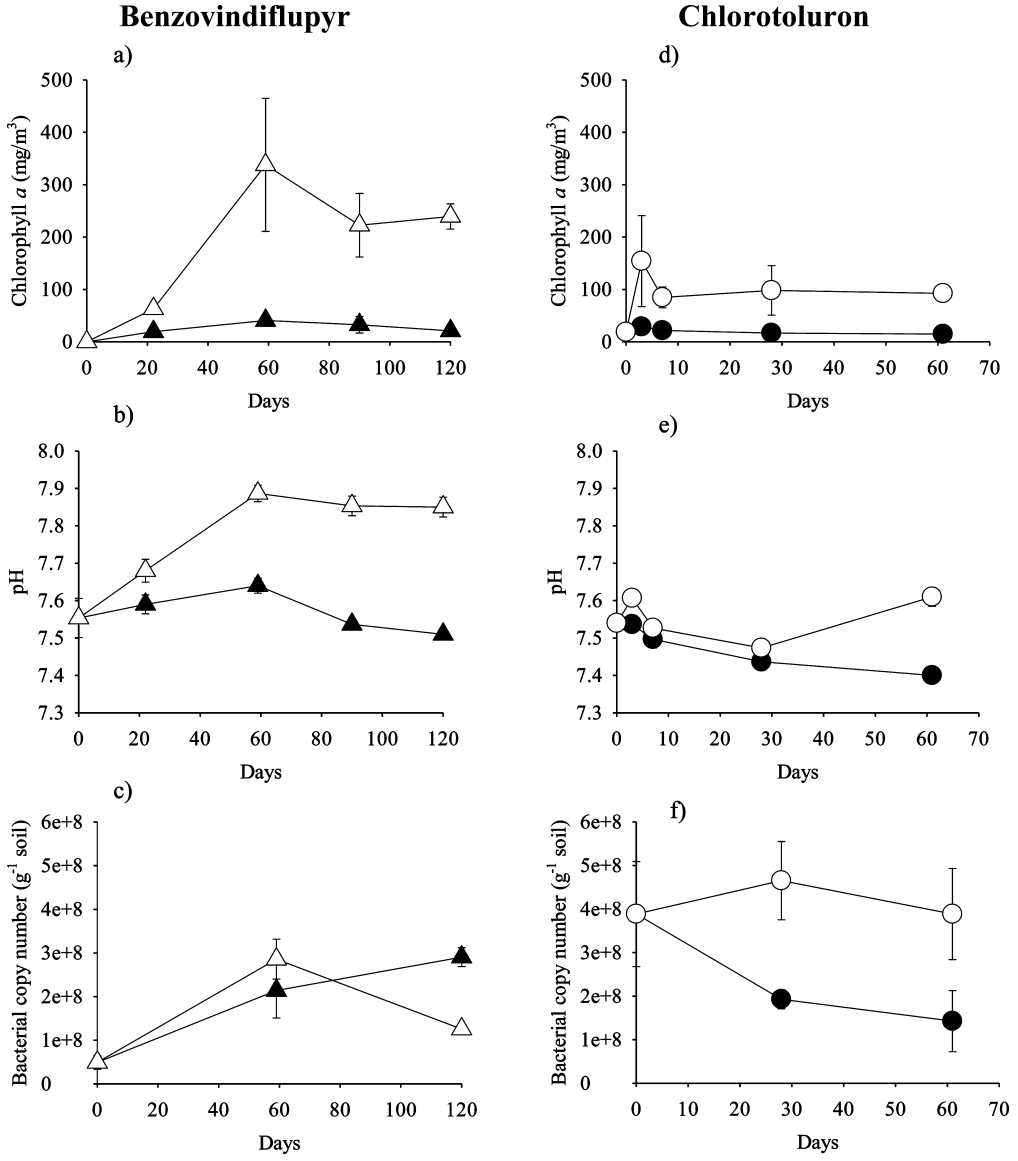

Figure 3. Chlorophyll *a*, pH, and bacterial (16S rRNA gene) copy number for soil incubated under light (open symbols) and dark (closed symbols) conditions during a time course of benzovindiflupyr (triangles) and chlorotoluron (circles) degradation. Error bars are ±1 SE.

screen showed the effect of light on the transformation of CPPs to be compound specific (Figure 4). Extractable parent compound was significantly lower under light relative to dark conditions for prometryn (6.4% ± 0.4% and 10.3 ± 0.5%; $p \leq 0.01$), fludioxonil (41.6% ± 1.3% and 65.7% ± 0.4%; $p \leq 0.01$), and imidacloprid (29.8% ± 1.1% and 48.1% ± 1.5%; $p \leq 0.001$). Cinosulfuron behaved atypically, with an increase in compound persistence under light conditions, with 42.2% ± 2.3% and 28.4% ± 0.7% under light and dark conditions, respectively ($p \leq 0.05$). There were no significant differences in the rate of transformation between light and dark treatments for propiconazole and lufenuron.

NER and mineralization results from the compound screen (Figure 5) were typically consistent with those from the $DegT_{50}$ degradation study (Figures 1 and 2). NERs were significantly higher under light for prometryn ($p \leq 0.05$), fludioxonil ($p \leq 0.01$), propiconazole ($p \leq 0.001$), lufenuron ($p \leq 0.05$), and imidacloprid ($p \leq 0.01$). However, no significant difference was observed for cinosulfuron. Particularly large differences in NER were observed for imidacloprid and fludioxonil, with an approximate 3.5-fold increase under light. Mineralization was significantly higher under dark conditions for prometryn ($p \leq 0.01$), cinosulfuron ($p \leq 0.01$), lufenuron ($p \leq 0.001$), and propiconazole ($p \leq 0.05$) (Figure 5). In contrast, a significantly greater proportion of fludioxonil mineralized under light compared to dark conditions ($p \leq 0.01$) (Figure 5). Imidacloprid mineralization did not differ significantly between light and dark conditions (Figure 5) (full details of compound screen results are shown in Table 4 of the SI).

■ DISCUSSION

The inclusion of non-UV light to the OECD guideline 307 had a compound specific effect on the rate of CPP degradation. Light was found to significantly increase the rate of transformation for five out of the eight CPPs tested and reduced the rate of degradation of one CPP. Light treatment also resulted in an increase in the rate of NER formation for seven CPPs. A variety of mechanisms could be responsible for the "light effect", including the proliferation of phototroph communities

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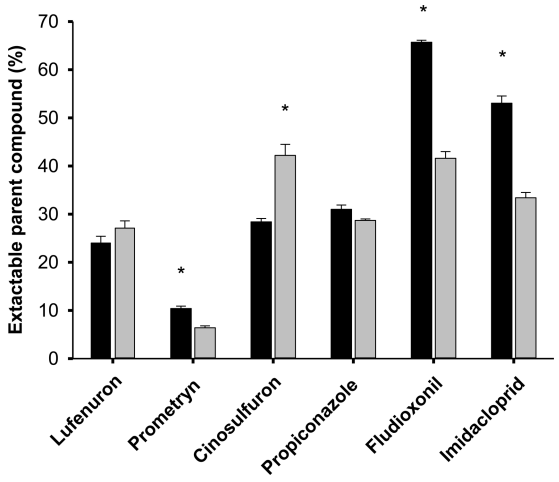

Figure 4. Extractable parent compound for a variety of crop protection products under light (gray) and dark conditions (black). Sampling times were as follows: lufenuron (25 DAT), prometryn (32 DAT), cinosulfuron (34 DAT), propiconazole (43 DAT), fludioxonil (69 DAT), and imidacloprid (102 DAT). Significant differences are indicated by an asterisk (*) ($p \leq 0.01$). Error bars are ±1 SE.

and pH shifts. This is the first study to report that non-UV light affects the rate of CPP degradation in soil.

The transformation rates of chlorotoluron, benzovindiflupyr, prometryn, imidacloprid, and fludioxonil significantly increased under light relative to dark conditions (Figures 1, 2, and 4). For example, the inclusion of light resulted in an approximate halving of the $DegT_{50/90}$ of benzovindiflupyr (Figure 1), a halving of the $DegT_{90}$ of chlorotoluron (Figure 2), and significantly lower extractable parent compound for prometryn (4%), imidacloprid (18%), and fludioxonil (24%) under light conditions compared to in the dark (Figure 4). A similar impact of light has also been observed in water−sediment systems containing algae or macrophytes; for example, Thomas and Hand showed considerable reductions in the persistence of chlorotoluron, fludioxonil, prometryn, pinoxeden metabolite (diketone), and propiconazole under light compared to dark controls.[16] The study showed a 4-, 5-, 7-, and 20-fold enhancement of transformation for chlorotoluron, prometryn, propiconazole, and fludioxonil.

In this study, light clearly influenced the rate of CPP transformation for six out of eight CPPs tested, but the mechanisms responsible may be compound specific. First, phototrophs proliferated under light conditions for all CPPs tested, with the exception of cinosulfuron (Figure 3a,b, and Table 5 of the SI). A direct effect of phototrophs on CPP degradation is supported by several studies indicating the potential for algae and cyanobacteria to degrade CPPs in axenic cultures.[13−17] Phototrophs could also indirectly affect CPP degradation through a shift in soil pH. Indeed, a significant increase in pH under light compared to dark conditions during the degradation of benzovindiflupyr and chlorotoluron (Figure 3c,d) would have been caused by the uptake of CO_2, which is acidic, by phototrophs during photosynthesis.[16] Both light[11] and pH[27] have previously been shown to influence microbial community composition in soil, which could in turn impact the rate of CPP transformation in the current system. For the CPPs tested, the pH increase is not thought to impact CPP dissociation as the soil pH is not within 2 pH units of the pK_a values of the CPPs tested. The fact that no correlation was found between the difference in CPP transformation between light and dark treatments and chlorophyll *a* abundance (Figure 5a of the SI) suggests that potential shifts in heterotroph communities caused by the impact of phototrophs on soil pH could be important (Figure 3c,d). The mechanisms responsible for enhanced degradation rates of cinosulfuron in the dark are unknown; however, it could be due to light preferentially selecting against organisms that are directly or indirectly important in cinosulfuron degradation.

An additional impact of light on soil communities may be driven by an input of C by photosynthesis, which could potentially increase viable biomass or microbial activity and consequently the rate of CPP transformation. If true, this mechanism would be analogous to "the rhizosphere effect", which has been documented for several organic compounds.[28] For example, Marchland et al. observed that under 4-week-old maize seedlings, 61% of atrazine had mineralized compared to only 48% in nonplanted soil.[29] However, our results do not support this theory, as there were no significant differences in bacterial, fungal, or archaeal copy numbers between light and dark treatments during a time course of benzovindiflupyr and chlorotoluron degradation (Figure 3, and Figure 4 of the SI).

A lag phase is often observed in the degradation of growth-linked catabolized compounds to allow microorganisms time to induce specific degradative enzyme systems to catabolize the compound.[30] In the current system, there may also be a lag phase to allow the development of phototrophs. At the end of the lag phase, phototrophs are able to directly or indirectly impact the rate of CPP transformation. Indeed, this is supported by the time course of chlorotoluron degradation, where the $DegT_{90}$ was halved under light compared to dark conditions, but no impact was observed on the $DegT_{50}$ (Figure 2).

The physicochemical properties or mode of action of the CPPs may also play a role; however, the difference in CPP transformation between light and dark treatments was not correlated with the environmental persistence ($DegT_{50}$) or bioavailability (K_{oc} value or water solubility) of the CPPs tested (Figure 5b−d of the SI). Further, the mode of action of the CPPs tested did not have as dramatic effect as expected. For example, prometryn and chlorotoluron both inhibit the electron transport system in photosytem II.[18] Therefore, an application of prometryn or chlorotoluron to soil may inhibit the growth of photosynthetic organisms, thereby reducing any contribution of phototrophs to CPP transformation. However, both CPPs were found to have significantly faster rates of transformation under light conditions.

It is also important not to overlook potential abiotic differences between light and dark treatments, such as indirect photolysis. Indirect photolysis refers to the absorbance of light by photosensitizers to form reactive intermediates such as hydroxyl radicals, which react with CPPs.[31] In soil, nitrate and humic acids could be considered to be the most likely photosensitizers. UV is thought to be primarily responsible for the photosensitization of humic acids,[32] and nitrate does not absorb light at >350 nm,[33] and therefore, neither would photosensitize in a non-UV light system (see Figure 3 of the SI for the spectrum and intensity of light). Therefore, indirect photolysis is unlikely to be major factor responsible for the "light effect"; however, unknown photosensitizers cannot be eliminated from contributing to the effect. Overall, the "light effect" and the mechanisms responsible for the effect appear to be CPP specific. For example, phototrophs proliferated for all

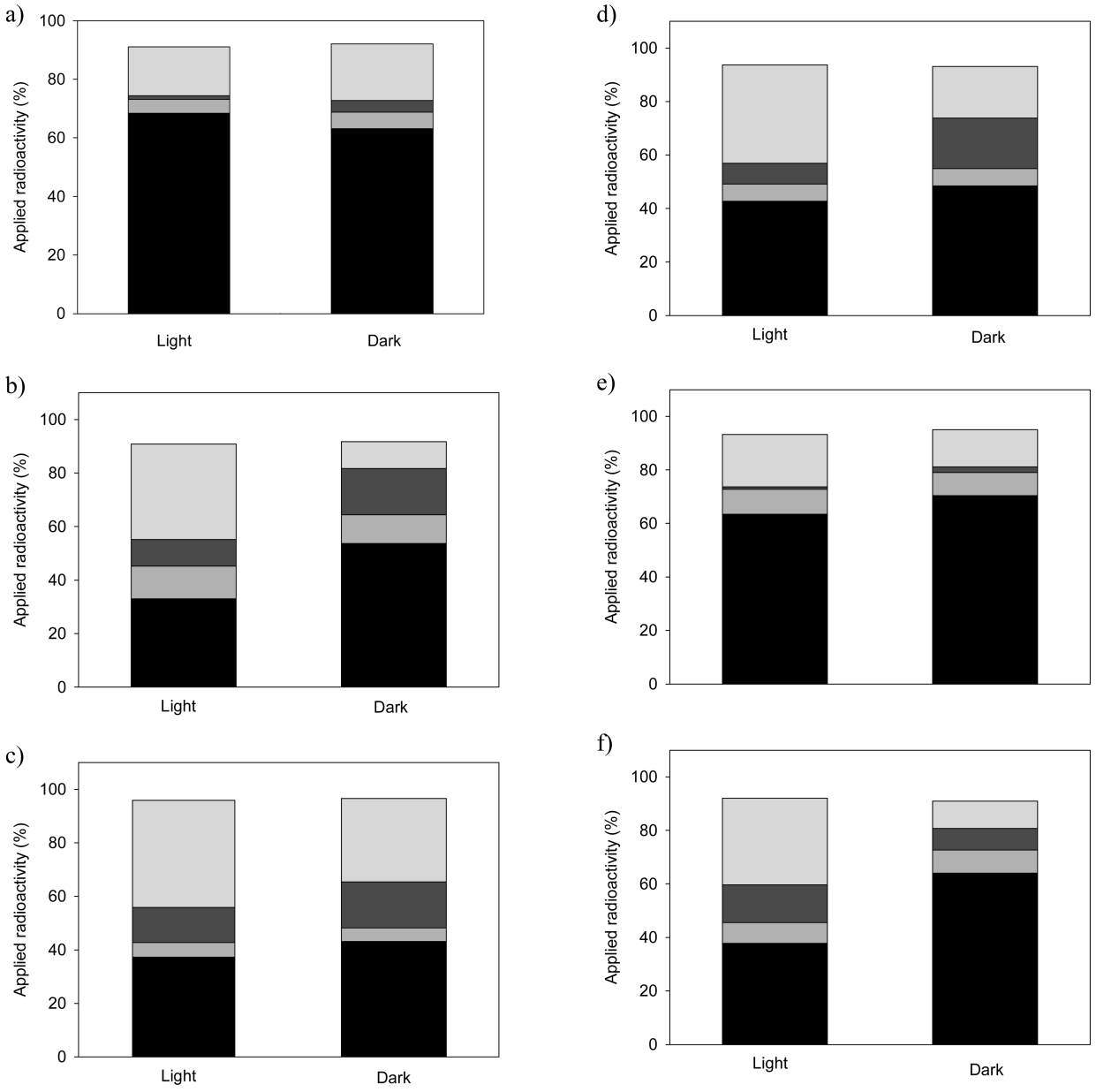

Figure 5. Recovery of partitioned radioactivity: (a) cinosulfuron (34 DAT), (b) imidacloprid (102 DAT), (c) prometryn (32 DAT), (d) propiconazole (43 DAT), (e) lufenuron (25 DAT), and (f) fludioxonil (69 DAT); main extract (black), ASE extract (light gray), mineralization (dark gray), and nonextractable residues (white).

CPPs that degraded faster under light; however, accelerated degradation rates were not observed for all CPPs that showed phototroph proliferation. Further, the contribution of heterotrophs to the "light effect" could also be CPP specific.

The use of light in laboratory degradation test systems has the potential to improve the accuracy of estimated CPP persistence in degradation studies. OECD guideline 307 states that degradation studies can only be conducted for a maximum of 120 d, as after this time, microbial biomass and activity are reduced considerably. The $DegT_{50}$ of highly persistent compounds are therefore estimated using regression analysis. However, the rate of CPP degradation may change from first-order kinetics following 120 d, and thus $DegT_{50}$ estimations by regression may not be accurate. The addition of C to the system via phototrophs may act to maintain microbial biomass and activity for a longer duration. Hence, studies could be carried out for longer than 120 d, potentially increasing the accuracy of $DegT_{50}$ values for persistent CPPs or other xenobiotics. In addition, the presence of phototrophs in an agricultural soil crust may affect CPP mobility, particularly for less polar CPPs such as lufenuron and fludioxonil (Table 1). These compounds may be more likely to sorb to the BSC rather than dissolve in the aqueous phase of soil, potentially accelerating rates of degradation through an extended exposure to phototrophs, heterotrophs, and photolysis at the soil surface.

Although CPP transformation rates were typically faster in the light, mineralization was considerably greater under dark conditions for all CPPs tested with the exception of imidacloprid and fludioxonil (Figures 1, 2, and 5). Phototrophs may be utilizing $^{14}CO_2$ for photosynthesis, reducing the overall amount collected in NaOH traps. Therefore, mineralization results may not be reliable under light conditions. Fludioxonil

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behaved atypically by being the only compound tested that showed higher rates of mineralization under light conditions. Transformation under light may be increased to such an extent as to offset any uptake of $^{14}CO_2$ by phototrophs. This is supported by fludioxonil showing the greatest difference in transformation between light and dark conditions compared to all other CPPs (Figure 4). Moreover, fludioxonil has been shown to be degraded by eight green algae and four cyanobacteria in pure culture.[34]

NER formation was greater under light for all CPPs with the exception of cinosulfuron. The increase in NER could be due to a variety of reasons, for instance (i) a change in the chemical properties of soil incubated under light increasing the sorption of the CPP to soil, (ii) a greater rate of CPP degradation and incorporation into biogenic residues by photosynthetic or nonphotosynthetic organisms under light, or (iii) the assimilation of $^{14}CO_2$ into the biomass of phototrophs. The third explanation was tested by accounting for the potential uptake of the additional $^{14}CO_2$ found in dark systems by phototrophs in light systems. It was found that NER were still higher under light conditions and significantly higher for fludioxonil ($p \leq 0.01$), imidacloprid ($p \leq 0.01$), lufenuron ($p \leq 0.05$), and benzovindiflupyr ($p \leq 0.001$). Therefore, it is likely that an increase in CPP degradation and incorporation into biomass and/or an increase in sorption are responsible for greater NERs under light conditions. NERs are not typically viewed as bioavailable, as particular forms of NER do not pose a short- or long-term threat to ecology, rather they are relatively stable and slowly degraded over an extended period.[35] Therefore, an increase in NERs under light conditions could translate to greater CPP sorption under agricultural cropping systems when a soil crust has developed.

This is the first study to investigate the effect of non-UV light on the degradation of CPPs. The inclusion of non-UV light to standard laboratory studies increased the rate of CPP transformation for five CPPs and NER formation for seven out of the eight CPPs tested. This effect may have been driven by the presence of soil phototrophs, which could directly degrade CPPs or indirectly impact heterotroph community composition and/or alter soil chemical properties such as pH. Phototrophs represent the first point of contact for CPPs applied to the soil surface, and therefore, the results have important implications for pesticide legislation. It is important to further investigate the mechanisms responsible, test if the "light effect" is soil-specific and if natural light also has a similar effect, and continue to alter additional variables, with the ultimate aim of bridging the gap between laboratory studies and field applications and improving the risk assessment of CPPs.

■ ASSOCIATED CONTENT

Supporting Information

Tables 1−5, Figures 1−5, and eqs 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org/

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Notes

The authors declare no competing financial interest.

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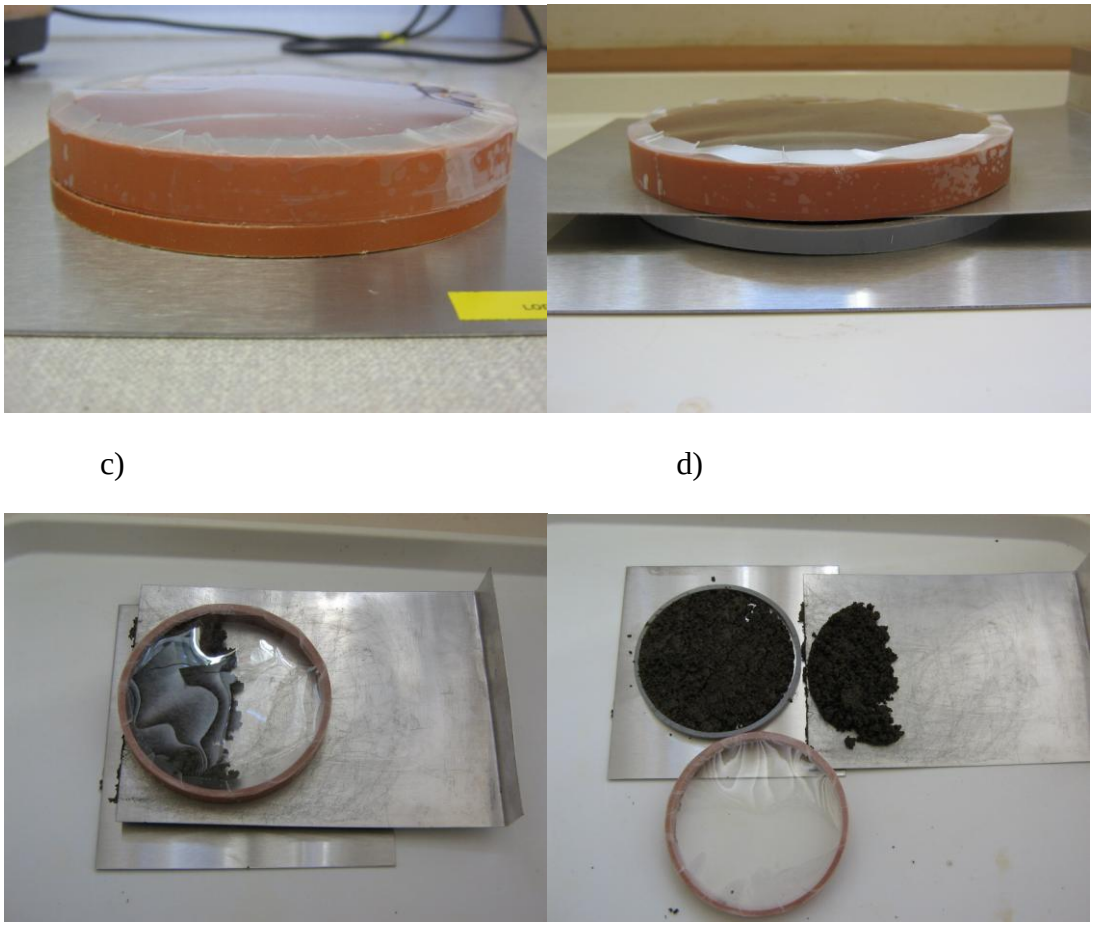
APPENDIX III

SOIL SURFACE SAMPLING FOR METATRANSCRIPTOME ANALYSIS

The sampling design used to generate soil samples used in metatranscriptome analysis are shown in Figure 1.

a) b)



c) d)

Figure 1: Sampling the soil surface; (a) Core sections; (b-d) Sampling top 3 mm of soil from the underlying 6 mm.

APPENDIX IV

CHLOROTOLURON DEGRADATION UNDER LIGHT AND DARK CONDITIONS IN OPEN SYSTEMS

1. CHLOROTOLURON DEGRADATION EXPERIMENTS

Additional chlorotoluron degradation experiments were conducted in the soil incubation systems setup in Chapters III and IV. These experiments aimed to reproduce the effect of light on chlorotoluron degradation rates shown in Chapter II in slightly modified test systems.

1.1. CHAPTER III: THE IMPACT OF ESTABLISHED PHOTOTROPH COMMUNITIES ON CHLOROTOLURON DEGRADATION IN SOIL

1.1.1. Method

The test system used was described in Section 3.3.2. Soil surface communities were allowed to develop for 85 days prior to chlorotoluron application. Chlorotoluron was applied at the same concentration as described in Section 2.3.3.3. Chlorotoluron was dissolved in 1 ml acetonitrile and 200 ml water was then added prior to applying dropwise to the soil surface. Day 0 and 20 DAT samples were taken. At each sampling point, soil was homogenised and chlorotoluron was extracted from 5 g soil using 20 ml acetonitrile:water (80:20 v/v) and analysed by HPLC as described in section 2.3.3.3.

1.1.2. Results

Chlorotoluron was applied at ~94% of the desired application rate. After 20 days, ~17% and ~14% of chlorotoluron had degraded under light and dark conditions, respectively (Table 1).

Table 1: Chlorotoluron transformation after a surface application to soil incubated under light and dark conditions for 85 days

Day	Treatment	Chlorotoluron (%)
0	Dark	93.9%±S.E 7.8
20	Light	16.9%±S.E 2.0
	Dark	14.1%±S.E 1.6

1.2. CHAPTER IV: THE IMPACT OF LIGHT ON THE DEGRADATION RATE OF CHLOROTOLURON APPLIED TO THE SOIL SURFACE

1.2.1. Method

1.2.1.1. Chlorotoluron application and extraction

Chlorotoluron was applied at a field application rate of 600 g ai ha^{-1} (0.8 µg/g) assuming a soil bulk density of 1.5 g cm^{-3} and the compound was distributed to a

maximum depth of 5 cm (OECD Guideline 307). 500 ml of chlorotoluron stock solution was prepared by dissolving 13.00 mg ^{12}C-chlorotoluron in 1 ml acetonitrile before adding 500 ml sterile, deionised water. The stock was filter sterilised (0.45 µM). A plastic matrix was laid over the soil surface containing 100 holes evenly spaced over the soil surface. Chlorotoluron stock was applied by pipetting 20 µl into each hole across the entire surface (2 ml total). Chlorotoluron was extracted from 10 g fresh soil within an hour of sampling for all samples. Soil was added to 50 ml falcon tubes and 2 x 20 ml acetonitrile: U.P H_2O (80:20 v/v) was added. Samples were shaken at 300 rpm for 1 hr using a shaker that rotated samples 360°. Extractions were pooled and 1 ml extract was transferred to a 2 ml glass HPLC vial and sealed with a septum and plastic screw top lid (Greyhound Chromatography, Birkenhead, UK) before being stored at -20°C prior to analysis.

1.2.1.2. Chlorotoluron analysis by liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed using an Agilent 1100 series quaternary pump, degasser and column oven, an API 4000 triple quadrupole mass spectrometer detector (Applied Biosystems, Warrington, UK) with Analyst software version 1.4.1, and Agilent 1100 series autosampler. An ACE5 C18 column (5 µM particle size, 3.0 mm diameter and 100 mm length) with a column oven temperature of 30°C± 3°C was used for liquid chromatography. A turboionspray interface, a temperature of 450°C and an ionspray voltage of 5500V was used for mass spectrometry. A run time of 6 mins was used with a flow rate of 1 ml min^{-1} using 20% acetonitrile and 80% acetic

acid in U.P H_2O (0.1% v/v), ramping up to 80% acetonitrile after 4 mins, and back to 20% after 5 mins. 25 µl sample was injected and chlorotoluron had a retention time of approximately 3 mins 20 secs. Chlorotoluron detection was suppressed by substances extracted from soil during the extraction process. This was accounted for by comparing chlorotoluron standards in acetonitrile:U.P H_2O (80:20 v/v) with those from extracts from soil incubated under light conditions for 14 days which were then spiked with known chlorotoluron standards.

1.2.2. Results

1.2.2.1. Chlorotoluron transformation

Almost 100% of chlorotoluron had been transformed after 30 days incubation under both light and dark conditions (Figure 1). The $DegT_{50}$ of chlorotoluron was similar under light and dark conditions at 8 and 9 days, respectively.

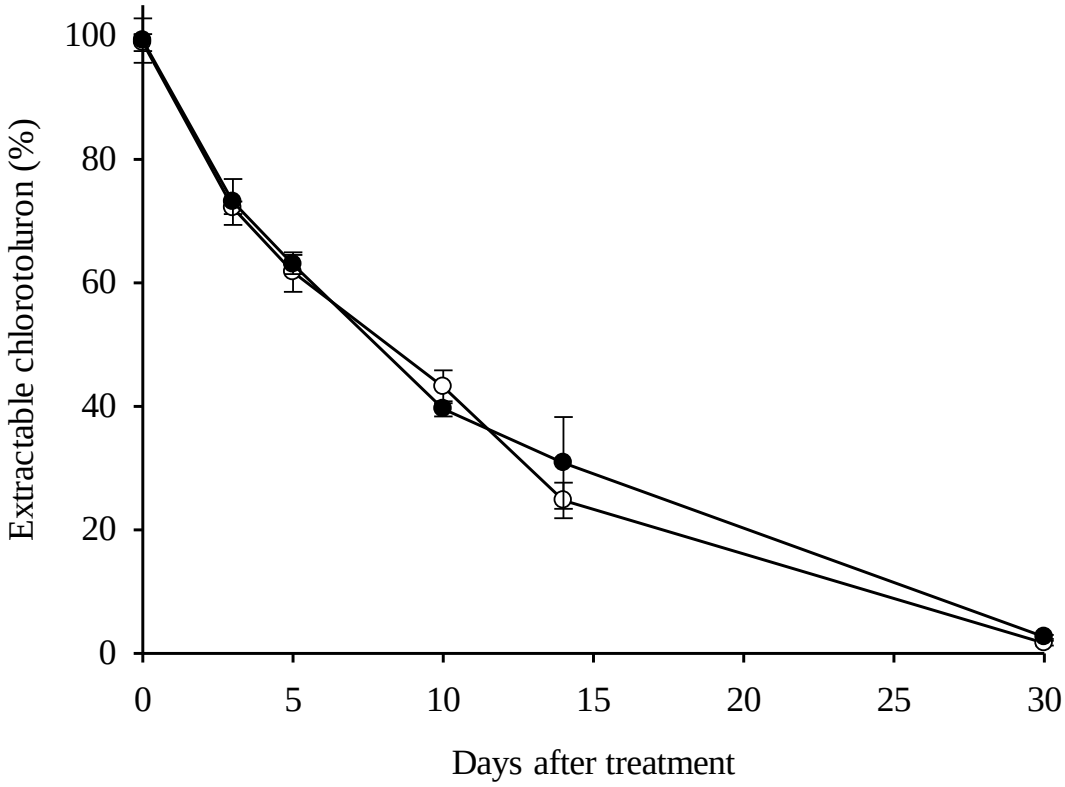


Figure 1: Timecourse of chlorotoluron transformation in Gartenacker soil incubated under light (open symbols) and dark (closed symbols) conditions. Error bars are ±1 S.E.

1.3. CONCLUSION

The impact of light on chlorotoluron transformation was tested in open systems, both with and without established soil surface phototroph communities. Light did not have an impact on the rate of chlorotoluron transformation in either test system, relative to dark conditions.