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The Link between Fungal Nutrition and Fungal Phenotype

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January 2014

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Abstract

Carbon and nitrogen are key macronutrients affecting growth and fitness particularly in heterotrophic organisms. A considerable body of evidence suggests that many organisms including mammals, insects and slime moulds have a target intake that is optimal in the sense of maximising growth or ecological fitness. In nutritionally heterogeneous environments, these organisms show an ability to regulate the intake of carbon (C) and nitrogen (N) compounds by selecting different food types to reach the target ratio. This is now a major focus of research in diet-related chronic disease in humans including obesity. Fungi are one of the most important components of the terrestrial ecosystem, and play a key role in nutrient cycling, structural genesis, water infiltration and carbon storage in soil. The manner in which the fungal phenotype emerges in response to the complex nutritional environment of soil is fundamental to the persistence of these functions across space and time. We explore the extent to which the fungal phenotype can be understood in terms of a target ratio of C: N. We used the fungus *Mucor mucedo* as the model species, and studies its growth in different nutrient regimes by varying the C: N ratio, and including both organic and inorganic sources of nitrogen. There is evidence for a target C: N ratio in a homogeneous environment, although growth rate remains high over a relatively broad range in the ratio by comparison with other organisms. We attribute this to the capacity of fungi to recycle and translocate internal sources of nutrients to regions of high demand. In a heterogeneous environment, we provide evidence that this is the case, although nitrogen is more readily translocated than carbon in this species. In this study, a comparison of growth rate for different C: N ratios and nutrient concentrations indicates efficiency, the amplitude of the oscillations is a measure of stability. This provides an important constraint for our understanding of underlying regulatory pathways linking C and N to growth. A hyphal-level
model for fungal growth is developed to study the consequences of our findings for the emergence of the fungal phenotype, and is used to generate new hypotheses for future testing. Finally, a metabolic model is constructed that synthesises existing knowledge of carbon and nitrogen pathways in cells. We built two versions of this network model corresponding to the case of organic and inorganic sources of nitrogen respectively. Both models reproduced oscillatory growth observed in the laboratory experiments and, consistent with observation, the amplitude of the oscillations is positively correlated with the C: N ratio only for the inorganic N version of the model. A peak C: N ratio for fungal growth is also only predicted for inorganic sources of N, as we saw in the observed behaviour. The networks we built for this project may be highly conserved across the kingdom of life, therefore the models may be broadly applicable with certain modifications.
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Chapter 1. Introduction

1.1 Nutritional Heterogeneity in the Environment

Most organisms typically are faced with heterogeneously distributed resources across a wide range of temporal and spatial scales. Resources are not only heterogeneous in their spatial distribution, but also in their nutritional composition. Even in apparently homogenous resources such as wood, the nitrogen content of wood ranges from 0.03 to 0.1% in surface to inner core layer of the wood (Cowling and Merrill, 1966). On the other hand, the demands of growth of organisms require that they obtain a wide range of nutrients from their diet. Nutritional components such as protein, carbohydrate and fat that are required in relatively large amounts are defined as macronutrients. Organisms also require micronutrients such as vitamins and trace elements. A single source of food seldom contains all nutrients in ideal concentrations. Therefore, organisms require food choices. At the most obvious level, the availability of nutrients is a primary factor defining the geographic distribution and temporal pattern of activity for many organisms (Raubenheimer, 2010). This is because nutrient requirements for a certain physiological needs such as fitness and reproduction differ between species, and even for the same species over time and space; nutrient requirements vary with age and sex, and physiological state and developmental stage (Raubenheimer and Simpson, 1999). Nutrient requirements also vary with environmental factors such as temperature. Because of the requirement of various nutrients, and the differing rates of use and depletion of nutrients, persistence in nutritionally homogenous environments is almost impossible for most organisms.
1.2 Energy and Glycolysis

Provision of energy is perhaps the most important and basic benefit of nutrition. Without energy, organisms are simply unable to function and access other resources. Because of their fundamental importance, for the most basic functions of living systems, the earliest organisms must have possessed these pathways.

Glycolysis is an energy releasing pathway. Many characteristics of the glycolytic pathway suggests establishment early in the history of life (Storey, 2004, Webster, 2003). Glycolytic enzymes operate within an aqueous medium, and adenosine 5′-triphosphate (ATP) production does not require a membrane (Storey, 2004). Glycolytic enzymes and their sequences are also some of the most ancient and highly conserved proteins and genes, even between higher mammals and Eubacteria (Lonberg and Gilbert, 1985, Peak et al., 1994, Poorman et al., 1984). Glycolysis functions independently of oxygen and would have been well-suited to an anaerobic ancient earth. The two substrate-level phosphorylation reactions of glycolysis would have provided enough energy to meet the needs of a primitive cell (Storey, 2004).

The glycolytic pathway is strongly conserved across different kingdoms of life (Fothergill-Gilmore, 1986), and this is also likely to be true for other key energy pathways. These metabolic pathways have undergone lengthy and strong selection while life was still single-celled (Storey, 2004), and therefore for about 75% of the time that there has been life on Earth. This can be the reason why organisms belonging to different kingdoms of life can share one generic set of nutritional pathways.
1.3 Macronutrition and the Importance of Carbon and Nitrogen

Supplies of key chemical elements may constrain outcomes of physiological processes in organisms and thus their growth and reproduction. Carbon is the most important and abundant element in living organisms. Carbohydrates provide energy and are also a major building block for many biological compounds, such as chitin, lipids, amino acid. Heterotrophic organisms live on organic compounds produced by other organisms (Campbell et al., 2009). Much attention has focussed on exactly how these organisms select from various sources of nutrition to ensure they have all of the compounds they require for growth. One hypothesis, most explicitly articulated in optimal foraging theory (Stephens and Krebs, 1986), has been to assume that a single nutritional currency, usually energy, is sufficiently important from the organism’s perspective to be the sole target for uptake (Ydenberg et al., 1994). However this theory has serious limitations because it is now known that the mix of different sources of energy are important and therefore the theory cannot deal with the central nutritional question of how organisms integrate the intake to optimise fitness (Raubenheimer and Simpson, 1999). Diverse energy sources may be consumed because of the organism’s requirements for nutrients not related directly to energy (Pulliam, 1975). Organisms are frequently forced to ingest excesses of other nutrients in order to limit the shortfall of a deficient one (Raubenheimer and Simpson, 1999). Therefore, nutritional models that implicate multiple nutritional elements are needed.

After carbon (and hydrogen), nitrogen is the next most abundant element in living systems. It is essential for the biosynthesis of complex molecules in cells such as amino acids, nucleic acids and some vitamins. Carbohydrate and nitrogenous compounds are closely linked in metabolic systems organisms, and indeed the carbon to nitrogen ratio is considered as an important stoichiometric relationship in ecology (Elser and Hamilton, 2007).
1.4 The Geometric Model for Nutrition

Studies on challenges in animal nutrition are more advanced than on other living organisms. The methodology used in animal study may inspire further nutrient studies on other living organisms. The geometric model is a useful representation of how organisms satisfy nutrient uptake requirements for two or more compounds simultaneously (Simpson and Raubenheimer, 2001). The geometric model describes how organisms must ingest a particular amount and combination of different nutrients, called the intake target, in order to perform optimally e.g. to maximise fitness or growth rate (Simpson and Raubenheimer, 1996, Raubenheimer and Simpson, 1993). In a nutritionally heterogeneous environment, where a number of unbalanced food items are distributed, many organisms show an ability to make food choices such that they regulate the intake of multiple nutrients independently to reach their intake target (Raubenheimer and Simpson, 1993). When nutritionally balanced or complementary foods are unavailable and the organism cannot balance its nutrient requirements solely by intake, they can achieve the target by selectively excreting excesses that they ingest.

1.5 Unitary and Modular Organisms

Organisms can be classified as either unitary or modular (Townsend et al., 2008). Growth of unitary organisms follows a determinate pathway of development from juvenile to adult form. All arthropods and vertebrates are unitary organisms. They have a fixed lifespan and size. Growth of modular organisms occurs by the indeterminate iteration of repeated units of structure (modules). They have an indefinite lifespan and size. Fungi, and slime mould are exemplars of modular organisms.
1.6 Examples of Unitary Organisms and How They Conform to the Geometric Model

Larvae of *Spodoptera littoralis* conform to the geometric model (Simpson et al., 2004). Final-instar larvae were provided with one of 35 foods varying in the ratio and concentration of protein and digestible carbohydrate. Seven dilutions of each protein to carbohydrate ratio (5:1, 2:1, 1:1, 1:2 or 1:5) were made available to the larvae. A fitness index was calculated as % survival within a treatment multiplied by mean rate of development (wet mass grown divided by stadium duration). Larvae reached the highest fitness index when the protein to carbohydrate ratio was close to 1:1.

Following on from the single food, no choice, experiment, final-instar larvae were provided with a pair of nutritionally complementary food blocks, containing the following protein to carbohydrate ratios (%P:%C): 35P:7C with 21P:21C; 28P:5.6C with 21P:21P; 28P:5.6C with 16.8P:16.8C; 21P:4.2C with 21P:21C; or 21P:4.2C with 16.8P:16.8C. The larvae selectively fed from the two available sources in such a way that the intake points converged in nutrient space, indicating strong regulation of uptake of both energy and protein. This point lay at 150 mg P, 125 mg C, on the ridge of the performance landscape close to the predicted position of the summit point from the no choice experiment.

The geometric model has been tested on other unitary organisms including other insects (van der Zee et al., 2002, Simpson and Raubenheimer, 2001), and mice (Sorensen et al., 2010) with similar results. The geometric model has also been tested on one modular organism, the slime mould *Physarum polycephalum* (Dussutour et al., 2010), which also conformed to the expectations of the geometric model. The geometric model could be a general model that is applicable to all heterotrophic organisms (Simpson et al., 2009).
1.7 Fungi as Exemplar Modular Systems

Fungi, like any other organisms, must survive in nutritionally heterogynous environment. However, it is not clear how these organisms grow in multiple nutrients.

1.7.1 Fungal Wall Structure

The filamentous growth form is an important property of fungi. Fungi form a mycelium consisting of an indeterminate system of tubes termed hyphae. The fungal wall at the tip is a fine layer over the plasma membrane (Figure 1.1). The wall is much thinner than the fungal wall behind tip. For example in *Neurospora crassa*, the thickness of the wall at the tip is approximately 50 nm, at 250 µm behind tip is approximately 125 nm thick.

1.7.2 Vegetative Fungal Extend and Branch

Fungal hyphae can extend (Deacon, 2006), elongate and branch (Figure 1.1). The fungal mycelium uptakes food mainly at the hyphae tips. Therefore hyphal branching, which produces more tips, can increase the uptake of nutrients by the fungal colony, consequently accelerating biomass production. Hyphal branching is therefore necessary for efficient colonisation and utilisation of the substrate upon which the fungus is growing.
Figure 1.1: Confocal image showing tip-growing and branched, multinucleate hyphae of Neurospora crassa. (A) *Neurospora crassa* hypha stained with FM4-64 showing sub-apical branch formation. Note the initiation of the Spitzenkörper beneath the plasma membrane (arrow) that has appeared just before the branch emerged. (B) Sclerotinia sclerotiorum hyphae stained with FM4-64 showing apical branching. Note the negatively stained nuclei (n) and less stained core region within the Spitzenkörper. Bars = 10 µm (Hickey et al., 2005).

1.7.3 Fungal Physiology of Nutrition

1.7.3.1 Nutrient Uptake, Transport and Storage

The plasma membrane at the fungal tip is the major selectively permeable barrier that dictates nutrient entry (Walker, 2011). Nutrient uptake may be an active and/or passive process at the plasma membrane. ATP is required for active transport of nutrients (Jennings, 1987).

Once in the hyphae, relatively little is known of the mechanisms that determine how nutrients are translocated (Cairney, 2005). Nutrients may be translocated in fungal hyphae by mass flow, diffusion, generalized cytoplasmic streaming and specific vesicular transport (Cairney, 2005).
Translocation through the vacuole has received particular attention because the vacuole in fungi provides a conduit in which material could be translocated independently of the cytoplasm (Ashford and Alloway 2007). The possibility of nutrient translocation over long distances in the vacuole was demonstrated in rapidly growing cultures of *Phanerochaete velutina* (Darrah et al., 2006). In filamentous fungi, the vacuole forms a constitutive, physically contiguous, extended organelle that can span a significant fraction of the colony (Fricker et al., 2008). All filamentous fungi tested so far contain a vacuolar system potentially enabling the translocation of nutrient, or at least the minerals N and P, through the mycelium (Ashford, 1998, Ashford and Allaway, 2002).

Nutrients such as carbohydrates and minerals can be stored in the mycelium (Kirk et al., 2008). Stored carbohydrates in the form of lipids, glycogen, and polyols such as glycerol, and trehalose can be used as energy source. When N availability exceeds fungal N requirement, the storage products will include various complex compounds of N. The stored nutrients can be allocated through the translocation system (Cowling and Merrill, 1966, Levi and Cowling, 1969, Gadd, 1995, Jin et al., 2012, Darrah and Fricker, 2014).

### 1.7.4 Different Groups of Fungi

Fungi is one of the most species-rich kingdoms. Fungal species can be divided into different taxa.

- **Mucoromycotina**: Mainly as saprotrophs in soil, on animal dung, or on various other substrates over-ripe fruits. Mucoromycotina, like all true fungi, produce cell walls containing chitin (Deacon, 2006). Most Mucoromycotina form hyphae that are
coenocytic: they lack cross walls or septa. Secondary septa may form at irregular intervals throughout the older parts of the mycelium (Gow and Gadd, 1995).

- Ascomycota: The Ascomycota are the most diverse division of true fungi. Hyphae have septa with a simple pore (Deacon, 2006). These fungi are important in the decomposition of plant litter and dead animals. The body of Ascomycota consists of a typical eukaryotic cell surrounded by a wall. Members of the Ascomycota have both asexual and sexual reproduction (Deacon, 2006).

- Basidiomycota: The Basidiomycota phylum contains about 30,000 described species, which is 37% of the described species of true Fungi (Kirk et al., 2008). The most conspicuous and familiar are those that produce mushrooms, which are structures for sexual reproduction. Basidiomycota are found in virtually all terrestrial ecosystems, as well as freshwater and marine habitats (Kohlmeyer and Kohlmeyer, 1979; Manohar and Raghukumar, 2013).

Three primary life strategies now are recognized in fungi. They are competitive (C-selected), Stress-tolerant (s-selected), and Ruderal (r-selected). The majority of Mucoromycotina are ruderal (r-selected) fungi. They have fast growth rates, short life spans. Stress, imposed either by poor access to nutrients in the substratum or by abiotic factors, tends to limit interspecific fungal competition. Some basidiomycetes are stress tolerant (s-selected) fungi. They have high enzymatic competence for resource exploitation, temporal persistence. Under a situation of either reduced stress or reduced availability of unexploited resources, C-selected species may predominate. These fungi are persistent, long-lived, and capable of defending captured
resources by interspecific antagonism, and they exhibit good enzymatic capabilities (Zak and Willig, 2004).

1.7.5 Fungal Adaptation of Natural Environment

Both fungal methodology and physiology are enable them adapted in some complicated natural environments. The typical fungal hypha is 2 to 4 µm in diameter (Stoops, 2010). This fine-scale filamentous structure contributes to the capacity for fungi to grow across surfaces, through small pores and across air gaps (Boswell et al., 2003).

Accessing a wide range of resources is an advantage for fungi adapted in different environment. Typically soil contains a low proportion of organic matter. The heterotrophic fungi utilize small molecular weight nutrients and large complex nutrients such as carbon polymers and proteins. Fungi release hydrolytic enzymes: digestion takes place outside the cell, and nutrients are absorbed at the hyphal tip (Russell, 2008). In this way, the fungi access resources for maintenance, growth and reproduction.

The fungal mycelium may redistribute nutrients (Govindarajulu et al., 2005, Tlalka et al., 2007, Martin et al., 1984). Redistribution of nutrients within the mycelium enables certain fungi to grow in patchy environments (Boswell et al., 2003). In particular redistribution allows fungi to use some resources at one location to invest in exploratory growth in another location (Fitzsimons, 2011, Ritz and Crawford, 1990). The filamentous fungal growth form can change from an exploratory growth form to an exploitative growth form where nutrients are located (Ritz and Crawford, 1990).
Last but not least, storage of energy and minerals enables fungi to survive periods of low nutrient availability and to grow in habitats where nutrients are unevenly distributed (Whipps, 1993).

1.8 Models of Fungal Growth

The study of fungal growth is fraught with difficulties stemming from the complexity of the both the organism and its environment (Falconer et al., 2011). Mathematical modelling provides a powerful and efficient method of investigation that complements experiments that test mechanistic explanations of phenomena (Davidson, 2007). Numerous mathematical models have been developed to clarify behaviours of fungi (Meskauskas et al., 2004, Cohen, 1967, Boswell et al., 2003, Boswell et al., 2007, Bull and Trinci, 1977, Prosser and Trinci, 1979, Davidson et al., 1996, Edelstein, 1982).

Many of these mathematical models focus on describing fungal morphology in the environment. In the first discrete model of morphology of mycelia (Cohen, 1967), the growth only occurs at the tips of hyphae. The amount and angle of growth are determined in the model by the extant hyphal density in the field. This approach, now termed a ‘vector-based’ model (Meskauskas et al., 2004), has been extended with the addition of many sub-models (Lindenma.A, 1968a, Lindenma.A, 1968b, Hutchinson et al., 1980, Bell, 1986, Kotov and Reshetnikov, 1990). The Neighbour-Sensing model (Meskauskas et al., 2004) aims to simulate mycelial morphology in three dimensions. The Neighbour-Sensing model considers non-planar growth and includes the fungal fruiting body. Though these models can often successfully simulate patterns of fungal growth observed in the same conditions, the current vector models typically neglect nutrition as a factor affecting growth of the fungus. Therefore, those models are unable to describe fungal growth in nutritionally heterogeneous environments or any other than the nutrient setting used...
in the experimental system. Furthermore, the underlying equations in those models are often derived from the statistical properties of one experimental system under investigation (Boswell et al., 2007). Therefore, the same equations may not be suitable to apply broadly. To more generally understand patterns of fungal morphology, different approaches need to be followed.

Continuum models have been built to study the influence of nutrient availability on fungal growth (Boswell et al., 2003, Falconer et al., 2005, Edelstein, 1982, Edelstein and Segel, 1983). Continuum models include many key physiological processes. Boswell et al. (2003) presented a model using three variables: active hyphae, inactive hyphae, and hyphal tips, involved in nutrient uptake and translocation (Boswell et al., 2003). Falconer et al. (2005) added recycling of biomass within the fungal colony (Falconer et al., 2005). The ‘recycling’ model successfully simulates a range of observed phenotypes including the fungal oscillation patterns that are widely observed in laboratory experiments. Continuum models provide good descriptions of mass and substrate distributions for growth in both homogeneous and physically heterogeneous environments. However, because they are continuous in space and time, these models do not incorporate mechanisms such as hyphal branching, anastomosis, and death. As with vector models, continuum models do not consider growth in more than one nutrient environment.

More advanced models have been developed based on the continuum models. Boswell et al. (2007) extended their previous continuum model (Boswell et al., 2003) to a multiple ‘cell’ model. Hao et al. (2009) extended the continuum model of Falconer et al. (2005) to a discrete hypha model. In Hao et al. (2009), each hypha has two kinds of biomass associated with it: mobile and immobile biomass. The model incorporates fundamental physiological processes such as nutritional uptake, translocation and re-mobilization. The current state in each hypha depends on local nutritional status, physiological processes associated with uptake and local recycling,
and the nutrient status of connected hyphae. These advanced continuum models can describe fungal growth in physically and nutritionally heterogeneous environments. However the fungal phenotype in these models, as in all the other models, is assumed to be limited by a single well-mixed nutrient source. Nutritionally heterogeneous environments in these models corresponds with a spatially-variable concentration of food in different locations, the ratios between different nutrients are constant. Constraining growth to a single nutrient environment is an important limitation for current mathematical models.

The carbon to nitrogen ratio of a resource is an important environmental factor affecting fungal growth. Therefore, the study of growth in a single nutrient environment will not address the central nutritional question of how fungi integrate the uptake of various nutrients (Raubenheimer and Simpson, 1999). Current mathematical models need to be extended to answer these central and important questions.

1.9 Fungal Growth and the C: N Ratio

In optimal environmental conditions, fungal growth may be unlimited. However fungal growth is modified by many environmental factors. Important amongst these include nutrient availability, pH value and moisture availability. Indeed, the specific requirements of a fungus may change over time.

The pH value in the environment is an important factor influencing growth of fungi. A Study in the Hoosfield acid strip at Rothamsted Research, United Kingdom, showed peak growth rates of soil fungi occurred above pH 4.5. The growth rate and biomass increased more than five-fold from pH at 8.3 to 4.5. Between pH 4.0 and 4.5 the growth rate and biomass declined
significantly (Rousk et al., 2009). Thus, keeping the pH constant in the substrate is crucial for the study of fungal nutrition.

Nutrients have an overwhelming influence on the growth of fungi. Among nutrient factors, the balance between total carbon and nitrogen uptake is critical for fungal growth and development. The impact of the ratio of carbon to nitrogen in the environment is thought to be greater than the concentration of carbon alone (Gao et al., 2007). In industry, maximum rates of production require the control of the ratio of carbon to nitrogen in media to ensure specific physiological conditions are maintained. Carbon content can be 40% of the fungal dry weight and the nitrogen content approximately 3% (Rajini, 2004). Young hyphae generally contain higher nitrogen content. Thus the C to N ratio in fungal biomass ranges from 5:1 to 15:1 (Strickland and Rousk, 2010). The carbon to nitrogen ratio of fungal growths is commonly assumed to be at approximately 10:1 (Strickland and Rousk, 2010, Gow and Gadd, 1995).

Wood-decaying fungi can be found in environments where the ratio of carbon to nitrogen ratio reaches 8000:1 (Merrill and Cowling, 1966). Three mechanisms have been found to support fungi living in nitrogen-poor conditions. First, wood decay fungi can physiologically adapt their nitrogen metabolism. When wood decay fungi grow on wood with high C: N ratios, their mycelia had reduced nitrogen content. Second, wood-decaying fungi can reuse nitrogen from old hyphae in conditions of nitrogen deprivation (Merrill and Cowling, 1966). Thirdly, wood-decaying fungi take up nitrogen from soil and translocate N to wood (Levi and Cowling, 1969, Lilly et al., 1991, Watkinson, 2006). These three physiological mechanisms indicate a wide tolerance of the ratio of C to N among fungi.
Most fungi are able to utilize both organic and inorganic nitrogen sources. If the organic matter in the substrate contains protein or other organic nitrogen, then fungi can use these as a source of N and organic carbon (Jennings, 1987, Rygiewicz et al., 1986). Indeed, the basidiomycetes *Agaricus bisporus*, *Corinus cinereus* and *Volvariella volvacea* all showed the ability to utilize protein as the sole source of carbon and nitrogen (Jennings, 1987). This capacity can enhance the ability of at least some fungi to function in nutritionally complicated environments.

The optimal carbon to nitrogen ratio for growth of fungi may change during the life cycle. For example, spores have a limited supply of N (and C) and following germination will only continue to grow if exogenous sources become available. During the life cycle, formation of the fruiting body is also likely to modify requirements for nutrients. Nutrient translocation and storage can also change nutrient demands, as fungi may allocate or/reuse their nutrients from internal stores at the growing site. In addition, many oscillatory phenomena that are widely found in fungi, such as nutrient transport in mycelia (Tlalka et al., 2007, Fricker et al., 2008) and the production of concentric mycelia rings observed in growth in agar plates (Loros and Dunlap, 2001), change in different growth conditions. Nutrient requirements for fungal growth are strongly dependent on culturing time.

Apart from carbon and nitrogen, fungal growth needs other nutrients. Phosphorus is an element for all cells, being present in nucleic acids and phospholipids. Sulphur is containing in some of the amino acids. When sulphur is lacking, methionine transport in *Neurospora crassa* is depressed. Concerning requirements for minerals, potassium, and magnesium are also necessary for fungal growth. Those nutrients required by fungal growth must be present in the substrate (Jennings, 1987).
1.10 Carbon and Nitrogen Metabolism in Fungi

Fungi are heterotrophic organisms. The energy absorbed from the substrates is used in maintenance, growth and reproduction. Fungi can utilize a wide range of carbon sources: from monosaccharides to carbon polymers. Glucose is a readily accessible carbon source (Jennings, 1987) that can be directly absorbed by the mycelium of most fungi. Glycolysis and the pentose phosphate pathway are two pathways of glucose metabolism that are widely found in filamentous fungi. Both pathways release energy and produce the carbon skeleton used in biosynthesis. In anaerobic conditions, depending on the fungal species, secondary products can include ethanol and/or lactic acid (Gleason and Price, 1969) which inhibit fungal growth.

Fungi can utilize nitrate, nitrite, ammonia and wide range of organic nitrogen compounds. As with all the other organisms, fungi assimilate nitrate in two steps. Firstly nitrate is reduced to nitrite, and then nitrite is reduced to ammonium. The first stage is mediated by nitrate reductase and the second by nitrite reductase. Ammonium is an easily assimilated inorganic nitrogen source for fungi. Ammonium and carbon skeletons together synthesize amino acids.

Fungi can break down complex organic sources of nitrogen such as protein and peptone, prior to uptake. They are able to absorb some amino acids directly from substrate, and these organic nitrogens then either catabolised on entry (for utilization as a nitrogen and carbon /energy source) or incorporated directly into macromolecules (Jennings, 1995, Oso, 1975, Vylkova et al., 2011).

1.11 Do Fungi Conform to the Geometric Model of Nutrition?

The geometric model has not only been applied to unitary species but also to the modular species, slime moulds. Both fungi and slime molds are modular species. However, there is distinct
difference between slime mould and fungi. Like animals, slime moulds can move and select the nutrients they need. On non-nutrient substrates, they can migrate a few centimeters per hour (Halvorsrud and Wagner, 1998), directed by external stimuli including gradients of nutrients such as sugars and proteins (Knowles and Carlile, 1978, Kincaid and Mansour, 1978). Mobility is a shared characteristic of all organisms studied in the geometric modelling framework. It is the primary mechanism for foraging and exploitation of nutrients in the environment by these organisms. Fungi, on the other hand, do not move in the same sense, however the remobilization and translocation of the cytoplasmic contents to areas of new growth is fundamentally a form of movement though the underlying processes and mechanisms are different. The question of whether the fungi conform to the geometric model remains an intriguing possibility.

1.12 Ecological Consequences of Fungal Foraging

Growing fungi play an important role in ecosystems, especially in the carbon and nitrogen cycles. They form associations with roots of approximately 80% of land plant species (Smith and Read, 2008) can utilize up to 20% of net plant photosynthates (Drigo et al., 2010, Jakobsen and Rosendahl, 1990). Those are the consequences of fungal foraging. Through translocation of nutrients inside the mycelium, mycorrhizal fungi may allocate carbon sources originating in plants and deposit them in soil away from the root while, in turn, allocating nitrogen (and phosphorus) from soil to the plant (Smith and Read, 2008, Kiers et al., 2011). However, little is known on the quantity of fungi growth in complex nutrient conditions.
1.13 Aim and Objectives of this Project

The aim of this project is to understand the influence of nutritional composition on the fungal phenotype (primarily growth and biomass distribution). In particular, the impact of the major macronutrients carbon and nitrogen on fungal growth will be examined. The aim is approached through the following main objectives:

- To determine whether tested fungi conforms to the geometric model for nutrition
- To extend an existing single-nutrient fungal network model to a two nutrient model and use this model to interpret consequences of nutritional composition for the fungal phenotype
- To develop a systems biology model for the co-metabolism of carbon and nitrogen to improve on models for fungal growth and the dynamics of carbon and nitrogen in the environment

1.14 Proposed Approaches for the Above Objectives

The Geometric model has been applied in a range of animals including locusts, caterpillars, nematodes and mice, and the modular slime mould *Physarum polycephalum*. The geometric model has not been tested in fungi, modular organisms with a mode of nutrition, growth and development that differs from slime moulds.

Following the method on testing animal growth by geometric model, the first step is to determine if there is an optimal nutrient condition for fungal growth, and to identify those conditions. In this project Chapter 2, we will look for an optimal carbon to nitrogen ratio in particular. To achieve this, tested fungus, *Mucor mucedo*, will be grown in a spatially homogeneous environment. In different treatments, media contains various carbon to nitrogen
ratio. This experiment on growing fungus in media will consider several variables will consider several variables including inorganic/organic nitrogen sources, concentration, and pH. As organic nitrogen can be used as nitrogen and carbon source, fungal growth under both organic and inorganic nitrogen sources will be compared. In addition, as we discussed above, time can be an important factor. These treatments need to be measured as a time series.

The second step is to find out whether *Mucor mucedo* can regulate their uptake of multiple nutrients to achieve optimal nutrient intake. To answer this question, in Chapter 3, the fungus is tested in a nutritionally heterogeneous environment. In each sample, the fungus is presented with a pair of mixed carbon and nitrogen resources in spatially isolated locations. The ratio of C: N in each location is changed to study the response of the mycelium to nutritional heterogeneity. All the treatments are measured as part of a time series.

Different kinds of oscillatory behaviour are found in fungal development. They can be an important diagnostic of the nonlinear feedbacks in underlying regulatory networks. In the experiments testing fungal growth according to the geometric model, we measure fungal growth as a time series. As well as the usual exponential increase in biomass, in Chapter 4, we discovered a superimposed oscillation in growth rate. To quantify these oscillations, data corresponding to the exponential growth phase are fitted by both an exponential and a harmonically-modulated exponential function to discover and understand oscillation in growth.

It is challenging to infer the integrated behaviour of the fungal mycelium, using laboratory experiments alone. Mathematical models provide an important way of extending intuition to link pieces information from laboratory experiments to study the integrated behaviour of the fungal network. Numerous fungal models (Boswell et al., 2003, Falconer et al., 2005, Edelstein,
1982, Edelstein and Segel, 1983) have been built to study fungal phenotype in both structurally and nutritionally complex environment. However, in all these models, the fungal phenotype is limited by a single resource even though the ratio of different nutrients can significantly impact on fungal growth. More importantly, models based on single nutrient limitation are not able to answer the central question of how organisms integrate the intake of different nutrient species. In this project, in Chapter 5, we extend the current single nutrient model developed by Hao (2009) to one limited by both carbon and nitrogen.

The extension to a two nutrient model requires the characterisation of the behavior of a single hypha and how these combine to create the fungal phenotype. Internal nutrients are involved in complex biochemical reactions during metabolism. Laboratory experiments have provided details of the individual metabolic networks for each of carbon and nitrogen. However these descriptions have not been combined to understand the integrated behavior of carbon and nitrogen metabolism. In this project, in Chapter 6, we built a mathematical model for the co-metabolism of carbon and nitrogen. In the model, we include genes and enzyme modification and key pathways for assimilation of carbon and nitrogen. Because fungi can utilize both organic and inorganic nitrogen sources and the pathways are different, models for using inorganic and organic sources of nitrogen are built separately. A set of coupled differential equations is written to describe the dynamics of the network and solved using Matlab R2011a. We applied a sensitivity analysis to understand how the parameters affect the emergent behaviour.
Chapter 2. Optimal Carbon to Nitrogen Ratio for Fungal Growth in Single Well Laboratory Experiments

2.1 Introduction

The study of the impact of nutrition on fungal growth in a homogeneous environment is an essential precursor to the study of more complex nutritional environments (Raubenheimer and Simpson, 1999, Dussutour et al., 2010, Sorensen et al., 2010). By growing organisms in nutritionally homogeneous environments with different ratios of key macronutrients, the optimal nutrient condition for a specific purpose, such as reproduction or fitness can be determined. This information can be used to help interpret whether the organism is able to achieve that optimal intake requirement in a nutritionally heterogeneous environment. In a homogenous environment, a range of different animal, insect and unicellular species behave in a manner that optimises growth and/or reproduction through selective intake of a specific carbohydrate to protein ratio from the environment (Dussutour et al., 2010, Sorensen et al., 2010, Simpson and Raubenheimer, 2001, Raubenheimer and Simpson, 1999, Dussutour et al., 2010).

The growth of the fungus M. mucedo in resources of different ratios of carbohydrate and protein will not follow the animal model. Protein is an organic nitrogen source. Fungi can utilize both organic and inorganic nitrogen sources through different pathways within the metabolic network (Jennings, 1987)(More details see Chapter 6 Figure 6.1, Figure 6.2). They have a capacity to break down the organic N and use it as a source of N and carbon source (Jennings, 1987, Rygiewicz et al., 1986). Indeed, fungi can use protein as sole source of carbon and nitrogen (Jennings, 1987). This extra carbon source from organic nitrogen substrate may
influence the optimal carbohydrate to nitrogen ratio. Comparison of fungal growth on organic and inorganic nitrogen source with the same ratio of carbon to nitrogen may indicate the relative importance of the form of nitrogen.

Fungi grow optimally in a resource where the carbon to nitrogen ratio is close to 10:1 (Gow and Gadd, 1995). In some species the optimal ratio shifts from lower to higher over time due to the different nutrient demands resulting from the production of a fruiting body (Moorelandecker, 1992). Other physiological processes such as internal nutrient recycling, translocation and nutrient storage may also change the optimal ratio. Fungi can translocate minerals through mycelia via mechanisms that include mass flow and diffusion (Jennings, 1987), and generalized cytoplasmic streaming or specific vesicular transport (Cairney, 2005). All filamentous fungi so far examined have motile tubular vacuolar systems (Ashford, 1998, Ashford and Allaway, 2002) that enable independent movement of different materials through the cytoplasm. For example, wood decay fungi can survive in conditions with extremely high C:N ratio (Cowling and Merrill, 1966). This is almost certainly due to their capacity to recycle and translocate internal nitrogen reserves to regions of high demand (Merrill and Cowling, 1966, Levi and Cowling, 1969, Lilly et al., 1991). Fungi can store carbon in various forms, such as glycogen, fatty acid, and lipid, and these stores can subsequently be used for structural organization and energy (Martin et al., 1984). In the higher ratios of carbon to nitrogen, fungal growth may be limited by the scarcity of nitrogen. Nitrogen from old hyphae may be translocated to those parts of the mycelium that have a high requirement for N (Gow and Gadd, 1995). Therefore the nitrogen limitation on growth can be reduced and the extra carbon compounds in the higher carbon to nitrogen ratio may increase fungal growth.
Our test species is the fungus *Mucor mucedo* (de Bary & Woron.). *M. mucedo* is an aseptate fungus that forms. Anastomose rarely appears in *M. mucedo* at its growth phase. They only produce small and simple fruiting bodies (Gow and Gadd, 1995). We chose this species because of its physiological simplicity (i.e. lower fungus, aseptate, simple and small fruiting bodies requiring less energy investment) that will aid in the interpretation of the experimental results and develop fungal network model in Chapter 5. Another advantage is that *M. mucedo* is a fast-growing species (Ellis, 2014). In its preferred environments, it can produce abundant biomass within a few days.

A series of experiments on *M. mucedo* have been undertaken in nutritionally homogeneous environments to confirm the presence and determine the location of an optimal C: N ratio for the growth of *M. mucedo*. Recognising that the results may depend on the form of nutrition, growths on organic and inorganic nitrogen sources were compared. Finally, because a growing fungus may require different ratios of C: N over time, harvests from at least three culture times were taken for each experiment and treatment.

### 2.2 Prior to Experiment Work

The fungal colony was cultured onto fresh sterile solid 15 g·L⁻¹ malt extract agar in petri dish (supplier: Livingstone International Pty Ltd) for 10 days in 25 °C room. All experimental treatments were inoculated with 10⁶ spores in suspension. Spores were harvested from cultures in sterile water, rinsed with sterile de-ionized water, diluted and pipetted to 25 ml solutions in 50 ml falcon tubes (supplier: Sarstedt Australia Pty Ltd).
2.2.1 Basal Mineral Medium, Culture Condition and Harvest

The basal mineral medium contained yeast extract 0.01 g l\(^{-1}\); CaCl\(_2\), 0.05 g l\(^{-1}\); KCl, 0.5 g l\(^{-1}\); MgSO\(_4\) · 7H\(_2\)O, 0.5 g l\(^{-1}\); FeSO\(_4\), 0.01 g l\(^{-1}\). The solutions were buffered with phosphate buffer.

In all experiments, five replicate tubes of each treatment were incubated in the dark on a rotary shaker at 10 rpm at 25 \(^{\circ}\)C (Chavant et al., 1981). At harvest, thalli were washed on a 52 µm grid with de-ionized water then placed in pre-weighed aluminium foil dishes, dried overnight at 80 \(^{\circ}\)C, and then weighed (Gleason et al., 2010).

2.2.2 Pilot Experiments

Many environmental conditions significantly modify fungal growth. Because pH may influence enzyme activity (Gleason et al., 2010), the first pilot experiment aimed to determine the optimum pH at which complete the main experiment. The nutrients, especially glucose, may influence osmotic pressure in the growth solution; high osmotic pressure may inhibit fungal growth (Borowitzka, 1985) and low nutrients limit ultimate fungal biomass. The second pilot experiment aimed to determine the concentration of carbon at which fungal growth was maintained.

2.2.2.1 Pilot Study 1: Determine Appropriate Buffer Concentration

*M. mucedo* is known to grow in a pH of 6.5 (Sethi et al, 2010). The addition of glucose, ammonium or other nitrogen sources can alter the pH of the growth medium. By using phosphate buffer, namely 2 units of KH\(_2\)PO\(_4\) to 1 unit of Na\(_2\)HPO\(_4\), the pH value of the substrate can be held at 6.5 independently of the levels of added nutrients. However, a high concentration
of phosphate buffer can be toxic to fungi, whilst a low concentration can result in failure to regulate the pH. This step aimed to identify a suitable buffer concentration.

Method for Determine Appropriate Buffer Concentration

*M. mucedo* was grown in six different media: 10 mM KH$_2$PO$_4$ and 5 mM Na$_2$HPO$_4$; 15 mM KH$_2$PO$_4$ and 7.5 mM Na$_2$HPO$_4$; 20 mM KH$_2$PO$_4$ and 10 mM Na$_2$HPO$_4$. Each of the buffers was supplemented with two carbon and nitrogen concentrations 7 g l$^{-1}$ glucose and 0.7 g l$^{-1}$ ammonium; and 32 g l$^{-1}$ glucose and 3.2 g l$^{-1}$ ammonium. Samples from 7 g l$^{-1}$ glucose and 0.7 g l$^{-1}$ ammonium were destructively harvested every 12 hours from 24hrs to 96 hrs and mycelia dried and weighed. The pH values in each medium were measured in each harvest.

Result from Determine Appropriate Buffer Concentration

At concentrations of 20 mM KH$_2$PO$_4$ and 10 mM Na$_2$HPO$_4$ the pH of the growth medium remains close to the initial pH value of 6.5 for the duration of the experiment. For concentrations of 10 mM KH$_2$PO$_4$ and 5 mM Na$_2$HPO$_4$, and 15 mM KH$_2$PO$_4$ and 7.5 mM Na$_2$HPO$_4$, the value of the pH dropped significantly after 60 hours. The fungus produces similar biomass in all media at both the 36 hour and 60 hour harvest. However the fungus produced significantly more biomass at 84 hours when growing in the medium with 20 mM KH$_2$PO$_4$ and 10 mM Na$_2$HPO$_4$ (Figure 2.1).

A concentration of 20 mM KH$_2$PO$_4$ and 10 mM Na$_2$HPO$_4$ did not reduce fungal growth (Figure 2.1). The buffer also maintained the pH, from 6.6 at the start of the experiment to 6.3 after 96 hrs, in the medium containing 7 g l$^{-1}$ glucose and 0.7 g l$^{-1}$ ammonium. pH dropped to 6.1 in the medium containing 32 g l$^{-1}$glucose and 3.2 g l$^{-1}$ ammonium medium. pH values in the other two buffer concentrations dropped statistically significantly after 96 hours.
In the following experiments, 20 mM KH$_2$PO$_4$ and 10 mM Na$_2$HPO$_4$ were added to the mineral growth medium.

Figure 2.1: Fungi were grown in liquid medium with one of the three buffer concentration: 10 mM KH$_2$PO$_4$ and 5 mM Na$_2$HPO$_4$, 15 mM KH$_2$PO$_4$ and 7.5 mM Na$_2$HPO$_4$, 20 mM KH$_2$PO$_4$ and 10 mM Na$_2$HPO$_4$ with 7 g l$^{-1}$ glucose and 0.7 g l$^{-1}$ ammonium. Fungal dryweights were measured from 24 hrs to 96 hrs in every 12 hrs. In each harvest, with 5 replicates for each treatment, the error bars show the standard deviation of the five replicates, and the curve has been fitted using the least-squares method in excel 2007.
2.2.2.2 Pilot Study 2: Determine Appropriate Carbohydrate Concentration

Method of Determine Appropriate Carbohydrate Concentration

*M. mucedo* was grown in each of 4 glucose concentrations in order to determine the concentration of glucose at which the growth is not delayed by osmotic pressure: 16 g l\(^{-1}\), 32 g l\(^{-1}\), 64 g l\(^{-1}\), 128 g l\(^{-1}\) with 0.9 g l\(^{-1}\) ammonium. Treatments were harvested every 12 hours between 24 and 96 hrs after inoculation.

Result from Testing Appropriate Carbohydrate Concentration

The increase in dry weight was similar in fungi growing in glucose at 16 g l\(^{-1}\) and 32 g l\(^{-1}\). Dry weight was significantly lower in 128 g l\(^{-1}\) glucose at all harvests after 24 hrs (Figure 2.2). Dry weight at 64 g l\(^{-1}\) was significantly lower than biomass production at 16 g l\(^{-1}\) between 36 hrs and 84 hrs (Figure 2.2).
Figure 2.2: Dry Weight (±sd) of *M. mucedo* at different glucose concentration: 16 g l\(^{-1}\), 32 g l\(^{-1}\), 64 g l\(^{-1}\), 128 g l\(^{-1}\) with 0.9 g l\(^{-1}\) NH\(_4\)Cl. In each harvest, there are five replicates for each treatment. Fungal dry weights were measured every 12 hours from 24 hrs to 96 hrs following inoculation.

2.3 Main Experiment Method

Three main experiments were undertaken to study the growth of *M. mucedo* in a homogenous environment. The first experiment studies the growth rate of *M. mucedo* in both organic and inorganic sources of nitrogen for a range of ratios of carbon to nitrogen, while the total concentration of carbon and nitrogen were constant. The second experiment tests for the existence of an optimal carbon to nitrogen ratio using inorganic nitrogen source, the individual concentrations of both nitrogen and carbon change, while the total concentration of C plus N were constant. The third experiment tested the optimal ratio for growth of *M. mucedo* by keeping the carbon concentration constant and varying the concentration of nitrogen.
2.3.1 Organic or Inorganic Nitrogen

Ammonium and glutamine were chosen as they were considered as preferred inorganic and organic nitrogen source respectively (Jennings, 1995). The effect of the extra carbon source in organic N on growth of *M. mucedo* is unknown. It may use organic N as both nitrogen and energy source. Our hypothesis is when using organic N source, the optimal C in glucose: N for fungal growth is lower than using inorganic N source. To test that, the fungus was grown in the same ratio of carbon compounds in glucose to nitrogen compounds under either organic N or inorganic N at a total concentration of glucose and nitrogen source at 8 g l\(^{-1}\). In this study, glutamine used as organic N source. NH\(_4\)Cl is used as inorganic N source.

*M. mucedo* was grown in liquid culture in one of 14 different treatments. Specifically, the treatments comprised seven different ratios of glucose to nitrogen, and inorganic or organic nitrogen. The total concentration of glucose and glutamine, or glucose and ammonium was 8 g l\(^{-1}\). For each source of nitrogen (glutamine or NH\(_4\)Cl), there were seven treatments corresponding to ratios of carbon in glucose to N at 15:1, 6:1, 4:1, 2:1, 1:1, 7:10, and 3:10. Each treatment was harvested after 48, 72 and 96 hrs.

2.3.2 Higher Carbon to Nitrogen Ratio using Inorganic Nitrogen Source

In the experiment described in section 2.3.1, when ammonium is used as the nitrogen source, the growth rate is significantly greater at 15: 1 than at other C: N ratios at 96 hrs. Therefore at 96 hrs, the optimal ratio of biomass production was at a ratio of carbon to nitrogen of 15:1. Thus, the higher carbon to nitrogen ratio was included in this experiment.
Carbon and nitrogen were supplied as glucose and ammonium chloride respectively. We studied the effect of varying the total concentration ([C] + [N]) in the growth medium using two concentration treatments and 10 C: N ratio treatments. The concentration treatments were 8 g l⁻¹. The C: N ratio treatments were 140:1, 80:1, 50:1, 30:1, 15:1, 10:1, 6:1, 3:1, 2:1, and 1.2:1. Each treatment was harvested after 48, 72 and 96 hrs.

2.3.3 Varying C: N with Constant Carbon Concentration:

Carbon and nitrogen were supplied as glucose and ammonium chloride respectively. In all the treatments, the concentration of glucose is 7 g l⁻¹. By changing the concentration of ammonium chloride, we tested carbon to nitrogen ratios of 8:10, 7:5, 12:5, 4:1, 7:1, 13:1, 22:1, 120:1, and 210:1. The medium without ammonium was used as the control. Treatments were harvested every 12 hours from 24 hours to 96 hours.

2.4 Statistics for Main Experiments

All the statistical analysis was conducted using GraphPad Prim 6. For each of the three main experiments written on section 2.3.1 and 2.3.2, normality was assessed using a Kolmogorov Smirnov test. All of the data were normally distributed. At many harvest times, the dry weights of M. muceda are similar across a range of carbon to nitrogen ratios (Figure 2.3 A, B and C). One way ANOVA with Tukey comparison was used to test the significance of any differences between the treatments using Graphpad Prism 6.

For experiments utilizing ammonium as the nitrogen source, we calculated the carbon to nitrogen ratio that maximized growth at each harvest time by fitting the data using polynomial equations. Polynomial equations are a series of nested models. To determine which degree of polynomial that is necessary to describe, data from each harvest were tested by both 2nd and 3rd
degree of polynomial. Comparison of the 2\textsuperscript{nd} and 3\textsuperscript{rd} degree of polynomial equation used the extra sum of squares F test (Ahmed and Rahman, 2012):

\[
F = \frac{(SS1 - SS2) / SS2}{DF2} \cdot \frac{DF1}{DF1 - DF2} \tag{2.1}
\]

where SS1, SS2 are the sum of squares from 2\textsuperscript{nd} degree of polynomial equation and 3\textsuperscript{rd} degree of polynomial equation respectively; DF1, DF2 are the degrees of freedom from 2\textsuperscript{nd} degree of polynomial equation and 3\textsuperscript{rd} degree of polynomial equation respectively.

The P value, which indicates the degree to which the variance in the data was calculated via the values F, DF1 and DF2 by Graphpad Prism 6 (Motulsky and Christopoulos, 2003). All the curves showed in Figure 2.4 Figure 2.5 is the suitable polynomial degree for the corresponding data.

Correlations were tested between optimal ratio and time for experiment 2.3.2.

2.5 Results from Main Experiments

2.5.1.1 Results for Organic vs. Inorganic Nitrogen

When glutamine was used as the nitrogen source, at 48 hours, biomass was the same for all the treatments (carbon to nitrogen ratio ranging from 3:10 to 4:1). No significant difference was found between the dry weight of \textit{M. mucedo} grown on media with carbon to nitrogen ratios between 7: 10 to 6:1 at 72 hrs, and between 7:10 to 15:1 at the 96 hours harvest (Figure 2.3).

For the treatments with ammonium as the nitrogen source, significant differences were observed and a peak growth rate was observed at an ‘optimal’ ratio of C: N. The optimal ratio
for growth was 4:1 at 72 h. At 96 h the biomass at a carbon to nitrogen ratio of 15:1, which is
the highest ratio we tested here, was significantly larger than at 6:1 and 4:1 (glucose:
ammonium N; $R^2$ = 0.70 and 0.81 respectively, Fig 2.3).
Figure 2.3: Dry weights (mg ± SD) of fungi grown in nutrient solution with varying ratios of glucose as the carbon source (Cg) to either glutamine(A, B and C) or ammonium(D, E and F) as the nitrogen source. Five replicates were harvested after 48 hrs (A and D), 72hrs (B and E), and 96hrs (C and F).
2.5.1.2 Results for Ratio Increased with Time Using an Inorganic Nitrogen Source

When the total concentration of glucose and ammonium was 8 g l\(^{-1}\) (Figure 2.4), at 48 hrs fungal biomass was highest for the C: N ratio of 4:1 \((R^2 = 0.8)\). This increased to 12:1 \((R^2 = 0.8)\) at 72 hrs and to 27:1 \((R^2 = 0.8)\) at 96 hrs. Thus the optimal ratio was observed to increase with time (Pearson correlation \(r=0.9)\).

![Figure 2.4: growth of M. mucedo with combined carbon and nitrogen concentration at 8 g l\(^{-1}\). Mean (±SD) dry weight (mg) of Mucor mucedo after 48, 72 or 96 hours in nutrient solution with a combined carbon and nitrogen concentration of 8 g l\(^{-1}\). The value of x-axis is log10(C: N). The curve was fitted using the least-squares method. For each harvest time, optimal C:N ratio is calculated from the x-axis value of the peak in the corresponding polynomial curve.](image-url)
2.5.1.3 Results for Varying C: N with Constant Concentration of C

When carbon was constant at 7 g l⁻¹, and the amount of ammonium was changed in the different treatments, the fungal growth at 24 hrs and 36 hrs was limited. Data from these two harvests poorly fit either a cubic or quadratic curve. A quadratic equation provides a satisfactory fit to the data after 48 hours ($R^2 \in [0.72, 0.84]$). The optimal C: N ratio for biomass production was fixed at 13:1 between 48 hours and 96 hours (see Figure 2.5).

![Graph](image)

Figure 2.5: Mean (±SD) dry weight (mg) of Mucor mucedo in media where the carbon was held constant and the N was changed. Value of x-axis is log10(C: N). From 60 to 96 hrs, the optimal C: N ratio for growth at 13:1. The curve was fitted using the least-squares method. For each harvest time, optimal C: N ratio is calculated from the x-axis value of the peak in the corresponding polynomial curve.

2.6 Discussion

Results supported our hypothesis fungus *M. mucedo* behaves differently to animals with respect to optimal protein to carbohydrate nutrition even though both fungi and animals are heterotrophic. An optimal carbon to nitrogen ratio for growth of *M. mucedo* was determined only when ammonium was used as the (inorganic) nitrogen source. When *M. mucedo* utilized an organic nitrogen source, it grew equally well across a broad range of carbon in glucose to nitrogen ratios (Figure 2.3).
Fungi may use amino acids as both a nitrogen and a carbon source (Oso, 1975, Vylkova et al., 2011). These extra carbon components can be used as an energy source and for biosynthesis (Jennings, 1995). Carbon may become a factor limiting fungal growth if inorganic nitrogen is the source of nitrogen when fungi are growing in substrates with a lower carbon: nitrogen ratio. However, the extra carbon components in organic nitrogen can supplement the source of energy. The link between fungal growth and the carbon to nitrogen ratio may be masked by the extra carbon in organic sources of nitrogen.

Our results may indicate nitrogen was been translocated inside of mycelium. When the total concentration of carbon and ammonium is constant, the optimal carbon to nitrogen ratio for fungal growth increased over time. Increasing optimal carbon to nitrogen over time can be explained by the nutrient needs of fungal reproduction (Moorelandecker, 1992). However sporangia were absent in the experiments. The observation that the optimal carbon to nitrogen ratio depends on time can be explained by nitrogen recycling, nitrogen translocation and local storage of carbon. Through nitrogen from old hyphae may be translocated to those parts of the mycelium that have a high requirement for N (Gow and Gadd, 1995), the scarcity of nitrogen in the higher ratios of carbon to nitrogen can be resolved. The extra carbon compounds in the higher carbon to nitrogen ratio may increase fungal growth.

When carbon was constant and the C: N ratio varied by altering the concentration of nitrogen, the optimal ratio was independent of time (Figure 2.5). In higher carbon to nitrogen ratios (nitrogen limited), recycling or translocation of nitrogen may not result in the boost to fungal growth because nitrogen is extremely limited across the mycelium. Thus, carbon concentration and nitrogen recycling may be the key reasons for the increase in the optimal ratio over time when both concentrations are changed simultaneously.
Chapter 3. Growth in a Heterogeneous Environment

3.1 Introduction

Many heterotrophic organisms from mice to slime moulds balance their intake of protein or carbohydrate by selecting different foods such that the outcome is optimised growth and/or fecundity (Dussutour et al., 2010, Sorensen et al., 2010, Warbrick-Smith et al., 2009, Simpson and Raubenheimer, 2001, Dussutour et al., 2010). Fungi are different from these heterotrophic organisms because of their indeterminate growth form (Russell, 2008). The fungal colony does not move directly but reforms its boundary through the emergence and extension of hyphal tips and autolysis (Deacon, 2006). Fungi appear to have a unique suite of adaptations to obtain nutrients. Various nutrients can be uptaken from different locations and re-organised through recycling and translocation inside of mycelium (Govindarajulu et al., 2005, Tlalka et al., 2007, Martin et al., 1984). The different models of nutrient translocation found in fungi include diffusion, mass flow and cytoplasmic streaming (Jennings, 1987, Cairney, 1992). Nutrients can also be stored in different forms in the mycelium (Martin et al., 1984). The capacity to store, recycle and translocate nutrients enables the fungi to pervade in nutritionally challenging and heterogeneous environments.

The translocation of various nutrients has been examined in only a few groups of fungi. Arbuscular mycorrhizal (AM) fungi are present in all arable soils and colonize most crops (Newman and Reddell, 1987, Hendrix et al., 1995). AM fungi rely entirely on the plant for energy used in growth and development. In other words, carbon is distributed from a single source to all parts of the mycelium. In addition, AM fungi may access phosphate in soil and translocate appreciable quantities to the host plant. Some basidiomycetous ectomycorrhizal fungi digest
organic matter in soil and transport the resultant nitrogen to the host plant (Smith and Read, 2008). Significant bi-directional transport of energy and nitrogen takes place in these symbiotic fungi.

Saprotrophic fungi rely on energy and minerals found in the environment. If these fungi translocate energy, then an entire mycelium might be supported by single, point sources of energy. However, an entire mycelium may require relatively large quantities of energy for maintenance, growth and development. Alternatively, exploitation of sources of energy around fungal tips would enable proliferation of the mycelium where energy is available, especially if cytoplasm is removed from areas where energy has been exploited. Nitrogen is an essential mineral required by fungi. It is required for the formation of amino acids and purines. Less N than energy is required for fungal growth and development. Indeed, support for translocation of N throughout a mycelium has been found in studies using tracers and quenching (Fricker et al., 2008, Darrah et al., 2006). Nitrogen is apparently carried in the system of vacuolar tubules found in all filamentous fungi examined (Cole et al., 1998). Co-location of N and energy would enable a fungus to thrive. However, soil is heterogeneous, and energy and minerals may be differently depleted. Maintenance of the mycelium would require translocation of either energy or mineral depending on the relative concentration of each nutrient. The process of translocation of more than one nutrient is poorly understood.

Growth of the fungus *Mucor mucedo* was examined in an environment where the available nutrients placed in spatially separated locations, and at each location the balance of C and N may be different: a situation analogous to a heterogeneous distribution of different nutrients. The hypothesis was that the fungus would translocate both C and N when the two nutrients
were physically separated in order to sustain growth of mycelia. Thus, we also hypothesise that *M. mucedo* can maximize their growth in nutritional heterotrophic environment.

### 3.2 Materials and Methods

#### 3.2.1 Experimental System

In this experiment, *M. mucedo* was grown in 6-well microtitre plates (Supplier: Sigma- Aldrich). In each plate, two replicates of different treatments were grown in each of the end pair of wells (Figure 3.1). The middle pair of wells is left empty to reduce the chance of spread of the fungus.

The fungal colony was maintained on 15% malt extract agar (MEA) for 10 days in 25 °C room in the dark. Spores of *M. mucedo* were then inoculated on sterile steel mesh (1x1.5 cm) placed on a fresh plate of MEA, and the plates incubated in the dark at 25 °C for 12 hrs to enable germination of the spores. Each mesh with germinated spores was placed on the bridge between adjacent wells of an end pair of a 6 well microtitre plate (Figure 3.1). This mesh provided a bridge between adjacent wells for the fungus but did not allow passage of nutrients (Figure 3.2).

Glucose and ammonium are used as the sources of carbohydrate and nitrogen respectively. Fungal growth rate is maximised in environments containing a ratio of carbon to nitrogen of approximately 10: 1. When the total glucose and ammonium concentration was at 8 g l⁻¹, the optimal carbon to nitrogen ratio for *M. mucedo* growth was 12: 1 at 72 hrs (Chapter 2 section 2.3.2). Though the optimal ratio was at 4:1 at 48 hours, and increased to 27:1 at 96 hours, the medium with a carbon to nitrogen ratio at 10:1 always supported good fungal growth. On the contrary, fungal growth was poor when the carbon to nitrogen ratio was 100: 1 and 4: 5.
In every pair, 17 ml of liquid medium was added to each well. All media contained nutrients: yeast extract 0.01 g l\(^{-1}\); CaCl\(_2\), 0.05 g l\(^{-1}\); KCl, 0.5 g l\(^{-1}\); MgSO\(_4\) \(\cdot\) 7H\(_2\)O, 0.5 g l\(^{-1}\); FeSO\(_4\), 0.01 g l\(^{-1}\) and was buffered to pH 6.5 (Sethi et al., 2010) by 20 mM KH\(_2\)PO\(_4\) and 10 mM Na\(_2\)HPO\(_4\). The carbon and nitrogen concentration varied across the treatments.

(1) To test whether carbon and nitrogen can be transferred in both directions, media containing high (100:1, total C+N concentration: 7.0 g l\(^{-1}\)) and low (4:5, 1.8 g l\(^{-1}\)) C: N were placed in adjacent wells.

(2) To determine the maximum growth of the fungus media containing C: N (10: 1, 4.4 g l\(^{-1}\)) was placed in each well.

(3) To determine whether growth was related to the total quantity of C and N, media containing C: N (10:1, 8.8 g l\(^{-1}\)) in one well, and only minerals in the adjacent well.

Two controls were included: Control 1: media contained high C: N ratio (100:1, 7.0 g l\(^{-1}\)) and basal mineral medium in the adjacent well. Control 2: media containing low C: N (4:5, 1.8 g l\(^{-1}\)) in one well and only minerals in the adjacent well.

Seven replicates of each treatment were randomly distributed across microtitre plates. After inoculation, each of those 6-well microtitre plates was placed in a 15 x 15 cm glass petridish. The fungi were incubated in the dark at 25 °C and harvested after 48, 72 and 96 hrs. At harvest, the thalli in each well were cut off from the colony and washed on 52 µm mesh with sterile water, placed on pre-weighed aluminium foil dishes, dried for 12 hrs at 80 °C and then weighed. The experiment was repeated and data from the second experiment is presented here. The entire mycelium in the well was removed, dried and then weighed.
Figure 3.1: Experimental system comprising two replicates from different treatments operated in each of the end pair of wells. The middle pair of wells was kept empty. Mesh with germinated spores was placed in between the paired wells.

Figure 3.2: Fine mesh with germinated fungal spores placed in the middle of a pair of wells. This fine mesh can provide bridge between the two wells and it is not in contact with the medium in wells.

3.3 Data Analysis

For each experiment, normality was assessed using a Kolmogorov Smirnov test. It was conducted with IBM SPSS Statistics for Windows, version 20. All the reported data are normally distributed.

We used One-way ANOVA test tukey's honestly significant difference (HSD) to test difference between hyphal growths in adjacent wells and compare the growth of entire fungal colony
between treatments. T-test is used to test the ratio of dry weights in adjacent wells. One-way ANOVA and T-test were analysed by Graphpad Prism 6.

3.4 Results

In broad terms (Table 3.1), a balanced supply (C: N at 10: 1) of carbon and nitrogen maximised total growth of _M. mucedo_, regardless of whether the nutrients were placed in one or two wells. When the fungus had access to adequate combined nutrition, but the greatest extreme in spatial separation of carbon and nitrogen, fungal growth was significantly reduced in both wells, and more so in the well lacking carbon. The fungus did not grow in mineral media, and only minor growth, similar to treatment 1, in a C: N of 4: 5, at all harvests.

Fungal growth in treatment 1 high (10: 1) carbon: nitrogen was significantly greater than the growth in the high C: N well in control 1. Biomass production in the well of treatment 1 (low (4: 5) carbon: nitrogen) and in the corresponding wells in control 2 are the same (Table 3.1).

When the total carbohydrate and nitrogen sources were constant and the environment was homogenous, the optimal carbon to nitrogen ratio increased with time (Chapter 2, Figure 2.4). When C and N are spatially separated, or the separated substrates were nutritionally unbalanced, a heterogeneous environment, with time the mycelium invested progressively more biomass in the C rich compared with the N rich resource (Table 3.1). In treatment 1, the ratio of hyphal dry weight in the high C: N well to that in the low C: N well was increasing over time. At 72 hours, the dry weight ratio in the two wells was significantly smaller than that ratio at 96 hours (p=0.0036, at 72hours the ratio is between 8:5 and 16: 5, at 96 hours the ratio was between 16: 5 and 9:2). Neither well had measurable biomass production at 48 hours.
Table 3.1: Mean dry weight (±SEM) of *Mucor mucedo* in media either deficient in carbon or nitrogen after 48, 72 or 96 hours. The a, b, c and d in each column indicate statistically significant differences at P<0.05. An asterix indicates statistically significant differences between the pair of wells in each row at each harvest.

<table>
<thead>
<tr>
<th>Control Or Treatment NO.</th>
<th>Treatment: C/N, concentration of C + N mg l⁻¹</th>
<th>Harvest (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Well 1</td>
<td>Well 2</td>
</tr>
<tr>
<td>Control 1</td>
<td>100/1, 7</td>
<td>nil</td>
</tr>
<tr>
<td>Control 2</td>
<td>nil</td>
<td>4/5, 1.8</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>100/1, 7</td>
<td>4/5, 1.8</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>10/1, 4.4</td>
<td>10/1, 4.4</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>10/1, 8.8</td>
<td>nil</td>
</tr>
</tbody>
</table>

3.5 Discussion

Interestingly, the fungus can only translocate nitrogen to regions of the mycelium where N is depleted, but not translocate carbon to regions where C is depleted. As the fungus proliferates in energy-rich locations, translocation of N enables more effective utilisation of energy. Fungi may autolyse (Shoji and Craven, 2011) after depletion of resources. Autolysis may be important for the maintenance of local supplies of energy within the mycelium: minerals are potentially translocated and recycled. *Fungus M. mucedo* have a much greater requirement for energy than minerals in growth and respiration. Local uptake and use of energy along with reuse and translocation of minerals such as N, indicate a profoundly different approach among the heterotrophic fungi to the searching for and uptake of C and N from that indicated in the
Geometric model. The Geometric model (Simpson and Raubenheimer, 2001) may be used to explain growth of the fungus *M. mucedo* only when the fungus has access to media where the ratio of carbon to nitrogen is approximately 10:1. The fungus appears unable to translocate significant quantities of C and will proliferate only when carbon is locally available. A new model is required to explain the differential exploitation of C and N in the environment by fungi.

A study of the growth of a slime mould in different nutrient regimes showed that when the ratio of carbon to protein was at 1:2, the growth rate was maximized. In an environment where those carbohydrate and protein were spatially separated, the slime mould adapted the investment of biomass such that 1 unit of biomass grows on carbohydrate while 2 units of biomass grows on protein. In this way, the slime mould optimized uptake and maximized growth rate in a similar manner to other, complex heterotrophic organisms (Dussutour et al., 2010).

Our results are consistent with the second hypothesis that the fungus invests biomass in a manner that optimizes the uptake rate of the different nutrients such that it maximizes its growth rate. Furthermore, these results also indicate the fungus *M. mucedo* can regulate uptake of external nutrients by investing in different amounts of hyphae, and through physiological processes, fungal growth can be less dependent on one of the nutrients (nitrogen in this case) over time. These results are consistent with results from Chapter 2 nitrogen has been translocation internally. Through nitrogen translocation, the cytoplasmic concentration of carbon and nitrogen can be balanced in a carbon rich but nitrogen depleted condition, therefore nitrogen, but not carbon, translocation appears to be the strategy used by *M. mucedo* to survive in a nutritionally heterogeneous environment.
In a well with a carbohydrate to nitrogen ratio that facilitates growth, the resulting growth rate is the same (Table 3.1) whether the total quantity of nutrient is in one location or equally separated into two locations. A number of factors may modify the growth rate. The total quantity of nutrient is important. High concentrations of nutrients may lead to delayed growth because of osmotic effects on hyphal tips (Peksel and Kubicek, 2003). Indeed, according to our results, when the C to N ratio is unbalanced, and even although the total quantity of nutrient is the same, fungal growth may be slower. This inferred the translocation of nitrogen may not maximize fungal growth. In this experiment, nutrient translocation between two wells is via hyphae that connect the two wells. The fungus *M. mucida* is aseptate and fusion between hyphae is rare (Gow and Gadd, 1995). As fungal growth started between the paired wells, slowed growth may be due to loss of cytoplasmic connections. Overtime old hyphae may die and collapse, or become vacuolated (Gow and Gadd, 1995), the number of links between the two wells then decreased overtime. Mycelium network can be fragile. Fragility of mycelia can reduce the translocation efficiency between two locations.

*M. mucida* as an r-selected fungus is usually found in nutrient rich environments, such as dung, or composted leaf litter. Those fungi have little capacity to deal with environmental stress including poor nutrient availability and long-term exploitation of scarce resources. The growth response to C and N should be tested among a wider range of fungal species especially those have other ecological strategies. Arbuscular mycorrhizal fungi translocate both carbon and nitrogen (Fellbaum et al., 2012, Cairney, 2005). In particular fungi which exist in two different habitats, such as endophytic fungi may elucidate broader nutritional concepts.
Chapter 4. Influence of Nutrient Availability on Growth Oscillation in *Mucor Mucedo*

4.1 Introduction

Periodic behaviour is found in a wide range of organisms (Weber, 2009) and the periodicity can range from years to seconds (Goldbeter, 2008, Kim et al., 2010). Biological oscillations can be an important diagnostic of underlying complex nonlinear feedbacks in regulatory networks, and include neuronal oscillations in humans, photoperiodism in plants, and the ovarian cycle in mammals. They have also been observed among fungi (Loros and Dunlap, 2001, Tlalka et al., 2007, Brandt, 1953). The circadian clock is a particularly important example and has been studied in *Neurospora* for more than fifty years. The periodic pattern of spore formation observed in *Neurospora* is known to be tightly linked with the circadian rhythm (Brandt, 1953). The oscillatory period is approximately 24 h, although it is sensitive to light (Lauter et al., 1997) and is temperature compensated (Brandt, 1953). Other kinds of rhythmic behaviour in fungi include nutrient transport in mycelia (Tlalka et al., 2007, Fricker et al., 2008) and the production of concentric mycelial rings observed in growth on agar plates (Loros and Dunlap, 2001), and these behaviours have not been directly associated with the circadian pathway (Wijnen and Young, 2006, Kozma-Bognar and Kaldi, 2008). The periodicity of these oscillations is markedly different from 24 h and they are not entrained by light (Tlalka et al., 2003, Falconer et al., 2005). The origin of these oscillations is not fully understood, but it is well known that organisms possess multiple oscillator systems, and that they can yield insight into underlying regulatory processes (Goldbeter, 2008).
Environmental factors can influence periodic behaviours (Portoles and Mas, 2010, Yang et al., 2011). As well as the shift in periodicity in the circadian clock induced by light and temperature, (Brunner and Kaldi, 2008), the oscillation in nitrogen translocation has displayed a marked temperature sensitivity with a self-sustaining metabolic rhythm (Tlalka et al., 2003).

Nutritional quality of the substrate can impact on the observed periodic behaviour. Oscillatory regimes in fungi grown in contrasting nutrient regimes have been observed that affect both the amplitude and period of the oscillations (Lakin-Thomas, 2006, Baker et al., 2012, Suzuki et al., 1996, Dunlap and Loros, 2006). The periodicity of condition is known to vary with the source of carbon or nitrogen and their concentration (Sargent and Kaltenbo.Sh, 1972, Lakin-Thomas, 2006). A physiologically-based model of the fungal phenotype suggests that mycelial rings are only observed above a critical nutrient concentration (Falconer et al., 2005).

A considerable body of work has examined the growth of fungi on substrates of varying carbon to nitrogen ratio (Levi and Cowling, 1969). Fungi are remarkable amongst other kingdoms, in their capacity to grow in conditions of extreme nitrogen deficiency relative to carbon. This is almost certainly due to their capacity to recycle internal nitrogen reserves and allocate them to regions of high demand (Levi and Cowling, 1969, Lilly et al., 1991). However, the metabolic processes and feedbacks involved are not well understood. A study of oscillatory behaviour of mycelial growth under regimes of contrasting nutritional quality may provide clues to these regulatory feedbacks.

In this chapter, we reanalyzed the fungal growth data corresponding to the exponential growth phase under the different nutrient conditions observed in Chapter 2, and studied the oscillatory behavior that resulted. We attempt to reconcile the exponential and oscillatory characteristics
used to describe fungal growth dynamics under different nutrient environments. Because organic forms of nitrogen supply both carbon and nitrogen, we will examine whether the ratio of carbon to nitrogen affects the growth qualitatively and quantitatively using both organic and inorganic forms of nitrogen.

4.2 Non-linear Curve Fitting for Organic and Inorganic Sources of Nitrogen

Growth data observed at Chapter 2, section 2.3.1, of utilizing organic and inorganic nitrogen sources were analysed separately, and oscillations in growth are clearly apparent. Here we defined oscillation in growth as instant growth rate is repeatable. To quantify the statistical significance of the periodic behavior, the periodic behaviour, biomass data corresponding to the exponential growth phase for each treatment from organic or inorganic nitrogen sources were fitted by both an exponential equation 4.1 and a harmonically-modulated exponential function (equation 4.2) using GraphPad Prim 6. The value of the parameter, c, in equation 4.2, (corresponding to the oscillatory period) is assumed to be independent of the carbon to nitrogen ratio, but is dependent on whether N is in organic or inorganic form.

\[ M(t) = Y_0 \times e^{(k+t)} \]  \hspace{1cm} 4.1

\[ M(t) = Y_0 \times e^{(k+t)} + b \times \sin(c \times t + d) \]  \hspace{1cm} 4.2

\( M(t) \) denotes the dry weight of the fungus (mg), \( t \): denotes growing time (hours), and \( Y_0, k, b, c, d \) are parameters. The parameter, \( d \), represents the phase of oscillations in equation 4.2. In fitting equation 4.2 to the data, we have made the assumption that the replicates in any given
treatment are in phase. This is a reasonable assumption, given that the replicate experiments were carried out simultaneously and that each replicate was initiated with inocula of spores having biomass an order of magnitude smaller than the biomasses measured in the experiment. We made no such assumption about the phase of oscillations between treatments, and these were allowed to vary with C: N ratio and the source of N.

4.3 Analysis of the Results from the Fitted Curves

Equation 4.1 and 4.2 were compared using the extra sum of squares F test. Using Equation

\[
F = \frac{(SS_1 - SS_2) / DF_2}{SS_2 * (DF_1 - DF_2)}
\]

where SS1, SS2 are the sum of squares from equation 4.1 and 4.2 respectively; and DF1, DF2 are the degrees of freedom from equation 4.1 and 4.2 respectively.

The P value, which indicates the degree to which the variance in the data is explained by equations 4.1 and 4.2, was calculated via the values F, DF1 and DF2 using GraphPad Prim 6 (Motulsky and Christopoulos, 2003). The relationships between the parameter values and the ratios of C: N were compared using SPSS v 20 to test the correlation between parameter values including best fit and confidence interval, and the ratios of C: N.

4.4 Results Curve Fitting for Organic and Inorganic Sources of Nitrogen

The overall pattern of growth when both glutamine and ammonium chloride were used as a nitrogen source was best described by a harmonically modulated exponential curve (Figure 4.1, Figure 4.2) with a period of 29 h (95% CI: 28.5-32 h and 28 - 31 h respectively). The data corresponding to the use of glutamine as the nitrogen source can be fitted using both an exponential and a harmonically modulated exponential (Equation 4.1 and 4.2 resp.) when the C:
N ratio is between 15:1 and 7:10 ($R^2 \in [0.76, 0.89]$ and [0.78, 0.94]: Figure 4.1). *M. mucedo* did not produce significant biomass at a C: N ratio of 3:10 (Figure 4.1). The data corresponding to the use of ammonium chloride as the nitrogen source can be fitted using both equation 4.1 and 4.2 when the C: N ratio is between 15:1 and 7:10 ($R^2 \in [0.57, 0.80]$ and [0.72, 0.86]: Figure 4.2). *M. mucedo* did not produce significant biomass at a C: N ratios of 3:10 (Figure 4.2).
Figure 4.1: Oscillatory growth of *Mucor mucedo* in organic nitrogen source up to 96 h. Data observed from Experiment 1 in Chapter 2; 2.3.1. Graphs (a) to (g) show growth of the fungus for each ratio of carbon in glucose to nitrogen in glutamine in the growth medium. The broken and solid lines show the best fit using equations 4.1 and 4.2 respectively, and the corresponding R² is shown on the graph. Graph (g) shows the fungus does not have significant growth at a C: N ratio of 3:10.
Figure 4.2: Oscillatory growth of *Mucor mucedo* in inorganic nitrogen source up to 96 h. Data observed from Experiment 1 in Chapter 2; 2.3.1. Graphs (a) to (g) show the details of the growth of the fungus in each ratio of carbon to nitrogen (as ammonium chloride), as indicated on the graph. The curves are observed by fitting data using both equations 4.1 and 4.2, and the corresponding R2 is shown on the graph. Graph (g) shows the fungus does not have significant growth at a C: N ratio of 3:10.
Across the entire range of the ratio of glucose carbon to nitrogen, equation 4.2 described the growth of *M. mucedo* significantly better than equation 4.1, using either glutamine or ammonium chloride as the nitrogen source (For glutamine the condition ratio F = 4.7; for ammonium the condition ratio F = 4.9; both P < 0.001). Therefore we can conclude that the data are best explained by periodic oscillations in dry weight in the treatments with C: N ratios ranging from 15:1 to 7:10 (Figure 4.1, Figure 4.2). Although the period is assumed to be constant within each nitrogen treatment, the best-fit value between treatments was not significantly different. Therefore our results are consistent with the conclusion that the period is independent of the source of nitrogen used in the study.

When glutamine was used as the nitrogen source, the growth rate parameter, k, in equation 4.2 was positively correlated with the C: N ratio (r = 0.69: Figure 4.3). In contrast, the absolute value of the amplitude, b, of the oscillations was not correlated with the C: N ratio (r = 0.165: Figure 4.3). When ammonium was used as the nitrogen source, both the growth rate and amplitude were positively correlated with the C: N ratio (r = 0.681 and r =0.598 respectively). The growth rate, k in equation 4.2 is not significantly different when utilizing organic nitrogen or inorganic nitrogen (P = 0.53: Figure 4.4), regardless of the C: N ratios between 15: 1 and 7:10.
Figure 4.3: Trade-off between amplitude (b) and the ratio of glucose carbon to nitrogen at total glucose and nitrogen at 8 gl⁻¹. The absolute value of parameter b is derived from fitting equation 4.2 to data from the corresponding nutrient condition. The absolute value of parameter b vs. the ratio of carbon in glucose to nitrogen in the form of both glutamine (Gln) and ammonium chloride (NH₄Cl). A significant trend is observed for inorganic, but not organic sources of N.

Figure 4.4: Dependence of growth rate on total C: N ratio at glucose and nitrogen at 8 gl⁻¹. Growth constant k (growth rate by a factor e) vs. the ratio of total carbon contents to nitrogen in the form of both glutamine (Gln) and ammonium chloride (NH₄Cl). Growth constant k is derived from fitting equation 4.1 to data from the corresponding nutrient condition.
4.5 Non-linear Regression on Data Observed in Chapter 2, 2.3.2 Varying C: N with Constant Carbon Concentration:

Data from the previous experiments utilizing the inorganic nitrogen source, a carbon concentration at 7g l$^{-1}$, and a range of carbon to nitrogen ratios for each treatment, were fitted with equation 4.2 using GraphPad Prim 6. Previously, the value of the parameter, $c$, (corresponding to the oscillatory period) was assumed to be independent of the carbon to nitrogen ratio, but could depend on whether N was in organic or inorganic form. The above results showed no significant difference between the different sources of N. Therefore the value of ‘c’ was set equal to the value we obtained from the above experiments corresponding to the inorganic nitrogen source (Period is 29h).

4.5.1 Results from the Analysis of Chapter 2, 2.3.2 Varying C: N with Constant Carbon Concentration:

The growth patterns observed when the nitrogen concentration was varied for constant carbon concentration changing ammonium amount with constant carbon source is satisfactorily modelled by the harmonically modulated exponential curve corresponding to equation 4.2 ($R^2 \notin [0.84, 0.97]$) as shown in Figure 4.5. The maximum amplitude was observed for a C: N of 8:1 (Figure 4.6). The maximum growth rate was observed for a C: N of 14:1 (Figure 4.7).

Although the period is assumed to be constant at 29 h within all the treatments and allowed to vary between treatments, the best-fit value between treatments was not significantly different. Therefore, our results in this section are consistent with the conclusion that the period is independent of the source of nitrogen used in the study.
Figure 4.5: Growth of *Mucor mucedo* in an inorganic nitrogen source for 96 h across a range of values for the C: N ratio from 120: 1 to 7: 5, with a constant concentration of glucose at 7 g l$^{-1}$. Data observed from Chapter 2; 2.3.3. Graphs (a) to (g) shows details of growth in each ratio. The curves are from fitting data using equation 4.2, and the corresponding $R^2$ is shown on the graph.
Figure 4.6: Trade-off between amplitude (b) and the ratio of glucose carbon to nitrogen where concentration of glucose is held constant at 7 g l$^{-1}$. The absolute value of parameter b is derived from fitting equation 4.2 to the data presented in Section 2.3.2 for the corresponding nutrient conditions.
Figure 4.7: Dependence of growth rate on total C: N where concentration of glucose at 7 g l⁻¹. Growth constant k (growth rate by a factor e) vs. the log of ratio of total carbon contents to nitrogen. The value of parameter k is from the best fitting of data from Chapter 2; 2.3.3 to equation 4.2 in the corresponding nutrient condition. Curve in this figure use the least-squares method in excel 2007.

4.6 Discussion

Oscillatory growth of biomass was observed in this species of fungus, regardless of nitrogen sources. During a single oscillatory period, the growth of *M. mucedo* appears to increase dramatically, slow to zero, and then in most cases the dry weight declines (Figure 4.1, Figure 4.2). The period is independent of nutrient quality and is 29 h (95% CI: 28.5-32 h and 28 - 31 h respectively) for all treatments. For inorganic sources of N we find that, when total carbon and nitrogen concentrations constant at 8 g l⁻¹, the amplitude of oscillation increased with an increase in the carbon to nitrogen ratio (Figure 4.3), but not for organic sources. Although the data point at a C: N ratio of 15:1 has a strong leverage, this conclusion is robust even if this data
point is removed from both data sets. When using inorganic nitrogen source with concentration of glucose was at 7 g l⁻¹, a peak value of amplitude of oscillation was observed with increased ratio of carbon to nitrogen (Figure 4.6).

When fungi uptake amino acids, fungi can either utilise them as a nitrogen source and catabolise them on entry, or incorporate them directly into macromolecules (Jennings, 1995). Fungi also show the ability to use amino acid as both a nitrogen and carbon source (Oso, 1975, Vylkova et al., 2011). These extra carbon components may mask any link between fungal growth and the C: N ratio.

Carbon metabolism via the glycolytic pathway is highly conserved in a range of biological systems and has been known for a long time to undergo oscillations with periodicity ranging from minutes to hours (Ghosh and Chance, 1964). These oscillations have stimulated a considerable body of work on models for glycolysis (Goldbeter, 2008) and result from the numerous feedbacks in the pathways central to carbon metabolism. However, the period of glycolytic oscillations is significantly smaller than the period measured in the growth here. In their recent and fascinating theoretical analysis of glycolytic oscillations, Chandra et al. (Chandra et al., 2011) propose that oscillations occur as a by-product of a trade-off between stability and efficiency in metabolism. Using a specific model for glycolysis they show that allosteric inhibition of phosphofructokinase (PFK) and pyruvate kinase (PK) by ATP produced in the reaction can lead to instability when the strength of the regulation is sufficiently high. Importantly, they generalise this result to any autocatalytic network using a universal model that is independent of most of the details of the specific pathways. They argue that efficiency is a key target in the evolutionary dynamics of metabolism, and that the feedbacks that are required to ensure this also give rise to oscillations as a side effect. Indeed, in studies of glycolysis in yeast cells, during
the conversion of glucose to pyruvate, the oscillatory state dissipates less energy and produces higher ATP/ADP ratio than the coexisting (unstable) steady state (Schell et al., 1987, Liu et al., 1997). The general theory of Chandra et al. (Chandra et al., 2011) predicts that the amplitude of the oscillations increases with the strength of regulation in their model.

An alternative explanation is that the oscillations we see are circadian, given the proximity of the measured period to 24 h. This may indeed be the case, but still leaves the question of their origin and behaviour unanswered. There are many processes that produce oscillations in biochemical networks (Goldbeter, 2008). The widely accepted model of the circadian clock implicates very specific processes involving the so-called clock genes and feedbacks on transcription in the cellular nucleus. However, 24 h oscillations can occur without transcription feedback, and indeed without a nucleus (O’Neill and Reddy, 2011). Therefore, periodicity approaching 24 h may convey an adaptive advantage to the pathways concerned, but can originate from many different causes.

These considerations lead us to the following hypotheses for the behaviour that we observe. Oscillations in dry matter production are a by-product of internal regulations that have evolved to optimise energetic efficiency. The source, and complexity, of nutrition affects the trade-off between efficiency and stability in the underlying metabolic pathways. The regulatory regime involved in biomass production has evolved enabling the metabolisms of the complex forms of carbon found in the environment. The following observations from our data support these hypotheses. In the experiments where we added carbon and nitrogen in separate sources and dynamically changing ratios, amplitude of oscillation increased as more glucose was added (i.e. as C: N increased) (Figure 4.3, Figure 4.4). This indicates the existence of a trade-off between stability and efficiency that increased as additional carbon was added in simple form. By
contrast, when carbon and nitrogen were added in complexed form, there is no such trend and the stability of the system is independent of C: N. Meanwhile, the efficiency of the conversion of carbon and nitrogen to dry biomass is the same for both simple and complex sources of nitrogen (Figure 4.4). Therefore, independent of whether the resource is organic or inorganic, the efficiency of the system is the same and depends only on the ratio of total carbon to total nitrogen.

Thus for the fungal species and conditions we have used, when dynamically changing C: N ratios, the growth rate is independent of the organic or inorganic origin of the resource, and depends only on the ratio of total carbon to nitrogen (Figure 4.4). A distinction between organic and inorganic forms is manifest in oscillations in growth rate that reflects a trade-off between efficiency and stability in the regulatory pathways connecting carbon and nitrogen metabolism. In order to maintain growth rate for simpler sources of carbon and nitrogen (i.e. inorganic sources of N) compared with more complex sources (i.e. organic sources of N), compensation in the associated regulatory pathways sacrifices stability.

When the concentration of glucose is constant, and more inorganic nitrogen source added, both amplitude of oscillation and growth rate is quadratic correlated with C: N ratio (Figure 4.6, Figure 4.7). The peak values of amplitude and growth rate is at carbon to nitrogen ratio at 8:1 and 14:1 respectively. The optimal carbon to nitrogen ratio was at 13:1 (Chapter 2, Figure 2.5). However, we are not sure if these differences between optimal ratios are significantly difference from each other. In fact, for data from Chapter 2, 2.3.1 using inorganic nitrogen source, optimal ratio after 72 h and 96 h was at 15:1, which is the same as growth rate and maximum amplitude here (Chapter 2, Figure 2.3). Our results showed correlation between amplitude of oscillation and growth rate when using inorganic nitrogen source.
These results should be tested for a wider range of species and resource qualities. In particular the relation between amplitude, C: N ratio and resource quality (Figure 4.3) should be verified. Our results also suggest that there could be a point where the sacrifice of system stability becomes critical and efficiency begins to decline in tandem. The identification and quantification of this critical point for different species will be informative. Furthermore, these results will be a sensitive test of models for the linked metabolism of carbon and nitrogen. Such models are an important synthesis for understanding the links in the regulatory apparatus involving carbon and nitrogen metabolism (Commichau et al., 2006). A similar dynamical approach, focussing on the stability and efficiency of carbon and nitrogen metabolism, may shed new light in other organisms.
Chapter 5. Discrete Two-Nutrient Model for the Fungal Phenotype

5.1 Introduction

In earlier theoretical work, fungal network models have been built based on its physiological processes. They have shown how a wide range of observed fungal phenotypes can emerge from different realisations of the same underlying processes of biomass recycling and translocation (Falconer et al., 2005). However, that work considered a simple resource base in which only a single nutrient component is limiting. An understanding of how the fungal phenotype responds to an environment in which the nutritional composition of the resource varies spatially requires an extension of previous mathematical models to help interpret the consequences of the complex interactions that are involved. Models for fungal growth are usually continuous models that describe biomass distribution in response to a single nutrient (see Chapter 1. Introduction). The significance of these continuous models is that they assume a scale of homogeneous mixing of nutrients in the environment that is significantly larger that the scale of hyphae. For example, a macroscopic scale model (Lamour et al., 2001, Lamour et al., 2002) describes the colonization and decomposition of a substrate, the subsequent uptake of nutrients, and incorporation into fungal biomass.

The models of Edelstein (1982) and Edelstein and Segel (1983) describe mycelial growth at the microscopic scale and include explicit properties of single hyphae. In Edelstein (1982), tip growth rates and branching rates are constant. The model of Edelstein and Segel (1983) includes nutrient as a growth factor. These authors were the first to consider the effects of hyphal death, hyphal fusion and different forms of branching on mycelial growth and development. Whilst this model reproduces the qualitative and some quantitative behaviours of the growing mycelium, it
is a phenomenological model that does not incorporate underlying physiological mechanisms. It is therefore not easy to extend to include the effects of nutritional quality on the phenotype.

In the present study, we wished to understand how acquisition and redistribution of nutrients by fungi result in spatial mixing of nutrients in the environment, and the distribution of hyphal biomass of relevance to soil aggregation. It is therefore inappropriate to impose a scale of mixing in advance and so for that purpose we require a hyphal-scale model. We developed a two-nutrient hyphal-level model for fungal growth from an existing single-nutrient model based on the work of Falconer et al. (2005). The model needs to simulate growth in a two-dimensional environment. We validated the model with data from the earlier experiments of Chapter 2 and 3, which strictly correspond to growth in a three dimensional spatial domain. This is justified because the growth medium is well mixed in the single-well experiments by use of the orbital shaker. Therefore, although the growth is in three dimensions, the resource should be homogeneously distributed and therefore the geometry of the fungi should not constrain uptake. Nevertheless, the geometric constraints on the distribution of biomass will play a role and so the comparison between model and experiment should be regarded as qualitative rather than quantitative. For the purpose of reproducing the observed behaviour to test for self-consistency of the interpretation of those experiments, this was considered adequate.

5.2 The Single Nutrient Discrete Model for the Fungal Phenotype Developed by Hao (Hao et al., 2009)

5.2.1 Model Frame Work

The physiologically-based model of Falconer et al. (2005) describes fungal growth by integrating both microscopic and macroscopic processes. It assumes that biomass transport and biomass
recycling can influence fungal growth patterns, and can therefore regulate fungal network structures. Although the model is continuous, it implicates uptake, transport and recycling processes at the hyphal level. The fungal growth phase in this model includes elongation, dieback and reallocation of biomass to centres of high demand.

A discrete version of this continuous model was developed by Hao (Hao et al., 2009). The conceptual framework, processes that were included, and the functional forms were equivalent to Falconer et al. (2005) but modified to describe the discrete state. In the discrete model, the fungal growth environment was set as a 200 by 200 hexagonal lattice of grid points (Figure 5.1). The mycelium was modelled as a network of hyphal segments that connect adjacent grid points on the lattice. A hyphal segment that did not connect to an adjacent grid point is defined as a hyphal tip. Resource was distributed on the grid. A homogenous environment was represented by resource that is equally distributed on the grid points. Resource that was unevenly distributed on the grid points was used to represent a heterogeneous environment. The mycelium was initiated with a single unit of hyphal length, representing a spore. During a single time step in the model, hyphal tips can extend one unit in length to an adjacent grid point, the dimensions of which are set by the values of the parameters in the model described below. The unit of growth extends in one of five possible random directions to the nearest neighbour grid point on the hexagonal lattice in 2D (Figure 5.2).
Fungal colonies are assumed to grow from a fungal spore in the centre at time step 0.

When a new hypha is produced from an existing hyphal tip at location ‘a’, it has 5 potential direction denoted 1-5 in the figure. The angle between each possible hyphal position is 60°.

Hyphal segments can be either added or deleted in the model at each time step. The model uses a look-up table to determine the development of the hyphal network at each time step. An example is shown in Table 5.1 and comprises a list of all hyphal segments that make up the fungal network, with all the information relevant to the model recorded for each hyphal segment in the present time step. In each time step, the programme checks the status of every
segment in the current look-up table and processes hyphal growth, hyphal dieback, uptake, biomass diffusion, and biomass recycling appropriately. The new status of each hyphal segment is then used to update the look-up Table 5.1.

Table 5.1 An example of a look-up table describing the status of each hyphal segment in the modelled network.

<table>
<thead>
<tr>
<th>No.</th>
<th>Start point</th>
<th>End point</th>
<th>Mobile biomass</th>
<th>Immobile biomass</th>
<th>Is it a tip?</th>
<th>Neighbours list</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(100,100)</td>
<td>(100,101)</td>
<td>20</td>
<td>10</td>
<td>No</td>
<td>2, 3</td>
</tr>
<tr>
<td>2</td>
<td>(100,100)</td>
<td>(99,100)</td>
<td>30</td>
<td>10</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>(100,101)</td>
<td>(101,101)</td>
<td>0</td>
<td>10</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
</tbody>
</table>
Figure 5.3: A model logic diagram representing the processes incorporated in the model to represent the dynamics of hyphal growth during a single time step.
5.2.2 Physiological Processes Included in the Model

The fungal mycelium in the model developed by Hao et al. (2009) included two components of biomass: immobile and mobile. Immobile biomass is the structural material of hyphae such as the hyphal wall and membrane. The integrated uptake and any remobilised biomass is moved within the mycelium via the mobile biomass component.

The fungal mycelium has three phases: spore, tip and hyphae behind tip. Fungi typically grow as filaments, termed hyphae (singular: hypha). Hyphae are initially generated from a fungal spore in the first time step.

5.2.2.1 Uptake

Local uptake in each time step is $U b f$, in which $b_i$ is the local immobile biomass concentration, $f$ is the local concentration of resource (food) in the environment, and $U$ is the uptake coefficient. The value for $U$ depends on whether or not the hypha is a tip. Equation 5.1 shows this relationship. In which $U_s$ is the uptake rate behind the tips, and $U_t$ is the rate at the tips. Fungal mycelia uptake resource mainly from the hyphal tips and so $U_t >> U_s$.

$$U = \begin{cases} U_s & \text{not at tip} \\ U_t & \text{at tip} \end{cases}$$

5.1
5.2.2.2 Extension and Branching

Whether a hyphal tip will stop growing, extend, or branch is assumed to depend on the immobile biomass concentration in the tip segment and the distance, \( l \), between the tip and the previous branching point according to:

\[
B_n = \begin{cases} 
0 & \frac{b}{B} l < B_1 \\
1 & B_1 \leq \frac{b}{B} l < B_2 \\
2 & \frac{b}{B} l \geq B_2 
\end{cases}
\]

5.2

where \( B_n \) is the number of hyphal lengths added in the time step. When \( B_n \) is equal to 0, the tip will not grow in that time step. When \( B_n \) equals 1, the tip will grow one unit length in that time step. When \( B_n \) equals 2, the tip will produce two branches. Each of which will grow one unit in length in that time step. \( B, B_1 \) and \( B_2 \) are constants. Each branch will grow one unit of length. It is assumed that every new unit of growth requires the transfer of \( N \) units of mobile biomass from the former tip, which is converted into \( N \) units of immobile biomass. Therefore each new growth segment contains \( N \) units of immobile biomass and no mobile biomass at the time of initiation. In subsequent time steps, mobile biomass can be transferred by diffusion from neighbouring nodes as set out in the following section.

5.2.2.3 Translocation

In this model, it assumed that mobile biomass diffusion is the only way to translocated biomass. It occurs between neighbouring hyphae where neighbours of a hypha are here taken as those hyphae that are directly connected to it. The diffusion between two neighbouring hyphal
segments is from the higher mobile biomass concentration to lower at a rate given by

\[ D(b_{m1} - b_{m2}) \], where \( b_{m1} \) and \( b_{m2} \) are the mobile biomass concentrations of the two neighbouring hyphal segments. \( D \) is the effective mobile biomass diffusion rate. The value of \( D \) is determined by \( b_{m1} \) and \( b_{m2} \) and is given by:

\[
D = \begin{cases} 
D_1 & \text{ if } b_{m1} > b_0 \quad \text{ or } \quad b_{m2} > b_0 \\
D_2 & \text{ if } b_{m1} < b_0 \quad \text{ and } \quad b_{m2} < b_0 
\end{cases} 
\]

where \( D_1 < D_2 \). This dependency of the diffusion coefficient on the mobile biomass follows the model of Falconer et al. (2005) and mimics the non-linear behaviour of diffusion in a medium with limited transport pathways.

5.2.2.4 Biomass Recycling

Biomass can interconvert between mobile and immobile forms. The net rate depends on the local mobile biomass concentration. In every time step, mobile biomass transfer into immobile biomass at a rate of \( \gamma \alpha \lambda^\theta \). Similarly, immobile biomass transfers into mobile biomass at a rate of \( \gamma \beta \lambda \). Where \( 0 \leq \gamma \leq 1 \) is the rate of transformation, \( \alpha \) is the coefficient for the rate of immobilization, while \( \beta \) is the coefficient for the rate of mobilization. \( \theta \) is a constant indicating the nonlinear process of immobilization. \( \lambda \) is the ratio of mobile to immobile biomass, given by:

\[
\lambda = \frac{b_m}{b_i} 
\]

5.2.2.5 Biomass Consumption

In each time step, mobile biomass in hyphal segments will reduce due to biomass consumption representing maintenance respiration. The reduction rate in one time step of one unit of hypha
is \( C \). Mobile biomass in each time step of one unit of hypha mobile biomass reduces by an amount given by \( C b m \), where \( b m \) is the local mobile biomass concentration.

5.2.2.6 Dieback

Physiological processes will change the amount of mobile biomass and immobile biomass in each hypha. It is set that if the immobile biomass concentration in a hypha is lower than \( b_d \), then this results in hyphal dieback. The result is removal of the hyphal segment, along with its mobile biomass, from the modelled network.

5.2.3 Branch Model and Anatomises Model

Hyphae can be classified as lower and higher, with the higher fungi being capable of of a number of distinct functions, including the capacity for hyphae to fuse, or anastomose, with one another (Gow and Gadd, 1995). There are two types of hyphal fusion, the tip-to-tip and the tip-to-side fusion, although the latter occur in 80% of cases (Aylmore and Todd, 1984). Lower fungi, such as Oomycetes and Mucoromycotina, rarely support hyphal fusion (Gow and Gadd, 1995).

In the model without anastomosis, new hyphae growth cannot overlap or connect to any existing hyphae (Figure 5.4a) and this restricts the opportunity for growth of a new hyphal segment, and the choice of direction. When anastomoses are included in the model, the direction of a new hyphal segment is chosen randomly from the centre of a hexagon located at the tip of the hyphae to any of corners of the hexagon (Figure 5.4b) irrespective of whether there is a hyphal segment located there or not.
5.3 Development of the Two Nutrient Fungal Model:

In this section, we extend the previous single nutrient hyphal level model for fungal growth developed by Hao (Hao et al., 2009) to a two-nutrient (carbon and nitrogen) model where fungal growth is assumed to be limited by two nutrients, namely both carbon and nitrogen sources.

Since *Mucor* forms an aseptate mycelium, in this project, we did not consider anastomoses so that we could compare the output to our experimental results from using *Mucor mucedo* (Mucoromycotina) (Edgar et al., 2012, John, 2012).

Hyphal branching is not always from the apex. The ability of rapidly growing hyphae to generate new polarity axes is the most important yet least understood aspects of fungal cell biology (Harris, 2008). In the development of this model we added a new functionality that allows tips to form laterally from pre-existing hyphae. Even although Mucor is an aceptate mycelium, it is capable of producing lateral hyphae like this (Harris, 2008). Otherwise the model development follows closely the approach described in the previous section, with certain differences that are described below to accommodate the two-nutrient limitation.
5.3.1 C and N Limited Fungal Growth Model

5.3.1.1 Nutrient Setting in the Environment

As in the single nutrient model, fungi are assumed to grow in a two dimensional environment. Again, the environment is comprised of an N x N hexagonal array of grid points. Because we do not consider anastomoses, which result in an indeterminate number of hyphal segments being associated with each grid point, we do not need an elaborate look-up table to capture the hyphal network. Each hyphal segment is associated with the grid point at the leading end, and information on the location of the grid points to which it is connected is stored along with the values of other variables associated with the hyphal segment. The environmental resources are assumed to contain a mixture of carbon and nitrogen and the values are distributed across the grid points in the array. The initial nutrient distribution can become depleted due to uptake by the growing fungal mycelium.

5.3.1.2 Fungal Structure and Nutrient Composition

The fungal colony is grown from a single hyphal segment (fungal spore) at time 0. Spores can germinate and sustain growth of the the germ tube for at least a short time in the absence of exogenous nutrients, indicating that they use endogenous supplies of carbon and nitrogen to support this early growth and development (Shaw and Hoch, 2007). In this model, we assume that the fungal spore contains immobile biomass $I_{ms}$, and mobile carbon and nitrogen compounds $C_{ms}$, and $N_{ms}$. The values of $I_{ms}$, $C_{ms}$ and $N_{ms}$ are assumed to have the same constant value in all the numerical experiments.

As before, the biomass contained in hyphae has mobile and immobile components. In this model, mobile biomass includes both mobile carbon ($C_{m}$) and mobile nitrogen ($N_{m}$). We also
assumed that the immobile biomass \((Im)\) is formed from carbon and nitrogen at a ratio of \(Pc : Pn\).

5.3.1.3 Uptake of Nutrient Resources

In this implementation of the model, we assume that uptake occurs only in tip segments. A hyphal segment uptakes in each time step an amount \(Efc\) of carbon resource and \(Efn\) of nitrogen resource, where \(E\) is the uptake rate. For simplicity in the first instance, we assumed that the uptake coefficient is the same for both carbon and nitrogen sources. The carbon and nitrogen resource concentrations in the substrate are denoted by \(fc\) and \(fn\) respectively.

5.3.1.4 Hyphal Transformation

A new tip can be generated from an established hypha through sub-apical branching (Harris, 2008). This process involves enzymatic degradation of part of the hyphal wall material. In this model, if the immobile biomass in a hyphal segment is larger than \(\alpha\) and the total mobile biomass is greater than \(\beta\), where \(\alpha\) and \(\beta\) are constants, then the properties of the hyphal segment will transform to those of a hyphal tip segment. At the same time, the immobile biomass in the original hyphal segment is reduced by \(Ti\), where \(Ti\) is a constant, and an amount \(Ti\) is added to mobile carbon and mobile nitrogen components in the ratio of \(Pc : Pn\). These new tips behave in the same way as all other tip segments and so can uptake nutrients and generate new hyphal segments.

5.3.1.5 Hyphal Growth

Following the previous model, hyphal growth includes extension and branching. Each tip can split into a maximum of two branches in each time step and hyphal growth occurs only at the
hyphal tip. Both extension and branching depend on the local mobile biomass. In this model, the growth condition is limited by both carbon and nitrogen in the mobile biomass.

\[
B_n = \begin{cases} 
1 & Cm > BR_1 \text{ and } Nm > \frac{PnBR_1}{Pc} \\
2 & Cm > BR_2 \text{ and } Nm > \frac{PnBR_2}{Pc} 
\end{cases}
\]

5.5

Where \( Cm \) and \( Nm \) are mobile carbon component and mobile nitrogen component in the tip, \( B_n \) is the number of new hypha, and \( BR_1 \) and \( BR_2 \) are constants. The growth requirement for mobile carbon components are \( Pc : Pn \) times greater than nitrogen. The tip will extend when \( B_n \) is 1 and will produce an apical branch when \( B_n \) is 2. Each of the new hyphae are one unit in length. The tip will not grow during a time step (i.e. \( B_n =0 \)), if neither of the conditions in equation 5.5 are met. The direction of growth of the new tip(s) is chosen randomly from one of the available directions at 60° intervals in the hexagonal array of grid points.

In this model a newly produced hyphal tip segment will inherit an amount, \( Si \), of immobile biomass from the parent hypha, where \( Si \) is a constant value. It also inherits proportion of mobile biomass from its parent hypha which will explain in next section: biomass translocation-local translocation.
5.3.1.6 Biomass Translocation

Local Translocation

During hyphal growth, the mobile biomass can move from older parts of the mycelium to newer parts through the process of translocation (Kues, 2000). In this model, each newly produced hypha will inherit an amount, $S_c$ and $S_n$, of $C_m$ and $N_m$ respectively from the parent tip where $S_c$ and $S_n$ are constant parameters.

Non-local Translocation

Nutrient translocation in individual hyphae is thought to be mediated by a combination of mass flow, diffusion, generalized cytoplasmic streaming and specific vesicular transport (Cairney, 2005). Following the single nutrient model, we assumed diffusion is the only process of translocating internal nutrients. Experiments have shown internal nutrients can be translocated across distances ranging from millimetre scale to colony scale (Fricker et al., 2008). As in the single nutrient model, we assume that mobile biomass diffuses between connected hyphal segments, however both carbon and nitrogen diffuse and are assumed to do so independently. As before, diffusion between two neighbouring hyphae is from higher carbon or nitrogen mobile biomass concentration to lower concentrations at a rate $D_c(C_{m_1} - C_{m_2})$ and $D_n(N_{m_1} - N_{m_2})$ respectively where the mobile carbon concentrations of the two neighbouring hyphae are denoted by $C_{m_1}$ and $C_{m_2}$, and $D_c$ is the diffusion rate of the mobile carbon component.

Throughout the simulations reported below, we assume that carbon is not translocated i.e. $D_c = 0$. Similarly, the mobile nitrogen concentration of two neighbour hyphae are $N_{m_1}$ and $N_{m_2}$, and $D_n$ is the mobile biomass diffusion rate for nitrogen. In the modelling results
reported below, we simulate two fungal phenotypes, one that can translocate nitrogen and one that cannot i.e. $Dn = 0$.

In each time step, the concentrations of internal biomass of all hyphae are updated according to the above scheme. By this mechanism, mobile biomass can be translocated a long distance over time.

5.3.1.7 Biomass Recycling

Both carbon and nitrogen compounds can interconvert between mobile biomass and immobile forms. Immobile biomass is mainly from fungal walls. They are fibrillar materials bound together by sugars, proteins, lipids and a variety of polysaccharides. Though the C: N ratio of fungal walls is unknown, those wall materials are complex organic matters containing high C: N ratio. Therefore we assumed immobile biomass contained 10 C: 1 N. We use a modified scheme for biomass recycling compared with the single nutrient model to accommodate for the fact that there are two nutrients to consider. For each hypha in each time step, if $(Cm + Nm) / Im < R_1$, where $R_1$ is constant, an amount $R_1 Im$ of immobile biomass transforms to mobile carbon and nitrogen, where $R_1$ is a constant. The relative amounts of carbon and nitrogen are in accordance with the ratio assumed for the immobile biomass i.e. $Pc : Pn$.

Where $(Cm + Nm) / Im > R_1$ and $Nm > \frac{PnR_2Cm}{Pc}$, $R_2Cm$ of mobile carbon components and $\frac{PnR_2Cm}{Pc}$ of mobile nitrogen components are transformed into immobile biomass, where $R_2$ is a constant. Again, this ensures that the mobile biomass is incorporated in to immobile biomass at the ratio $Pc : Pn$. 

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5.3.1.8 Biomass Consumption

As in the previous model, in each time step, the mobile biomass in hyphae is assumed to be consumed as part of maintenance respiration. In this model, mobile biomass reduction rates for carbon and nitrogen compounds are $Mc$ and $Mn$ respectively. Therefore, in each time step, the reduction in mobile biomass in each hypha is:

$$\Delta Cm = Mc Cm$$

$$\Delta Nm = Mn Nm$$

where $Cm$ and $Nm$ are the carbon and nitrogen concentrations in the mobile biomass respectively.

5.3.1.9 Dieback

In each time step both mobile and immobile biomass will change in the hypha. We assume a hypha will die back, i.e. be removed from the modeled hyphal network, if the carbon components in the mobile biomass falls below $b_c$, or the nitrogen in mobile biomass falls below $b_n$, or immobile biomass is less then $b_d$, where $b_c$, $b_n$ and $b_d$ are parameters of constant value. The immobile biomass associated with the hyphal segment is removed from the system.

5.3.1.10 Collection of Equations

In summary, mobile biomass is potentially influenced by uptake, translocation, biomass consumption, the production of sub-apical branches and biomass recycling. Immobile biomass is changed by growth, the production of sub-apical branches, and biomass recycling.
The full set of equations is written below. For compactness, we introduce the constants $K_1 - K_4$ which have values equal to either 0 or 1.

\[
\Delta Cm = K_1 Efc + \Sigma[Dc(Cm_{\text{neighbour}} - Cm)] - B_n ScCm - McCm + \frac{K_2 Pc TiIm}{Pc + Pn} + \left(\frac{K_3 Pc R_1 Im}{Pc + Pn} - K_4 R_2 Cm\right)
\]
\[
\Delta Nm = K_1 Enn + \Sigma[Dn(Nm_{\text{neighbour}} - Nm)] - B_n SnNm - MnNm + \frac{K_2 Pn TiIm}{Pc + Pn} + \left(\frac{K_3 Pn R_1 Im}{Pc + Pn} - K_4 Pn R_2 Cm\right)
\]
\[
\Delta Im = B_n SiIm - K_2 TiIm + \left(\frac{K_4 (Pc + Pn) R_2 Cm}{Pc} - K_3 R_1 Im\right)
\]
\[
\Delta fc = -K_1 Efc
\]
\[
\Delta fn = -K_1 Enn
\]
\[
K_1 = \begin{cases} 
0 & \text{not at tip} \\
1 & \text{at tip}
\end{cases}
\]
\[
B_n = \begin{cases} 
1 & \text{Cm > BR}_1 \text{ and } Nm > \frac{Pn^* BR_1}{Pc} \\
2 & \text{Cm > BR}_2 \text{ and } Nm > \frac{Pn^* BR_2}{Pc} \\
0 & \text{Bn} \neq 1 \text{ or } 2
\end{cases}
\]
\[
K_2 = \begin{cases} 
0 & \text{a tip or Im} > \alpha \text{ or } Cm + Nm > \beta \\
1 & \text{not a tip and Im} > \alpha \text{ and } Cm + Nm > \beta
\end{cases}
\]
\[
K_3 = \begin{cases} 
0 & (Cm + Nm) / Im \leq R_1 \\
1 & (Cm + Nm) / Im > R_1
\end{cases}
\]
\[
K_4 = \begin{cases} 
0 & Nm < \frac{Pn R_1 Cm}{Pc} \\
1 & Nm \geq \frac{Pn R_1 Cm}{Pc}
\end{cases}
\]
5.3.2 Nondimensionalization

The rewriting of equations in non-dimensional form is a common technique that is useful for reducing the number of parameters. The result is a more compact form of the equations and a smaller set of new parameters that are functions of the original model parameters. These functions express equivalencies between the original parameters, in the sense that their values can be changed without affecting the value of the new parameter and therefore the qualitative behaviour of the model predictions. Non-dimensional forms are particularly useful when the values of the original parameters are unknown or highly uncertain, as is the case here.
Nodimentsionalization equations: \( P_c \) : \( P_n \) is the ratio of carbon to nitrogen in immobile biomass

\[
C_m = C_{m_0} C_m^*
\]
\[
N_m = N_{m_0} N_m^*
\]
\[
I_m = I_{m_0} I_m^*
\]
\[
f_c = f_{c_0} f_c^*
\]
\[
f_n = f_{n_0} f_n^*
\]
\[
C_{m_0} = f_{c_0} = B_{r_1}
\]
\[
N_{m_0} = f_{n_0} = \frac{P_n}{P_c} B_{r_1}
\]
\[
I_{m_0} = \frac{P_c + P_n}{P_c} B_{r_1}
\]

\[
\Delta C_m^* = K_1 E_{f_c}^* + \Sigma [D_c (C_m^* - C_m^*)] - B_n S_c C_m^* - M_c C_m^* + K_2 T_i I_m^* + K_3 R_i I_m^* - K_4 R_r C_m^*
\]
\[
\Delta N_m^* = K_1 E_{f_n}^* + \Sigma [D_n (N_m^* - N_m^*)] - B_n S_n N_m^* - M_n N_m^* + K_2 T_i I_m^* + K_3 R_i I_m^* - K_4 R_r C_m^*
\]
\[
\Delta I_m^* = B_n S_i I_m^* - K_2 T_i I_m^* + K_4 R_r C_m^* - K_3 R_r I_m^*
\]
\[
\Delta f_c = -K_i E_{f_c}^*
\]
\[
\Delta f_n = -K_i E_{f_n}^*
\]
5.3.3 Computer Implementation and Visualization

Visual C++ is chosen to implement this model in the computer. The visualization tool is OpenGL. The C++ programme code is presented in appendix 1.

5.3.4 Parameters

In all cases where the same relations are used as in the single nutrient model, the same parameter values are used. Otherwise a search of the parameter space was conducted to seek the parameter values that sustained growth using a genetic algorithm using Galib version 247, GAlib is a C++ library of genetic algorithm objects. We tested 20 and 100 generations. Code of seeking parameter values is presented in appendix 2. Code of genetic algorithm searching for value of parameters is presented in appendix 2. A sensitivity analysis is performed as part of the research and is outline below. Figure 5.5 shows the nominalised data observed from current two nutrient fungal model using parameter values in at C: N 15:1 and from previous experiment (chapter 2, 2.3.1) when ammonium used as a nitrogen source. C: N at 15:1 ($R^2=0.96$). 15:1 (C: N) was the optimal ratio observed at last harvest in that experiment.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Definition of Parameter</th>
<th>Values or range of value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungal spore</td>
<td>$I_{ms}$</td>
<td>Immobile biomass</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$C_{ms}$</td>
<td>Carbon component in mobile biomass</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$N_{ms}$</td>
<td>Nitrogen component in mobile biomass</td>
<td>40</td>
</tr>
<tr>
<td>Ratio</td>
<td>$P_c: P_n$</td>
<td>Ideal C:N ratio</td>
<td>10:1</td>
</tr>
<tr>
<td>Uptake</td>
<td>$E$</td>
<td>Uptake coefficient</td>
<td>0.015</td>
</tr>
<tr>
<td>Mobile biomass Translocation</td>
<td>$S_c$</td>
<td>Translocation coefficient of Mobile carbon during growth</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>$S_n$</td>
<td>Translocation coefficient of Mobile nitrogen during growth</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>$D_c$</td>
<td>effective mobile carbon diffusion rate</td>
<td>0</td>
</tr>
<tr>
<td>Category</td>
<td>Parameter</td>
<td>Description</td>
<td>Value</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td><strong>Effective Mobile Nitrogen Diffusion Rate</strong></td>
<td>$D_{n}$</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Requirement for Hyphal Extension in the Time Step</strong></td>
<td>$BR_{1}$</td>
<td>Requirement for hyphal extension in the time step</td>
<td>8</td>
</tr>
<tr>
<td><strong>Requirement for Hyphal Branch in the Time Step</strong></td>
<td>$BR_{2}$</td>
<td>Requirement for hyphal branch in the time step</td>
<td>12</td>
</tr>
<tr>
<td><strong>Constant for Inherit of Immobile Biomass from the Parent Hypha</strong></td>
<td>$Si$</td>
<td>Constant for inherit of immobile biomass from the parent hypha</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>The Reduction Rate of Mobile Carbon in One Unit of Hypha</strong></td>
<td>$Mc$</td>
<td>The reduction rate of mobile carbon in one unit of hypha</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>The Reduction Rate of Mobile Nitrogen in One Unit of Hypha</strong></td>
<td>$Mn$</td>
<td>The reduction rate of mobile nitrogen in one unit of hypha</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Constant for Requirement of Recycling</strong></td>
<td>$R_{1}$</td>
<td>Constant for requirement of recycling</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Recycling Rate of Immobile Biomass</strong></td>
<td>$Rr_{1}$</td>
<td>Recycling rate of immobile biomass</td>
<td>0.2</td>
</tr>
<tr>
<td>Parameter</td>
<td>Description</td>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>$Rr_2$</td>
<td>Recycling rate of mobile biomass</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>$b_c$</td>
<td>Standard of mobile carbon for dieback</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$b_n$</td>
<td>Standard of mobile nitrogen for dieback</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$b_d$</td>
<td>Standard of immobile biomass for dieback</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Standard of immobile biomass for sub-apical tip</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>$\beta$</td>
<td>Standard of mobile biomass for sub-apical tip</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>$Ti$</td>
<td>Reduce rate of immobile biomass for sub-apical tip</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.5: Data from laboratory experiment vs data from current fungal model. Line is nominalization immobile biomass from using parameters value as Table 2 at C: N15:1. O is nominalization dry weight of *M. muceda* from chapter 2, 2.3.1.2. at C: N15:1.
Figure 5.6: Two nutrient model flow chart. In each time step every hypha need to go through all the physiology processes.
5.4 The Nutritional Environments Used in the Model

We used previous data from the single- and two-well experiments to validate the model and test the hypothesis that the observed behaviour can be interpreted as being due to nitrogen translocation within the organism. The model is therefore run to simulate the effects of different carbon and nitrogen conditions in both homogenous and heterogeneous environments. The simulated homogeneous environment corresponds to the nutrient conditions to the second experiment in Chapter 2 that is discussed in section 2.3.1 and 2.3.2. For the heterogeneous environment, the nutrient conditions correspond with control 1, 2 and treatment 1 as in the experiment in Chapter 3.

To test the hypothesis that the experimental results can be explained by differential C and N translocation, we simulated to contrasting ‘phenotypes’. One phenotype has the same coefficients movement of mobile resource for both C and N forms, while the second phenotype does not translocate nitrogen (\(Dn\) and \(Sn\) are 0). The other parameter values are set to those listed in
Table 5.2. All of the numerical experiments began with a spore (single hyphal segment) and were run for 90 time steps.

5.4.1 Numerical Experiment 1: Nutritionally Homogenous Environment

The model was run to simulate growth in a nutritionally homogeneous environment, where the resources are equally distributed throughout the entire environment. Seven treatments were tested with a carbon to nitrogen ratio at 55:1, 50:1, 25:1, 19:1, 15:1, 10:1, 7:1, 4:1, 3:1, 5:3, 6:7 and 3:5. From the pilot study, we knew the optimal ratio C:N is located within the C:N range of these treatments for all parameter sets we tested. For the phenotype that is unable to translocate nitrogen, the model predicts no growth for carbon to nitrogen ratio above 50:1. In that case we used a carbon to nitrogen ration of 40:1 for the highest ratio. For all the treatments, the initial total carbon and nitrogen at each lattice point in the environment is set at 800.

5.4.2 Experiment 2 Nutritional Heterogeneity Environment

To model the fungal response in a nutritionally heterogeneous environment, the modelled resource base (dimensions 200 x 200 grid points) is initialized separately for the left and right rectangles of dimension 100 x 200 grid points (Figure 5.7). Resources are equally distributed in the left 100 x 200 rectangle with carbon resource $f_{c_1}$ and nitrogen resource $f_{n_1}$ in each grid point. Similarly, $f_{c_2}$ and $f_{c_2}$ indicate carbon and nitrogen resources in each grid point on the right 100x 200 rectangle of the culture environment (Figure 5.7).
To test whether the model for fungi where nitrogen, but not carbon, translocation occurs is consistent with the data from the lab experiments described in Chapters 3, control 1, 2 and treatment 1, the following numerical experiments were undertaken.

- **Control 1** media on left side of the environment contained high (65:1, total C+N concentration in each grid: 800 units) C: N ratio and no carbon or nitrogen source on the other side of the environment.

- **Control 2** media on left side of the environment contained low (5:3, total C+N concentration in each grid: 800 units) C: N ratio, and no carbon and nitrogen source on the other side.

- **Treatment 1** media containing high (65:1, total C+N concentration: 800 units) and low (5:3, total C+N concentration : 800 units) C: N were placed on two sides of the environment.

### 5.4.3 Statistics:

One way ANOVA is used to test the difference between fungal growth in the different environments using GraphPad Prisim 6.
5.4.4 Results

In a homogeneous environment, for a modeled mycelium with an ability to translocate nitrogen (parameter $Sn = 0.1$, diffusion coefficient $Dn = 0.5$), the optimal ratio of carbon to nitrogen for producing immobile biomass is 20:1 (Figure 5.8C) at 60 time steps, and this increases to 35:1 at 90 time steps (Figure 5.8D). By comparison, for a modeled mycelium that is unable to translocate nitrogen (both $Sn = 0$ and $Dn = 0$) the optimal carbon to nitrogen ratio for producing immobile biomass is 15:1 at both 60 and 90 time steps (Figure 5.8 A,B).

In a homogeneous environment, for the same culture times and for C: N ratios higher than 20:1, the mycelium that is able to translocate nitrogen can produce significantly more immobile biomass than the mycelium that is unable to translocate nitrogen (P<0.05, Figure 5.9). We did not find this phenomenon for lower C: N ratios.

In a homogeneous environment, the growth pattern at the optimal C: N ratio is larger in size and denser for the mycelium that is able to translocate nitrogen compared with the mycelium that cannot translocate nitrogen (Figure 5.10).

In a heterogenous environment, the mycelium that is unable to translocate nitrogen (both $Dn$ and $Dc = 0$), no biomass is produced in the half of the environment where the media contains high (65:1; well1 for control 1 and treatment 1) C: N ratio regardless the nutrient conditions in the other side of environment (Figure 5.7). For the mycelium that is able to translocated nitrogen, the immobile biomass is produced in the high (65:1) C: N ratio side of environment in treatment 1 when it is connected to a well containing high nitrogen. In contrast no immobile biomass is produced high (65:1) C: N ratio side of environment in control 1, when it is connected to a well with no nitrogen. For both mycelia (translocating and non-translocating), immobile
Biomass is produced in equal amounts in low (5:3; well 2 of control 2 and treatment1) C: N ratio, regardless of the nutrient status of the connected well (P<0.05).

Figure 5.8: Immobile biomass vs. C: N ratio. A to D shows immobile biomass production vs C: N ratio in a homogenous environment. A and B shows the results assuming the mycelium is unable to translocate nitrogen. C and D shows the results assuming the mycelium can translocate nitrogen. A and C correspond to a simulated time of 60 time steps, and B and D correspond to a simulated time of 90 time steps.
Figure 5.9: A comparison of the simulated biomass production with and without nitrogen translocation in a homogeneous environment. Results are shown for a resource with a carbon to nitrogen ratio of 25:1 and 50:1 at 90 time steps.

Figure 5.10: Growth pattern of a mycelium that can translocate nitrogen. The environment is homogeneous with a carbon to nitrogen ratio of 15:1 at 60 time steps.
Figure 5.11: Growth pattern of a mycelium that can translocate nitrogen. The environment is homogeneous with a carbon to nitrogen at 3:5 at 60 time step.

Table 5.3: Fungal growth in a heterogeneous environment. Mean immobile biomass (±SEM) of two fungi: one unable translocate C and N; the other fungus able to translocate N, but not C in media either deficient in carbon, nitrogen after 60 or 90 time steps. a, b, c and d in each column indicate statistically significant differences at p<0.05. An asterix indicates statistically significant differences between right/ left side of environment in each row at each harvest.

<table>
<thead>
<tr>
<th>Control Or Treatment NO.</th>
<th>Treatment: C/N, Fungi unable translocate C and N</th>
<th>Harvest (hrs) 60 (well 1)</th>
<th>Harvest (hrs) 60 (well 2)</th>
<th>Harvest (hrs) 90 (well 1)</th>
<th>Harvest (hrs) 90 (well 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Well 1</td>
<td>Well 2</td>
<td>60 (well 1)</td>
<td>60 (well 2)</td>
<td>90 (well 1)</td>
</tr>
<tr>
<td>Control 1</td>
<td>65/1</td>
<td>nil</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>Control 2</td>
<td>nil</td>
<td>5/3</td>
<td>243±12 b</td>
<td>0 a</td>
<td>712±11 b*</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>65/1</td>
<td>5/3</td>
<td>252±9 b</td>
<td>0 a</td>
<td>734±13 b*</td>
</tr>
</tbody>
</table>
5.4.5 Discussion

The model is parameterized using data corresponding to fungal growth in a homogeneous environment at a single C: N ratio (15:1) and then compared with experiments corresponding to different carbon and nitrogen nutrient environments for both a homogeneous and heterogeneous environment. We model two fungal phenotypes. The first is able to translocate nitrogen, and the second cannot.

The ratio of carbon to nitrogen significantly impacts on fungal growth in terms of immobile biomass production. In both homogeneous and heterogeneous environments, the key results from laboratory experiments can be interpreted by the model corresponding to the case where the fungus lacks the ability to translocate carbon, but is able to translocate nitrogen.

The first numerical experiment in this chapter corresponds to the physical experiment in Chapter 2 section 2.3.2. There, the mycelium grew in a homogenous environment, where the total concentration of carbon and nitrogen was constant of C: N varied. The results from the laboratory experiments show that the optimal carbon to nitrogen ratio for growth is time dependent. At 48 hrs fungal biomass was highest for the C: N ratio of 4:1, then for 12:1 at 72 hrs and then for 27:1 at 96 hrs. Results from the modelling experiments simulating growth in a homogenous environment, and where the mycelium translocate internal nitrogen resources also showed that the optimal carbon to nitrogen ratio for growth is time dependent, though the optimal ratio observed from this model is higher than in the physical experiment. The simulated optimum increased from 20: 1 at 60 time steps to 35: 1 at 90 time steps (Figure 5.8). For the simulated mycelia that lacked the ability to translocate nitrogen, the predicted optimal carbon
to nitrogen ratio is time-independent and fixed at 15:1. This optimal carbon to nitrogen ratio is lower than in mycelia that has the ability to translocate N.

Results corresponding to fungal growth in a heterogeneous environment obtained in chapter 3 control 1, 2 and treatment 1 shows that in a high C: N ratio well, *M. muceda* can produce significantly more biomass if the connected well contains a high nitrogen concentration. On the contrary, in a low C: N ratio well, *M. muceda* produces an equal amount of biomass regardless of the carbon concentration in the connected well. In this model, the simulated mycelium that could translocate N but not C, could reproduce the qualitative behaviour of both the experiments. When running the model under the assumption that neither C nor N could be translocated, only the second result can be reproduced.

By translocating minerals through the mycelium, fungi can overcome local depletion of specific minerals. For wood decaying fungi, where conditions are usually highly nitrogen limited, there is clear evidence of nitrogen translocation from the soil in to the wood (Levi and Cowling, 1969, Lilly et al., 1991, Watkinson, 2006). In our model, we observed similar results. The fungus achieved that by relocating internal nitrogen to the active hyhal growth region or/and translocated nitrogen from outside of the carbon rich location. In other words, the ability to translocate nitrogen is an advantage in both homogeneous and heterogeneous environments where nitrogen is limiting.

In this chapter, we have extended a previous single-nutrient fungal model to a two nutrient model. To our best knowledge, this is the first fungal model that includes multiple nutrient elements. However in this model, the metabolism of carbon and nitrogen in to biomass production is represented by a very simple heuristic function. In reality, normal morphogenesis
involves major changes in biochemical composition of many parts of the cell and considerable turnover of existing cellular components. Biomass production is a consequence of the co-metabolism of nitrogen and carbon in a complex network of biochemical reactions. Given the importance of the impact of nutritional quality on fungal growth, and the importance of fungi in ‘averaging out’ the nutritional heterogeneity of the environment, an important priority for future research is to better understand the pathways of carbon and nitrogen metabolism. This is the subject of the next chapter.
Chapter 6. Network Models for the Co-Metabolism of Carbon and Nitrogen in *Mucor mucedo*

6.1 Introduction

Carbohydrate and nitrogenous compounds are closely linked in the metabolic systems of organisms. The growth of organisms can be optimized by the balance of these two nutrients (Simpson and Raubenheimer, 1996, Raubenheimer and Simpson, 1993). However, exactly how the nutritional cues are integrated within the cellular matrix of organisms is not clear. The system will also change in response to nutrient balance at the physiological level such as in nutrient translocation, recycling of nutrients and in autolysis. The use of controlled laboratory experiments has revealed considerable detail in the corresponding metabolic pathways (Jennings, 1995), but it is difficult to carry out studies of the metabolic network in colony scale laboratory experiments. Computational cell biology provides a useful tool to unpick the interconnected mechanisms underlying the dynamics of metabolic pathways.

The aim of the work is to develop a model for the co-metabolism of carbon and nitrogen to better understand the impact of nutrition on the growth of fungi. Fungi can use both organic and inorganic nitrogen sources. The pathways for organic nitrogen and inorganic are different, therefore we developed two different metabolic network models for the effect of the ratio of carbon to nitrogen on fungal growth depending on whether the nitrogen is in organic or inorganic form. A set of coupled differential equations is written to describe each of the networks and solved using Matlab R2011a. These models are based on the results of observations of the growth of the fungus *Mucor mucedo* in different nutrient regimes. Our experimental studies found oscillatory growth in a wide range of carbon to nitrogen ratios. In
addition, the ratio of glucose to ammonium, not glutamine, modified the amplitude of the oscillatory growth. Biological oscillations can be an important diagnostic of underlying complex nonlinear feedback in regulatory networks. Therefore, these oscillations in fungal growths are an important test of the models for the underlying regulatory network describing here. We applied a sensitivity analysis to understand how the parameters affect the emergent behaviour.

6.2 Development of the Methodology

The metabolic network assumes that the metabolites are spatially well-mixed. In absence of information to the contrary, the reactions are described by Michaelis-Menton or mass-action kinetics as appropriate. The conversion of metabolites to biomass depends only on the rate-definable sources and sinks in the network. As a consequence, the rate of change in concentration of each molecular species in the cell can be written as an ordinary differential equation, which obeys the conservation of mass within the network.
6.3 Model

6.3.1 Inorganic Nitrogen Source

In the model (Figure 6.1) glucose and ammonium are used as carbon and inorganic nitrogen sources respectively. Both sources can cross the membrane without enzymatic pre-processing. This network only included primary metabolism, no secondary metabolites are included in the model at this stage.

6.3.2 Nitrogen

6.3.2.1 Concentration of Nitrogen in Substrate

Depending on the ammonium concentration in the substrate, uptake of ammonium by certain mycorrhizal fungi is consistent with two different mechanistic systems: high-affinity transport systems (HATS) or the low-affinity transport systems (LATS) (Javelle et al., 2001, Perez-Tienda et
al., 2012). For the fungus *Rhizophagus irregularis*, low-affinity transport predominates when the ammonium concentration exceeds 1mM (Perez-Tienda et al., 2012). Knowledge of ammonia transport into fungal cell membrane is relatively scarce. Fluorescent labelling technology on ammonium transceptors in live cells indicates that the process in concentrations between 0 to 10mM can be described by Michaelis-Menten kinetics (Michele et al., 2013). Here we use the Michaelis-Menten function to summarize these two uptake processes in fungi. \( N_s \) is the concentration of ammonium in the substrate and we assume Michaelis–Menten kinetics with a limiting rate \( V_{na} \) and a Michaelis constant \( k_{na} \). The differential equation for ammonium concentration in the substrate is therefore:

\[
\frac{dN_s}{dt} = -V_{na} \frac{N_s}{N_s + K_{na}}
\]

6.1

Table 6.1 presents the units of the various parameters and state variables used in the model. All concentrations are defined with respect to fresh fungal biomass.

6.3.2.2 mRNA and Ammonium-assimilating Enzymes

Genes for controlling amino acid synthesis have been identified in different fungi. For example, in fungus *Saccharomyces cerevisiae*, the process is co-regulated by more than 20 genes (Jennings, 1987). However, the detailed nature of the co-regulation mechanism is not known. Therefore, in this model amino acid biosynthesis mRNA is combined. We assumed the concentration of combined mRNA to be produced proportional to the concentration of internal nitrogen, with a constant rate coefficient denoted by \( k_2 \). The process of bio-synthesis of amino acids is also regulated by ammonium-assimilating enzymes (Kersten et al., 1997). This model includes the combined concentration of ammonium-assimilating enzyme \( E_2 \). In this model, we
assumed directly proportional by the concentration of combined mRNA from carbon
components and ammonium in a percentage of $ka_1$ and $ka_2$ respectively. The rate of
downstream degradation of the mRNA and ammonium-assimilating enzymes are assumed to be
governed by Michaelis-Menten kinetics with velocities $V_{dm}$ and $V_{de}$ respectively and

$K_{dm}$ and $K_{de}$ are the corresponding Michaelis constants. Downstream degradation of
combined mRNA and enzymes are outside this network.

$$\frac{dN_m}{dt} = k_2N_{in} - V_{dm} \frac{N_m}{N_m + k_{dm}}$$ \hspace{1cm} 6.2

$$\frac{dE_z}{dt} = k_3Nm - V_{de} \frac{E_z}{K_{de} + E_z}$$ \hspace{1cm} 6.3

6.3.2.3 Concentration of Combined Amino Acids.

The only route of ammonium assimilation which can be considered as universal in fungi is the
synthesis of glutamate from ammonium and 2-oxoglutarate by the enzyme glutamate
dehydrogenase followed by glutamate synthesis to other amino acids (Moore, 1998). The
general control of amino acid biosynthesis implicates many genes and ammonium-assimilating
enzymes (Jennings, 1995, Kersten et al., 1997). The reaction rate of amino acid biosynthesis in
this model is determined by the concentration of combined mRNA, the ammonium-assimilating
enzyme, internal ammonium, and the carbon component. Carbon and nitrogen components are
the main constituents of amino acids. In this model, a percentage $ka_1$ of biosynthesised amino
acid is assumed to be from carbon components, and a percentage $ka_2$ of biosynthesised amino
acid is from internal ammonium, where \( ka_1 + ka_2 = 1 \). The change in concentration of combined amino acids \( Aa \) depends on the rate of biosynthesis of amino acid and the cost of the biosynthesis of biomass.

\[
\frac{dAa}{dt} = (ka_1 + ka_2)(Ez + knKm) \frac{Ni}{(Kr + Ni)(Kc + Cc)} - fnivb \quad 6.4
\]

where \( fni \) is the cost of nitrogen to produce a gram of fungal biomass, and \( vb \) is the relative mycelium growth rate.

6.3.2.4 Relative Growth Rate

In this model, apart from the carbon and nitrogen sources, all the fungal growth factors are assumed to be readily available. The relative rate of fresh fungal biomass \( vb \) depends on the concentration of carbon component \( Cc \), the concentration of amino acid, \( Am \), and energy availability. The additive model in (Oneill et al., 1989), which forms the first part of equation 6.5,

\[
\frac{VbCcAm}{K_1Cc + CcAm + K_2Am},
\]

\( K_1 \) and \( K_2 \) are Michael's constant, and \( Vb \) is the potential relative growth rate of the mycelium.

In addition, the equation for the fungal biomass production rate utilises energy available in the mycelium. Both the pentose and glycolysis pathways in glucose metabolism release energy. Here we use the amount of carbon compounds as a surrogate to represent the available energy from glucose metabolism, which gives rise to the production of biomass. Relative growth rate is therefore calculated by equation 6.5.
\[ vb = \frac{VbCcAm}{K_iCc + CcAm + K_zAm (1 + Kn_2 / Cg)} \]  

Equation 6.5

\( Kn_2 \) is activation constant for energy effect on biomass production. Equation 6.5 can be also written as equation 6.6. Therefore, fungal fresh biomass can be calculated as equation 6.7. \( B \) is fresh biomass.

\[ vb = \frac{dB}{Bdt} \]  

Equation 6.6

\[ B = \exp(\int_0^t vb dt) \]  

Equation 6.7

6.3.2.5 Internal Ammonium

The concentration of internal ammonium \( Ni \) is determined by the rate of uptake of ammonium and the equivalent cost of the assimilating enzyme and amino acid. Details of each function have been discussed in the above sections.

\[ \frac{dNi}{dt} = V_{na} \frac{N_i}{N_i + k_{na}} - (1 - kc)k3Nm - ka_i(1 - ka) \frac{(Ez + knKm) Ni}{(Kr + Ni)} \frac{Cc}{Kc + Cc} \]  

Equation 6.8

6.3.2.6 Carbon

Some fungal species utilize a wide range of carbon sources from monosaccharide to polysaccharide (Sati and Bisht, 2006). Glucose is readily utilised by many fungi (Jennings, 1995). Fungal uptake of glucose is described by Michaelis–Menten kinetics (Carlsen et al., 1996, Vrabl et al., 2008). Internal osmotic pressure will increase when the internal carbon concentration
increases, which can slow down the rate of uptake of glucose (Beever and Laracy, 1986).

Therefore, in this model, the glucose uptake rate depends on the concentration of glucose in the substrate $Cs$ and is inhibited by the concentration of pentose $Cp$ and carbon components $Cc$.

It is used as both a carbon and an energy source for growth (Jennings, 1995).

$$\frac{dCs}{dt} = -Vc_1 \frac{Cs}{Cs + Kn_1} \frac{Kn_3}{Cp + Cc}$$

6.9

$Vc_1$ is a limiting rate and $Kn_1$ is Michaelis constant. $Kn_3$ is the inhibition constant value corresponding to the negative feedback from $Cp$ and $Cc$.

6.3.2.7 The Glucose Pathways

Glycolysis and pentose phosphate pathway and are found in all fungi (Vaseghi et al., 1999, Jennings, 1995, Zikmanis and Kampenusa, 2012). The evidence of pentose phosphate pathways widely distributed in fungi is less compelling than that for presence of glycolysis. The conversion of Glucose to glucose-6-phosphate is the first step for both of these two pathways. Both pathways release energy and can provide carbon components for biosynthesis of amino acid and biomass. However, additional unknown pathways are likely presented in fungi (Jennings, 1995).

The word glycolysis describes the conversion of glucose to pyruvate (Moore, 1998). The most common glycolysis pathway is the Embden-Meyerhof-Parnas (EMP) pathway. It comprises nine enzymic steps. Through EMP pathway, one glucose is converted to two molecules of pyruvic acid and two molecules of Adenosine-triphosphate (ATP) and two molecules of nicotinamide-adenine dinucleotide (reduced) ($\text{NADH}_2$) (Moore, 1998). $\text{NADH}_2$ is an enzyme, and $\text{ATP} + \text{NADH}_2$ can produce energy.
The pentose Phosphate Pathway (PPP) also called the hexose monophosphate pathway (HMP). PPP provide pentose sugars for biosynthesis and nicotinamide-adenine dinucleotide phosphate (reduced) (NADPH2). NADPH2 is the coenzyme which is most often used in biosynthetic reactions that require reducing power, especially fat and oil synthesis. Comparing with the EMP pathway, that produces intermediate energy, PPP is more likely to be involved in furnishing biosynthetic intermediates (Moore, 1998).

In this model, the effects of the unknown pathways were assumed to be incorporated in a description of the glycolysis pathway. The rate of producing carbon components from these pathways depends on the concentration of glucose-6-Phosphate $C_g$. For the pentose pathway, pentose concentration $C_p$ depends on the production rate from G6p and the cost of producing carbon components. $C_c$ is concentration of carbon components.

$$\frac{dC_g}{dt} = V_c \frac{C_s}{C_s + Kn_1} \frac{Kn_3}{C_p + C_c} - V_g \frac{C_g}{C_g + Kg_1} - V_c \frac{C_g}{C_g + Kg_2}$$  \hspace{1cm} 6.10$$

where $V_g$, $V_c$ are the potential velocities of producing $C_p$ and $C_c$ from the glycolysis pathway respectively, and $Kg_1$ and $Kg_2$ are their Michaelis constants.

Pentose is produced from glucose-6-Phosphate and used to produce carbon components, and $V_p$ and $K_p$ are the potential velocities and their Michaelis constants, respectively.
\[
\frac{dC_p}{dt} = V_g \left( \frac{C_g}{C_g + K_g} - V_p \frac{C_p}{C_p + K_p} \right) \quad \text{(6.11)}
\]

\(C_c\) are produced by glucose metabolism pathways and are used to synthesise enzymes, amino acids and biomass. The glucose pathways can therefore be described by

\[
\frac{dC_c}{dt} = \frac{V_c C_g}{C_g + K_{g2}} + \frac{V_p C_p}{C_p + K_p} - f_{cv} - \frac{k_a (E_z + K_{Nm}) N_i}{(K_r + N_i)} \frac{C_c}{K_c + C_c} \quad \text{(6.12)}
\]

where \(f_c\) is the cost of carbon to produce a gram of fungal biomass.

### 6.3.3 The Organic Nitrogen Model

![Carbon and nitrogen co-metabolic network for glutamine used as nitrogen source](image)

Figure 6.2: Carbon and nitrogen co-metabolic network for glutamine used as nitrogen source

When amino acids are used as a nitrogen source, fungi can either utilise them as a nitrogen source and catabolise them on entry, or incorporate them directly into macromolecules.
Fungi also use amino acids as both nitrogen and carbon sources (Oso, 1975, Vylkova et al., 2011). In this model, the rate of uptake of amino acid was assumed to be dependent on the concentration of amino acid in the substrate Nos according to Michaelis-Menten kinetics. Internal amino acid is then directly used as an amino acid in biomass biosynthesis. The maximum rate and Michaelis constant of this process is

\[ V_a \] and \( K_a \) respectively. Stores of internal amino acid can also be catabolised as ammonium and a carbon source. In this model we assumed that the carbon source is g6p. The maximum rates of conversion of internal glutamine to ammonium and g6p are denoted by \( Vn_1 \) and \( Vn_2 \) respectively. \( K_{nc} \) is the Michaelis constant for internal gln decomposition into nitrogen and carbon. Equation 6.13 describes glutamine concentration in substrate. Equation 6.14, 6.15, and 6.16 describe internal concentration of glutamine, ammonium and glucose-6-phosphate respectively. Concentration of glucose in substrate, pentose, carbon component, mRNA, enzyme, combined amino acids and biomass production can be described by the same equation as their corresponding concentration in inorganic nitrogen network.

\[
\frac{dN}{dt} = -V_{o} \frac{N}{N + K} 
\]

\[
\frac{dN_{i}}{dt} = V_{o} \frac{N}{N + K} - (V_{n_{1}} + V_{n_{2}}) \frac{N_{i}}{K_{nc} + N_{i}} - V_{a} \frac{N}{N + K}
\]

\[
\frac{dN_{i}}{dt} = V_{n_{i}} \frac{N_{i}}{N_{i} + K_{nc}} + \frac{V_{n_{1}} N_{i}}{N_{i} + K_{nc}} - (1 - k_{c}) k_{3} N - k_{a_{2}} (1 - ka_{a}) \frac{(E_{z} + kn_{K_{m}}) N_{i}}{(K_{r} + N_{i})} \frac{C_{c}}{K_{c} + C_{c}}
\]
\[
\frac{dC_g}{dt} = \frac{V_{c_1}C_s}{C_s + Kn_1} \frac{Kn_3}{C_p + Cc} + \frac{V_{n_2}Noi}{Noi + Kn_2} - V_{g_6} \frac{C_g}{C_g + K_{g_1}} - V_c \frac{C_g}{C_g + K_{g_2}}
\]

As with the glucose and ammonium network, this network also ignores secondary metabolites.

6.4 Parameters and State Variables

The parameter values in table Table 6.1 are determined by searched for the best fit of equation 4.2 with consideration of the experimental results in Chapter 4. For simplicity, many metabolic steps in this model are either combined or neglected, so some parameter values may not reflect the biologically realistic values.

Table 6.1: Normal values of parameters and initial values of state variables.

<table>
<thead>
<tr>
<th>Parameter symbol</th>
<th>Definition</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( f_c )</td>
<td>Cost of carbon to produce a unit of fungal biomass</td>
<td>( \mu )mol g(^{-1} )</td>
<td>680</td>
</tr>
<tr>
<td>( f_{n} )</td>
<td>Cost of nitrogen to produce a unit of fungal biomass</td>
<td>( \mu )mol g(^{-1} )</td>
<td>180</td>
</tr>
<tr>
<td>( ka_1 )</td>
<td>Reducing power for producing unit of amino acid from ammonium</td>
<td>h(^{-1} )</td>
<td>0.19</td>
</tr>
<tr>
<td>( ka_2 )</td>
<td>Reducing power for producing unit of amino</td>
<td>h(^{-1} )</td>
<td>0.68</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Unit</td>
<td>Value</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------</td>
<td>--------</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Rate constant for combined mRNA synthesis</td>
<td>h⁻¹</td>
<td>0.035</td>
</tr>
<tr>
<td>$k_3$</td>
<td>Rate constant for combined ammonium assimilating enzyme synthesis</td>
<td>h⁻¹</td>
<td>2</td>
</tr>
<tr>
<td>$K_1$</td>
<td>Michaelis constant for amino acid determined growth biomass</td>
<td>µmol g⁻¹</td>
<td>90</td>
</tr>
<tr>
<td>$K_2$</td>
<td>Michaelis constant for carbon components determined growth biomass</td>
<td>µmol g⁻¹</td>
<td>42</td>
</tr>
<tr>
<td>$K_{dm}$</td>
<td>Michaelis constant for degradation combined mRNA</td>
<td>µmol g⁻¹</td>
<td>0.1</td>
</tr>
<tr>
<td>$K_{de}$</td>
<td>Michaelis constant for degradation combined ammonium assimilating enzyme</td>
<td>µmol g⁻¹</td>
<td>1.5</td>
</tr>
<tr>
<td>$K_r$</td>
<td>Michaelis constant for assimilating enzyme from Ni</td>
<td>µmol g⁻¹</td>
<td>5</td>
</tr>
<tr>
<td>$V_{dm}$</td>
<td>Maximum relative rate of degradation combined mRNA</td>
<td>µmol g⁻¹ h⁻¹</td>
<td>0.52</td>
</tr>
<tr>
<td>$V_g$</td>
<td>Maximum relative rate of fungal growth in biomass</td>
<td>µmol g⁻¹ h⁻¹</td>
<td>0.36</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Unit</td>
<td>Value</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>$V_{na}$</td>
<td>Maximum relative rate of uptaking ammonium</td>
<td>μmol g$^{-1}$ h$^{-1}$</td>
<td>2.8</td>
</tr>
<tr>
<td>$V_{de}$</td>
<td>Maximum relative rate of degradation ammonium assimilating enzyme</td>
<td>μmol g$^{-1}$ h$^{-1}$</td>
<td>5.8</td>
</tr>
<tr>
<td>$K_{na}$</td>
<td>Michaelis constant for uptaking ammonium</td>
<td>μmol g$^{-1}$</td>
<td>300</td>
</tr>
<tr>
<td>$V_{c_1}$</td>
<td>Maximum relative rate of uptaking glucose</td>
<td>μmol g$^{-1}$ h$^{-1}$</td>
<td>100</td>
</tr>
<tr>
<td>$V_{c}$</td>
<td>Maximum relative rate of producing carbon compounds from glucose apart from pentose pathway</td>
<td>μmol g$^{-1}$ h$^{-1}$</td>
<td>50</td>
</tr>
<tr>
<td>$K_{n_0}$</td>
<td>Michaelis constant for producing carbon compounds from glucose apart from pentose pathway</td>
<td>μmol g$^{-1}$</td>
<td>1600</td>
</tr>
<tr>
<td>$K_{n_1}$</td>
<td>Michaelis constant for uptaking glucose</td>
<td>μmol g$^{-1}$</td>
<td>2060</td>
</tr>
<tr>
<td>$K_{n_2}$</td>
<td>Activation constant for energy effect on biomass production</td>
<td>μmol g$^{-1}$</td>
<td>180</td>
</tr>
<tr>
<td>$K_{n_3}$</td>
<td>Constant of inhibition of uptake glucose from osmotic effect</td>
<td>μmol g$^{-1}$</td>
<td>50</td>
</tr>
<tr>
<td>$V_{g_6}$</td>
<td>Maximum relative rate to producing pentose</td>
<td>μmol g$^{-1}$ h$^{-1}$</td>
<td>7</td>
</tr>
<tr>
<td>$K_{g_1}$</td>
<td>Michaelis constant for producing pentose</td>
<td>μmol g$^{-1}$</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Unit</td>
<td>Value</td>
</tr>
<tr>
<td>-----</td>
<td>------------------------------------------------------------------------------</td>
<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>$K_p$</td>
<td>Michaelis constant for producing carbon compounds from pentose pathway</td>
<td>$\mu$mol g⁻¹</td>
<td>200</td>
</tr>
<tr>
<td>$k_c$</td>
<td>Reducing concentration of carbon components for producing unit of assimilating enzyme</td>
<td>h⁻¹</td>
<td>0.9</td>
</tr>
<tr>
<td>$K_{g_2}$</td>
<td>Michaelis constant rate of producing carbon compounds from glucose apart from pentose pathway</td>
<td>$\mu$mol g⁻¹</td>
<td>30</td>
</tr>
<tr>
<td>Organic nitrogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_o$</td>
<td>Maximum relative rate of uptaking glutamine</td>
<td>$\mu$mol g⁻¹ h⁻¹</td>
<td>8</td>
</tr>
<tr>
<td>$K_o$</td>
<td>Michaelis constant for uptaking glutamine</td>
<td>$\mu$mol g⁻¹</td>
<td>180</td>
</tr>
<tr>
<td>$K_{nc}$</td>
<td>Michaelis constant for internal gln decomposition to nitrogen and carbon source</td>
<td>$\mu$mol g⁻¹</td>
<td>50</td>
</tr>
<tr>
<td>$V_{n_1}$</td>
<td>Maximum relative rate of producing G6p from internal glutamine</td>
<td>$\mu$mol g⁻¹ h⁻¹</td>
<td>2.8</td>
</tr>
<tr>
<td>$V_{n_2}$</td>
<td>Maximum relative rate of producing ammonium from internal glutamine</td>
<td>$\mu$mol g⁻¹ h⁻¹</td>
<td>5.6</td>
</tr>
<tr>
<td>$V_a$</td>
<td>Maximum relative rate of gln directly used as amino acid for producing biomass</td>
<td>$\mu$mol g⁻¹ h⁻¹</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Ka</strong></td>
<td>Michaelis constant for gln directly used as amino acid for producing biomass</td>
<td>$\mu$mol g$^{-1}$</td>
<td>140</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Constant and variety initials</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ns</strong></td>
<td>Ammonium concentration in substrate</td>
<td>$\mu$mol g$^{-1}$</td>
<td>*</td>
</tr>
<tr>
<td><strong>Ni</strong></td>
<td>Ammonium concentration in mycelium</td>
<td>$\mu$mol g$^{-1}$</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Nm</strong></td>
<td>Concentration of combined mRNA concentration</td>
<td>$\mu$mol g$^{-1}$</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Ez</strong></td>
<td>Concentration of combined ammonium assimilating enzymes</td>
<td>$\mu$mol g$^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td><strong>Aa</strong></td>
<td>Concentration of combined amino acid for directly producing biomass</td>
<td>$\mu$mol g$^{-1}$</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Cs</strong></td>
<td>Carbon concentration in substrate</td>
<td>$\mu$mol g$^{-1}$</td>
<td>*</td>
</tr>
<tr>
<td><strong>Cg</strong></td>
<td>Carbon concentration in g6p</td>
<td>$\mu$mol g$^{-1}$</td>
<td>1</td>
</tr>
<tr>
<td><strong>Cp</strong></td>
<td>Carbon concentration in pentose</td>
<td>$\mu$mol g$^{-1}$</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Cc</strong></td>
<td>Concentration of carbon components</td>
<td>$\mu$mol g$^{-1}$</td>
<td>3</td>
</tr>
<tr>
<td>vb</td>
<td>Relative growth rate in biomass</td>
<td>h⁻¹</td>
<td>5.2</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Nos</td>
<td>Total carbon and nitrogen concentration from glutamine in substrate</td>
<td>µmol g⁻¹</td>
<td>*</td>
</tr>
<tr>
<td>Noi</td>
<td>Total carbon and nitrogen concentration from glutamine in mycelium</td>
<td>µmol g⁻¹</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*is nutrient concentration in the substrate which varies amongst treatments.

This model simplified many metabolic steps (such as carbon storage, downstream steps of mRNA and enzyme metabolism) are neglected, so some parameter values may not exactly reflect the biologically realistic values.

### 6.5 Sensitivity Analysis

By varying the values of individual parameters in the metabolic network model described above, the parameters that most sensitively affect the periodicity of biomass production were determined. Sensitivity analysis is applied to the inorganic nitrogen network model by increasing or decreasing the value of each individual parameter by 20% of its normal value, and the rest of parameter at normal value when the initial carbon to nitrogen ratio of 7:1. The predicted temporal patterns of biomass production from each parameter change were then fit to the same equation 4.2 (see Chapter 4) as the data to allow a direct comparison to results from the set of normal parameter values.

Significant oscillations were observed for all values of the parameters used in the sensitivity analyses. To quantify the statistical significance of the periodic behaviour, simulated data for
each treatment using either organic or inorganic nitrogen sources were fitted by equation 4.2 using GraphPad Prim 6.

Figure 6.3 shows the effect of parameter variation on amplitude and period of the oscillation in growth. It turns out that parameters related to uptake of both glucose and ammonium \((Vc_1, Vna, Kn_3)\), the rate of combined mRNA synthesis \((k_2)\), and the rate of production of pentose \((Vg_6)\) most strongly influence the oscillation in growth.

![Graph showing sensitivity of predicted period and amplitude of oscillations in growth to parameter variations](image)

Cost of carbon and nitrogen to produce a unit of fungal biomass respectively. \(Vg\) : Maximum relative rate of fungal growth in biomass. \(Vc_1\) : Maximum relative rate of uptaking glucose. \(Kn_3\) : Constant of slowing down of uptake glucose from osmotic effect. \(Vg_6\) : Maximum relative rate of g6p producing pentose. \(K_2\) : Michaelis constant for carbon components determined growth biomass. \(ka_2\) : Reducing power for producing unit of amino acid from carbon components.
6.6 Numerical Experiment

This experiment examined fungal growth in a nutritionally homogenous environment using both the inorganic and organic nitrogen network models. In each treatment, a range of initial values of carbon concentration in glucose $Cs$ and nitrogen concentration in either ammonium $Ns$ or glutamine $Nos$ were used. Values of the rest of variables were displayed in table 6.1 and remained the same among treatments.

For all the treatments, initial values of combined carbon concentration in glucose and nitrogen concentration are $8000 \, \mu\text{mol g}^{-1}$. The initial value of all the remaining constant variables and parameters are normal values displayed in Table 6.1. Fungal growth is calculated to 100 hours.

6.7 Statistics

The numerical solutions of the equations describing the metabolic network were carried out using Ode15s solver by Matlab R2011a. In the same way as described in Chapter 4, the statistical significance of the modeled periodic behavior we observed in our simulated data was quantified for each treatment corresponding to organic or inorganic nitrogen sources. The simulated data were fitted to equation 4.2 (see Chapter 4) using GraphPad Prim 6. The significance of the correlation between the ratio of carbon to nitrogen and the value of the parameters in equation 4.2 was tested using SPSS v20.
Figure 6.4: Simulated fungal growth corresponding to the case when ammonium is the nitrogen source. Graphs A to D show simulated fungal growth in different glucose carbon to nitrogen ratios with a total carbon and nitrogen concentrations at 8000 µmol g⁻¹.
Figure 6.5: Simulated fungal growth corresponding to the case when glutamine is the nitrogen source. Graphs A to D shows simulated fungal growth in different glucose carbon to nitrogen ratios with a total glucose and nitrogen in glutamine concentration at 8000 µmol g⁻¹.
Figure 6.6: Simulated fungal growth over a 100 hour time period in different ratios of glucose carbon to nitrogen. Observed data in each ratio was fitted into a harmonically modulated exponential curve as in Chapter 4 (equation 4.2). Abs b denotes the fitted amplitude of the oscillations in the simulated fungal growth. When ammonium is used as the nitrogen source, the predicted amplitude is positively correlated with glucose carbon to nitrogen ratio. In contrast, the predicted amplitudes of the oscillations are independent of the glucose carbon to nitrogen ratio when glutamine is used as the nitrogen source.
Figure 6.7: The simulated change in nutrient concentration with time, showing the decrease in the concentration of nutrients with time. Blue lines show the change of glucose carbon concentration in the substrate with time. The green lines show the change in the concentration of nitrogen in ammonium/gln in the substrate.

Figure 6.8: Predicted fresh fungal biomass in grams (y-axis) source at different times as a function of C: N ratio (x-axis) when ammonium is the nitrogen source. Graphs A to D show the predicted fungal biomass corresponding to ratios of carbon to nitrogen ranging from 0 to 100 at different times in culture. The graphs show the predicted increase in the apparent optimum growth rate with culture time.
Figure 6.9: Predicted fresh fungal biomass in grams (y-axis) at different times as a function of C: N ratio (x-axis) when glutamine is the nitrogen source. Graphs A to D show the predicted fungal biomass corresponding to ratios of carbon to nitrogen ranging from 0 to 100 at different times in culture. The graphs show an absent, or weak, apparent optimum value of the ratio at all culture times.

6.8 Simulation Results

The increase in fungal biomass over time for the cases where inorganic or organic sources of nitrogen are provided behaves as a harmonically modulated exponential with period of approximately 29 h (Figure 6.4, Figure 6.5). The resulting growth curves predicted by the model can be well fitted using equation 4.2 (see Chapter 4) with all $r^2$ values of more than 0.9 in all cases. There is no corresponding harmonic behaviour in the predicted uptake curves for either the nitrogen or carbon resources (Figure 6.7).

As shown in Figure 6.6, where the carbon to nitrogen ratio is between 0 and 10:1, the amplitude of the oscillation in the growth curve is significantly positively correlated with the C: N ratio (Pearson correlation $r=0.8$, $P=0.004$) but only when inorganic nitrogen is used as a nitrogen source. There is no significant corresponding trend when organic nitrogen is used as a source ($P>0.05$, Figure 6.6).
As shown in Figure 6.8, the range in the values of the C: N ratio that produce significant growth narrows over time when ammonium is used as the nitrogen source. By contrast, when glutamine is used as nitrogen source, the fungus is predicted to continue to grow well across a wide range of carbon in glucose to nitrogen ratios (from 0.3 to 20).

6.9 Discussion

The co-metabolism of carbon and nitrogen is a fundamental process in the growth of organisms. In this study, two versions of a mathematical model for fungal co-metabolism of carbon and nitrogen were developed. One is based on the case where inorganic nitrogen (ammonium) is the source of nitrogen, and the other where organic nitrogen (glutamine) is the source of nitrogen. Oscillatory growth is predicted by both models, and the amplitude of the oscillations is positively correlated with the C: N ratio only in the inorganic N version of the model.

Biochemical and biophysical rhythms are a common characteristic of living organisms (Tyson, 2010) and arise out of the non-linear feedbacks present in most metabolic and signalling networks operating across scales from the cell to the whole organism. Many mathematic models have successfully reproduced these rhythms (Tyson, 2010, Falconer et al., 2005).

Circadian oscillations in fungi have been known for more than half a century (Brandt, 1953). Metabolic oscillations in fungi has also been studied (Falconer et al., 2005, Tlalka et al., 2007) but with significantly less attention. The model here reproduced the results of the experiments described in Chapter 4. In particular, it reproduces the link between optimal growth rate and C: N ratio, the oscillatory growth behaviour, and the correlation between the amplitude of the oscillations and the C: N ratio, only for inorganic sources of N. For organic source N version of model, it reproduces no strong link between the amplitude of the oscillation and the C: N ratio. Both the experiments and the model are set in constant environmental conditions. In other
words, the observed and predicted oscillatory behaviours are endogenous. The oscillation in growth arises from specific feedbacks in metabolic network.

Though periodicity in biomass production appears across a wide range of nutrient conditions, the results from the model show there are no corresponding oscillations in the uptake of nutrient from the environment (Figure 6.7). This means that the total nutrient storage in the system also undergoes oscillation in anti-phase with the oscillation in growth.

The only difference between these two model versions occurs at the first step of organic nitrogen utilisation. Organic nitrogen can be used directly as an amino acid or it can be transformed to nitrogen plus a carbon source. It is consistent with the hypothesis (Chapter 5), that when fungi uptake amino acids, fungi may use these as both a nitrogen and a carbon source (Oso, 1975, Vylkova et al., 2011). The extra carbon source in glutamine also masked the optimal growth response to the ratio of carbon to nitrogen (Figure 6.9), which is consistent with the result observed in Chapter 2.

However, the curve of ‘biomass vs C: N ratio’ was flat in the early harvests as compared with the curves from later harvests (see Chapter 2 section 2.3.2 Figure 2.4. This model reproduces this phenomenon (Figure 6.8). It can be explained as the carbon and nitrogen conditions in the mycelium (Equation 6.7). Initial internal nutrient conditions are the same in all treatment (and given by the initial value in Table 6.1). This may explain the absent of optimal growth during early growth. Overtime, the internal nutrient conditions in different treatments changed differently, responding to the different nutrient concentrations in the substrate. The internal nutrient conditions may explain why the range of ratios producing growth close to the optimum narrowed over time while ammonium was the nitrogen source.
Chapter 7. Conclusions and Future Work

7.1 Summary

The overall aim of this thesis is to understand the influence of the nutritional composition of the environment on the fungal phenotype, and particularly the impact of different abundances and forms of carbon and nitrogen sources. We used both experimental and mathematical modelling approaches to deal with the resulting complexity. The first objective tested whether fungi conform to the geometry model for nutrition as described by (Raubenheimer and Simpson, 1993). This involved experiments to search for evidence of an optimal ratio of protein to carbohydrate corresponding to maximum growth rate, followed by experiments to discover whether the fungal phenotype emerged to optimize growth in heterotrophic environments. To help interpret the laboratory experiments, we extended a single-nutrient fungal network model (Hao et al., 2009) to include two-nutrient limitation. This model demonstrated the importance of internal nutrient dynamics, and our experimental results suggested the existence of internal regulatory apparatus in the co-metabolism of carbon and nitrogen. Therefore, we developed a systems biology model for the co-metabolism of carbon and nitrogen to improve on models for fungal growth and the dynamics of carbon and nitrogen in the environment.

*M. mucedo* is the test species. Early stages of this species have relatively simple structure and physiological processes, which can simplify the interpretation of the experimental results. First, in chapter 2, *M. mucedo* was grown in a homogenous environment. In addition to nutritional effects, the fungal phenotype can be impacted by pH and the osmotic potential of the environment. To ensure the results we observe are due to the fungal response to nutritional differences, we operated two pilot studies to choose a suitable buffer and nutrient
concentration. In the main experiment, fungal growth is compared using both inorganic and organic sources of nitrogen across a range of carbon in glucose to nitrogen ratios. When using an organic nitrogen source, the growth rates were similar across a broad range of carbon in glucose to nitrogen ratios. By contrast when the fungus was grown in an inorganic nitrogen source (ammonium) the growth rate was strongly dependent on the ratio of carbon to nitrogen. However, unlike the slime mould and other organisms that have been tested, the optimum ratio increased over time. This time dependence disappeared when the carbon to nitrogen ratio was changed with the concentration of the carbon source held constant. This behavior can be explained by translocation of nitrogen in the mycelium.

Following this part of the study, *M. mucedo* was grown in a heterogeneous environment. In different treatments, the fungus was presented with different pairs of resources with different carbon to nitrogen ratios. These results show the geometric model (Simpson et al., 2009) may be used to explain the growth of the fungus *M. mucedo* only when the fungus has access to media where the ratio of carbon to nitrogen can provide sufficient growth. The fungus behaves in a manner consistent with relatively high rates of nitrogen translocation, but relatively low rates of carbon translocation and so will proliferate only when carbon is locally available. In a heterogeneous environment, *M. mucedo* is not able to maximize growth under conditions of extreme contrast in carbon and nitrogen distribution. However, it showed the ability to recycle internal nitrogen and with that ability, the optimal carbon to nitrogen ratio for growth increased over time. The intake target in the geometric model is dynamic, when the physiological needs for nutrients change in different life stage. In the case here, the mechanism is internal recycling of nutrients, suggesting that growth of *M. mucedo* does not fully conform to the geometric model.
In Chapter 4, we conducted a time series analysis of fungal growth during the exponential growth phase under different nutrient conditions, and studied the observed oscillatory behaviour that resulted. We reconciled the exponential and oscillatory characteristics used to describe fungal growth dynamics under different nutrient environments. The result show that the period of the oscillations is independent of nutrition, while the amplitude is correlated with the carbon to nitrogen ratios in the substrate, but only when ammonium is used as the nitrogen source.

Following this work, we extended a single nutrient fungal model developed by Hao to a two nutrient model. The network in this model included the main physiological processes: hyphal growth, biomass consumption, hyphal dieback, uptake, biomass diffusion, and biomass recycling. The model predictions are consistent with *M. mucedo* having the ability to translocate nitrogen but not carbon. The results were sensitive to the dynamics of internal carbon and nitrogen that were described only heuristically in the model. In reality carbon and nitrogen are co-regulated by complex biochemical reactions during metabolism.

To explore the details of internal carbon and nitrogen regulation, we developed a model for the co-metabolism of carbon and nitrogen in chapter 6. Two versions of the model were built to represent inorganic (ammonium) and organic sources of nitrogen (glutamine). Glucose was assumed as the carbon source for both versions of the model. This model reproduced the observed oscillatory behaviour in growth. When ammonium is used as a nitrogen source, the model predicts that growth is positively correlated with C: N ratio. It also confirmed that the oscillations in growth can be endogenous. The results from this model confirm the observation that when glutamine is used as the nitrogen source, the fungus can grow well in a broad range
of carbon in glucose to nitrogen ratios. In the ammonium version of the model, optimal growth was observed in a narrow range of C: N ratios.

7.2 Further Work

This thesis covers a wide range of topics related to nutrition and the fungal phenotype, ranging from the molecular- to organism-scale. Each study opens up intriguing avenues for future research. The main opportunity is to test the generalization of these results using species other than *M. mucedo*. It was beyond the scope of the PhD to repeat the work for other species, but clearly the protocols and modelling approaches that have been developed in this work are now ready for application to other species. Even with *M. mucedo* as the chosen species, there are opportunities to undertake additional experiments and modelling to improve our understanding. Perhaps the most immediate priority is to develop experimental approaches capable of quantifying the nutrient uptake in the two-well experiment to confirm the extent to which the phenotype can compensate for physical and nutritional heterogeneity to maintain growth rate. Finally, the model for the co-metabolism of carbon and nitrogen can be applied to other organisms and other kingdom of life to test the extent to which the modelled processes are generic. These ideas are explored in more detail in the following sections.

7.1.1 Further Work Based on Other Fungal Species

The fungal kingdom has rich diversity. The outcomes of this research on *M. mucedo* may not apply to other fungi. *M. mucedo* readily grows in nutrient rich dung until the energy is depleted. Translocation through mycelium is considered as ability can assist fungi coping with poor nutrient condition (Boswell et al., 2003). The evolution of carbon translocation may provide an ecological advantage to organisms such as mycorrhizal fungi, which co-habit in both roots of
plants where carbon is abundant and in soil where carbon can be limiting. For some of other fungal groups such as mycorrhizal fungi, who co-habit in both roots of plants where carbon is abundant and in soil where carbon can be limiting, the evolution of carbon translocation can produce ecological advantage for organisms surviving in these environment. Therefore, fungi from other habitats should be tested following the experimental designs used in this study.

The structure and growth of the mycelium and is different across different fungal group. In this project we only extended the branching version of the single-nutrient model in Chapter 5. The physiological processes described in Chapter 5 could also include the process of anastomosis as represented in the version of single nutrient model developed by Hao (2009).

7.1.2 Further work based on Mucor mucedo

Firstly, in the laboratory experiments described in Chapters 2 and 3, we measured fungal growth in biomass. Future work can repeat the experiments in this project and measure the percentage of carbon and nitrogen components in biomass. Those data can help understand physiological responses to different carbon and nitrogen conditions. The data will also be useful for improving the models in chapter 5 and chapter 6. In addition, in this project we tested fungal nutrient translocation indirectly. Carbon and nitrogen translocation in M. mucedo should be test directly to confirm our conclusions.

The two-nutrient fungal model was restricted to describing two dimensional growth patterns, and for direct comparison with experiments this should be extended to three dimensions. The restriction is less important for modelling growth in soil, which is predominantly on the 2-D surface of particles. The current implementation of the model can accommodate this kind of growth by describing the growth surface appropriately, and indeed has been used in such a way
by a colleague in our group. In addition, new hyphae can only grow in every 60° growth direction interval because of the assumed hexagonal geometry of the array. This is only a limitation where significant curvature in growth is observed over scales of the order of the distance between the nodes in the array. Fungi have been observed to grow curve in either a left- or right handed direction, forming a spiral growth pattern in the mycelia (Trinci, 1984). Figure 7.3 shows such a ‘spiral’ growth of hyphae.

Figure 7.3: ‘Spiral’ growth of hyphae (Trinci et al., 1979)

Future fungal growth models that are implemented at the level of hyphae may need to consider simulating such growth of hyphae by setting more appropriate rules.

The metabolic network model in Chapter 6 used glucose and ammonium or glutamine as carbon and nitrogen sources respectively. Fungi can use other carbon and nitrogen sources, and so for future work, the model will have to be modified accordingly.
Carbon and nitrogen appear in a single formula in the two-nutrient fungal model in Chapter 5. A future model should try to link the metabolic and hyphal-level models together and explore the importance of translocation as a means of overcoming osmotic and other stresses that single-cell organisms may face. This would be an important precursor to applying the metabolic model to higher organisms including humans.

7.1.3 Generalisation of the Co-Metabolic Model to Protein and Carbohydrate Metabolism in Organisms from Other Kingdoms of Life

Carbon and nitrogen are probably the two most important components for heterotrophic organisms. Some of the metabolic pathways will have undergone lengthy and strong selection while life was still single-celled. They are conserved cross in all the kingdom of life. Therefore with certain modifications, the model developed in Chapter 6 may be applied to organisms from other kingdoms of life.
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Appendix 1 C++ Code for Fungal Growth Modelling

/*
* fungi.h
*/

#include <iostream>
#include <sstream>
#include <cstdlib>
#include <vector>
using namespace std;

//CF NF changing in each treatment
#define CF1 750
#define NF1 50
#define CF2 750
#define NF2 50

class Hypha;
class NofGraph;
  // class Graph;
  //class Environment;

struct Location
{
  int mX;
  int mY;
  bool mbInvalid;
  Location(bool bInvalid=false){mX=mY=0; mbInvalid = bInvalid;}
  Location(int x, int y, bool bInvalid=false){mX = x; mY = y; mbInvalid = bInvalid;}
  Location& operator=(Location const& that) {mX = that.mX; mY = that.mY; mbInvalid = that.mbInvalid;
    return *this;}
  bool operator==(Location const& that) {return ((mX == that.mX) && (mY == that.mY)); }
};
class Hypha
{
  private:
    static const int PEAK_RATIO = 10;
    bool mbTip;
    bool mbHyphea;
    bool mbFusion;
    bool mbSpore;
    double mCF;
    double mNF;
  public:
    double mIMB;
    double mCB;
    double mNB;
    double GetCF() { return (mLocation.mbInvalid?0.0:mCF);}
    void SetCF(double cf) {mCF = cf;}
    double GetNF() { return (mLocation.mbInvalid?0.0:mNF);}
}
void SetNF(double nf) {mNF = nf;}

Location mParent;
vector<Location> vecOffspr;
vector<Location> vecHadDifu;
Location mLocation;
vector<Location> vecNeighbor; //new for both kids+ parents
Hypha(){
    //mbNewtip =
    mbTip = mbHypha = mbFusion = mbSpore = false;
    mIMB = mCB = mNB = mCF = mNF = 0;
    mParent.mbInvalid = true;
}
Hypha(int x, int y){
    Init(x,y);
}
void Init(int x, int y);
void SetSpore(bool bSpore=true){mbSpore=bSpore;}
void Uptake(double EC, double EN);
void SetTip(bool bTip, bool bHypha);
void SetHypha (bool); // mbHypha=true);
double RealNB(){double realNB; return realNB=mNB/vecNeighbor.size();} 
double RealCB(){double realCB; return realCB=mCB/vecNeighbor.size();}
void SetNeighbors(Location N);
void Metabolized(double MN, double MC); 
void Recycling(double R1,double Rr1,double Rr2); 
bool Tip() const{ return mbTip; }
bool Hypha() const {return mbHypha; }
void SetParent(Location loc) {mParent = loc; }
void SetOffspr(Location loc)
    {if (loc==true)
        vecOffspr.clear();
    else
        vecOffspr.push_back(loc); }
bool Spore() const{ return mbSpore; }
void HypheaTip(double A, double B, double Ti); 
vector<Location> vecdifued;
}

class Graph{
p
protected:
    int hypha_num;
    vector<vector<Hypha>> mHyphas;
    vector<Location> vecgraph;
    int mMaxX;
    int mMaxY;
    double mTTL, mTTR;
    double mCL, mNL, mCR, mNR;
    //double gEC, gEN;

public:

    Graph(int size); 
    Hypha& Element(int x, int y);

    double GetTTL() { return mTTL; }
double GetTTR() { return mTTR; }
double GetmCL(){ return mCL; }
double GetmNL() { return mNL; }
double GetmCR(){ return mCR; }
double GetmNR(){ return mNR; }
void SetTTR() { mTTR=0; }
void SetTTL() { mTTL=0; }
void SetmMR() { mCL=0; mNL=0; }
void SetmML() { mCR=0; mNR=0; }
bool Graph::overboundary(int x, int y);
void Spore(int x, int y);
void Initfood();
vector<Location> RanLocation(vector<Location>);

//group of pure virtual functions start
virtual void Setm_imb(int i, int j, double bc, double bd) = 0;
virtual void Growth(int i, int j, double br1, double br2, double si, double sc, double sn, double db, double Ec, double En, double Mn, double Mc, double R1, double Rr1, double Rr2, double A, double B, double Ti) = 0;
virtual void Branch(int i, int j, int nBranches, double bc, double bd) = 0;
virtual void Dieback(int i, int j, double bc, double bn, double bd) = 0;
virtual void React(double BR1, double BR2, double si, double Sc, double Sn, double Dn, double Dc, double bc, double bn, double bd, double EC, double EN, double MN, double MC, double R1, double Rr1, double Rr2, double A, double B, double Ti) = 0;
virtual void Translocation(int i, int j, double bc, double bd) = 0;
virtual void Diffuse_food(double DIR);
Location& Direction(int i, int j);

void DrawGraph();
void DrawLineToParent(int x, int y);
vector<Location> GetAdjacentNodes(int i, int j);
bool IsAdjacent(Location const & loc1, Location const & loc2);
void WriteRecord(Hypha const & srcHypha, Hypha const & desHypha);
void WriteRecord(const stringstream& s);
void WriteRecord(double, double, double, double);
void Graph::WriteRecord(double TT);
void Graph::WriteRecord(double TTL);
void Graph::WriteRecord(double TTR);

};

class NofGraph : public Graph
{
public:
  NofGraph(int size):Graph(size) {}  
  ~NofGraph() {}  
  void Setm_imb(int i, int j, double bc, double bd) { }  
  void Growth(int i, int j, double br1, double br2, double si, double sc, double sn, double db, double Ec, double En, double Mn, double Mc, double R1, double Rr1, double Rr2, double A, double B, double Ti) { }  
  void Branch(int i, int j, int nBranches, double bc, double bd) { }  
  void Dieback(int i, int j, double bc, double bn, double bd) { }  
  void React(double BR1, double BR2, double si, double Sc, double Sn, double Dn, double Dc, double bc, double bn, double bd, double EC, double EN, double MN, double MC, double R1, double Rr1, double Rr2, double A, double B, double Ti) { }  
  vector<Location> vecNewGrewed;
class Environment {
    private:
        int num;
        NofGraph mGraph;//(num);
        int mnTick;
        double eTTL; double eTTR;
        double eCL; double eNL; double eCR; double eNR;
    public:
        Environment(int num);
        void IncTick() {mnTick++;}
        int GetTick() { return mnTick;}
        void Replay() {mnTick = 0;}
        void ShowGraph() {mGraph.DrawGraph();}
        void WriteRecord1(double);
        void WriteRecord(double);
        void WriteRecordtip(int tick, int i, int j, double, double, double);
        void Play();
        void WriteRecord(const stringstream& s);
};

//////////////////////////////////////////////////////////
#include <stdlib.h>
#ifdef __APPLE__
#include <GLUT/glut.h>
#else
#include <GL/glut.h>
#endif

#include <ctime>
#include <math.h>
#include <algorithm>
#include <fstream>
#include "fungi.h"

void Hypha::Init(int x, int y) {
    mLocation.mX = x;
    mLocation.mY = y;
    mbNewtip = mbTip = mbHyphea = mbFusion = mbSpore = false;
    mLIMB = mCB = mNB = mCF = mNF = 0;
    //mEC=mEN=0;
    mParent.mbInvalid = true;
}

void Hypha::Uptake(double EC, double EN) {
    //mCB/mCB
    if (mbTip) {//double inh=(mCB+1)/(mCB+2);
        mCB = mCB + EC*mCF;
    }
mNB = mNB + EN*mNF;
mCF = (1-EC)*mCF;
mNF = (1-EN)*mNF;

}
}

void Hypha::SetTip(bool bTip, bool bHyphea) {
    mbSpore = false;
    mbTip = bTip;
    mbHyphea = bHyphea;
}
void Hypha::SetNeighbors(Location N) {
    vecNeighbor.push_back(N);
}
void Hypha::SetHyphea(bool bHyphea) {
    //mbNewtip = false;
    mbTip = false;
    mbHyphea = bHyphea;
}
void Hypha::Metabolized(double MN, double MC) {
    mCB=mCB-MC*mCB;
    mNB=mNB-MN*mCB;
}
void Hypha::Recycling(double R1, double Rr1, double Rr2) {
    R1=1; Rr1=0.1;
    if(((mCB + mNB)/(mIMB) < R1)) {
        mCB = mCB + mIMB *Rr1;//Rr1/2;
        mNB = mNB + mIMB * Rr1*0.1;//Rr1/2;
        mIMB =mIMB*(1-1.1*Rr1);
    }
    else {
        Rr2=0.001;
        if((mNB-Rr2*mCB)>0)
            mIMB= mIMB+11*Rr2*mCB;//Rr2:2*Rr2*mNB
        mCB=mCB-10*Rr2*mCB;//Rr2*mNB
        mNB = mNB-Rr2*mCB;
    }
}
void Hypha::HypheaTip(double A, double B, double Ti) {
    if(mbHyphea==1&&(!mbTip)&&(mIMB>A)&&(mNB+mCB)>B)
mNB = mNB + Ti/11;
mCB = mCB + Ti*10/11;
mIMB = mIMB - Ti;
SetHyphea(false);
SetTip(true, true);
//note: changed order

//////////////// Class Graph functions ///////////////////////////
Graph::Graph(int size)
{
    //mTL1 = mTL2 = mTL3 = mTR1 = mTR2 = mTR3 = 0;
    mHyphas.resize(size);
    vecgraph.resize(size * size);
    for (int i = 0; i < size; i++)
    {
        mHyphas[i].resize(size);
    }
    mMaxX = mMaxY = size;
    for (int x = 0; x < size; x++)
    {
        for (int y = 0; y < size; y++)
        {
            mHyphas[x][y].Init(x, y);
            vecgraph[x * mMaxY + y] = Location(x, y);
        }
        Initfood();
    }
    //gEN = gEC = 0;
}

void Graph::Initfood()
{
    int i, j;
    for (i = 0; i < mMaxX; i++)
    {
        for (j = 0; j < mMaxY; j++)
        {
            if (i < mMaxX / 2)
            {
                mHyphas[i][j].SetCF(CF1);
                mHyphas[i][j].SetNF(NF1);
            }
            else if (i > mMaxX / 2)
            {
                mHyphas[i][j].SetCF(CF2);
                mHyphas[i][j].SetNF(NF2);
            }
            else
            {
                if (CF1 == 0 && CF2 == 0)
                {
                    double CF3 = (CF1 + CF2) / 2;
                    double NF3 = (NF1 + NF2) / 2;
                    mHyphas[i][j].SetCF(CF3);
                    mHyphas[i][j].SetNF(NF3);
                }
                else if (CF1 == 0)
                {
                    mHyphas[i][j].SetCF(642);
                }
                else if (CF2 == 0)
                {
                    mHyphas[i][j].SetCF(642);
                }
                else
                {
                    mHyphas[i][j].SetCF(642);
                    mHyphas[i][j].SetNF(642);
                }
            }
        }
    }
}
mHyphas[i][j].SetNF(157);
}
else if(CF2==0){
mHyphas[i][j].SetCF(642);
mHyphas[i][j].SetNF(157);
}

void Graph::Spore(int x, int y)
{
  mHyphas[x][y].SetSpore();
  //mHyphas[x][y].SetNewTip();
  mHyphas[x][y].SetTip(true, true);
  mHyphas[x][y].mCB = 100;//check?50
  mHyphas[x][y].mNB = 40;//check?10
  mHyphas[x][y].mIMB = 100;//check50
  mHyphas[x][y].mParent.mbInvalid = true; //initially no parent
}

Hypha& Graph::Element(int x, int y)
{
  Hypha aHypha;  //initialized as all zero
  if (x>=0 && x<mMaxX && y>=0 && y<mMaxY)
  {
    return (Hypha&) mHyphas[x][y];
  }
  else
  {
    aHypha.mLocation.mbInvalid = true;
  }
  return aHypha;
}

vector<Location> Graph::RanLocation(vector<Location>L){
  vector<Location> vecVisited;
  Location N;
  do
  {
    N=L[rand()%L.size()];
    if (find(vecVisited.begin(), vecVisited.end(), N)==vecVisited.end())
    {
      vecVisited.push_back(N);
    }
  }while(vecVisited.size()!=L.size());
  return vecVisited;
}

bool Graph::overboundary(int x, int y){
  if(x<0|| y<0||x>mMaxX||y>mMaxY){
    return true;
  }
  else{
    return false;
  }
}
```cpp
void Graph::Diffuse_food(double DIR) {
    // check neighbor?!
    //double tf[4];
    int nPivot = mMaxX/2 + (mMaxX%2 ? 0 : 0);
    int nOverBoundary = 0;
    for(int i=0; i<nPivot; i++)
    {
        for(int j=0; j<mMaxY; j++)
        {
            Element(i,j).GetCF() +
            DIR * ((overboundary(i-1,j))?0:Element(i-1,j).GetCF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetCF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetCF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetCF()) +
            ((overboundary(i+1,j-1))?0:Element(i+1,j-1).GetCF()) -
            (6-nOverBoundary)*Element(i,j).GetCF();
            Element(i,j).SetCF( )
            Element(i,j).GetNF() +
            DIR * ((overboundary(i-1,j))?0:Element(i-1,j).GetNF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetNF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetNF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetNF()) +
            ((overboundary(i+1,j-1))?0:Element(i+1,j-1).GetNF()) -
            (6-nOverBoundary)*Element(i,j).GetNF();
        }
    }
    for(int i=nPivot+1; i<mMaxX; i++)
    {
        for(int j=0; j<mMaxY; j++)
        {
            Element(i,j).GetCF() +
            DIR * ((overboundary(i-1,j))?0:Element(i-1,j).GetCF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetCF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetCF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetCF()) +
            ((overboundary(i+1,j-1))?0:Element(i+1,j-1).GetCF()) -
            (6-nOverBoundary)*Element(i,j).GetCF();
            Element(i,j).SetNF( )
            Element(i,j).GetNF() +
            DIR * ((overboundary(i-1,j))?0:Element(i-1,j).GetNF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetNF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetNF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetNF()) +
            ((overboundary(i+1,j-1))?0:Element(i+1,j-1).GetNF()) -
            (6-nOverBoundary)*Element(i,j).GetNF();
            //tf[1]=Element(i,j).GetNF();
        }
    }
const float point_width = 1.0;
```
const float sizeX = 10.0;
const float sizeY = sqrt(3.0)*sizeX/2.0;
const float originX = 10.0;
const float originY = 10.0;

bool operator==(const Location& lhs, const Location& rhs)
{
    return ((lhs.mX==rhs.mX) && (lhs.mY==rhs.mY));
}

bool Graph::IsAdjacent(Location const& loc1, Location const& loc2)
{
    bool ok = false;
    vector<Location> vecLocations = GetAdjacentNodes(loc1.mX, loc1.mY);
    vector<Location>::iterator it = find(vecLocations.begin(), vecLocations.end(), loc2);
    if (it!=vecLocations.end())
    {
        ok = true;
    }
    return ok;
}

void Graph::DrawLineToParent(int x, int y)
{
    float cordX = originX + x*sizeX; //shifted half length
    float cordY = originY + y*sizeY;
    if (Element(x,y).Hyphea())
    {
        //link to one of these (x-1, y), (x, y-1), (x+1, y-1), (x+1, y), (x, y+1), (x+1, y+1)
        Location locParent = Element(x,y).mParent;
        if (IsAdjacent(Location(x,y), locParent))
        {
            glBegin(GL_LINES);
            glVertex2f(cordX, cordY);
            float parentX = originX + locParent.mX*sizeX; //shifted half length
            float parentY = originY + locParent.mY*sizeY;
            glVertex2f(parentX, parentY);
            glEnd();
        }
    }
}

void Graph::DrawGraph()
{
    for(int x=0; x<mMaxX; x++)
    {
        float cordX = originX + x*sizeX;
        float newCordX = cordX;
        for(int y=0; y<mMaxY; y++)
        {
            float cordY = originY + y*sizeY;
            newCordX = (y%2==0)? cordX:(cordX + sizeX/2); //shifted half length
            glColor3f(0.0, 0.0, 0.0); //new
            glBegin(GL_QUADS);
            glVertex2f(newCordX-point_width/2, cordY-point_width/2);
            glVertex2f(newCordX+point_width/2, cordY-point_width/2);
            glEnd();
        }
    }
}
glVertex2f(newCordX+point_width/2, cordY+point_width/2);
glVertex2f(newCordX-point_width/2, cordY+point_width/2);
gEnd();

DrawLineToParent(x, y);

/*
   glBegin(GL_LINES);
   double lx=originX+(mMaxX /2*10);
   double ly=originY+mMaxY *17;
   glVertex2f(lx,originY);
   glVertex2f(lx, ly);
   glEnd();*/
}
}
}

vector<Location> Graph::GetAdjacentNodes(int i, int j)
{
    vector<Location> vecLocations;
    vecLocations.push_back(Location(i, j-1));
    vecLocations.push_back(Location(i-1, j));
    vecLocations.push_back(Location(i, j+1));
    vecLocations.push_back(Location(i+1, j));
    if (j%2==1)
    {
        vecLocations.push_back(Location(i+1, j-1));
        vecLocations.push_back(Location(i+1, j+1));
    }
    else
    {
        vecLocations.push_back(Location(i-1, j-1));
        vecLocations.push_back(Location(i-1, j+1));
    }
    return vecLocations;
}

Location& Graph::Direction(int i, int j)
{
    Location loc;
    vector<Location> vecLocations = GetAdjacentNodes(i, j);
    for (vector<Location>::iterator it = vecLocations.begin(); it!=vecLocations.end();)
    {
        if (Element(it->mX, it->mY).mLocation.mbInvalid|| Element(it->mX, it->mY).Hyphea())
        {
            //invalid or already hyphea, remove this location from list
            it = vecLocations.erase(it);
        }
        else
        {
            ++it;  //move to next item
        }
    }
}
if (!vecLocations.empty())  //now randomly select one from the rest
{
    loc = vecLocations[rand()%vecLocations.size()];
}
else  //return an invalid location
{
    loc.mbInvalid = true;
}
return loc;

const string RecordFileName = "biomass.txt";

void Graph::WriteRecord(const stringstream& ss)
{
    ofstream ofile(RecordFileName.c_str(), ios::out | ios::app);
    ofile << ss.str().c_str() << endl;
    ofile.close();
}

void Graph::WriteRecord(Hypha const& srcHypha, Hypha const& desHypha)
{
    ofstream ofile(RecordFileName.c_str(), ios::out | ios::app);
    ofile << "From Hypa, i=" << srcHypha.mLocation.mX << ", j=" << srcHypha.mLocation.mY << ", CB:" << srcHypha.mCB << " to: i=" << desHypha.mLocation.mX << ", j=" << desHypha.mLocation.mY << ", CB:" << desHypha.mCB << endl;
    ofile.close();
}

void Graph::WriteRecord(double mCL, double mNL, double mCR, double mNR)
{
    ofstream ofile(RecordFileName.c_str(), ios::out | ios::app);
    ofile << " " << mCL << " " << mNL << " " << mCR << " " << mNR << endl;
    ofile.close();
}

const string RecordFileName3 = "Tottle IMB.txt";

void Graph::WriteRecord(double TT)
{
    ofstream ofile(RecordFileName3.c_str(), ios::out | ios::app);
    ofile << TT << endl;
    ofile.close();
}

const string RecordFileName4 = "Tottle ImbL.txt";

void Graph::WriteRecordr(double TTL)
{
    ofstream ofile(RecordFileName4.c_str(), ios::out | ios::app);
    ofile << TTL << endl;
    ofile.close();
}

const string RecordFileName5 = "Tottle ImbR.txt";

void Graph::WriteRecordr(double TTR)
void NofGraph::Translocation(int i, int j, double Dn, double Dc)
{
    for (vector<Location>::iterator it = Element(i,j).vecNeighbor.begin(); it!=Element(i,j).vecNeighbor.end(); it++) {
        if (find(Element(it->mX, it->mY).vecdifued.begin(), Element(it->mX, it->mY).vecdifued.end(), Location(i,j)) == Element(it->mX, it->mY).vecdifued.end()) {
            int m = it->mX; int n = it->mY;
            Element(m, n).mCB = Element(m, n).mCB + (Element(i,j).mCB - Element(m, n).mCB) * Dc;
            Element(i,j).mCB = Element(i,j).mCB - (Element(i,j).mCB - Element(m, n).mCB) * Dc;
            Element(m, n).mNB = Element(m, n).mNB + (Element(i,j).mNB - Element(m, n).mNB) * Dn;
            Element(i,j).mNB = Element(i,j).mNB - (Element(i,j).mNB - Element(m, n).mNB) * Dn;
            Element(i,j).vecdifued.push_back(Location(m, n));
        }
    }
}

void NofGraph::React(double BR1, double BR2, double si, double Sc, double Sn, double Dn, double Dc, double bc, double bn, double bd, double EC, double EN, double MN, double MC, double R1, double Rr1, double Rr2, double A, double B, double Ti)
{
    vector<Location> myLocation = RanLocation(vecgraph);

    for (vector<Location>::iterator it = myLocation.begin(); it!=myLocation.end(); it++) {
        if (Element(it->mX, it->mY).Tip() && (!Element(it->mX, it->mY).mLocation.mbInvalid)) {
            if (find(vecNewGrowed.begin(), vecNewGrowed.end(), Location(it->mX, it->mY)) == vecNewGrowed.end()) {
                Growth(it->mX, it->mY, BR1, BR2, si, Sc, Sn);
            }
        }
    }

    for (vector<Location>::iterator it = myLocation.begin(); it!=myLocation.end(); it++) {
        if (Element(it->mX, it->mY).Hyphea()) {
            Element(it->mX, it->mY).Uptake( EC, EN);
        }
    }

    //Diffuse_food( DIR);
    for (vector<Location>::iterator it = myLocation.begin(); it!=myLocation.end(); it++) {
    
    
}
if( Element(it->mX,it->mY).Hyphea()) {
    Translocation(it->mX,it->mY, Dn,Dc);
}
for (vector<Location>::iterator it = myLocation.begin();it!=myLocation.end();it++) {
    if( Element(it->mX,it->mY).Hyphea()) {
        Element(it->mX,it->mY).Metabolized(MN, MC);
    }
}
for (vector<Location>::iterator it = myLocation.begin();it!=myLocation.end();it++) {
    if( Element(it->mX,it->mY).Hyphea()) {
        Element(it->mX,it->mY).Recycling(R1, Rr1, Rr2);
    }
}
for (vector<Location>::iterator it = myLocation.begin();it!=myLocation.end();it++) {
    if( find(vecNewGrowed.begin(), vecNewGrowed.end(), Location(it->mX,it->mY)) == vecNewGrowed.end()) {
        if( Element(it->mX,it->mY).Hyphea()) {
            Element(it->mX,it->mY).HypheaTip( A, B, Ti);
        }
    }
}
vecNewGrowed.clear();
myLocation.clear();
//biomass caculation
int i, j;
for( i = 0; i<mMaxX; i++) {
    for( j=0; j <mMaxY; j++) {
        if( (i>(mMaxX/2-1) && Element(i,j).Hyphea()==1) {
            mTTL= mTTL+Element(i,j).mIMB;
            mCL=mCL+Element(i,j).mCB;
            mNL=mNL+Element(i,j).mNB;
        } else if ( i<(mMaxX/2+1) && Element(i,j).Hyphea()==1) {
            mTTR= mTTR+Element(i,j).mIMB;
            mCR=mCR+Element(i,j).mCB;
            mNR=mNR+Element(i,j).mNB;
        }
    }
    Element(i,j).vecdifued.clear();
}
WriteRecord( mCL,mNL, mCR,mNR);
WriteRecord(mTTL+mTTR);
WriteRecordr(mTTR);
WriteRecordl(mTTL);
//WriteRecordE( gEC, gEN);
}

void NofGraph::Dieback(int i, int j, double bc, double bn, double bd) {
if( Element(i,j).Hyphea()){

if(!(Element(i,j).Tip()==0) {
   if ((Element(i,j).mCB < 0) || (Element(i,j).mNB < 0 || Element(i,j).mIMB<bd))
   {//if ((Element(i,j).mIMB<DI&&(!Element(i,j).Tip())))

   //Element(i,j).SetNewTip(false);
   Element(i,j).SetTip(false,false);
   Element(i,j).SetHyphea(false); //change length;
   Element(i,j).SetParent(Location(true));
   Element(i,j).SetOffspr(Location(true));
   }
   }
   }
}

void NofGraph::Branch(int i, int j, int nBranches,double si, double Sc,double Sn) {
if (nBranches > 0) {
   Location loc(i,j);
   Location locNext = Direction(i, j);
   //WriteRecord(ss);
   
   if (!locNext.mbInvalid) //there is a valid branch
   {
       // WriteRecord( Element(i,j), Element(locNext.mX,locNext.mY) );
       --nBranches;
       if (!Element(i,j).mParent == locNext) && !(Element(locNext.mX, locNext.mY).mLocation==Location(i,j))
       {
       //Element(locNext.mX, locNext.mY).SetNewTip();
       Element(locNext.mX,locNext.mY).SetTip(true,true);
       vecNewGrowed.push_back(locNext);
       Element(locNext.mX, locNext.mY).SetParent(Location(i,j));
       Element(locNext.mX, locNext.mY).SetNeighbors(loc);
       Element(i,j).SetNeighbors(Location(locNext.mX, locNext.mY));

       Setm_imb(locNext.mX,locNext.mY, si, Sc, Sn);
       }
   if (nBranches>0) //branch again
   {
      Branch(i, j, nBranches,si, Sc, Sn);//recall Branch function with one less nBranches
   }
   }Element(i, j).SetHyphea(true);
}
void NofGraph::Setm_imb(int i, int j, double si, double Sc, double Sn)
{
    if (!Element(i, j).mParent.mbInvalid)
    {
        Element(i, j).mIMB = si*Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mIMB;
        Element(i, j).mCB = Sc*Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mCB;
        Element(i, j).mNB = Sn*Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mNB;
    double m=Element(i, j).mCB;
    // Element(i, j).mParent.mIMB = 1.1;
    Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mNB = Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mNB *(1 - Sn);
    Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mCB = Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mCB *(1 - Sc);
    Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mIMB = Element(Element(i, j).mParent.mX, Element(i, j).mParent.mY).mIMB *(1 - si);
    }
}

void NofGraph::Growth(int i, int j, double BR1, double BR2, double si, double Sc, double Sn)
{
    double BRN1 = BR2*0.1; double BRN = BR1*0.1;

    if(Element(i, j).Tip() && Element(i, j).mCB > BR2 && Element(i, j).mNB > BRN1)
    { Branch(i, j, 2, si, Sc, Sn); }
    else if(Element(i, j).Tip() && Element(i, j).mCB > BR1 && Element(i, j).mNB > BRN)
    { Branch(i, j, 1, si, Sc, Sn); }
}

//////////////////////////////// Class Environment functions ///////////////////////////
Environment::Environment(int num) : mGraph(num)
{
    srand(time(0));
    mGraph.Spore(num*0.5, num*0.5);
    //mGraph.Spore(75,75);
    mnTick = 0;
}
const string RecordFileName = "Tottle eTTL2.txt";
void Environment::WriteRecord1(double eTTL)
{
    ofstream ofile(RecordFileName.c_str(), ios::out | ios::app);
    //ofile << "At Tick ="<<tick<<"Total biomass in left side is:" << eTTL << "Total biomass in right side is:" << eTTR << endl;
    ofile << eTTL << endl;
    ofile.close();
}

const string RecordFileName2 = "Tottle eTTR2.txt";
void Environment::WriteRecord(double eTTR)
{
    ofstream ofile(RecordFileName2.c_str(), ios::out | ios::app);
    //ofile << "At Tick ="<<tick<<"Total biomass in left side is:" << eTTL << "Total biomass in right side is:" << eTTR << endl;
    ofile << eTTR << endl;
}
const string RecordFileNameT = "Tip.txt";
void Environment::WriteRecordtip(int tick, int i, int j, double CB, double NB, double imb)
{
    ofstream ofile(RecordFileNameT.c_str(), ios::out | ios::app);
    ofile << "At Tick =" << tick << " tip =" << i << " j =" << j << " CB =" << CB << " NB =" << NB << " imb =" << imb << endl;
    ofile.close();
}

void Environment::Play()
{
    double EC=0.015; double EN=EC;
    double MN=0.001; double MC=0.01; double R1=0.2;
    double Rr1=0.2;
    double Rr2=0.2; double A=2; double B=5; double Ti=1;
    double Dn=0.5; double Dc=0;
    double bc=0; double bn=0; double bd=0.1;
    double Sc=0.1; double Sn=0.1;
    double BR1=8; double BR2=12; double si=0.2;
    double Para[21]={BR1,BR2,si, Sc, Sn, Dn, Dc, bc, bn, bd, EC, EN, MN, MC, R1, Rr1, Rr2, A, B, Ti};

    //vector<double> VecPara; VecPara.push_back(mEC);
    // Enumerate all the hypha
    // Reset the environment variables
    // Tick the time counter
    /* Step 1 */
    /* Step 2 */
    mGraph.React(BR1,BR2,si, Sc, Sn, Dn, Dc, bc, bn, bd, EC, EN, MN, MC, R1, Rr1, Rr2, A, B, Ti);
    /* Step 4 */
    IncTick();
    int tick=GetTick();
    eTTL = mGraph.GetTTL(); // mGraph.SetTL3(1.0);
    eTTR = mGraph.GetTTR();
    eCL = mGraph.GetmCL(); eNL = mGraph.GetmNL(); eCR = mGraph.GetmCR(); eNR = mGraph.GetmNR();
    WriteRecord1(eCL+eNL);
    WriteRecord(eNR+eCR);
    for (int i = 0; i < 50; i++)
    {
        for (int j= 0 : j<50; j++)
        {
            if(mGraph.Element(i,j).Tip())
            {
                WriteRecordtip(tick, i,j,mGraph.Element(i,j).mCB, mGraph.Element(i,j).mNB, mGraph.Element(i,j).mIMB);
            }
        }
    }
    //WriteRecord(tick, eCL, eNL, eCR, eNR);
mGraph.SetTTL();
mGraph.SetTTR();
mGraph.SetmML();
mGraph.SetmMR();

/* Step 5 */

// ExecuteInfo ret_info(mTick, 0, EXECUTE_SUCCESS);

// return ret_info;
}

////////////// Main ///////////////////////////////////

#include <stdlib.h>

#ifdef __APPLE__
#include <GLUT/glut.h>
#else
#include <GL/glut.h>
#endif
#include <iostream>
#include "fungi.h"

/* Change your grid size here */
Environment env(100);
int addtrick;
int submenu_id;

void display(void)
{
    glClearColor(GL_COLOR_BUFFER_BIT | GL_DEPTH_BUFFER_BIT);
    env.ShowGraph();
    glutSwapBuffers();
}

void reshape(int width, int height)
{
    glViewport(0, 0, width, height);

    /* Project the coordinator */
    glMatrixMode(GL_PROJECTION);
    glLoadIdentity();
    gluOrtho2D(0, width, 0, height);
    glMatrixMode(GL_MODELVIEW);
}

void idle(void)
{
    glutPostRedisplay();
}

void menu(int value){
    if(value == 0){
        glutDestroyWindow(addtrick);
        exit(0);
    } else {
        for(int i=0;i<value;i++) {
            env.Play();
        }
    }
}
static
}
}
glutPostRedisplay();
}
}
void createMenu(){
    /* Now create just a very simple menu here */
    submenu_id = glutCreateMenu(menu);
    glutAddMenuEntry("Exit",0);
    glutAddMenuEntry("One More Step",1);
    glutAddMenuEntry("Steps ...",90);
    glutAttachMenu(GLUT_RIGHT_BUTTON);
}
void setup() {
    glClearColor(1.0f, 1.0f, 1.0f, 1.0f);
}
int main(int argc, char** argv)
{
    glutInit(&argc, argv);
    glutInitDisplayMode(GLUT_RGBA | GLUT_DOUBLE | GLUT_DEPTH);
    glutInitWindowSize(1000,1000);
    glutCreateWindow("GLUT Program");
    glutDisplayFunc(display);
    setup();
    glutReshapeFunc(reshape);
    glutIdleFunc(idle);
    //InitializeParams(argc, argv);
    createMenu();
    glutMainLoop();
    return EXIT_SUCCESS;
}
Appendix 2 C++ Code for Using Galib Searching for Parameters’ Value in Fungal Growth Modelling

/*
 * fungi.h
 */
#include <iostream>
#include <sstream>
#include <cstdlib>
#include <vector>
using namespace std;
#define CF1 750
#define NF1 50
#define CF2 750
#define NF2 50

class Hypha;
class NofGraph;
// class Graph;
//class Environment;

struct Location
{
    int mX;
    int mY;
    bool mbInvalid;
    Location(bool bInvalid=false) {mX=mY=0; mbInvalid = bInvalid;}
    Location(int x, int y, bool bInvalid=false) {mX = x; mY = y; mbInvalid = bInvalid;}
    Location& operator=(Location const& that) {mX = that.mX; mY = that.mY; mbInvalid = that.mbInvalid;
    return *this;}
    bool operator==(Location const& that) { return ((mX == that.mX) && (mY == that.mY)); }
};
class Hypha
{
private:
    static const int PEAK_RATIO = 10;
    bool mbTip;
    bool mbHyphea;
    bool mbFusion;
    bool mbSpore;
    double mCF;
    double mNF;
public:
    double mIMB;
    double mCB;
    double mNB;
double GetCF() { return (mLocation.mbInvalid?0.0:mCF); }
void SetCF(double cf) { mCF = cf; }

double GetNF() { return (mLocation.mbInvalid?0.0:mNF); }
void SetNF(double nf) { mNF = nf; }

Location mParent;
vector<Location> vecOffspr;
vector<Location> vecHadDifu;
Location mLocation;
vector<Location> vecNeighbor;//new for both kids+ parents

Hypha(){
  //mbNewtip =
  mbTip = mbHypha = mbFusion = mbSpore = false;
  mIMB = mCB = mNB = mCF = mNF = 0;
  mParent.mbInvalid = true;
}
Hypha(int x, int y){
  Init(x,y);
}

void Init(int x, int y);
void SetSpore(bool bSpore=true){mbSpore=bSpore;}
void Uptake(double EC, double EN);
void SetTip(bool bTip, bool bHypha);
void SetHypha(bool bHypha):// mbHypha=true);
double RealNB(){ double realNB; return realNB=mNB/vecNeighbor.size();} 
double RealCB(){ double realCB; return realCB=mCB/vecNeighbor.size();}

void SetNeighbors(Location N);
void Metabolized(double MN, double MC);
void Recycling(double R1, double Rr1, double Rr2);
bool Tip() const{return mbTip; }
bool Hypha() const{return mbHypha; }
void SetParent(Location loc) {mParent = loc; }
void SetOffspr(Location loc)
  {if (loc==true)
     vecOffspr.clear();
   else
     vecOffspr.push_back(loc);}
bool Spore() const{return mbSpore; }
void HyphaTip(double A, double B, double Ti);
  vector<Location> vecdifued;
};

class Graph
{
 protected:
  int hypha_num;
  vector<vector<Hypha>> mHyphas;
  vector<Location> vecgraph;
  int mMaxX;
  int mMaxY;
  double mTTL, mTTR;
  double mCL, mNL, mCR, mNR;
  //double gEC, gEN;
 public:

Graph(int size);
Hypha& Element(int x, int y);

double GetTTL() { return mTTL; }
double GetTTR() { return mTTR; }
double GetmCL() { return mCL; }
double GetmNL() { return mNL; }
double GetmCR() { return mCR; }
double GetmNR() { return mNR; }
void SetTTR() { mTTR = 0; }
void SetTTL() { mTTL = 0; }
void SetmMR() { mCL = 0; mNL = 0; }
void SetmML() { mCR = 0; mNR = 0; }

bool Graph::overboundary(int x, int y);

void Spore(int x, int y);
void Initfood();

vector<Location> RanLocation(vector<Location>);

// group of pure virtual functions start
virtual void Setm_imb(int i, int j, double, double, double) = 0;
virtual void Growth(int i, int j, double, double, double, double, double, double) = 0;
virtual void Branch(int i, int j, int nBranches, double, double, double, double) = 0;
virtual void Dieback(int i, int j, double bc, double bn, double bd) = 0;
virtual void React(double BR1, double BR2, double si, double Sc, double Sn, double Dn, double Dc, double bc, double bn, double bd, double EC, double EN, double MN, double MC, double R1, double Rr1, double Rr2, double A, double B, double Ti) = 0;
virtual void Translocation(int i, int j, double, double) = 0;

virtual void Diffuse_food(double DIR);
Location& Direction(int i, int j);

void DrawGraph();
void DrawLineToParent(int x, int y);

vector<Location> GetAdjacentNodes(int i, int j);
bool IsAdjacent(Location const& loc1, Location const& loc2);
void WriteRecord(Hypha const& srcHypha, Hypha const& desHypha);
void WriteRecord(const stringstream& s);
void WriteRecord(double, double, double, double);
void Graph::WriteRecord(double TT);
void Graph::WriteRecord(double TTL);
void Graph::WriteRecordr(double TTR);

};

class NofGraph : public Graph
{
public:
    NofGraph(int size): Graph(size) { }
    ~NofGraph() {} 
    void Setm_imb(int i, int j, double, double, double);
    void Growth(int i, int j, double, double, double, double, double);
    void Branch(int i, int j, int nBranches, double, double, double);
    void Translocation(int i, int j, double, double);
    void Dieback(int i, int j, double, double, double);
void React(double BR1, double BR2, double si, double Sc, double Sn, double Dn, double Dc, double bc, double bn, double bd, double EC, double EN, double MN, double MC, double R1, double Rr1, double Rr2, double A, double B, double Ti);
    vector<Location> vecNewGrewed;
};

class Environment
{
    private:
        int num;
        NofGraph mGraph://(num);
        int mnTick;
        double eTTL; double eTTR;
        double eCL; double eNL; double eCR; double eNR;
    public:
        Environment(int num);
        void IncTick() {mnTick++;}
        int GetTick() {return mnTick;}
        void Replay() {mnTick = 0;}
        void ShowGraph() {mGraph.DrawGraph();}
        void WriteRecord1(double);
        void WriteRecord(double);
        void WriteRecordtip(int tick, int i, int j, double, double, double);
        void Play();
        void WriteRecord(const stringstream& s);
};

#include <stdlib.h>
#ifdef __APPLE__
#include <GLUT/glut.h>
#else
#include <GL/glut.h>
#endif
#include <ga.h>
#include <ctime>
#include <math.h>
#include <algorithm>
#include <fstream>
#include "fungi.h"

void Hypha::Init(int x, int y)
{
    mLocation.mX = x;
    mLocation.mY = y;
    //mbNewtip =
    mbTip = mbHyphae = mbFusion = mbSpore = false;
    mIMB = mCB = mNB = mCF = mNF = 0;
    //mEC=mEN=0;
    mParent.mbInvalid = true;
}

void Hypha::Uptake(double EC, double EN)
{  //mCB/(mCB
if (mbTip) //double inh=(mCB+1)/(mCB+2):
    mCB = mCB + EC*mCF;
    mNB = mNB + EN*mNF;
    mCF = (1-EC)*mCF;
    mNF = (1-EN)*mNF;

void Hypha::SetTip(bool bTip,bool bHyphea)
{
    mbSpore = false;
    mbTip = bTip;
    mbHyphea = bHyphea;
}
void Hypha::SetNeighbors(Location N)
{
    vecNeighbor.push_back(N);
}
void Hypha::SetHyphea(bool bHyphea)
{
    //mbNewtip = false;
    mbTip = false;
    mbHyphea = bHyphea;
}
void Hypha::Metabolized(double MN, double MC)
{
    mCB=mCB-MC*mCB;
    mNB=mNB-MN*mCB;
}
void Hypha::Recycling(double R1, double Rr1, double Rr2)
{  R1=1; Rr1=0.1;
    if((mCB + mNB)/(mIMB) < R1)
    {
        mCB = mCB + mIMB *Rr1; //Rr1/2;
        mNB = mNB + mIMB * Rr1*0.1; //Rr1/2;
        mIMB =mIMB*(1-1.1*Rr1);
    }
    else{ Rr2=0.001;
        if((mNB-Rr2*mCB)>0)
           mIMB= mIMB+11*Rr2*mCB; //Rr2:2*Rr2*mNB
        mCB=mCB-10*Rr2*mCB; //Rr2*mNB
        mNB = mNB-Rr2*mCB;
    }
void Hypha::HypheaTip(double A, double B, double Ti)
{
    if(mbHyphea==1&&(!mbTip)&&(mIMB>A)&&(mNB+mCB)>B)
    {
        mNB =mNB+ Ti/11;
        mCB =mCB + Ti*10/11;
        mIMB = mIMB-Ti;
        SetHyphea(false);
        SetTip(true,true);
    }  //note: changed order
}

/////////////// Class Graph functions ///////////////////////////
Graph::Graph(int size)
{
    //mTL1 = mTL2 = mTL3 = mTR1 = mTR2 = mTR3 = 0;
    mHyphas.resize(size);
    vecgraph.resize(size*size);
    for (int i=0; i< size; i++)
    {
        mHyphas[i].resize(size);
    }
    mMaxX = mMaxY = size;
    for (int x=0;x< size;x++)
    {
        for (int y = 0; y< size ; y++)
        {
            mHyphas[x][y].Init(x, y);
            vecgraph[x*mMaxY+y]= Location(x,y);
            Initfood();
        }
    }  //gEN=gEC=0;
}

void Graph::Initfood()
{
    int i, j;
    for( i = 0; i< mMaxX; i++){
        for (j=0; j < mMaxY; j++){
            if (i< mMaxX/2 ){
                mHyphas[i][j].SetCF(CF1);
                mHyphas[i][j].SetNF(NF1);
            }
            else if (i> mMaxX/2){
                mHyphas[i][j].SetCF(CF2);
                mHyphas[i][j].SetNF(NF2);
            }
            else{
                if(CF1!=0||CF2!=0){
                    double CF3=(CF1+CF2)/2;
                }
            }
        }
    }
}
double NF3 = (NF1 + NF2) / 2;
mHyphas[i][j].SetCF(CF3);
mHyphas[i][j].SetNF(NF3);
else if (CF1 == 0) {
mHyphas[i][j].SetCF(642);
mHyphas[i][j].SetNF(157);
} else if (CF2 == 0) {
mHyphas[i][j].SetCF(642);
mHyphas[i][j].SetNF(157);
}
}
}
}
}
}
}
}
}
}
}
}

void Graph::Spore(int x, int y) {
    mHyphas[x][y].SetSpore();
    //mHyphas[x][y].SetNewTip();
    mHyphas[x][y].SetTip(true, true);
    mHyphas[x][y].mCB = 100; //check?50
    mHyphas[x][y].mNB = 40; //check?10
    mHyphas[x][y].mIMB = 100; //check50
    mHyphas[x][y].mParent.mbInvalid = true; //initially no parent
}

Hypha& Graph::Element(int x, int y) {
    Hypha aHypha; //initialized as all zero
    if (x >= 0 && x < mMaxX && y >= 0 && y < mMaxY) {
        return (Hypha&) mHyphas[x][y];
    } else {
        aHypha.mLocation.mbInvalid = true;
    }
    return aHypha;
}

vector<Location> Graph::RanLocation(vector<Location>L) {
    vector<Location> vecVisited;
    Location N;
    do {
        N = L[rand()] % L.size();
        if (find(vecVisited.begin(), vecVisited.end(), N) == vecVisited.end()) {
            vecVisited.push_back(N);
        } while (vecVisited.size() != L.size());
    return vecVisited;
}

bool Graph::overboundary(int x, int y) {
    if (x < 0 || y < 0 || x > mMaxX || y > mMaxY){

return true;
else {
    return false;
}

void Graph::Diffuse_food(double DIR) {
    //check neighbor?!!
    //double tf[4];
    int nPivot = mMaxX/2 + (mMaxX%2 ? 0:0);
    int nOverBoundary = 0;
    for(int i=0; i<nPivot; i++) {
        for (int j=0; j<mMaxY; j++)
            nOverBoundary = ((i==0)?2:0) + ((j==mMaxY-1)?2:0) + ((j==0)?2:0) + ((i==mMaxX-1)?2:0);
            Element(i,j).SetCF(
            Element(i,j).GetCF() +
            DIR * ( ((overboundary(i-1,j))?0:Element(i-1,j).GetCF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetCF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetCF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetCF()) -
            (6-nOverBoundary)*Element(i,j).GetCF()));
            Element(i,j).SetNF(
            Element(i,j).GetNF() +
            DIR * ( ((overboundary(i-1,j))?0:Element(i-1,j).GetNF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetNF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetNF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetNF()) -
            (6-nOverBoundary)*Element(i,j).GetNF()));
    }
    for(int i=nPivot+1; i<mMaxX; i++) {
        for (int j=0; j<mMaxY; j++)
            nOverBoundary = ((i==0)?2:0) + ((j==mMaxY-1)?2:0) + ((j==0)?2:0) + ((i==mMaxX-1)?2:0);
            Element(i,j).SetCF(
            Element(i,j).GetCF() +
            DIR * ( ((overboundary(i-1,j))?0:Element(i-1,j).GetCF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetCF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetCF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetCF()) -
            (6-nOverBoundary)*Element(i,j).GetCF()));
            Element(i,j).SetNF(
            Element(i,j).GetNF() +
            DIR * ( ((overboundary(i-1,j))?0:Element(i-1,j).GetNF()) +
            ((overboundary(i,j+1))?0:Element(i,j+1).GetNF()) +
            ((overboundary(i+1,j))?0:Element(i+1,j).GetNF()) +
            ((overboundary(i,j-1))?0:Element(i,j-1).GetNF()) -
            (6-nOverBoundary)*Element(i,j).GetNF()));
    }
}
const float point_width = 1.0;
const float sizeX = 10.0;
const float sizeY = sqrt(3.0)*sizeX/2.0;
const float originX =10.0;
const float originY =10.0;

bool operator==(const Location& lhs, const Location& rhs)
{
    return ((lhs.mX==rhs.mX) && (lhs.mY==rhs.mY));
}

bool Graph::IsAdjacent(Location const& loc1, Location const& loc2)
{
    bool ok = false;
    vector<Location> vecLocations = GetAdjacentNodes(loc1.mX, loc1.mY);
    vector<Location>::iterator it = find(vecLocations.begin(), vecLocations.end(), loc2);
    if (it!=vecLocations.end())
    {
        ok = true;
    }
    return ok;
}

void Graph::DrawLineToParent(int x, int y)
{
    float cordX = originX + x*sizeX + ((y%2==0)?0:sizeX/2.0); //shifted half length
    float cordY = originY + y*sizeY;
    if (Element(x,y).Hyphea())
    {
        //link to one of these (x-1, y), (x, y-1), (x+1, y-1), (x, y), (x+1, y), (x+1, y+1)
        Location locParent = Element(x,y).mParent;
        if (IsAdjacent(Location(x,y), locParent))
        {
            glBegin(GL_LINES);
            glVertex2f(cordX, cordY);
            float parentX = originX + locParent.mX*sizeX + ((locParent.mY%2==0)?0:sizeX/2.0);
            float parentY = originY + locParent.mY*sizeY;
            glVertex2f(parentX, parentY);
            glEnd();
        }
    }
}

void Graph::DrawGraph()
{
    for(int x=0; x<mMaxX; x++)
    {
        float cordX = originX + x*sizeX;
        float newCordX = cordX;
        for(int y=0; y<mMaxY; y++)
        {
            float cordY = originY + y*sizeY;
            newCordX = (y%2==0)? cordX:(cordX + sizeX/2); //shifted half length
            }
glColor3f(0.0, 0.0, 0.0); // new

// can be removed later, drawing dots

void DrawLineToParent(x, y)
{
    glBegin(GL_LINES);
    double lx = originX + (mMaxX / 2)*10;
double ly = originY + mMaxY * 17;
glVertex2f(lx, originY);
glVertex2f(lx, ly);
glEnd();
}

vector<Location> Graph::GetAdjacentNodes(int i, int j)
{
    vector<Location> vecLocations;
    vecLocations.push_back(Location(i, j - 1));
    vecLocations.push_back(Location(i - 1, j));
    vecLocations.push_back(Location(i, j + 1));
    vecLocations.push_back(Location(i + 1, j));
    if (j%2==1)
    {
        vecLocations.push_back(Location(i+1, j-1));
        vecLocations.push_back(Location(i+1, j+1));
    }
    else
    {
        vecLocations.push_back(Location(i-1, j-1));
        vecLocations.push_back(Location(i-1, j+1));
    }
    return vecLocations;
}

Location& Graph::Direction(int i, int j)
{
    Location loc;
    vector<Location> vecLocations = GetAdjacentNodes(i, j);
    for (vector<Location>::iterator it = vecLocations.begin(); it!=vecLocations.end();)
    {
        if (Element(it->mX, it->mY).mLocation.mbInvalid || Element(it->mX, it->mY).Hyphea())
        {
            // invalid or already hyphea, remove this location from list
            it = vecLocations.erase(it);
        }
        else
++it;  //move to next item
}

if (!vecLocations.empty())  //now randomly select one from the rest
{
    loc = vecLocations[rand()%vecLocations.size()];
} else //return an invalid location
{
    loc.mbInvalid = true;
}
return loc;

const string RecordFileName = "biomass.txt";

void Graph::WriteRecord(const stringstream& ss)
{
    ofstream ofile(RecordFileName.c_str(), ios::out | ios::app);
    ofile << ss.str().c_str() << endl;
    ofile.close();
}

void Graph::WriteRecord(Hypha const& srcHypha, Hypha const& desHypha)
{
    ofstream ofile(RecordFileName.c_str(), ios::out | ios::app);
    ofile << "From Hypa, i=" << srcHypha.mLocation.mX << ", j=" << srcHypha.mLocation.mY << ", CB:" << srcHypha.mCB << " to: i=" << desHypha.mLocation.mX << ", j=" << desHypha.mLocation.mY << ", CB:" << desHypha.mCB << endl;
    ofile.close();
}

void Graph::WriteRecord(double mCL, double mNL, double mCR, double mNR)
{
    ofstream ofile(RecordFileName.c_str(), ios::out | ios::app);
    ofile << " " << mCL << " " << mNL << " " << mCR << " " << mNR << endl;
    ofile.close();
}

const string RecordFileName3 = "Tottle IMB.txt";

void Graph::WriteRecord(double TT)
{
    ofstream ofile(RecordFileName3.c_str(), ios::out | ios::app);
    ofile << TT << endl;
    ofile.close();
}

const string RecordFileName4 = "Tottle ImbL.txt";

void Graph::WriteRecordL(double TTL){
    ofstream ofile(RecordFileName4.c_str(), ios::out | ios::app);
    ofile << TTL << endl;
const string RecordFileName5 = "Tottle ImbR.txt";

void Graph::WriteRecordr(double TTR)
{
    ofstream ofile(RecordFileName5.c_str(), ios::out | ios::app);
    ofile << TTR << endl;
    ofile.close();
}

void NofGraph::Translocation(int i, int j, double Dn, double Dc)
{
    for (vector<Location>::iterator it = Element(i, j).vecNeighbor.begin(); it != Element(i, j).vecNeighbor.end(); it++)
    {
        if (find(Element(it->mX, it->mY).vecdifued.begin(), Element(it->mX, it->mY).vecdifued.end(), Location(i, j)) == Element(it->mX, it->mY).vecdifued.end())
        {
            int m = it->mX;
            int n = it->mY;
            Element(m, n).mCB = Element(m, n).mCB + (Element(i, j).mCB - Element(m, n).mCB) * Dc;
            Element(i, j).mCB = Element(i, j).mCB - (Element(i, j).mCB - Element(m, n).mCB) * Dc;
            Element(m, n).mNB = Element(m, n).mNB + (Element(i, j).mNB - Element(m, n).mNB) * Dn;
            Element(i, j).mNB = Element(i, j).mNB - (Element(i, j).mNB - Element(m, n).mNB) * Dn;
            Element(i, j).vecdifued.push_back(Location(m, n));
        }
    }
}

void NofGraph::React(double BR1, double BR2, double si, double Sc, double Sn, double Dn, double Dc, double bc, double bn, double bd, double EC, double EN, double MN, double MC, double R1, double Rr1, double Rr2, double A, double B, double Ti)
{
    vector<Location> myLocation = RanLocation(vecgraph);

    for (vector<Location>::iterator it = myLocation.begin(); it != myLocation.end(); it++)
    {
        if (Element(it->mX, it->mY).Tip() && (!Element(it->mX, it->mY).mLocation.mbInvalid))
        {
            if (find(vecNewGrowed.begin(), vecNewGrowed.end(), Location(it->mX, it->mY)) == vecNewGrowed.end())
            {
                Growth(it->mX, it->mY, BR1, BR2, si, Sc, Sn);
            }
        }
    }

    for (vector<Location>::iterator it = myLocation.begin(); it != myLocation.end(); it++)
    {
        if (Element(it->mX, it->mY).Hyphea())
        {
            Element(it->mX, it->mY).Uptake(EC, EN);
        }
    }
}
//Diffuse_food(DIR);
for (vector<Location>::iterator it = myLocation.begin(); it != myLocation.end(); it++)
{
    if (Element(it->mX, it->mY).Hyphea())
    { Translocation(it->mX, it->mY, Dn, Dc); }
}

for (vector<Location>::iterator it = myLocation.begin(); it != myLocation.end(); it++)
{
    if (Element(it->mX, it->mY).Hyphea())
    { Metabolized(MN, MC); }
}

for (vector<Location>::iterator it = myLocation.begin(); it != myLocation.end(); it++)
{
    if (Element(it->mX, it->mY).Hyphea())
    { Recycling(R1, Rr1, Rr2); }
}

for (vector<Location>::iterator it = myLocation.begin(); it != myLocation.end(); it++)
{
    if (find(vecNewGrowed.begin(), vecNewGrowed.end(), Location(it->mX, it->mY)) == vecNewGrowed.end())
    { HypheaTip(A, B, Ti); }
}

vecNewGrowed.clear();
myLocation.clear();

//biomass caculation
int i, j;
for (i = 0; i < mMaxX; i++)
{
    for (j = 0; j < mMaxY; j++)
    {
        if (i < (mMaxX/2 - 1) && Element(i, j).Hyphea() == 1)
        { mTTL = mTTL + Element(i, j).mIMB;
            mCL = mCL + Element(i, j).mCB;
            mNL = mNL + Element(i, j).mNB;
        }
        else if (i > (mMaxX/2 + 1) && Element(i, j).Hyphea() == 1)
        { mTTR = mTTR + Element(i, j).mIMB;
            mCR = mCR + Element(i, j).mCB;
            mNR = mNR + Element(i, j).mNB;
        }
        Element(i, j).vecdifued.clear();
    }
}
WriteRecord( mCL,mNL, mCR,mNR);
WriteRecord(mTTL+mTTR);
WriteRecordr(mTTR);
WriteRecordl(mTTL);
//WriteRecordE( gEC, gEN);

void NofGraph::Dieback(int i, int j, double bc, double bn, double bd)
{
  if( Element(i,j).Hyphea()){
    if(!Element(i,j).Tip()==0){
      if((Element(i,j).mCB < 0) || (Element(i,j).mNB < 0 || Element(i,j).mIMB<bd))
        //if ((Element(i,j).mIMB<DI&&(!Element(i,j).Tip())))
          
      {  
          //Element(i,j).SetNewTip(false);
          Element(i,j).SetTip(false, false);
          Element(i,j).SetHyphea(false); //change length;
          Element(i,j).SetParent(Location(true));
          Element(i,j).SetOffspr(Location(true));
        }
    }
  }
}

void NofGraph::Branch(int i, int j, int nBranches, double si, double Sc, double Sn)
{
  if (nBranches > 0)
  {
    Location loc(i,j);
    Location locNext = Direction(i, j);
    // WriteRecord(ss);
    if (!locNext.mbInvalid) //there is a valid branch
      
      // WriteRecord( Element(i,j), Element(locNext.mX,locNext.mY) );
      --nBranches;
    if (!(!Element(i,j).mParent == locNext) && !(Element(locNext.mX,
              locNext.mY).mLocation==Location(i,j)))
      
      {  
          //Element(locNext.mX, locNext.mY).SetNewTip();
          Element(locNext.mX,locNext.mY).SetTip(true, true);
          vecNewGrewed.push_back(locNext);
          Element(locNext.mX, locNext.mY).SetParent(Location(i,j));
          Element(locNext.mX, locNext.mY).SetOffspr(loc);
          Element(i,j).SetNeighbors(Location(locNext.mX, locNext.mY));
      
      Setm_imb(locNext.mX,locNext.mY, si, Sc, Sn);
      
      }
    if (nBranches>0) //branch again
      {
      Branch(i, j, nBranches,si, Sc, Sn);//recall Branch function with one less nBranches
    }
}
void NofGraph::Setm_imb(int i, int j, double si, double Sc, double Sn)
{
    if (!Element(i,j).mParent.mbInvalid)
    {
        Element(i,j).mIMB = si*Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mIMB;
        Element(i,j).mCB = Sc*Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mCB;
        Element(i,j).mNB = Sn*Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mNB;
        double m = Element(i,j).mCB;
        // Element(i,j).mParent.mIMB = 1.1;
        Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mNB = Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mNB*(1-Sn);
        Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mCB = Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mCB*(1-Sc);
        Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mIMB = Element(Element(i,j).mParent.mX, Element(i,j).mParent.mY).mIMB*(1-si);
    }
}
void NofGraph::Growth(int i, int j, double BR1, double BR2, double si, double Sc, double Sn)
{
    double BRN1 = BR2*0.1; double BRN = BR1*0.1;
    if (Element(i,j).Tip() && Element(i,j).mCB > BR2 && Element(i,j).mNB > BRN1)
    { Branch(i, j, 2, si, Sc, Sn); }
    else if (Element(i,j).Tip() && Element(i,j).mCB > BR1 && Element(i,j).mNB > BRN)
    { Branch(i, j, 1, si, Sc, Sn); }
}
//ofile << "At Tick ="<<tick<<"Total biomass in left side is:" << eTTL << "Total biomass in right side is:" << eTTR <<endl;
    ofile << eTTR <<endl;
    ofile.close();
}

const string RecordFileNameT= "Tip.txt";
void Environment::WriteRecordtip( int tick, int i, int j,double CB ,double NB, double imb ){
    ofstream ofile(RecordFileNameT.c_str(), ios::out | ios::app);
    ofile << "At Tick ="<<tick<<"tip" << i<<"  "<<j <<"  "<<CB <<"  "<<NB <<"  "<<imb<<endl;
    ofile.close();
}

double Environment::Play(GAGenome & c) {
    GABin2DecGenome & genome = (GABin2DecGenome &)c;
    double result; result=0;
    double MC=genome.phenotype(0);//0.01
    double R1=genome.phenotype(1);//0.2
    double Rr1=genome.phenotype(2);  //0.2
    double Rr3=genome.phenotype(3);  //0.2
    double A=genome.phenotype(4);    //2  
    double B=genome.phenotype(5);    //5
    double Ti=genome.phenotype(6);    //1
    double EC= genome.phenotype(7);//0.015;
    double BR1=genome.phenotype(8);  //8
    double si=genome.phenotype(9);   //0.2
    double Dn=0.5;
    double Dc=0;
    double bc=0.1;
    double bn=0.1;
    double bd=0.1;
    double Sc=0.1;
    double Sn=0.1;
    double EN=EC;
    //double DIR=0.1;
    double MN=MC*0.1;
    double BR2=1.5*BR1;
    /* Step 1
        if(this->tick == 20){
            ret_info.tick = this->tick
            return ret_info;
        }
    */
    /* Step 2 */
    //mGraph.React(BR0,BR1,STI,  STC,  STN,DIR, DR, DC, DN, DI, mEC, mEN, MN, MC,R1, R2, RR1, RR2, RR3, A, B, SI);
mGraph.React(BR1, BR2, si, Sc, Sn, Dn, De, bc, bn, bd, EC, EN, MN, MC, R1, Rr1, Rr2, A, B, Ti);
/* Step 4 */
IncTick();

result = mGraph.GetTTL() + mGraph.GetTTR(); //mGraph.SetTL1(1.0);

int tick = GetTick();

eTTL = mGraph.GetTTL(); //mGraph.SetTL3(1.0);
eTTR = mGraph.GetTTR();
eCL = mGraph.GetmCL(); eNL = mGraph.GetmNL(); eCR = mGraph.GetmCR(); eNR = mGraph.GetmNR();
WriteRecord1(eCL + eNL);
WriteRecord(eNR + eCR);
for (int i = 0; i < 50; i++)
{
  for (int j = 0; j < 50; j++)
  {
    if (mGraph.Element(i, j).Tip())
    {
      WriteRecordTip(tick, i, j, mGraph.Element(i, j).mCB, mGraph.Element(i, j).mNB, mGraph.Element(i, j).mIMB);
    //if (mGraph.Element(i, j).Hyphae())
    //WriteRecordHyphae(tick, i, j);
  }
  } //WriteRecord(tick, eCL, eNL, eCR, eNR);

mGraph.SetTTL();
mGraph.SetTTR();
mGraph.SetmML();
mGraph.SetmMR();
return result;
}

/* Class Environment functions */

#include <stdio.h>
#include <ga.h>
#include <std_stream.h>
#include <stdlib.h>
#include <ctime>
#include <math.h>
#include <algorithm>
#include <fstream>
#include "fungi.h"
#include <vector>
#include <stdio.h>

#define cout STD_COUT

float objective(GAGenome &);

int main(int argc, char **argv)
{
}
cout << "Example 9
";
cout << "This program finds the maximum value in the function
";
// cout << " y = - x1^2 - x2^2
";
cout << "Parameters
";
//cout << " -5 <= x1 <= 5
";
//cout << " -5 <= x2 <= 5
";
cout << "\n"; cout.flush();

// See if we've been given a seed to use (for testing purposes). When you
// specify a random seed, the evolution will be exactly the same each time
// you use that seed number.

unsigned int seed = 0;
for(int i=1; i<argc; i++) {
   if(strcmp(argv[i++], "seed") == 0) {
      seed = atoi(argv[i]);
   }
}

// Declare variables for the GA parameters and set them to some default values.

int p = 20;
int n = 100;
float p = 0.01; //0.01
float p = 0.6; //0.6

// Create a phenotype for two variables. The number of bits you can use to
// represent any number is limited by the type of computer you are using. In
// this case, we use 16 bits to represent a floating point number whose value
// can range from -5 to 5, inclusive. The bounds on x1 and x2 can be applied
// here and/or in the objective function.

GABin2DecPhenotype map;
map.add(16, 0, 0.02); //0.01
map.add(16, 0.1, 0.3); //0.2
map.add(16, 0, 0.4); //0.2
map.add(16, 0.1, 0.3); //0.2
map.add(16, 1, 3); //2
map.add(16, 3.7); //5
map.add(16, 0.2); //1
map.add(16, 0.01, 0.03); //0.015
map.add(16, 0.1); //8
map.add(16, 0.1, 0.4); //0.2

// map.add(16, -5, 5);
// Create the template genome using the phenotype map we just made.

GABin2DecGenome genome(map, objective);

// Now create the GA using the genome and run it. We'll use sigma truncation
// scaling so that we can handle negative objective scores.

GASimpleGA ga(genome);
GASigmaTruncationScaling scaling;
ga.populationSize(p);
ga.nGenerations(n);
ga.pMutation(pmut);
ga.pCrossover(pcross);
ga.scaling(scaling);
ga.scoreFilename("bog.txt");
ga.scoreFrequency(10);
ga.flushFrequency(50);
ga.evolve(seed);

// Dump the results of the GA to the screen.
cout << "the ga found an optimum at the point (";
cout << genome.phenotype(0) << ", " << genome.phenotype(1) << ", " << genome.phenotype(2) << ", " << genome.phenotype(3) << ", " << genome.phenotype(4) << ", " << genome.phenotype(5) << ", " << genome.phenotype(6) << ", " << genome.phenotype(7) << ", " << genome.phenotype(8) << "\n"
<< ga.scoreFilename() << "\n";
return 0;
}

// This objective function tries to maximize the value of the function
//
// float objective(GAGenome & c){

Environment env(60);
GABin2DecGenome & genome = (GABin2DecGenome & )c;
double ttm; double ttm1; double ttm2; double ttm3;
ttm3=0;
for(int i=0;i<91;i++)
{
    ttm= env.Play(c);
    if (i==41)
    {
        ttm1=ttm; //mGraph.SetTL1(1.0);
    }
    else if (i==65)
    {
        ttm2=ttm; // mGraph.SetTL2(1.0);
    }
    else if (i==89)
    {
        ttm3=ttm; // mGraph.SetTL3(1.0);
    }
    if(ttm3!=0 & & i ==89)
    {
        ttm1=ttm1/ttm3; ttm2=ttm2/ttm3; ttm3=1;
    }
}

    float fit;
    fit=-(0.327-ttm1)*(0.327-ttm1)+(0.421-ttm1)*(0.421-ttm1)+(0.327-ttm1)*(0.327-ttm1)+(0.339-
    ttm1)*(0.339-tpm1)+(0.3-tpm1)*(0.3-tpm1)+(0.811-tpm2)*(0.811-tpm2)+(0.744-tpm2)*(0.744-tpm2)+(0.717-
    tpm2)*(0.717-tpm2)+(0.72-tpm2)*(0.72-tpm2)+(0.693-tpm2)+(0.693-tpm2)+
\[(1.039 \times ttm3) \times (1.039 \times ttm3) + (1.02 \times ttm3) \times (1.02 \times ttm3) + (0.89 \times ttm3) \times (0.89 \times ttm3) + (1.01 \times ttm3) \times (1.01 \times ttm3) + (1.04 \times ttm3) \times (1.04 \times ttm3)\]

return (fit);
}