Sustainable biotechnological production of vitamin K_1 for human and animal health

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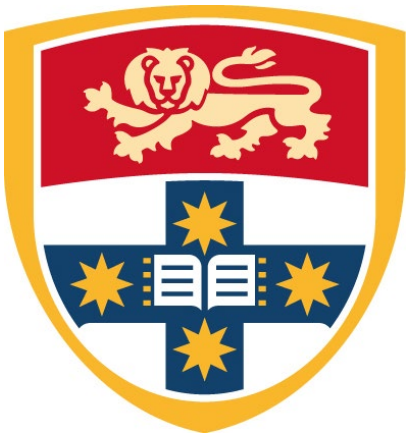

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Abstract

Vitamin K is an essential nutrient involved in the regulation of blood coagulation, tissue mineralization and immune response. Various types of vitamin K are manufactured, mainly by chemical synthesis, for use as dietary supplements and drugs in both humans and domestic animals. In fact, the largest single application of vitamin K supplements (in the form of the vitamin K analog menadione) is in intensive chicken farming. Vitamin K_1 is the most common form found in the natural diets of both humans and chickens. Research in humans has shown that vitamin K_1 has additional health benefits compared to other types of vitamin K, and there is evidence to suggest this is also the case in chickens. However, increasing consumer preferences for natural and sustainable products has increased the demand for biosynthesized vitamin K_2 in recent years. A natural vitamin K_1 product has obvious applications in both human and animal health. Therefore, the objective of this work was to develop a biotechnological process for the production of vitamin K_1.

The benefits of vitamin K_1 for the immune system were investigated in laying hens. Comprehensive blood biochemistry analyses were performed, including concentration of cytokines in white blood cells, concentration of cytokines in plasma and concentrations of different types of vitamin K in the blood. Vitamin K_1 (as Quinaquinone®) was shown to significantly increase blood vitamin K concentrations to more than 20 ng mL^{-1}, while vitamin K_2 (as MenaQ7®) and the analog menadione (as menadione nicotinamide bisulfite) achieved concentrations of less than 5 ng mL^{-1}. The range of concentrations observed was below those demonstrated to modulate inflammatory response. No significant differences in cytokine concentrations were observed. Therefore, higher concentrations and/or larger sample sizes are probably required to observe a difference in immune response. Nevertheless, vitamin K_1 is the best form for improving vitamin K status in chickens.

The biotechnological production of vitamin K_1 requires a microorganism that naturally contains a high concentration. Vitamin K_1 is produced by photosynthetic

organisms, and is usually known as phylloquinone in this context. A review of the literature showed that algae have the highest concentrations of phylloquinone, so seven strains of microalgae that are commonly used for scientific and commercial applications were tested. The cyanobacterium *Anabaena cylindrica* (ANACC strain CS-172) was found to have the highest concentration at 200 μg g^{-1} on a dry-mass basis. This is approximately six times higher than the concentration found in rich dietary sources such as parsley. One gram can provide almost three times the Australian daily adequate intake of 70 μg per day and four times the chicken intake of 60 μg per day. Subsequent nutritional analysis by an independent laboratory confirmed this value and revealed *A. cylindrica* is also a rich source of protein, iron and vitamin B_{12}. A mouse bioassay also demonstrated the short-term safety of diets including 5-15% *A. cylindrica* on a dry mass basis. No acute toxicity due (e.g. due to cyanotoxins) was observed. This strain of *A. cylindrica* is an excellent candidate for biotechnological production of vitamin K_1 and other health products.

Maximizing the volumetric productivity of *A. cylindrica* in the culture system is essential in developing a biotechnological process, as reactor productivity is usually the limiting step in photoautotrophic processes. Light, nitrogen and phosphorus are usually the key determinants of productivity; their effects were tested by growing *A. cylindrica* in a bench-top (5 L) bubble column photo-bioreactor (PBR). Increasing the Photosynthetic Photon Flux Density (PPFD; from 160 to 330 μmol m^{-2} s^{-1}) and the sodium nitrate concentration (from 170 to 1700 mg L^{-1}) led to a four-fold increase in phylloquinone productivity (from 4.9 to 22 μg L^{-1} d^{-1}) and final titer (from 34 to 129 μg L^{-1}). Unexpectedly, increasing the concentration of dipotassium hydrogen phosphate (from 34.8 to 348 mg L^{-1}) did not have any main effect but did have a weak negative interaction with the sodium nitrate concentration. This may indicate that the ratio of N:P is too low in the standard medium used here. The phylloquinone productivity of *A. cylindrica* in a bench-top PBR was successfully boosted by manipulating light and nitrate concentration.

Scale-up of biotechnological processes usually entails some loss of productivity; however, the magnitude of this loss can be the difference between commercial success and failure. Irradiance and mixing are usually the limiting factors in large reactors for the culture of microalgae. *A. cylindrica* was grown in a pilot-scale (50 L) bubble column photo-bioreactor in order to test the efficiency of scale-up, as well as strategies for improving mixing and irradiance. At 2 × concentration MLA medium and a PPFD of ~300 μmol m^{-2} s^{-1}, varying the air flow rate (superficial gas velocities in the range 0.62 – 6.2 cm s^{-1}) and the sparger design (ceramic with pore size of <0.3 mm or steel tube with 30 holes of diameter 2 mm) had no significant effect on phylloquinone productivity (~19 μg L^{-1}) or final titer (~130 μg L^{-1}). These values were very similar to those achieved in the bench-scale reactor, mainly due to the higher specific phylloquinone concentration (approximately 260 versus 150 μg g^{-1}) induced by light limitation in the pilot-scale reactor. The productivity and final titer were doubled by increasing the duration of light from 12 to 24 hours per day. However, this required more concentrated medium nutrients (5 × MLA). At a medium concentration of 2 × MLA with continuous illumination, nitrate was depleted by day 4 and specific phylloquinone concentration declined sharply from approximately 300 to less than 200 μg g^{-1} by day 7 (the final day).

The results of this research lay the foundations for the biotechnological production of vitamin K_1, a world first. New applications, sources and production methods were presented. Future work could include studies in larger populations of animals, investigation of genetic influences on phylloquinone biosynthesis in microalgae, further optimization of microalgal culture conditions and development of downstream processing methods for microalgae.

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Student Declaration

I declare that the work contained within this thesis is my own, unless stated otherwise. No part of this work has been submitted to any other institution for award of a degree.

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List of Publications and Presentations

Journal articles

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1 Introduction

Vitamin deficiencies are a major contributor to chronic disease, the most costly and debilitating category of diseases in the modern world [1]. Diseases are termed "chronic" when they emerge gradually and subsequently affect patients for many years. The most commonly reported chronic diseases in Australia in 2011 were cancer (19%), cardiovascular diseases (including ischemic heart disease, 15%), musculoskeletal disorders (including osteoporosis and rheumatoid arthritis, 12%) and mental health disorders (including depression and dementia, 12%) [2], which cost more than AUD 12 billion to treat. This is a common trend among developed nations.

The increasing incidence and severity of chronic disease has been attributed primarily to detrimental lifestyle behaviors, such as poor diet [1]. Research is beginning to reveal simple dietary choices that can help to reduce the burden of chronic disease. Two well-known examples are the Mediterranean and Okinawan diets, which can reduce the risk of morbidity and mortality due to heart disease, diabetes and cancer [3]–[5]. These diets are primarily made up of vegetables (leafy greens, roots and legumes) and are rich in plant-based chemicals [5].

Nutrient deficiencies and imbalances also contribute to reduced productivity and animal welfare in livestock systems [6]. This is a problem because the health of the poorest and fastest growing human populations is limited by the availability of nutritious animal food products, especially pork and chicken. In fact, chicken meat and eggs are the fastest growing and most widely consumed animal products in the world [6]. Optimal nutrition remains an unsolved problem in the poultry industry and micronutrient interventions could vastly improve animal welfare and productivity [7]. For example, increased vitamin levels are required when chickens are stressed or sick [8].

1.1 The need for vitamin K

The rationale for the research into vitamin K presented in this thesis is three-fold. Firstly, the most recent research suggests a much more important physiological role for vitamin K than has been understood since its discovery in the 1930s. If these findings prove true, then the potential benefits to human and animal health are enormous. Secondly, and as a corollary of the first point, there is very little available research on vitamin K (16,000 articles on Clarivate's Web of Science as of January 2019) compared to other vitamins, such as the fat-soluble vitamins A (approx. 33,000), D (approx. 79,000) and E (approx. 50,000). There is considerable work required on the physiological roles and synthesis of vitamin K to bring our knowledge to a level on par with knowledge of the other vitamins. Thirdly, the K vitamins typically sell for several thousand USD/kg, making them some of the most expensive nutritional commodities in the global market. The potential economic value of a new vitamin K product is also enormous.

Vitamin K refers to a family of structurally similar chemical compounds defined by their ability to facilitate the modification of vitamin K dependent proteins. True K vitamers (vitamins K_1 and K_2) are characterized by their ability to act as the enzyme cofactor in the vitamin K cycle. Natural vitamin K_1 is also known as phylloquinone, while vitamin K_2 consists of a group of compounds called the menaquinones (denoted MK-n). In contrast, vitamin K analogs (the menadione derivatives, sometimes known erroneously as vitamin K_3) are pro-vitamins that require transformation before they become active in the vitamin K cycle [9]–[11]. They exhibit lower activity [12] and can be toxic [13]–[17]. In fact, menadione and its derivatives have been banned in human products since 1963 [18].

In humans, vitamin K is essential for the activity of at least 15 proteins that regulate diverse biochemical processes. Vitamin K has long been used to prevent and treat certain types of coagulation disorders [19]–[21]. There are also many emerging health benefits of vitamin K. Increased vitamin K intake can help prevent

bone fracture [22], [23] arterial calcification [24], [25], inflammatory diseases [25], [26], and cognitive decline [27]–[29]. Despite this, public health agencies around the world have so far not attempted to encourage increased vitamin K intakes.

The most common sources of vitamin K are leafy green vegetables, which are a rich source of vitamin K_1. Unfortunately, most people in developed nations do not eat enough vegetables. In Australia, only 1% of 18-34 year olds eat at least the recommended amount of vegetables per day [30]. Seaweed, livers, and some fermented soy bean foods are also very rich sources of vitamin K, however their consumption is not widespread. Therefore, in order to boost vitamin K intakes, food fortification and/or supplementation may be necessary. Vitamin K could be added to oil-based spreads and dairy products like the other fat soluble vitamins A, D and E.

1.2 Thesis Aims

A lack of availability of micronutrient-rich foods is a major impediment to the good health of both humans and livestock. Vitamin K has historically been ignored in favor of vitamins A, C, E and, more recently, B-group vitamins and vitamin D. The development of effective, safe and relatively cheap vitamin K products has been neglected as research and development focused on these other vitamins. This is especially evident in animal nutrition, such as in commercial chicken feed, where menadione derivatives are still used despite demonstrated toxicity in humans. This research aims to develop a natural vitamin K_1 product using a biotechnological process. Such a product could have applications in both human and animal health.

The literature pertaining to the benefits and production methods of vitamin K are reviewed in Chapter 2. Chapter 3 examines the benefits of vitamin K supplementation for poultry health. Domestic laying hens were fed various vitamin K-supplemented diets; their blood was subsequently analyzed for immune function and vitamin K concentration. Chapter 4 presents a novel microalgal process for the biosynthesis of vitamin K_1. Several common microalgal strains were screened for vitamin K_1 content, and the nutritional value and safety of the best strain was tested.

Chapter 5 investigates the effects of nutrients on the productivity of the selected microalga. Light intensity, phosphate concentration and nitrate concentration were varied in five liter photo-bioreactor systems, and vitamin K_1 concentration and productivity were tested. Chapter 6 investigates the scaling-up of the microalgal process. Attempts to boost the productivity of cultures in a 50 liter bubble-column investigated effects of operating conditions such as superficial gas velocity, bubble flow, and day-length. Finally, Chapter 7 presents conclusions, discusses general trends in the present research, and proposes ideas for future research.

2 Literature review – the benefits and industrial production methods of vitamin K

The first half of this chapter answers the questions "what is vitamin K?" and "why do we care about vitamin K_1?" Vitamin K is defined and its characteristics are presented. The need for vitamin K is also reviewed in relation to known physiological roles and dietary sources. Subsequently, the known and putative benefits of vitamin K_1 for human and animal health are highlighted. The second half of this chapter answers the questions "how do we make vitamin K_1 now?" and "how might we make vitamin K_1 in the future?" The most common process for the chemical synthesis of vitamin K_1 is reviewed. Past, present and future efforts to improve the process are discussed. Alternatives to the chemical synthesis, namely biotechnological processes, are presented in light of the precedent for bacterial synthesis of vitamin K_2. Criteria are proposed for the selection of organisms that are rich in vitamin K_1, as well as methods for boosting production, processing the biomass and extracting the vitamin K_1. Finally, the chapter concludes with a summary of the key points from this literature review.

2.1 The vitamins

The bulk of the food we eat is made up of macronutrients - fats, proteins, carbohydrates and fiber. Our foods also contain micronutrients – the vitamins and minerals [31]. While the amounts required are small (much less than one gram per day), they are essential dietary ingredients as the human body cannot produce them [31]. Vitamins are distinguished by their organic nature (unlike minerals, which are inorganic) [31]. Severe vitamin deficiencies are known to cause a range of acute diseases such as beriberi, pellagra, scurvy and rickets. Vitamins are usually classified as either water or fat-soluble. The water-soluble vitamins include the B group vitamins and vitamin C, while the fat-soluble vitamins are A, D, E and K (Table 2.1).

Table 2.1: The vitamins and their natural sources, arranged from highest to lowest price.

Vitamin (common chemical form)	Rich dietary sources	Typical method of production	Indicative price range (USD/kg)	References
B_{12} (cyanocobalamin)	Livers, meat (red and white), dairy, eggs	Biosynthesis	1,000-10,000	[32], [33]
K (phytonadione)	Leafy green vegetables, plant and animal fats, livers	Chemical synthesis	1,000-2,000	[33], [34]
B_7 (biotin)	Egg yolks, peanuts, livers, chicken, yeast, mushrooms	Chemical synthesis	100-1,000	[32], [33]
B_1 (thiamin)	Wholegrains, seeds, legumes, nuts, yeast	Chemical synthesis	10-100	[32], [33]
B_2 (riboflavin)	Dairy, wholegrains, egg whites, leafy green vegetables, meat, yeast, livers, kidneys	Chemical synthesis OR biosynthesis	10-100	[32], [33]
B_5 (pantothenic acid)	Livers, meat (red and white), kidneys, eggs, yeast, legumes	Chemical synthesis	10-100	[32], [33]
B_6 (pyridoxine)	Wholegrains, legumes, leafy green vegetables, nuts, meat (red and white), livers	Chemical synthesis	10-100	[32], [33]
B_9 (folic acid)	Leafy green vegetables, legumes, seeds, livers, poultry, eggs, cereals, citrus fruits	Chemical synthesis	10-100	[32], [33]
A (retinol)	Livers, dairy, egg yolks, root vegetables, stone fruits, leafy green vegetables	Chemical synthesis	10-100	[33], [35]
D (cholecalciferol)	None (Vitamin D is naturally biosynthesized in the human body after sun exposure)	Chemical synthesis OR extraction from waste	10-100	[33]
E (α-tocopherol)	Vegetable oils, nuts, leafy green vegetables	Chemical synthesis OR extraction from waste	10-100	[33], [36]
B_3 (niacin)	Meat (red and white), dairy, eggs, wholegrains, nuts, mushrooms	Chemical synthesis	1-10	[32], [33]
C (ascorbic acid)	**Berries, citrus fruits, tomatoes, kiwifruit**	**Chemical synthesis OR biosynthesis**	**1-10**	**[33], [37]**

Although vitamins are naturally found in foods, thousands of tons of pure vitamin ingredients are made every year (~45,000 tons in 2016) for a variety of applications. The biggest category of vitamin use is animal feed, primarily for intensively farmed animals such as poultry [38], [39]. In manufactured animal feed, addition or fortification with a range of vitamins is normal practice [33]. This is due to the low micronutrient levels in raw feed materials, and micronutrient losses due to processing and storage. In human health, vitamin ingredients find a much greater application in human pharmaceuticals [40], [41], with a smaller fraction in cosmetics. Food fortification in the human population occurs only when there is a demonstrated severe and widespread deficiency [42]. For example, under Australian law, fortification is mandatory in wheat bread flour (vitamins B_1, B_9) and in edible oil spreads (vitamin D) [43].

Vitamins that cannot be synthesized chemically, due to unfavorable reactions in the synthetic pathway or structural complexity, are produced by a biotechnological means [44]. Biotechnological processes harness living cells, often microorganisms, to produce these chemicals. However, in such a process, the product is just one dilute component of a complex "broth" composed of cells, metabolites and growth medium. Concentration and purification of a dilute product is costly – as a rule-of-thumb, improvements to a biosynthetic process that can increase the product concentration ten-fold will enable a ten-fold reduction in market price [45]. Occasionally it is economical to extract and purify vitamins from food processing waste (as is the case for vitamin E) [44]. Prices for natural vitamins vary widely depending upon purity and form, however for close to 100% purity the cheapest is usually vitamin C at less than 10 USD/kg, while the most expensive are vitamin B_{12} and vitamin K at more than 1000 USD/kg [33].

The vitamin ingredient market is worth more than USD 5 billion annually [46]–[48]. By market value, the main applications for vitamin ingredients are animal feed and pharmaceuticals (Figure 2.1). Vitamins A, C and D account for around 50% of all vitamin ingredients. As the B group is comprised of 8 different vitamins, its market share is more than one-third. Vitamin market growth is strong at around 5% year on year; however, the biggest growth in demand is expected for vitamins D, K and some B-group. This is driven by their increasing use to improve animal welfare, fight chronic disease in the human population, and add value to cosmetic products [48]. Market price is strongly influenced by manufacturing cost, which is in turn influenced by manufacturing complexity [49]. Vitamin analogs (such as menadione) and many vitamins (such as vitamin C) can be made by cheap and simple chemical syntheses [44], [50]. Chemically synthesized vitamins usually cost less than 100 USD/kg.

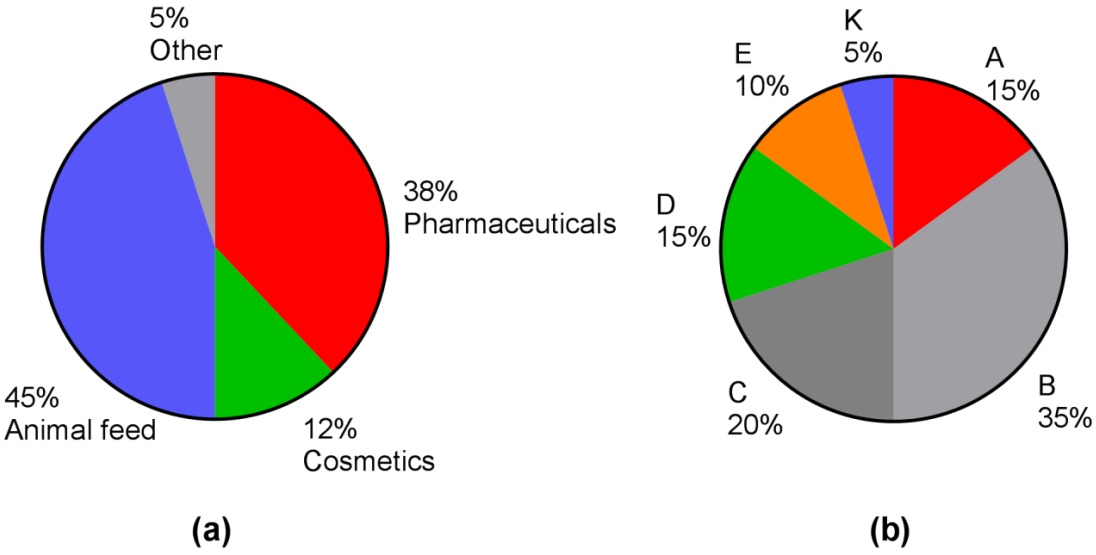


Figure 2.1: Global vitamin ingredient market split by application (a) and type (b) in 2015 [46]–[48].

2.1 Vitamin K

Vitamin K refers to a family of structurally similar chemical compounds characterized by their ability to act as the enzyme cofactor in the γ-carboxylation of certain proteins. In humans, vitamin K is essential for the activity of at least 15 proteins that regulate diverse biochemical processes. Vitamin K has been traditionally known for its essential role in the blood coagulation cascade [19]–[21], however it is also involved in maintaining bone, preventing arterial hardening, protecting the nervous system, and modulating inflammation [51]–[55].

Improved bone health has emerged in recent years as an additional benefit of increased vitamin K levels. Several cross-sectional studies [56]–[59] and randomized controlled trials [60], [61] have found that increased vitamin K intakes reduce the risk of bone fracture. Although some research has failed to demonstrate a statistically significant benefit [59], [62]–[64], the most recent systematic reviews and meta-analyses confirm that increased vitamin K intake reduces the risk of fracture [22], [23]. Whether this is due to increased bone mineral density, remodeling or some other mechanism is not clear [22], [23], [65], [66]. Other reviews of clinical and cross-sectional data demonstrate a possible role for vitamin K in preventing arterial calcification [24], [25], mitigating inflammatory diseases [25], [26], and preventing cognitive decline [27]–[29].

More rigorous research in future years will eliminate any remaining uncertainty about the health benefits of vitamin K. Most of the available data on the health benefits of vitamin K come from cross-sectional studies, which are limited by self-reporting, population biases (e.g. age or gender) and inability to prove cause-and-effect. Of the limited number of randomized controlled trials, many have been conducted over short durations with small samples sizes. Addressing these limitations will enable conclusive assessment of the health benefits of vitamin K.

2.1.1 Chemistry of K vitamers

Vitamin K is comprised of a 1,4-naphthoquinone group with a methyl group at the 2-position and an aliphatic side-chain at the 3 position (Figure 2.2(a)). The compound known as phylloquinone (vitamin K_1) is distinguished by its "phytyl" side-chain [67], [68] (Figure 2.2(b)). The compounds known as the menaquinones (vitamin K_2) consist of the same methylated naphthoquinone group [69], [70] and a polyunsaturated isoprenoid side-chain of varying length *n* [71] as in Figure 2.2(c). "Menaquinone-*n*" is usually abbreviated to MK-*n*. There are a number of artificial structural analogs of vitamin K (Figure 2.3) that exhibit vitamin K activity *in vivo* [72]–[76]. Although menadione and its derivatives are often referred to as vitamin K_3, they are not natural components of foods and therefore do not strictly meet the definition of a vitamin [31]. These compounds become active as vitamin K after the *in vivo* addition of an uncharged side-chain at the 3-position [9], [10], [12], [77], and should properly be known as pro-vitamins.

2.1.2 Physicochemical properties of vitamin K

The properties of K vitamers and their analogs can be attributed either to the 1,4-naphthoquinone moiety or the aliphatic side-chain. Generally, the 1,4-naphthoquinone group enables vitamin K to take different oxidation states, while the side-chain length and saturation affects the solubility and reactivity. The interplay between these two distinct parts of the molecule is also important, as overall effectiveness of the vitamin is determined by bioavailability, transport, metabolism and cofactor activity. The 1,4-naphthoquinone moiety is one of a large family known more generally as the quinones. The quinones are known to readily absorb visible and ultraviolet light, due to the presence of conjugated double bonds and carbonyl groups, and thus appear colored [78]. Menadione and the menaquinones exist as pale yellow solid crystals at ambient conditions [79], [80], while phylloquinone exists as a yellow liquid [81]. The yellow color is due to the absorption of ultraviolet and near-UV light by the 1,4-naphthoquinone group [82].

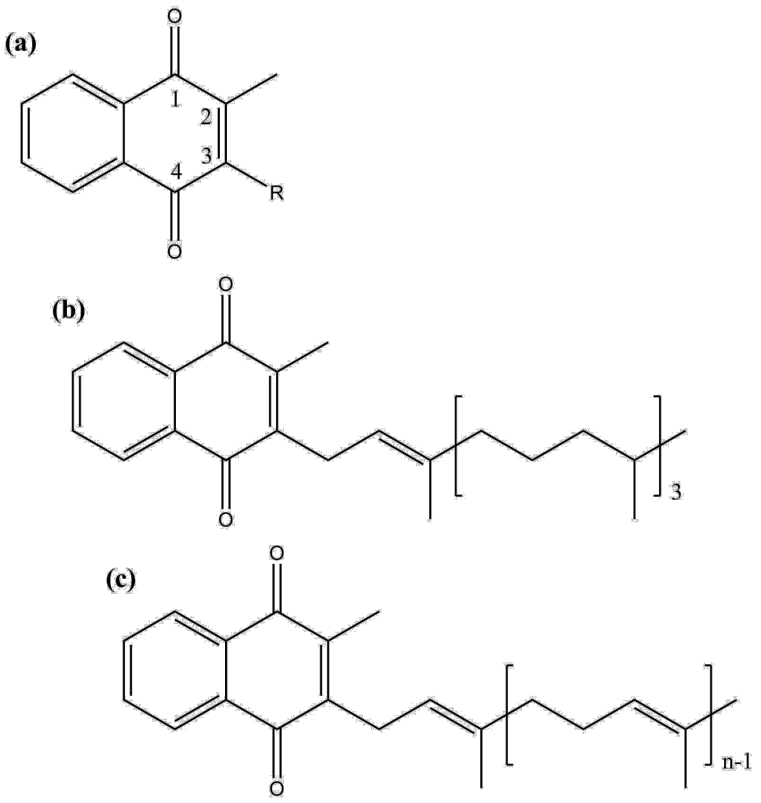


Figure 2.2: Structural diagrams of: (a) generic K vitamers; (b) phylloquinone; and, (c) menaquinone-n (MK-n).

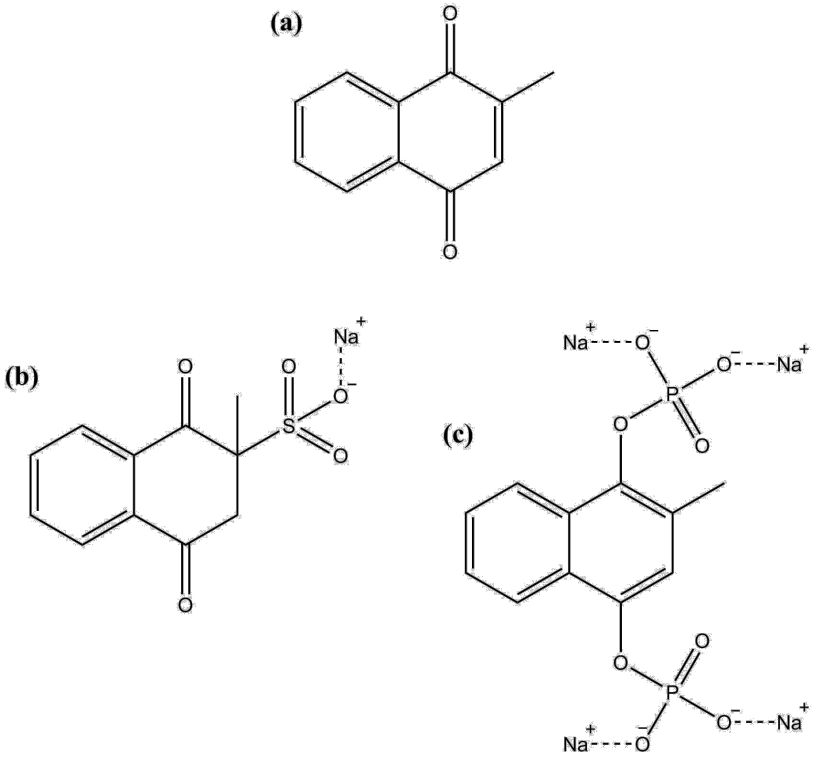


Figure 2.3: Structural diagrams of: (a) menadione; (b) menadione sodium bisulfite; and, (c) menadione sodium diphosphate.

Quinones are also well known for their redox cycling capability [78], which accounts for their existence in the electron transport chains of a wide variety of organisms [83]. The purpose of the electron transport chain is to transfer electrons across intracellular membranes, thereby creating the electrochemical gradient required for ATP synthesis. Phylloquinone is present in the electron transport chain of photosynthetic organisms [84] including plants, algae and cyanobacteria [85], while menaquinones are involved in the respiratory chains of many micro-organisms such as bacteria and archaea [86].

While the naphthoquinone group accounts for the redox cofactor activity of vitamin K, the side-chain is also essential for vitamin K activity [9], [10], [12], [77]. Possible reasons for this include its effects on redox potential and solubility in membranes. Vitamin K is usually found embedded in intracellular membranes, as phylloquinone and menaquinones are membrane-bound in both plants and bacteria [87], and probably localizes to the membrane of the endoplasmic reticulum in humans [88]. Molecules with longer side-chains tend to localize further towards the hydrophobic interior of the membrane bilayer, sometimes entirely within the midplane region [89], [90]. This could explain why the γ-carboxylase activity of menaquinones decreases as the side-chain length increases [10]. The side-chain also appears to prevent the generation of reactive oxygen species and depletion of glutathione [13], [91], harmful effects that are commonly associated with other naphthoquinone species [92] including menadione [13], [15], [17], [93]. The widespread belief that the side-chain does not contribute to vitamin K activity possibly originates from Henrik Dam's 1946 Nobel Prize lecture [94].

2.2 The requirement for vitamin K

2.2.1 Physiological functions and roles of vitamin K

The main function of vitamin K is as an essential cofactor for the enzymatic gamma-glutamate carboxylation of Vitamin K-Dependent Proteins (VKDPs) as depicted in Figure 2.4. The gamma-glutamyl carboxylase enzyme (GGCX) carboxylates the glutamate (Glu) residues of VKDPs [9], [95], [96]. The GGCX cofactor activity of compounds with vitamin K activity is different for all K vitamers and menadione derivatives [10], [12]. The protein-dependent effects of vitamin K are achieved by GGCX-mediated protein modification. Gamma-carboxylated glutamate (Gla) residues have a much higher calcium binding affinity than singly carboxylated glutamate (Glu) residues [97], [98], which is an important characteristic of many VKDPs. In fact, gamma-carboxylation is necessary for the normal function of all known VKDPs (Table 2.2) [99]–[108].

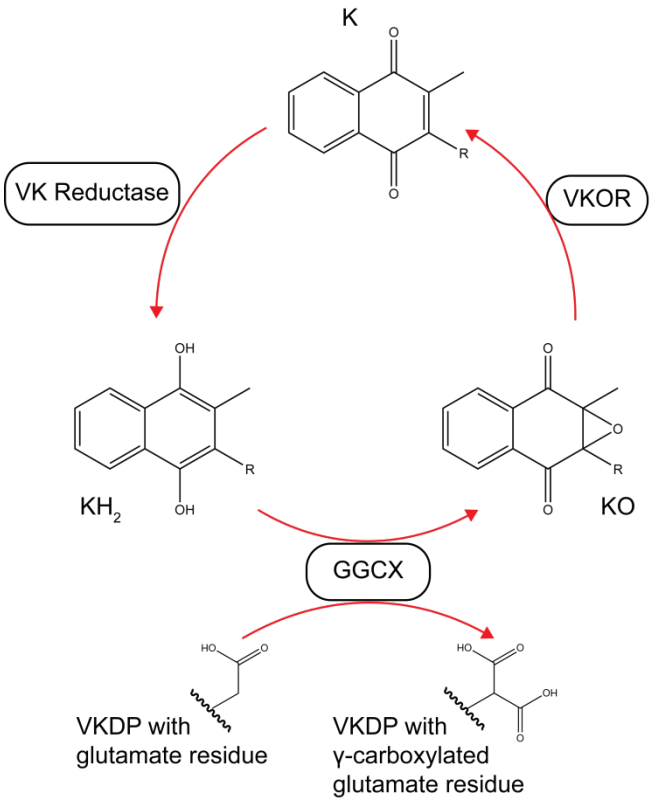


Figure 2.4: The vitamin K cycle (adapted from [109]). Acronyms: vitamin K hydroquinone (KH_2); vitamin K epoxide (KO); vitamin K epoxide reductase (VKOR); γ-glutamyl carboxylase (GGCX).

Table 2.2: The vitamin K-dependent proteins [54], [55], [110]–[113].

Protein Name, Synonyms	Known Roles	Main Location of Gene Expression
Factor II, prothrombin	Procoagulant	Liver
Factor VII	Procoagulant	Liver
Factor X	Procoagulant	Liver
Factor IX, Christmas factor	Procoagulant	Liver
Protein C	Anticoagulant	Liver
Bone Gla Protein (BGP), osteocalcin	Bone development, glucose homeostasis	Bone
Protein Z	Antithrombotic	Liver
Protein S	Cofactor for protein C, neuroprotective	Liver, cardiovascular, reproductive, respiratory, nervous, digestive and excretory systems
Matrix Gla protein (MGP)	Inhibition of calcification	Reproductive, cardiovascular and excretory systems
Growth-arrest specific protein 6 (Gas6)	Regulation of cell cycle, phagocytosis and inflammation	Cardiovascular, reproductive, respiratory, nervous, digestive and excretory systems
Trans-membrane Gla protein 1 (TMG1), Proline-rich Gla protein 1	Unknown, possibly trans-membrane signaling	Nervous, cardiovascular and reproductive systems
Trans-membrane Gla protein 2 (TMG2), Proline-rich Gla protein 2	Unknown, possibly trans-membrane signaling	Nervous, cardiovascular and reproductive systems
Trans-membrane Gla protein 3 (TMG3)	Unknown, possibly trans-membrane signaling	Nervous, cardiovascular and reproductive systems
Trans-membrane Gla protein 4 (TMG4)	Unknown, possibly trans-membrane signaling	Nervous, cardiovascular and reproductive systems
Gla-rich protein (GRP)	Fibrillogenesis, wound healing, strain repair	Reproductive, cardiovascular and respiratory systems

The best-known role of vitamin K is in coagulation. Although the relationship between vitamin K and clotting proteins has been known since the 1930s [114], it was not until the 1970s that the mechanism of vitamin K-dependent activation of the prothrombin protein was elucidated. The work of several researchers confirmed that the post-translational gamma-carboxylation of prothrombin enhanced its calcium-binding affinity [97], [115]–[119]. Another 6 vitamin K-dependent plasma proteins were isolated and characterized in the late 1970s [120]–[123]. Prothrombin, factor VII, factor IX and factor X are essential elements of the blood coagulation cascade, while protein C, protein S and protein Z have the ability to inhibit the cascade [112]. Probably the most well-known application of vitamin K is in neonatal medicine. Newborn babies usually have low vitamin K levels, and 5-10 in every 100,000 newborn babies suffer from Vitamin K Deficiency Bleeding (VKDB) [124]. This deficiency is impossible to detect at the time of birth so Vitamin K_1 is routinely given to newborn babies, usually by intramuscular injection, to prevent VKDB [125].

Aside from the role of vitamin K in coagulation, vitamin K also has an important role in calcium homeostasis. The vitamin K-dependent proteins osteocalcin, Matrix Gla Protein (MGP) and Gla-rich Protein GRP are essential regulators of tissue mineralization. Osteocalcin, initially known as Bone Gla Protein (BGP) [98], [126], regulates both calcium binding and cellular activity in bone. Gamma-carboxylated osteocalcin has a high affinity for calcium, which is greatly diminished in un-carboxylated osteocalcin. Osteocalcin has also been shown to stimulate the activity of both osteoblasts and osteoclasts; however, this activity is not wholly dependent upon carboxylation status [127].

While MGP and GRP are also found in bone, they do not appear to bind calcium like osteocalcin. Rather, they behave mainly as modulators of calcification. This is important because MGP and GRP are found in connective and soft tissues as well as bone. Gamma-carboxylated MGP is essential for normal cartilage mineralization – insufficiency of MGP will compromise the survival of chondrocytes, while undercarboxylation of MGP will lead to uncontrolled calcification of cartilage.

The role of GRP is less clear, however it stimulates deposition of aggrecan and collagen type II while delaying osteoblast maturation [55].

Protein-dependent roles of vitamin K in innate immunity have also been discovered. The ability of the Gas6 protein and protein S to bind to the Tyro3, Axl, and Mer (TAM) family of transmembrane cell receptors was discovered in the 1990s [70]. When Gas6 binds to the extracellular domain of a TAM receptor, the intracellular protein kinase domain of the receptor is activated. The protein kinase phosphorylates various signaling proteins that modulate the cell's behavior. Changes to the cell's excretion of cytokines may also lead to changes in the behavior of other cells. This mechanism has been shown to induce a wide variety of responses including inhibition of inflammatory response [129], [130], stimulation of phagocytosis and maturation of natural killer cells [131].

The roles of four vitamin K-dependent proteins, the transmembrane Gla proteins (TMGs) [132], [133], are yet to be determined. However, TMGs are expressed in many different tissues throughout the body including skeletal muscle, kidney, lung, liver, heart, brain, pancreas, colon, prostate, trachea, spinal cord, and thyroid. Furthermore, TMG extracellular domains include the Gla-residues while cytoplasmic domains display similarities with other signal transduction proteins [134]. It is therefore likely that the TMGs are involved in signal transduction throughout the body.

There are currently two proteins whose vitamin K-dependence is in doubt. Periostin and transforming growth factor-β induced protein (TGFBIp, also known as βig-h3 and kerato-epithelin) are closely related proteins with roles primarily in creation, maintenance and repair of connective tissues [135]. Their wide distribution in the extracellular matrix throughout the body highlights their physiological importance, and they have been implicated in a range of diseases, from corneal dystrophy to asthma. Their structural similarity with other known VKDPs has seen them tentatively categorized as vitamin K-dependent; however, evidence is conflicting [136], [137].

Vitamin K can confer benefits independent of its activity as a protein carboxylase cofactor. The correlation between vitamin K insufficiency and cognitive decline has been widely studied in recent years. This effect is partially attributed to the importance of MK-4 in the synthesis of sphingolipids [138] [28]. Sphingolipids form more mechanically and chemically stable bilayers than phospholipids, which protect the cells of the nervous system. Unfortunately, the mechanism by which vitamin K promotes sphingolipid synthesis is not yet known [139]. Research has also shown that K vitamers can protect against intracellular oxidative stress in developing oligodendrocytes and neurons [140], which can also contribute to cognitive decline.

There are some bone-related effects of vitamin K that are protein independent [22]. While the mechanisms are not conclusively known, it appears that vitamin K can inhibit the formation of osteoclasts and stimulate the formation of osteoblasts, thereby encouraging bone growth. Vitamin K is a known activator of the steroid and xenobiotic receptor (SXR), which is involved in both bone creation and resorption, so the effect of vitamin K is multi-faceted [141].

Finally, there is a putative role for vitamin K in mitochondrial electron transport chains. Vitamin K has been shown to rescue electron transport in the mitochondria of flies affected by knockout of the pink1 gene, which encodes the mitochondrial protein kinase implicated in early-onset Parkinson's Disease [142]. Apart from this study, the potential role of vitamin K in human and animal electron transport chains has received little attention, despite the known role of naphthoquinones in plant and microorganism electron transport chains.

2.2.2 Dietary intake of vitamin K in humans

As for all vitamins, ensuring a healthy dietary intake of vitamin K is essential. Leafy green vegetables are the richest dietary sources of phylloquinone, while fermented foods (e.g. natto) are rich sources of menaquinones (Table 2.3). There is evidence

from studies in mice that phylloquinone (which is the primary dietary source of vitamin K) is converted into MK-4 [143] by the prenylation of menadione [11]. Gastrointestinal bacteria are another possible source of menaquinones, though their contribution to total intake remains unknown. Studies of the gastrointestinal tract have demonstrated the presence of menaquinone-producing bacteria in the large intestine [144]–[146], as well as the absorption of menaquinones in the intestines [147]–[151]. The prevailing opinion among medical professionals is therefore that gastrointestinal menaquinones are an important source of vitamin K. However, it is not clear if the menaquinones of gastrointestinal origin are efficiently absorbed by the host, mainly because vitamin K is thought to be poorly absorbed in the large intestine compared to the small intestine [146], [150], [152]. The contribution of gastrointestinal menaquinones to vitamin K status has been reviewed and is generally considered insignificant [153], [154].

Guidelines in the US [155], EU [156], and many other regions provide an "adequate intake" (AI) figure for phylloquinone, which is approximately 1 μg phylloquinone per kg bodyweight per day. These are based upon typical population-wide phylloquinone intakes (such as in the US) or the requirement of phylloquinone for normal coagulation (such as in the EU). The extremely low prevalence of vitamin K deficiency bleeding, which is the only accepted clinical sign of vitamin K deficiency, implies that typical intakes are adequate. Nevertheless, these guidelines recognize that there is insufficient evidence to publish a "dietary reference value" (DRV) for vitamin K intake based upon optimal protein carboxylation. Furthermore, the guidelines recognize that there is insufficient evidence to determine an AI or DRV for any menaquinones.

Table 2.3: Dietary sources of phylloquinone (PK), menaquinone-4 (MK-4) and other menaquinones (MK). "n.d." denotes that no data was available.

Food	Vitamin K content (ng g^{-1})			References
	PK	MK-4	Other MK	
Vegetables				
Wakame (seaweed)	12,930	n.d.	n.d.	[157]
Kale	6,180-8,170	n.d.	n.d.	[34], [158]
Parsley	3,600-5,480	n.d.	n.d.	[34], [159]
Spinach	2,700-5,750	n.d.	n.d.	[34], [157]–[161]
Laver (seaweed)	4,130	n.d.	n.d.	[157]
Cabbage	1,270-3,390	0-10	n.d.	[34], [157]
Broccoli, boiled	1,100-3,070	n.d.	n.d.	[34], [157]–[161]
Brussels sprouts	1,220-2,890	n.d.	n.d.	[34], [159]–[161]
Plant oils and fats				
Soybean oil	1,310-2,340	n.d.	n.d.	[34], [157], [161]–[163]
Vegetable oil, mixed	1,340-1,640	n.d.	10 (MK-7)	[157], [161]
Rapeseed oil, refined	920-1,500	n.d.	0-30 (MK-7)	[34], [157], [161]–[163]
Margarine	120-1,100	0-3	0-1	[157], [158], [160]–[162]
Olive oil	300-800	0-4	n.d.	[34], [157], [158], [161]–[163]
Fruits				
Kiwi fruit	343	n.d.	n.d.	[159]
Black currant	300	n.d.	n.d.	[159]
Avocado	10-200	n.d.	n.d.	[34], [159], [160]
Grapes, green	83-190	n.d.	n.d.	[34], [159]–[161]
Fermented products				[157], [158], [164], [165]
Natto	200-450	0-10	9,000-12,300 (MK-7)	
Cheese, Jarlsberg	60	84	652 (MK-9 (4H))	[166]
Cheese, Edam	19	33	442	[167]
Cheese, Emmental	52	84	269 (MK-9 (4H))	[166]
Animal products				
Chicken meat	0-20	89-600	0-0.1	[157], [158], [167]–[169]
Chicken liver	0-25	40-141	0-2	[168], [169]
Egg, whole	3-25	56-250	n.d.	[157], [164], [169]
Egg yolk	7-70	155-640	0-7	[157], [158], [169]
Egg white	n.d.	4-10	n.d.	[157], [158], [169]
Beef liver	18-58	7-8	73-1148	[167], [168]
Beef meat	3-7	11-150	0-6	[157], [158], [167], [168]

Requirements for vitamin K are strongly influenced by bioavailability, especially from plant-based foods. Humans cannot efficiently extract nutrients from plant matter [170] as they do not produce the cellulase enzymes required to digest tough plant material, nor do they harbor microorganisms that produce these enzymes [171]–[173]. The typical plant cell is surrounded by a tough cell wall, comprised of a strong cellulose fabric embedded within a cross-linked matrix of hemicelluloses, pectins, glycoproteins and, sometimes, lignins [174]. Complex polysaccharides are almost impossible to digest due to high resistance to hydrolysis by mammalian enzymes [175]. Therefore, only a small fraction of total phylloquinone content is extracted from plant foods in the human gut.

In comparisons of vitamin K preparations, the bioavailability of MK-4 from fat was 40-80%, while bioavailability of phylloquinone from boiled spinach was only 4-18% [176], [177]. Food preparation methods that improve digestibility may help to release vitamin K [178]. Eating organisms with weak or absent cell walls (e.g. cyanobacteria) and/or processing of the material to break down the walls (e.g. by milling and enzymatic treatment) would also improve bioavailability [170]. Problems with the secretion of bile salts or a lack of dietary lipids can also cause a reduction in the amount of vitamin K absorbed [149], [176], [179], [180]. In fact, oily foods have been shown to approximately double the absorption of phylloquinone from plant-based foods [176], [179].

2.2.3 Dietary intake of vitamin K in chickens

Animal nutrition is a 20 billion USD global industry, and vitamin K is extensively added to the diets of poultry, swine and companion animals [181]. Despite this, *Gallus gallus domesticus* (the domestic chicken) is the only animal species for which vitamin K requirements have been extensively investigated. This research stemmed from the discovery in the 1930s of a hemorrhagic disorder in chicks [94], [114], [182], [183]. Unsurprisingly, the largest single use of fat-soluble vitamin supplements, including vitamin K, is in fortification of chicken feed [184].

Modern manufactured chicken rations typically use menadione derivatives such as menadione nicotinamide bisulfite (MNB). Addition of these vitamin K analogs stems from research in the 1950s and 60s studying the prevention of vitamin K deficiency bleeding in chicks [185]–[190]. This research found that 0.5 mg menadione (or equimolar equivalent of menadione derivatives) per kg of finished diet was adequate to prevent vitamin K deficiency bleeding. This addition rate remains in use today [39]. However, menadione is not a natural ingredient in the diet of wild birds and this addition rate does not reflect the normal intake. Prior to the adoption of menadione, vitamin K came primarily from cheap leafy crops, such as alfalfa, that were included in the feed mix [39].

The diets of the Red Jungle Fowl (*Gallus gallus*) and its domestic cousin (*Gallus gallus domesticus*, aka the chicken) are omnivorous and considerably varied in the wild. A study of a feral population of *Gallus gallus domesticus* on Northwest Island off the coast of Gladstone in Queensland, Australia, observed that the birds ate leaves, figs, berries, seeds, grass, insects, slugs, isopods, and even the remains of other wild birds [191]. A British study attempted to replicate the conditions of these observations by releasing domestic fowl into the wild on an Island off the west coast of Scotland [192]. They observed that during summer, grass leaves made up over 80% of the diet with 5-10% made up of invertebrates. In contrast, the lack of grass available during the winter forced the birds to eat more roots, stems and leaves. This seasonal cycle was observed in chicks as well.

A more recent, much more detailed study of wild *Gallus gallus* (Red Jungle Fowl) in Malaysia found that the birds commonly ate a range of invertebrates (notably ants, termites, beetles, leeches), vertebrates (snakes, other birds) and plants (seeds, leaves, oil palm fruit) [193]. From the available diet studies it can be inferred that chickens, in the absence of dietary restrictions, will consume vitamin K primarily as phylloquinone. A bird eating a wild diet consisting of 20-80% grass or leaves with a phylloquinone concentration of 250-500 μg per 100 g, would have a

phylloquinone intake of 50-400 mg per kg feed. Plant-eating gastropods such as slugs and snails may also be a rich source of phylloquinone for *Gallus gallus*.

The vitamin K intake of a natural diet is vastly different to the manufactured diet. Not only does a wild diet provide vitamin K mainly as phylloquinone, the concentration is at least 4 times higher on a molar basis compared to the menadione in manufactured diets. Given that phylloquinone is known to be safer and more effective than menadione, it is likely that birds on the wild diet may experience some additional health benefits over their domesticated cousins. However, no comparisons of natural and manufactured diets were found in the open literature.

2.3 Production of vitamin K_1

2.1.1 Global vitamin K production

Vitamin K makes up approximately 5% of the current value of the global vitamin ingredients market [47]. This market was worth USD 5.36 billion in 2016 and is forecast to reach USD 8.19 billion by 2025 [46]. Animal feed (44%), pharmaceuticals (38%) and cosmetics (12%) are the primary end uses [48]. Applications, production methods and prices differ for each of the K vitamers and analogs (Table 2.4). The vitamin K analogs (salts of menadione bisulfite and menadiol diphosphate) are chemically synthesized. They are widely used in livestock and pet food (especially for poultry), which is surprising considering that they have been banned from human products for more than 50 years. Chemically synthesized vitamin K_1 (known as phytomenadione or phytonadione) is used for a wide range of human nutrition, pharmaceutical and cosmetic products. There is no commercial biosynthetic process for vitamin K_1. Vitamin K_2 may be chemically synthesized or biosynthesized, however it is expensive and not widely used. Chemically synthesized MK-4 is usually known as menatetrenone rather than menaquinone-4.

Table 2.4: Comparison of some commercially available compounds with vitamin K activity [194]–[197].

Compound	Primary application	Method of production	Price range (USD/kg)
Menadione sodium bisulfite	Animal food VK replacement	Reaction of sodium bisulfite salt with menadione	10-20
Menadiol sodium diphosphate	Animal food VK replacement	Reduction and phosphorylation of menadione	50-100
Phytonadione (vitamin K_1)	Human supplements, pharmaceuticals & infant formula	Friedel-Crafts alkylation of menadione with phytol	1,000-2,000
Menatetrenone (vitamin K_2/MK-4)	Human supplements	Friedel-Crafts alkylation of menadione with geranylgeraniol	2,000-3,000
Menaquinone-7 (vitamin K_2/MK-7)	Human supplements	Fermentation of plant-based substrate using *B. subtilis* OR Friedel-Crafts alkylation	200,000-300,000

2.3.1 Chemical synthesis of vitamin K_1

For pharmaceutical and food products, a synthetic version of vitamin K_1 is manufactured by condensing naphthoquinoid and isoprenoid precursors [195], [198]. Chemical synthesis begins with menadione, which is itself synthesized by oxidation of 2-methylnaphthalene [197]. The most widely known method was developed by Roche [199] (Figure 2.5) and entails condensation of menadione with isophytol [200]. First, to prevent undesirable reactions during the condensation step, the menadione is reduced under acidic conditions and esterified at C1 [201] (commonly with benzoate [197]). The condensation of the menadiol monoester with phytol is then catalyzed with heat and acid (commonly a Lewis acid such as boron trifluoride etherate) [197], [200]. The product is then de-protected by saponification (traditionally with potassium hydroxide and methanol [200]) and oxidized (with a catalyst such as silver oxide [199] or in air [197]) to form vitamin K_1.

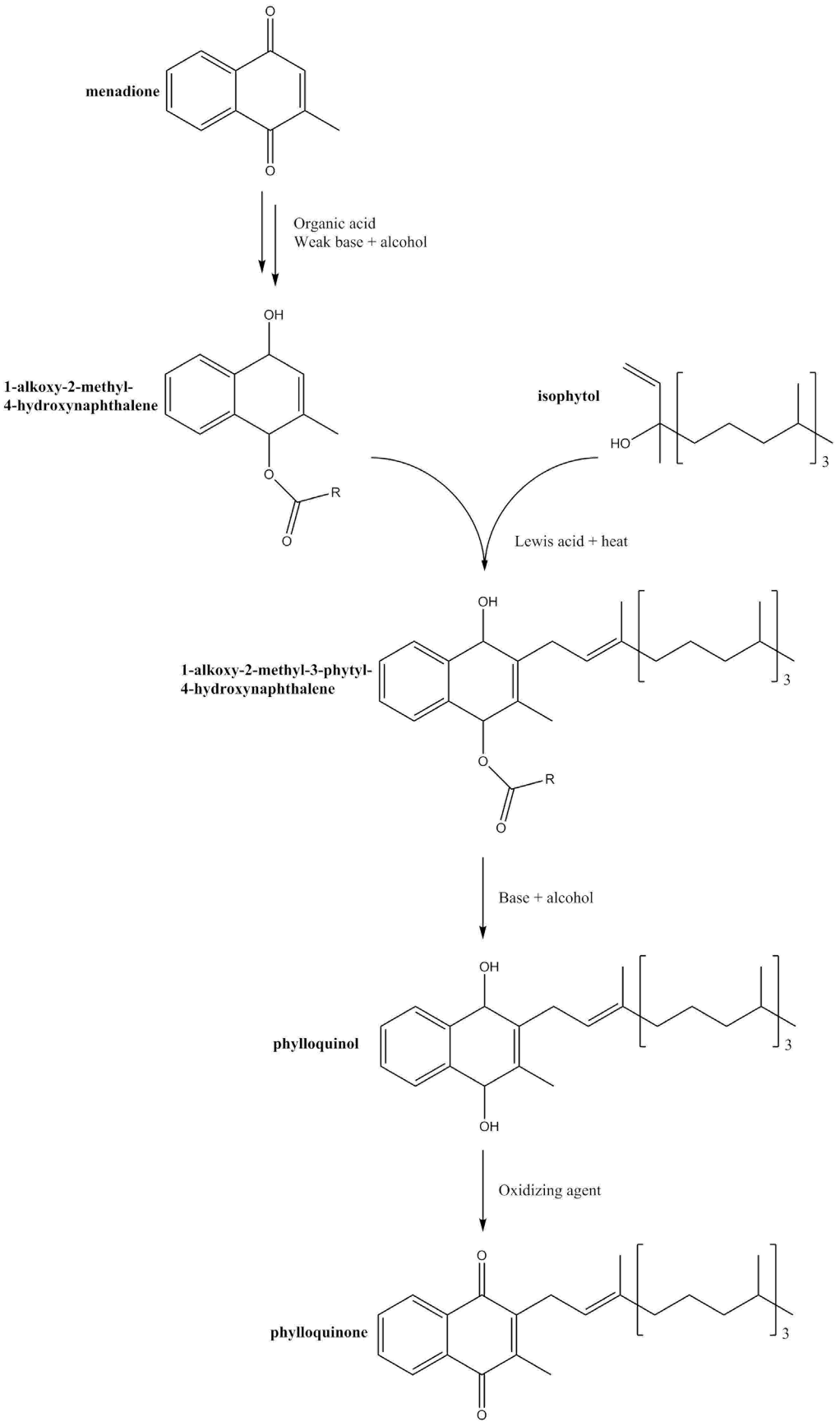


Figure 2.5: Scheme of industrial synthesis of vitamin K_1 [197]. Double arrows denote multiple steps, bold text denotes chemical compound, regular text denotes reagents and catalysts.

The commercial process for the chemical synthesis of vitamin K_1 has remained mostly unchanged since it was published in 1939 [68]. This is primarily due to the low cost and ready availability of raw materials and reagents. This process has two notable drawbacks. The first is that the condensation of menadione with phytol results in formation of 10-20% of the inactive *Z*-isomer [195]. Chemically synthesized vitamin K_1 is therefore a racemic mixture, and is often referred to as phytonadione or phytomenadione. The second drawback is that the catalysts and solvents are hazardous to both humans and the environment, necessitating costly treatment [50].

Research on chemical synthesis of vitamin K_1 has traditionally focused on improving the stereoselectivity of the phytylation step, as formation of the inactive *Z*-isomer reduces yields and the activity of the final product. Efforts from the 70s, 80s and 90s have been reviewed [195], and there has been little research into chemical synthesis of vitamin K_1 since then. These efforts can be summarized into three main categories: (1) condensation of protected naphthoquinone with phytyl where one moiety is organometallic (organocuprates, organolithiums or Grignard reagents) and the other a halide; (2) condensation of menadione directly with phytyl where one moiety is a silane or stannane and the other a halide; (3) condensation of menadione or protected naphthoquinone with a short-chain isoprenoid, followed by a secondary side-chain alkylation, using methods from category (1) or (2).

There is limited evidence of successful attempts to reduce or eliminate the use of toxic chemicals in vitamin K_1 synthesis. The method of Coman et al. (2010) employed heterogeneous, nanoscopic metal fluoride catalysts to reduce the amount of catalyst required to 1 g per 6.5 g vitamin K_1 [198]. A 2016 patent, by Tien et al. of Sunny Pharmatec Inc., claims that the replacement of metal catalysts with amines can reduce the environmental and health dangers of vitamin K_1 synthesis [202]. However, the proposed amine catalysts include 4-dimethylaminopyridine (DMAP), triethylamine (TEA) and pyridine, which are all hazardous chemicals. Processes based on enzymatic reactions are a "greener" alternative, as they are highly selective and proceed under mild conditions with reduced consumption of organic solvents

and other toxic reagents [50]. Although the biosynthetic pathway of phylloquinone is known, enzymatic chemical synthesis of vitamin K_1 has not yet been investigated.

2.3.2 Biosynthesis of vitamin K_1

No biotechnological process for vitamin K_1 has been proposed or commercialized, however bacterial biosynthesis of vitamin K_2 is already a commercial reality [203], [204]. Some bacteria of genus *Flavobacterium* synthesize menaquinones with side-chains as short as 5 isoprene units (MK-5), while strains of *Bacteroides oralis* and *Bacteroides ruminicola* produce side-chains of up to 14 isoprene units (MK-14) [205]. The production of menaquinones by liquid and solid state fermentation has been previously reviewed in detail [196]. The lack of interest in biotechnological production of vitamin K_1 could be due to the fact that phylloquinone is regarded as the least abundant of the terpenoid quinones [206] based on research from higher plants. Nevertheless, it is possible that there are higher phylloquinone concentrations in other photosynthetic organisms, such as algae.

Aquatic species are an attractive prospect for biosynthesis of vitamin K as many have been reported to have much higher phylloquinone concentrations than terrestrial plants. The phylloquinone content of macroalgae (seaweed) varies considerably, from 24.4 μg/100g dry mass in kelp [207] to the incredible figure of 75,000 μg/100g dry matter in *Sargassum muticum* [208]. The best candidates for biosynthesis of phylloquinone are microalgae because they have many industrially useful characteristics: high surface area to volume-ratio; high growth rates; diverse metabolic pathways; environmental adaptability, and; simplicity of screening and genetic manipulation [209]. Reported phylloquinone concentrations in microalgae also vary, from undetectable levels in the diatom *Chaetoceros calcitrans* to 2,800 μg/100 g in the green microalga *Tetraselmis suecica* [208].

The microalgal production of carotenoids has been successful for over 30 years, and provides some clues as to how production of phylloquinone might be achieved [210]. Carotenoids, such as β-carotene and astaxanthin, are a diverse group

of isoprenoids which are produced industrially using halotolerant microalgae [210], [211]. Establishment of the industry began with identification of the most carotenoid rich species [210]. Growth conditions, including climate and nutrients, were optimized to maximize productivity and process robustness. More recently, research has aimed to further improve the productivity of these "cellular factories" by genetic modifications [212], [213].

When formulating a potential biosynthetic process, it is important to understand why plants and algae contain vitamin K_1 (phylloquinone). As shown in Figure 2.6, phylloquinone is intimately associated with the photosynthetic apparatus; specifically photosystem I (PSI) [214]. Along with photosystem II (PSII), PSI is responsible for harvesting energy from light to facilitate electron transport. It is the reducing power of these electrons that enable all photosynthetic processes, including carbon fixation and oxygen evolution. Phylloquinone functions primarily as an electron acceptor from chlorophyll-*a* (chl-*a*) in Photosystem I (PSI) [85], [215], [216]. However, phylloquinone can also act as an electron acceptor during the formation of protein disulfide bonds in the thylakoid membrane [217], [218].

There are many strategies that can be used to increase the productivity of a biotechnological system. Selection of a highly productive organism (strain screening) is the simplest and usually the first step. Optimization of environmental conditions (such as light and nutrition) is more difficult but nevertheless essential for maximizing productivity. Metabolic (genetic) engineering is a much more complex strategy for increasing productivity. In many jurisdictions, the legal framework makes it difficult or impossible to engage in any genetic manipulation of microorganisms; however, it can be incredibly effective [219].

Strain screening

The simplest method of achieving a high phylloquinone yield is strain screening. The field of likely candidates can be narrowed by understanding phylogenetic differences in photosynthesis. Organisms that have a high PSI content can be used as a starting point for screening; other desirable traits may include a high growth rate,

salt or pH tolerance (favorable for preventing contamination) and a weak cell wall (which may be favorable for the extraction of the phylloquinone).

Light

Light is widely acknowledged as the most important factor affecting microalgal growth [220] and the productivity of an industrial microalgal system [170]. During light harvesting, electrons from PSII are transferred to PSI. It is important that the flux of electrons from PSII matches the demand from PSI to prevent harmful accumulation of reductants [221]. Regulation of this electron flux is primarily achieved by changing the amount of PSI or the size of the PSII light harvesting antenna [221].

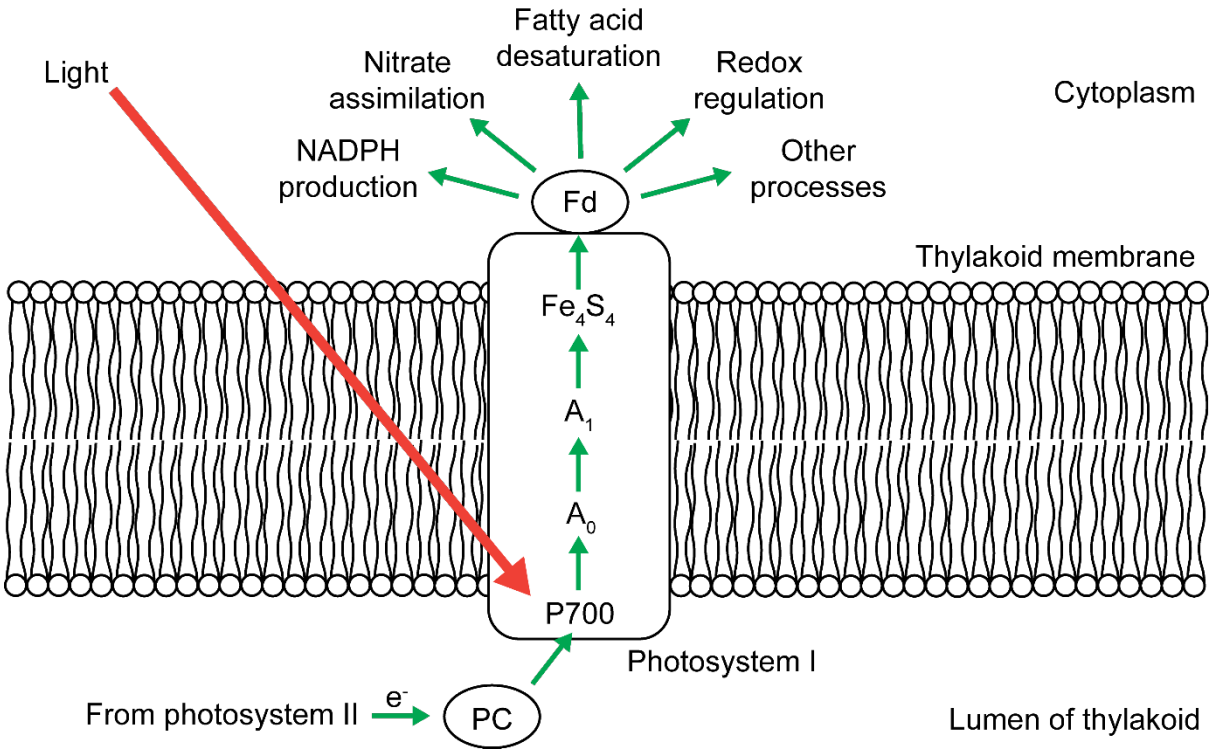


Figure 2.6: Electron transport in photosystem I. Primary inputs and outputs in bold, electron flux in green, photon flux in red. Acronyms: Plastocyanin (PC), modified antenna chlorophyll (P700), modified electron transport chlorophyll (A0), phylloquinone (A1), iron-sulfur protein complex (Fe_4S_4), ferredoxin protein (Fd) [221].

Photosynthetically Active Radiation (PAR) includes electromagnetic wavelengths from 400 to 700 nm (violet to red). The PAR intensity is usually expressed as irradiance in W m^{-2}. Total solar irradiance averages 1000 W m^{-2} at sea level, however only around 45% of this is in the wavelength range 400-700 nm, and irradiance depends upon atmospheric attenuation and latitude [222]. On average over the year, tropical PAR is steady at around 400 W m^{-2}, while it ranges between 0 and 550 W m^{-2} with an average less than 200 W m^{-2} inside the Arctic and Antarctic circles. The intensity of photosynthetically active light can also be expressed as the Photosynthetic Photon Flux Density (PPFD) in photon μmol m^{-2} s^{-1}, where 1 W m^{-2} is approximately 4.57 μmol m^{-2} s^{-1} [222].

At low light intensities (PPFD < 100 μmol m^{-2} s^{-1}), light-dependent chemical reactions (driven mainly by reductants from PSII) are the rate limiting step in photosynthesis. An increase in the rate of light-dependent reactions in response to higher light intensity necessitates an increase in PSII/PSI ratio [220]. In a long term process (hours to days) known as photoacclimation [223], cells will reduce the number of PSI to ensure that electron flux is balanced [221]. At very high light intensities (typically PPFD 500-1000 μmol m^{-2} s^{-1} [220], [223], [224]), PSII functionality is impaired and protective pigments are produced to prevent harmful photo-oxidation [224], [225]. This protective response is accompanied by a decrease in photosynthesis, a phenomenon known as photoinhibition. Low light intensity is favorable for PSI (and thus phylloquinone) synthesis.

Plants and algae harvest light at different wavelengths using different pigments, so the spectral power distribution also affects the light response in a phenomenon known as chromatic adaptation [223]. Cyanobacteria and rhodophytes contain light harvesting phycobilins, which are sensitive to spectral intensity distribution. Phycocyanin and phycoerythrin harvest in the green-red and blue-green portions of the visible spectrum, respectively. Shifting the spectral distribution towards shorter wavelengths will favor an increase in phycocyanin, while shifting toward longer wavelengths will favor phycoerythrin [224]. Of special relevance is

the fact that most photosynthetic organisms are sensitive to changes in the red portion of the spectrum. Specifically, the PSII red light absorption maximum is at a wavelength of approximately 680 nm, while the PSI maximum is at around 700 nm. Shifting the spectral distribution towards shorter wavelengths will induce an increase in number of PSI during photoacclimation [220], [221], favoring phylloquinone synthesis.

Temperature

Temperature also has an important role in microalgal growth. Temperatures below the optimum reduce the ability of the organism to photosynthesize, and therefore induce a response similar to high light [226]. Temperatures above the optimum induce a similar response to low light [226]. Generally, a 10 ˚C increase in temperature will double the rate of photosynthesis (though each organism has an optimum temperature, above which growth declines) [226]. Furthermore, photosynthesis proceeds optimally at a higher temperature and lower light compared to cell growth [223], [226]. Data on microalgal species commonly used in research and industry reveal that marine microalgae grow fastest between 20 and 25 ˚C [227], [228], while freshwater species typically grow fastest between 25 and 35 ˚C [229], [230].

Nutrient supply

Nutrient optimization is another strategy that can be employed to maximize productivity of microalgal systems. Photosystem I, which includes phylloquinone, ultimately transfers electrons to ferredoxin; reduced ferredoxin in turn drives many processes including NADPH generation, nitrate fixation, and fatty acid desaturation [221]. The most important nutrients for photosynthesis are nitrogen, phosphorus, and iron [170]. Nitrogen is an essential ingredient in all proteins, including biosynthetic enzymes, and accounts for 7-10% of algal dry cell weight (DCW). Phosphorus is required for many components of the cell, including DNA and thylakoid membrane phospholipids, and makes up around 1% of DCW. Iron is found only at trace levels in algal cells (0.2-34 mg/g DCW) [231] however it is

involved in electron transport and chlorophyll synthesis [231], [232]. Limitation of any one of these three nutrients will inhibit photosynthetic activity and growth rate [232]–[236] in most algae. Many cyanobacteria are able to overcome nitrogen limitation by fixing atmospheric nitrogen [237], however this activity is iron-dependent [232]. Ample supply of nitrogen, phosphorus and iron are required for an optimal yield of phylloquinone.

Metabolic engineering

Metabolic engineering could be used to increase the output of the phylloquinone biosynthetic pathway. Engineering efforts usually begin by identifying rate-influencing steps in the synthetic pathway [238]. The biosynthetic pathway of phylloquinone, as shown in Figure 2.7, is similar to that of menaquinones in bacteria [239] and has recently been reviewed in detail [214]. In this process, synthesis of the phytyl side-chain begins with isopentenyl pyrophosphate (IPP) from the methylerythritol (MEP) pathway [240]–[242], which is converted to dimethylallyl pyrophosphate (DMAPP) by the isopentenyl pyrophosphate isomerase (IPPI) enzyme [243]. Production of geranylgeranyl pyrophosphate (GGPP) from 3 IPP molecules and DMAPP is achieved in one step by geranylgeranyl pyrophosphate synthase (GGPPS) or in two sequential steps by geranyl pyrophosphate synthase (GPPS) [243]. Finally, three consecutive double bonds are reduced by geranyl pyrophosphate reductase (GGPR) in three sequential steps to yield phytyl pyrophosphate (phytyl-PP) [244].

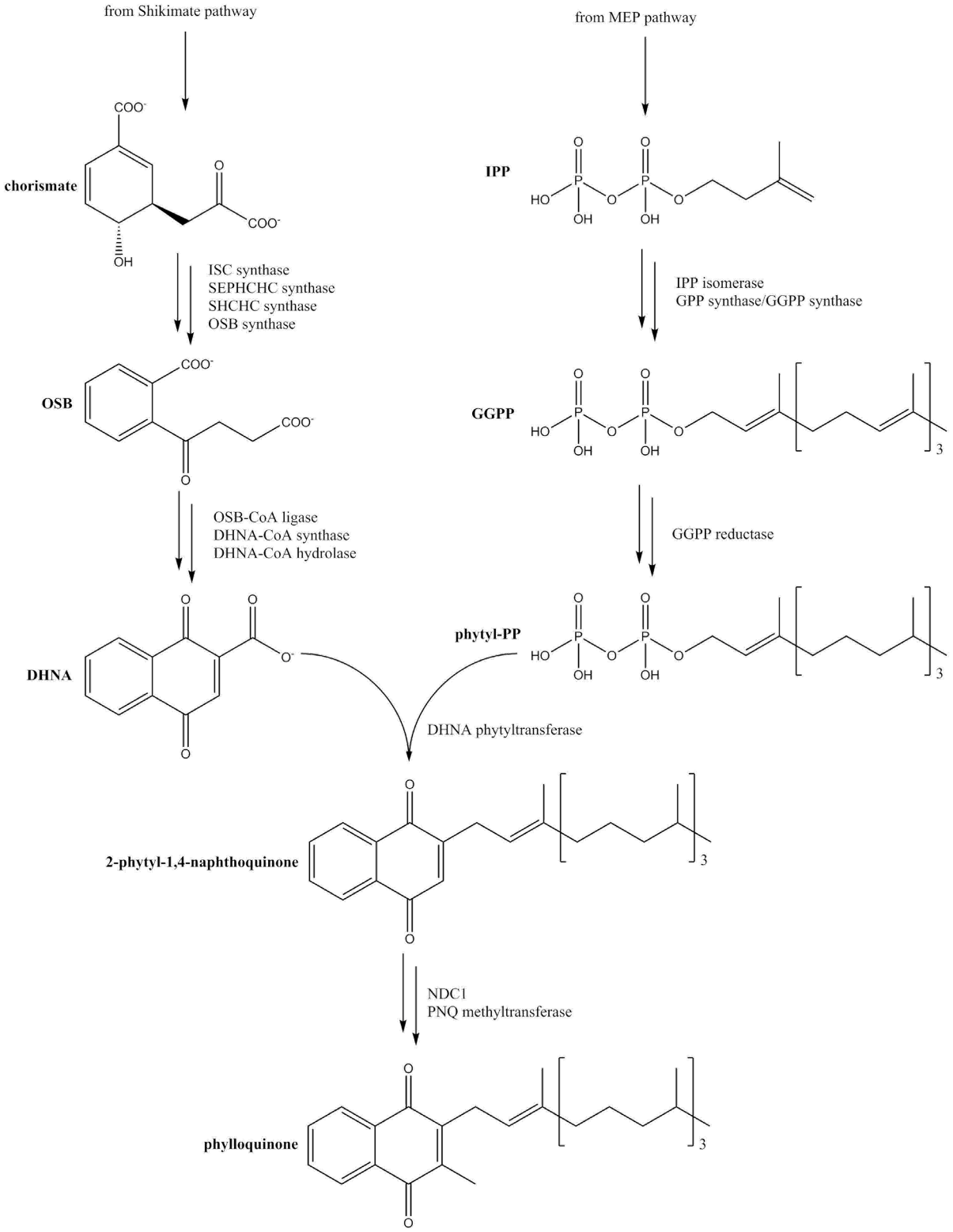


Figure 2.7: The biosynthetic pathway of phylloquinone. Only structures of the key intermediates are shown. Double arrows denote multiple steps, bold text denotes chemical compound, regular text denotes biosynthetic enzymes.

Synthesis of the naphthoquinone backbone begins with chorismate from the shikimate pathway in a process analogous to the bacterial process of menaquinone synthesis [245]–[247]. Chorismate is converted to *o*-succinyl-benzoate (OSB) via intermediates isochorismate (ISC), 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid (SEPHCHC) [248] and 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) [249]. The OSB molecule is converted to 1,4-dihydroxynaphthoate (DHNA) by esterification with coenzyme A (CoA), cyclization to produce DHNA-CoA and hydrolysis to remove CoA [250]. The DHNA and phytyl-PP are condensed to yield 2-phytyl-1,4-naphthoquinone (PNQ) [246]. The PNQ molecule is reduced by NADPH Dehydrogenase C1 (NDC1) [251] and methylated [252] to yield phylloquinol, which spontaneously oxidizes to yield phylloquinone [251].

Gene knockout or overexpression are powerful tools for identifying rate influencing genes, however simpler methods such as identifying mutants or supplementation with key precursor compounds [238] could also be used to determine the rate-limiting steps in the phylloquinone biosynthetic pathway. While there is relatively little literature specifically related to phylloquinone, many authors have examined genetic modification for the production of similar compounds such as ubiquinones [253], [254] and menaquinones [255].

Genetic manipulation can increase the synthesis of precursors, divert precursors to preferred biosynthetic pathways, prevent expression of genes encoding inhibitory enzymes and increase the storage capacity for the target compounds. Reviews of the topic suggest that targeting the genes of the biosynthetic pathway, especially the committing enzyme gene, is the most reliable method for boosting yield [213], [256], [257]. Typical approaches involve increasing the flux through the MEP pathway by overexpressing genes such as DXS and also diverting chorismate towards quinone pathways by overexpressing genes encoding biosynthetic enzymes [253].

Tools for the genetic modification of cyanobacteria and algae are not yet as well developed as those for other microorganisms (e.g. *Escherichia coli* or *Saccharomyces cerevisiae*). However, there have been many recent advances in this area (as reviewed elsewhere [258]–[260]). Cyanobacteria may be favorable hosts compared to eukaryotic species as their (relatively) simpler structure may mean the metabolic engineering process is less complex. Indeed, many authors have examined the use of engineered cyanobacteria for the production of a range of isoprenoids [256], [261], [262]. Metabolic engineering and synthetic biology offer promising approaches to the biosynthetic production of phylloquinone.

2.3.3 Culture systems for microalgae

In order to produce phylloquinone commercially using microalgae, an efficient culture system is required. Microalgal cultures are extremely diffuse, usually less than 10 g of solids per liter of liquid, so large culture volumes are required. Microalgal culture systems are designed to optimize the availability of light, carbon dioxide and dissolved inorganic compounds (such as nitrate and phosphate) that are essential for the rapid growth of microalgae. Ponds are simple to construct though they achieve very low culture densities (approximately 0.5 g per liter), sub-optimal irradiance, and are prone to contamination [263]–[265]. Most of the global supply of microalgal biomass is produced in open ponds, however closed Photo-Bioreactors (PBRs) are also used [263], [266]. Closed PBRs eliminate the problem of contamination, achieve culture densities of 2-10 g per liter, and use land much more efficiently. The extra expense of bioreactors is justified if the product is sufficiently valuable [263]–[265].

There are many types of photo-bioreactors and they are usually distinguished by their geometry. Common shapes include flat plate, tubular and bag reactors [263]. Reactors can also be distinguished by their mode of mixing, such as stirred, paddle-wheel or airlift [263]. The most commonly used photobioreactors in industry are the bubble column, raceway pond, flat panel and tubular (Figure 2.8) [170], [267]. Each

reactor has its advantages and disadvantages, meaning that each is used in specific circumstances. Most of the world's algal biomass is grown in raceway ponds (Figure 2.8(b)), which are simply long narrow ponds of algae culture mixed by paddle wheels [268]. At much less than 1000 USD per cubic meter of culture volume, raceways are by far the cheapest reactors on a volumetric basis [269], [270]. However, open ponds are inappropriate for many algal species and applications where more controlled conditions and/or a high degree of cleanliness is required.

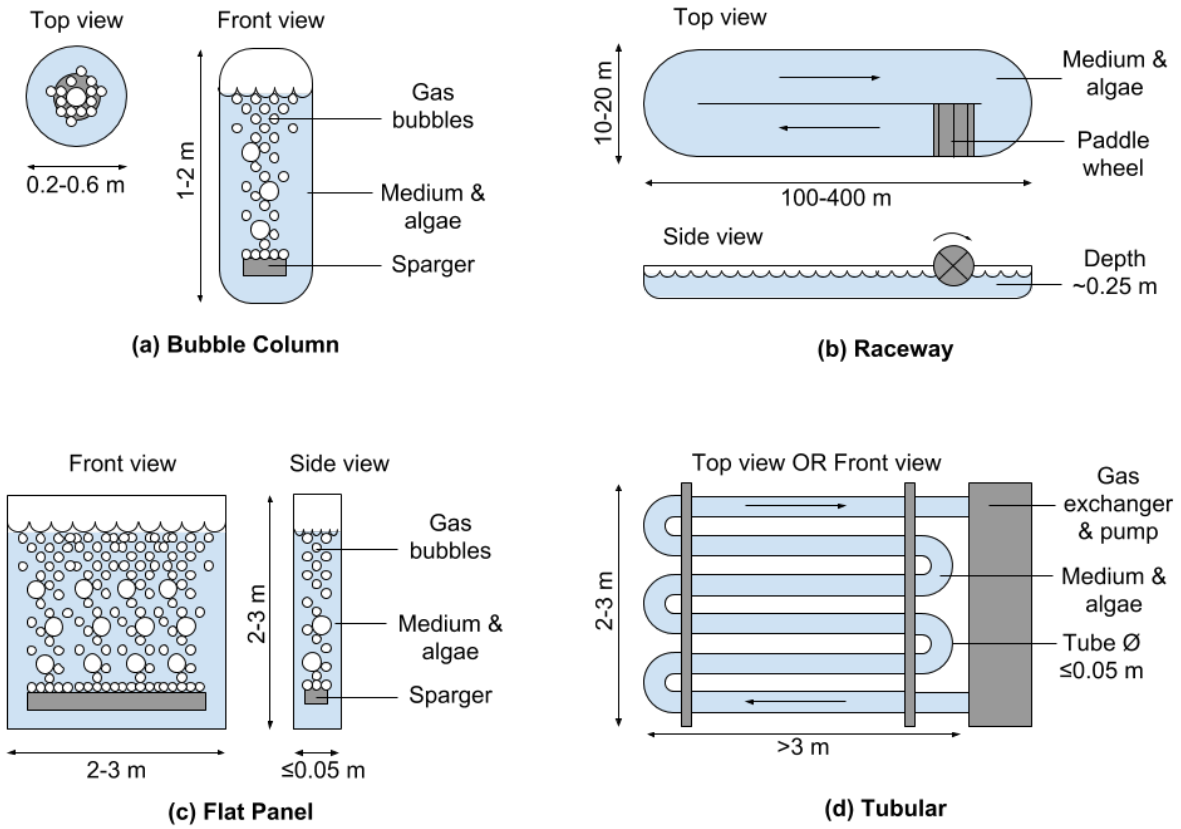


Figure 2.8: Diagrams of the most commonly used algal culture bioreactors. The top and/or front views face toward the light source.

The flat panel (Figure 2.8(c)) and tubular (Figure 2.8(d)) closed photobioreactors are the most commonly used types where cleanliness and the highest possible volumetric productivities are required [271]. The light path length through the algae culture in these reactors is seldom more than 5 cm, which minimizes shading. A flat panel reactor consists of a large, rectangular prism enclosing a narrow space [272]. The algae culture is grown within this space and mixing is provided by air bubbles, which also ensure that appropriate dissolved oxygen and carbon dioxide levels are maintained [272]. A tubular photobioreactor consists of tubes that are tens of meters long but only around 5 cm in diameter [273]. The algae culture is pumped from a gas exchanger through these tube manifold to maximize light exposure, then back to the gas exchanger where excess oxygen is removed and carbon dioxide is injected [273]. Flat panel and tubular reactors typically cost 5,000 to 10,000 USD per cubic meter of reactor volume [270], [273].

Bubble column reactors

Bubble columns (Figure 2.8(a)) are much cheaper and simpler closed alternatives to flat panels and tubular reactors [170]. A bubble column consists of a tall, usually cylindrical, vessel of 20 to 50 cm in diameter in which the algae is grown [274]. Like the flat panel, mixing and gas exchange is provided by air bubbles [274]. The simplest bubble column vessels are single-use sterile plastic bags that can be hung from a rack or installed in a mesh enclosure [170]. For this reason, bubble columns are the best closed reactors when low cost and flexibility of scale are more important than volumetric productivity. Nevertheless, the volumetric productivities of bubble columns are much closer to flat panel and tubular reactors than raceway ponds [275], and there is ample evidence of this from the last 20 years (Table 2.5). Bubble columns are therefore the most appropriate closed photobioreactors for the kind of prospective research proposed here.

Table 2.5: Summary of research into algal culture in bubble column photobioreactors. When no data was available the result was denoted n.d. * Values estimated for linear photoautotrophic growth regions of results in reference.

Species	Product	Column volume (L)	Column diameter (cm)	Superficial gas velocity (cm s^{-1})	PPFD (μmol m^{-2} s^{-1})	Photo-period (hours)	Max. DCW (g L^{-1})*	DCW Prod. (g L^{-1} d^{-1})*	Ref.
Botryococcus braunii	Hydro-carbons	0.8	7	0.17	150	n.d.	6.5	0.5	**[276]**
Agardhiella subulata	Bioactives	0.25	4.5	0.1	38	16:08	14.1	0.043	**[277]**
Phaeodactylum tricornutum	Eicosapenta-enoic acid	60	19	1	~ 1150	14:10	3.5	0.3	**[278]**
Haematococcus pluvialis	Astaxanthin	10	11	0.35	140	n.d.	2.6	0.25	**[279]**
Haematococcus pluvialis	Astaxanthin	1.6	6.9	0.23	48.5	n.d.	5	0.48	**[280]**
Anabaena variablis	Hydrogen	0.33	2.6	2	110	n.d.	4	0.67	**[281]**
Aphanothece microscopica Nageli	Biomass	3	7.5	1.1	150	12:12	3	1	**[282]**
Phaeodactylum tricornutum	Eicosapenta-enoic acid	60	19	1	~ 1000	14:10	4	0.48	**[283]**
Scenedesmus almeriensis	Lutein	2	6	0.59	1700	12:12	3	0.7	**[284]**
Anabaena sp.	Biomass	9	12	0.27	900	12:12	1	0.31	**[285]**
Chlorella sp.	Lipids for biofuel	2	10	1.9	37	n.d.	n.d.	0.11	**[286]**
Nannochloropsis oculata	Biomass	25	14	0.16	~ 750	12:12	0.36	0.051	**[287]**
Chlorella sorokiniana	Biomass	1.6	7	0.24	100	n.d.	4	0.5	**[288]**
Eustigmatophyceae spp.	β-carotene	0.42	3	4.7	300	n.d.	6	1	**[289]**

Bubble-column reactors are used in many chemical processes, primarily because of their excellent heat and mass transfer coefficients, minimal maintenance requirement, and low operating cost [274], [290], [291]. The main design considerations for bubble column reactors are the column aspect ratio (usually height >5 × diameter) and the sparger geometry (perforated plate, ring, arm) [274], [290], [291]. It is also important to consider at what gas flow, pressure, and temperature the column will be operated [274], [290], [291]. The primary parameters used to characterize the hydrodynamic state of the column are superficial gas velocity and column diameter, from which the bubble flow regime can be estimated [13]–[15]. Bubble flow regime is important because it affects the mixing, heat and mass transfer behavior of the system. Gas holdup and bubble size are also important for predicting mass transfer based upon the gas-liquid interfacial area [13]–[15].

The key considerations in BC-PBRs are slightly different to typical industrial BCRs because the bubble flow and column diameter greatly affects the irradiance of the algal culture. Specifically, high mixing rates between the walls and centre of the column are important to prevent light limitation of the culture, while bubbles can also reduce the transmission of light when the source is at an oblique angle to the reactor surface [275], [292]. High shear stress due to high air flow rates can also damage or destroy algal cells, however the shear tolerance is different for every species [27], [28]. On the other hand, gas-liquid mass transfer is just as important in bubble column PBRs as in conventional bubble column reactors because oxygen build-up and carbon dioxide deficiency can severely limit growth [275], [292].

2.3.4 Recovery of vitamin K_1 from biomass

Break-down or disruption of the cell wall

The first step in recovery of chemicals from biomass is usually to break down the microstructure of the raw material. This is necessary to reduce the particle size and to degrade the tough cell wall that surrounds the cells of most photosynthetic organisms [170]. The breakdown is most commonly achieved by mechanical treatment (grinding and milling), hydrolysis with chemicals (enzymes, acids and bases) or with high pressure saturated steam [293]. Other methods that have been tested for the disruption of microalgal cell wall include high pressure homogenization (HPH), high speed homogenization (HSH), ultrasound, microwave, pulsed electric field (PEF) [294]. Due to the instability of phylloquinone in acidic and alkaline solutions, digestion using acid or base is not likely to be a viable approach. Ultrasound, microwave, PEF and HSH have high specific energy consumption, which is a drawback for scale-up [294]. The high temperatures required for pressurized saturated steam treatment may not affect the phylloquinone yield [163], [295] but they will degrade other valuable bioactive molecules. This processing step may be eliminated or simplified by selecting an organism with weak or absent cell walls, such as a cyanobacterium [170].

Extraction of the intracellular components

The second step is extraction from the processed biomass. A search of the scientific literature and patent databases revealed few examples of industrial phylloquinone extraction processes. Patents filed in the 80s and 90s describe the extraction of phylloquinone from vegetable oil processing waste (the deodorizing distillate fraction) by esterification of fatty acids, followed by anion exchange resin separation [296], [297], molecular distillation [297] or solid-phase extraction on activated carbon [298]. These methods are useful for concentrating and purifying phylloquinone from oily or alcoholic solutions; however they are not designed for direct extraction from biomass.

It is important that new extraction processes should be designed to be sustainable, encompassing ideas from green chemistry including reducing waste generation, eliminating hazardous materials and minimizing energy consumption [299]. A comparison of "green" extraction solvents reveals that carbon dioxide (CO_2) has the best combination of properties for extraction of vitamin K – it readily dissolves non-polar compounds and is less hazardous than the alternatives [300]. The main drawbacks are the technical complexity of the processing equipment and the associated cost. The most promising "green" extraction methods are supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) [301]. Carbon dioxide can be used in SFE and PLE methods, making them preferable to microwave and ultrasound methods. Methods like SFE and MAE typically utilize organic solvents, are difficult to scale up and are, as previously stated, energy inefficient. Pressurized carbon dioxide can be used to extract lipophilic compounds from a range of biomasses, including plants and algae [301], [302].

A good example of CO_2-SFE is the recovery of vitamin E (*RRR*-α-tocopherol) from plants and algae. Phylloquinone and *RRR*-α-tocopherol have similar molecular weights, are lipophilic terpenoid compounds found in the chloroplasts of plants [303], and both exist as pale yellow viscous liquids at ambient temperature and pressure [197], [304]. A number of papers have been published on recovery of vitamin E from oil and solid biomass using SC-CO_2 [305]–[308] and these methods have been shown to work equally well for extraction of vitamin K (unpublished data).

2.4 Summary

Vitamin K is an essential nutrient involved in a range of physiological processes including ensuring proper function of blood coagulation, improving bone density and reducing arterial calcification, among others. Of the K vitamers, phylloquinone (Vitamin K_1) is the most common dietary source for humans and animals such as

chickens; additionally it is the only form used therapeutically. Although the benefits of K vitamers have been demonstrated in humans, it is unclear what benefits they confer in animals, especially in comparison to the industry standard menadione derivatives MNB and MDP. It is possible that natural K vitamers may be superior to menadione, with important benefits for animal welfare and consumer health.

Biotechnological production of vitamin K offers several advantages over the chemical synthesis: improved stereoselectivity; a 'natural' product preferred by consumers; the benign synthesis conditions; and, the use of renewable feedstocks. Biotechnological processes for menaquinones have been demonstrated, and industrial biosynthesis of menaquinones-7 is common. However, no such process has been demonstrated for phylloquinone. Phylloquinone is a key part of the electron transport chain in photosynthetic organisms. Hence it may be possible to produce phylloquinone via extraction from green waste or growth of photosynthetic microorganisms. The biotechnological production of phylloquinone offers a promising route to reducing the environmental costs of including vitamin K_1 in supplements and fortified foods.

3 The immune benefits of dietary vitamin K for chickens: a comparison of supplements

This chapter investigates the effects of different forms of vitamin K on the avian immune system. Some background is provided on the potential effects of vitamin K on white blood cells in chickens. The experimental methods for testing the effects of menadione, vitamin K_1 and vitamin K_2 on chicken white blood cells are presented. The results from both *in vitro* and *in vivo* experiments are discussed. Finally, the key points of the chapter are summarized.

3.1 Background

Commercial chicken feed is one of the single largest uses of vitamin K supplements. Despite evidence of toxicity in humans, menadione derivatives such as MNB are the industry standard in commercial chicken feed. Vitamin K is known to beneficially modulate the cell-mediated immune response by protein-dependent mechanisms [108], [130], [131], while menadione derivatives have been shown to directly suppress the inflammatory response [309], [310]. It is possible that menadione derivatives are detrimental for the immune system. Numerous papers have examined the behavior of menadione in protein thiol oxidation, impairment of calcium homeostasis and alteration of cell surface morphology [14]–[16]. This sparked interest in the use of menadione derivatives as redox-dependent tumor suppressants [311], [312], as they were found to suppress proliferation and up-regulate apoptosis.

Commercial chicken flocks are frequently exposed to factors that stress the immune system, from climatic and weather conditions (free-range birds) to frustration and behavioral restrictions (cage- and barn-raised birds) [313]. During these times, vitamin requirements are elevated [7], [8]. Numerous studies have observed marked immune responses in stressed birds [314], [315]. The primary immune cells found in avian blood are lymphocytes, thrombocytes, monocytes and

heterophils [316]. An elevated heterophil to lymphocyte ratio is the most obvious indicator of an immune response. Latimer et al. reported that the heterophil count, followed by the monocyte count, increased 3-4 times after an inflammatory injury [315]. In contrast, lymphocyte counts decreased immediately following the injury. In recent years, more specific markers of immune response, such as pro-inflammatory cytokines, have been preferred for probing the immune system [309], [310], [317].

The immune effects of different K vitamers and menadione derivatives have never been compared in the same experiment. Such a comparison could reveal great differences in the relative benefits of these supplements. Optimizing vitamin K supplementation has the potential to improve flock welfare and reduce the use of antibiotics. The aim of this experiment was therefore to examine the effects of menadione, phylloquinone and menaquinone-7 on inflammatory response in chickens. Specifically, to determine the effects of these compounds upon production of pro-inflammatory cytokines IL-1β and IL-6 by the WBCs.

Flow cytometry offers a quick and powerful method for analysis of cell suspensions, including blood samples. It can be used to distinguish between and assay characteristics of white blood cells (WBC), the immune cells found in blood. It can also be used to assay pro-inflammatory markers (such as the cytokines IL-1β and IL-6) using fluorescent staining. A paper published in 2010 by De Boever et al. describes a robust method for flow cytometric immunophenotyping of avian WBC [317]. Other research, such as in Bohls et al. from 2006 [318], provide additional information on flow cytometric analysis of avian leukocytes.

3.2 Method and Materials

3.2.1 Sample size calculation

The *Z*-statistic was calculated to test the difference between predicted means and standard deviations of the values of the control and test treatments. This test was used to determine the number of animals required to observe a statistically significant difference between treatments. If we assume that the number of birds in each group (n) and the standard deviations of the values in each group (σ) are equal then the required number of animals in each group is given by the following equation [319]:

$$n = \frac{2\sigma^2(z_{\alpha/2} + z_\beta)^2}{(\mu_{test} - \mu_{cont.})^2} \quad (3.1)$$

Where α is the false positive (type I error) rate and β is the false negative (type II error) rate. The *Z*-statistic for the type I error ($z_{\alpha/2}$) is set at 1.96 for a 5% level of significance and the *Z*-statistic for the type II error (z_β) is set at 0.84 at a power level of 80%:

$$n = \frac{2\sigma^2(1.96 + 0.84)^2}{(\mu_{test} - \mu_{cont.})^2} = 15.68\left(\frac{\sigma}{\mu_{test} - \mu_{cont.}}\right)^2 \quad (3.2)$$

From Checker et al. [309], we can predict a mean difference in IL-6 concentration of over 40% in response to menadione at 1 μmol L^{-1}:

$$\frac{\mu_{test} - \mu_{cont.}}{\mu_{cont.}} \geq 0.4 \quad (3.3)$$

Coefficients of variation were less than 20% in Checker et al. [309]:

$$\frac{\sigma}{\mu_{cont.}} \leq 0.2 \tag{3.4}$$

Substituting equations (3.3) and (3.4) into equation (3.2), we have:

$$n \geq 15.68\left(\frac{0.2}{0.4}\right)^2 = 3.92 \tag{3.5}$$

Therefore at least 4 birds per treatment group are required to observe a significant difference in inflammatory response with the control group. Allowing for potential attrition, 6 birds were assigned to each treatment group.

3.2.2 Animals, diet and sample collection

The project was approved by the University of Sydney Animal Ethics Committee (AEC). The project number was 2015/769 (see approval letter in Appendix A). Twenty-four Rhode Island Red x Australorp hens were separated into 4 groups during the 6-week *in vivo* diet study. Each group was kept in a separate compartment of a large cage for the duration of the experiment. The cage was constructed of galvanized mesh (25 mm × 25 mm × 4 mm for the floor and 35 mm × 35 mm × 4 mm for the walls). Half of the cage walls and the entire roof were covered with galvanized roofing sheets. Each compartment was 2 m (h) x 2.4 m (l) x 1.4 m (w), and included food and water vessels, a roosting perch, and a nesting box containing hay or straw.

Group 1 (control group) was fed the basal diet (Table 3.1 and Table 3.2), while groups 2-4 were fed the basal diet supplemented with equimolar amounts of menadione, vitamin K_1 (phylloquinone) or vitamin K_2 (menaquinone-*n*). Group 2 was fed the basal diet supplemented with 2 mg menadione nicotinamide bisulfite (MNB, DSM N.V., Heerlen, Netherlands) per kg feed, group 3 was fed the basal diet

supplemented with 3.45 mg menaquinone-7 (as MenaQ7 powder, NattoPharma ASA, Oslo, Norway) per kg feed, and group 4 was fed the basal diet supplemented with 2.40 mg phylloquinone (as Quinaquinone, "QAQ", Agricure Scientific Organics Pty Ltd, Braemar, NSW, Australia) per kg feed. Blood samples were collected after the 1st, 2nd, 4th and 6th weeks of the diet study. Blood was taken from the brachial artery of birds using hypodermic syringes (SUR-VET®, Terumo Corporation, Tokyo, Japan) with 23 gauge needles. Approximately 1 mL was transferred to a blood sample tube (Greiner Bio-One International GmbH, Kremsmünster, Austria) preloaded with 25 IU of sodium heparin. The anticoagulated whole blood samples were maintained at around 23 °C for the 2 hour duration of transport to the laboratory.

Table 3.1: Ingredients of complete vegetarian chicken diet.

Ingredient	Fraction (% w/w)
Ground wheat bulk	60.59
Soybean meal	10.00
Calcium carbonate (lime)	9.00
Wilpromil R soy protein conc.	5.00
Coconut meal	5.00
Mill run (wheat husk) bulk	3.00
Vitamin premix	2.50
Process water	1.85
Canola oil - crude	1.50
Dicalcium phosphate powder	1.50
Mastercube pellet binder	0.07

Table 3.2: Ingredients of vitamin premix.

Ingredient	Fraction (% w/w)
Mill run (wheat husk) bulk	61.30
Sodium bicarbonate	9.20
Sodium chloride	8.00
Nisso DL-methionine 99%	6.92
L-lysine HCl	6.28
Choline chloride 60%	3.33
L-threonine	1.72
Nicotinamide	0.99
Vitamin E 250 IU natural	0.80
Ferrous sulfate 30%	0.66
Manganous oxide 62%	0.48
Oxistat dry antioxidant	0.48
Zinc oxide 72%	0.30
Selenium premix 0.5% + ROVIMIX vitamin E50	0.12
Copper sulfate 25% Cu	0.10
Calcium pantothenate 98%	0.06
Vitamin A 1000 IU + vitamin D 3200 IU	0.04
Riboflavin (B2) 80%	0.03
Pyridoxine HCl (B6)	0.02
Potassium iodide 76% fat sol	0.01
Sodium molybdate 40%	0.01
Vitamin B12 FG 1%	0.01
Thiamine mononitrate	0.01
Folic acid 97%	0.01

3.2.3 Flow cytometric sample preparation

Flow cytometry samples were prepared as per the method of De Boever et al. [317]. Blood (500 μL) was transferred to a centrifuge tube. In order to trigger an immune response, 50 μL LPS (LPS from *Escherichia coli* 0127:B8, Sigma-Aldrich, St. Louis, MO, USA) solution was added to the blood, which was incubated for a further 3 hours. Prior to the diet study, an *in vitro* experiment was performed where the blood samples were pre-treated with a solution of each vitamin K compound. Solutions (50 μL) of menadione (analytical standard, 47775, Sigma-Aldrich), phylloquinone (V3501) or menaquinone-7 (analytical standard, ASB-00022822, Chromadex Inc., Irvine, CA, USA) in ethanol (for molecular biology, Sigma-Aldrich) were added to each sample and the tube placed on a rocker in an incubator for 1 hour at 37°C. The control was pure ethanol. The lower treatment concentration for each solution was set at 0.01 μmol L^{-1} because this corresponds to a physiological plasma baseline concentration of phylloquinone (4.51 ng/mL) [320]. Ten- and 100-times concentrations were also tested.

Red blood cells (RBC) were lysed by adding 4.5 mL BD FACS Lysing Solution (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to the tube, which was incubated at room temperature on a rocker for 10 minutes. The RBC debris was removed by centrifugation at 500 RCF and washing with stain buffer – 1% BSA (lyophilized powder BioReagent bovine serum albumin, Sigma-Aldrich) in RPMI1640 (RPMI1640 without phenol red or L-glutamine, Sigma-Aldrich). The pellet was resuspended in 50 μL of CD45 MAb conjugated with R-PE (Mouse anti-chicken CD45 MAb conjugated with R-phycoerythrin, AbD Serotec, Kidlington, UK) at 50 μg/mL in stain buffer. The pellet was incubated for 30 minutes at 4 °C in the dark. After washing, the pellet was resuspended in 500 μL permeabilizing reagent (BD FACS Permeabilizing Solution 2, Becton, Dickinson and Company) and incubated for 10 minutes at room temperature in the dark. The cells were again washed and the pellet resuspended in 50 μL of IL-1β or IL-6 antibody (rabbit anti-

chicken polyclonal antibody, AbD Serotec) at 50 μg/mL in RPMI, which was incubated for 30 minutes at room temperature in the dark.

After washing, blocking was performed by resuspending the pellet in 500 μL of 20% goat serum (Life Technologies, Carlsbad, CA, USA) in stain buffer. This was incubated for 30 minutes at room temperature in the dark. The cells were washed again and the pellet resuspended in 50 μL of secondary antibody ($F(ab')_2$ fragment of goat anti-Rabbit IgG conjugate with CF™488A, Sigma-Aldrich) at 5 μg/mL in stain buffer. This was incubated for 30 minutes at 4 °C in the dark. The cells were washed in DPBS (without Ca or Mg) then resuspended in 250 μL BD CytoFIX for 10 minutes at 4 °C. Finally, the cells were washed twice in DPBS and stored at 4 °C until analysis (within 24 hours). Immediately before analysis, the cell suspension was passed through a 40 μm filter and vortexed.

3.2.4 Flow cytometric sample analysis

A BD Accuri C6 flow cytometer (Becton, Dickinson and Company) was used to analyze the samples. Flow rate was set at 13 μL/second and samples were diluted if the event count exceeded 2,500 per second. Data acquisition continued until at least 10,000 $CD45^+$ events were recorded. All values for forward scatter, side scatter and fluorescence intensity were output in arbitrary units. These values could be reported as peak intensity (H) or time integral of intensity (A) for each cell counted. The gating strategy was adapted from the study by De Boever et al. [317]. Debris was eliminated by removing all events with a peak forward-scatter value (FSC-H) below 500,000, as shown in Figure 3.1a. This value is recommended by the Accuri C6 user manual [321] and proved to be a good threshold in preliminary studies.

The CD45 antibody (specific for WBC) was conjugated with the fluorescent dye PE so that white blood cell events were gated based on plots of CD45-PE fluorescence signal. The histogram of cell count versus CD45-PE signal in Figure 3.1b shows the distinction between CD45-negative and CD45-positive cells (debris shown in red). The $CD45^+$ and $CD45^-$ cells are shown in green and blue, respectively, on the

CD45-PE versus SSC plot in Figure 3.1c. The IL-1β and IL-6 antibodies were conjugated with the fluorescent dye CF488, and the CD45-PE signal was plotted against the IL-CF488 signals (Figure 3.1d). When the WBC contained more of the pro-inflammatory cytokines IL-1β or IL-6, the IL-CF488 signal increased and the WBC population shifted to the right.

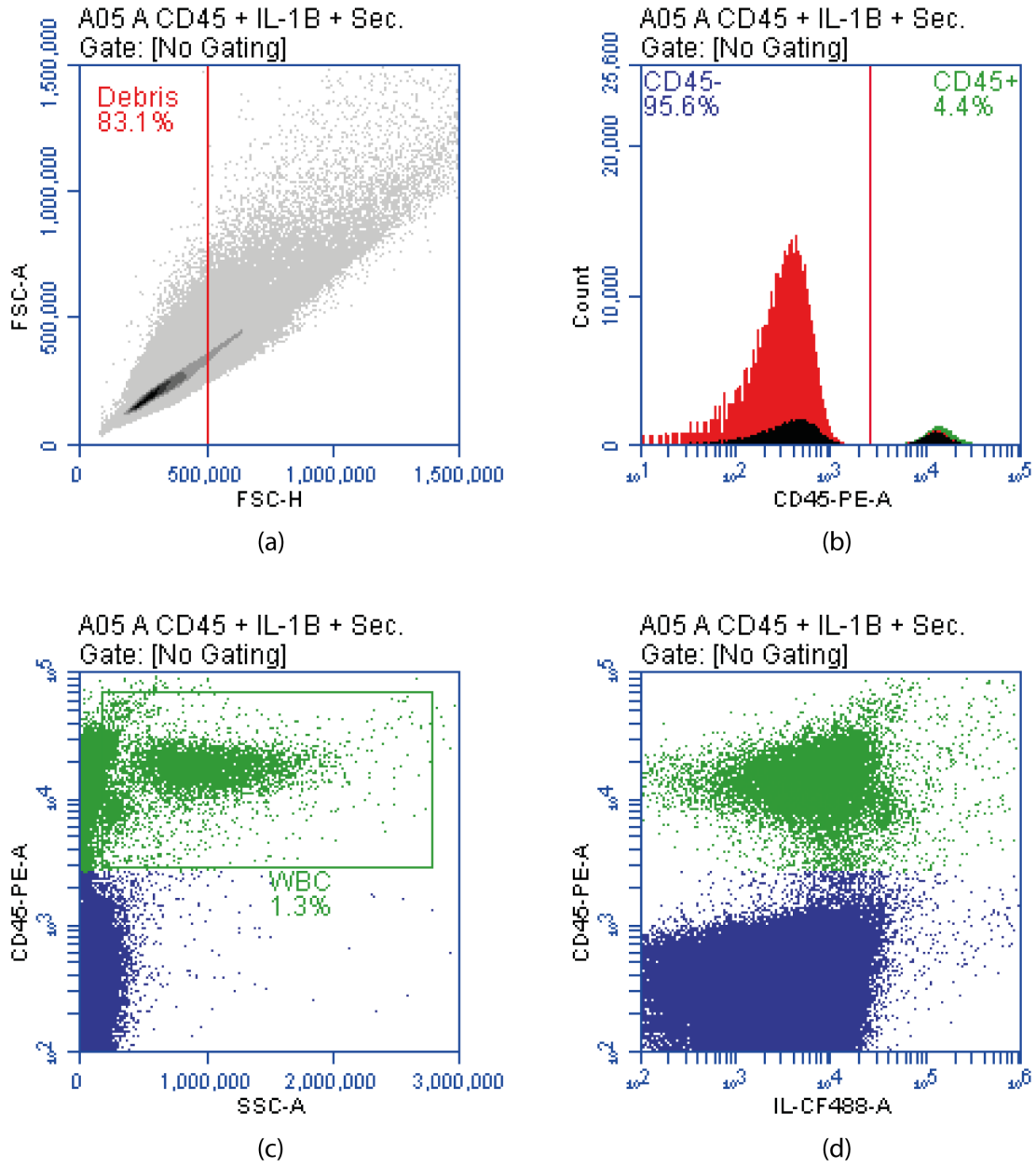


Figure 3.1: Gating strategy showing: elimination of cellular debris (a); selection of CD45+ events (b); identification of WBC (c), and; observation of IL-1β fluorescence (d).

3.2.5 Extracellular cytokine analysis

Extracellular pro-inflammatory cytokines were analyzed by Cardinal Bioresearch Pty. Ltd. (New Farm, QLD, Australia) using ELISA kits purchased from CUSABio (CUSABIO TECHNOLOGY LLC, Houston, TX, USA). Chicken IL-1β, (ELISA kit CSB-E11230Ch), IL-6 (ELISA kit CSB-E08549Ch) and TNF-α (ELISA kit CSB-E11231Chken) were assayed. All data were plotted and statistical analysis was performed using GraphPad Prism 7 for Windows (GraphPad Software, Inc.).

3.2.6 Blood vitamin K concentration analysis

The plasma (0.5 mL) was transferred to a centrifuge tube. Ethanol (1 mL) was then added to denature the protein and 3 mL of hexane was added. After shaking for 5 min, the solution was centrifuged at 3000 RCF for 5 min. The upper layer was aspirated and transferred to an amber glass vial. This solution was evaporated to dryness under a constant stream of nitrogen. The dried sample was reconstituted in 1 mL of methanol and sonicated for 10 minutes. The solutions were transferred to microvials and capped to await analysis. Analysis of samples was performed using an Agilent 1100 series system with fluorometric detection (Santa Clara, CA, USA). Flow was isocratic at a rate of 1 mL/min, injection volume was 50 μL, mobile phase was 90% methanol, 10% isopropanol with 1.37 g/L zinc chloride, 0.41 g/L anhydrous sodium acetate and 0.30 g/L glacial acetic acid. The column assembly consisted of a C_{18} guard column, C_{18} analytical column (150 mm x i.d. 4.6 mm) and empty column (50 mm x i.d. 4.6 mm) packed with zinc dust (particle size < 63 μm). The zinc stationary phase is preferentially oxidized as the mobile phase, including the vitamin K, is reduced. Reduced vitamin K (the hydroquinone oxidation state) excited at 243 nm produced fluorescence that was detected at 430 nm. All data were plotted and statistical analysis was performed using GraphPad Prism 7 for Windows (GraphPad Software, Inc.).

3.3 Results and Discussion

3.3.1 Effect of vitamin K concentrations on cytokine production *in vitro*

The concentrations of vitamin K substances necessary to suppress the synthesis of pro-inflammatory cytokines were assayed *in vitro*. Pure solutions of menadione, phylloquinone and menaquinone-7 in ethanol were tested. All treatment groups were expected to suppress inflammation, and therefore have lower fluorescence intensity, compared to the control group. However, no significant difference in intensity was observed for any treatment (Figure 3.2).

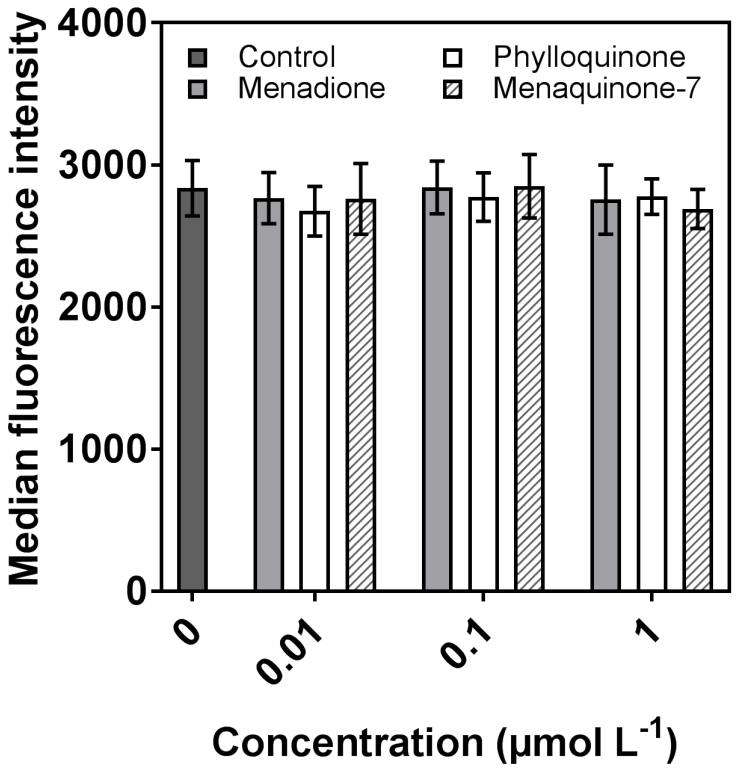


Figure 3.2: Median fluorescence intensity of pro-inflammatory cytokine signals for WBC incubated with different vitamin K compounds at different concentrations. Values are the mean of 3 repeats, all repeats are mean of duplicates and error bars are standard deviation of repeats.

The failure to observe any significant differences between the treatment groups may be due to the short incubation time used. It is possible that the WBC need to incubate

for longer than 1 hour before the vitamin K substances can take effect. It is also highly likely that the carboxylation of the Gas-6 protein, which is responsible for the vitamin K-mediated suppression of the innate pro-inflammatory response, cannot occur in an isolated blood sample [54], [131]. Furthermore, menadione has been shown to suppress cytokine expression at 10 μmol L^{-1} (4,510 ng/mL) and above [310], [311] so concentrations in excess of the physiological norm may be required to modulate immune response.

3.3.2 Effect of different vitamin K substances on cytokine production *in vivo*

The *in vivo* assay attempted to determine the effect of dietary supplementation on the intra- and extra-cellular pro-inflammatory cytokine concentrations in avian blood. Menadione was added as MNB, phylloquinone as QAQ®, and menaquinone-7 as MenaQ7®. Comparisons of the results between sample time points yielded no differences so they are presented here as repeats. Again, no significant difference was observed between the supplemented diets and the control diet. Both the intracellular cytokine fluorescence (Figure 3.3) and the plasma cytokine concentration (Figure 3.4) demonstrate that pro-inflammatory response on all diets was broadly the same. Recently published research has revealed that RBCs are a large reservoir of cytokines [322], [323]. Removal of the RBC fraction in the sample processing step possibly masked the true inflammatory response.

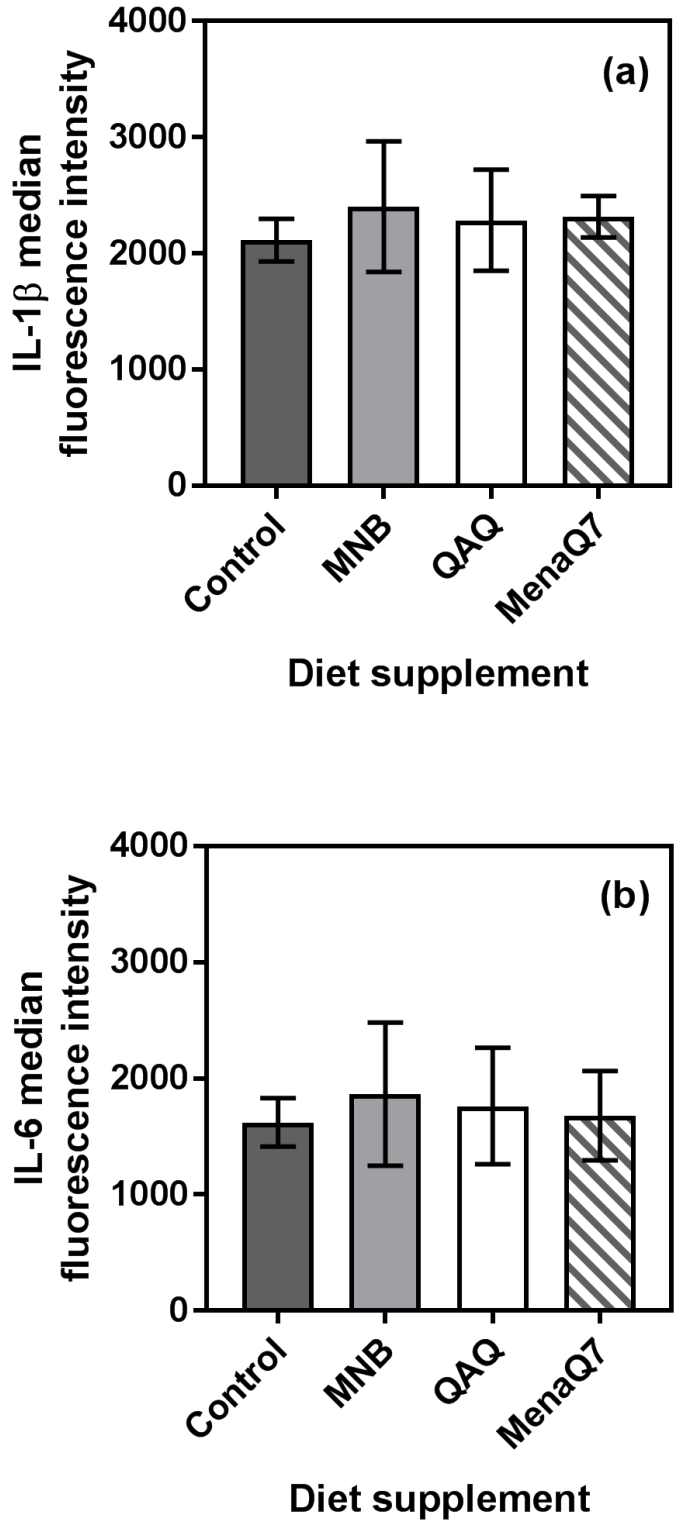


Figure 3.3: Fluorescence signal of (a) IL-1β and (b) IL-6 in WBC of birds fed diets supplemented with different vitamin K substances. Values are mean of 4 repeats, all repeats mean of 3 biological replicates, error bars are standard deviation of repeats.

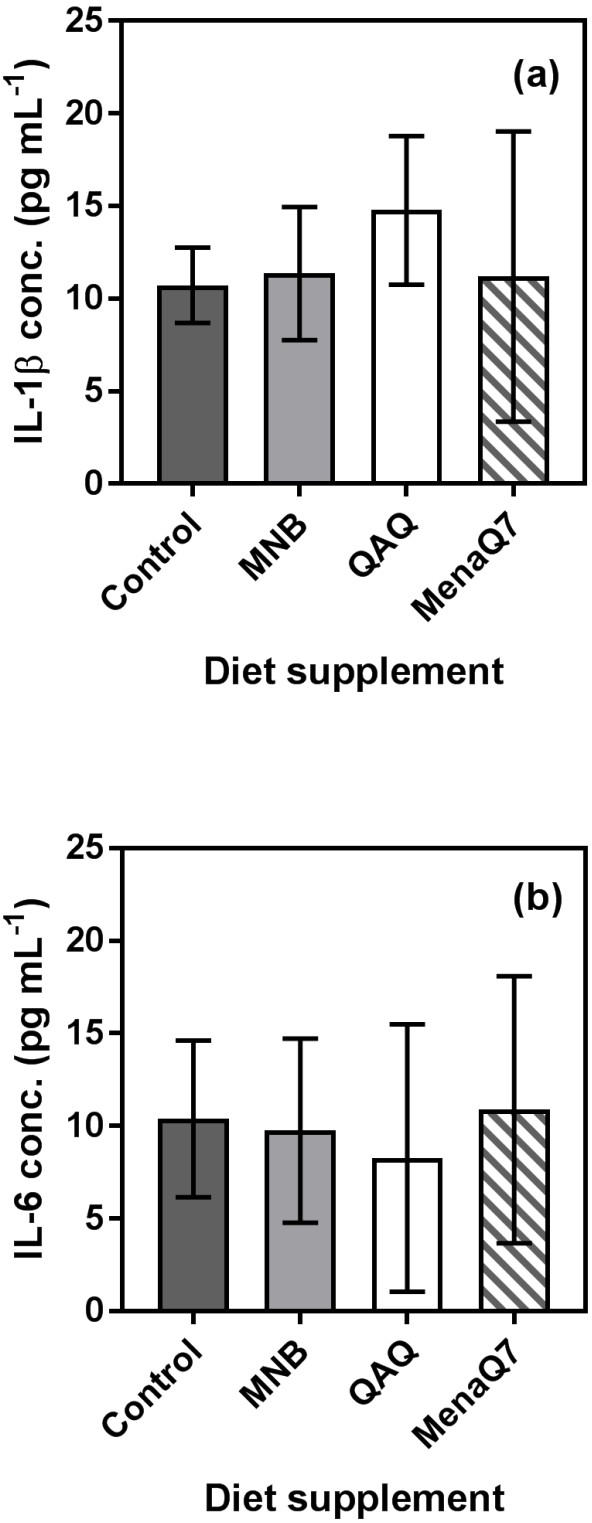


Figure 3.4: Pro-inflammatory extracellular cytokines in plasma of birds fed diets supplemented with different vitamin K substances. Values are mean of 4 repeats, all repeats mean of 2 or 3 replicates, error bars are standard deviation of repeats.

3.3.3 Replication of reference method for flow cytometric immunophenotyping

De Boever et al. used the CD45-PE versus SSC plot to distinguish between the $CD45^+$ populations of lymphocytes, thrombocytes, monocytes and heterophils. However, as shown in Figure 3.5, only the heterophils could be identified. Consequently only aggregate data for all WBC were used in our analysis of intracellular pro-inflammatory cytokines. It is possible that the inability to distinguish the WBC populations is due to the lower resolution of the BD Accuri C6 compared to more sophisticated machines, such as the FACSCanto used by De Boever et al. It is also likely that excessive cellular debris and un-lyzed erythrocytes contributed to increased non-specific binding as well as accounting for a large proportion of the recorded events [324]. The primary innate immune cells of avian blood are thrombocytes and monocytes [316]. They are more prolific producers of pro-inflammatory cytokines, as well as being more likely to respond to the vitamin K-mediated anti-inflammatory mechanism. An inability to distinguish between the WBC may be the reason that no differences between treatment groups were observed, as discussed later.

Another relevant difference in our study was the use of CF488A, rather than APC, conjugated secondary antibody. The CF488A fluorophore is fluorescein-based [325], which means that it has higher spectral overlap with the R-PE. Another possible source of error peculiar to the fluorescein-based fluorophores is cytoplasmic granule staining of the heterophils [326]. This phenomenon, where the fluorescein binds with the granules in the cytoplasm of avian heterophils, may lead to false positives and increased background for the IL-1β and IL-6 antibodies.

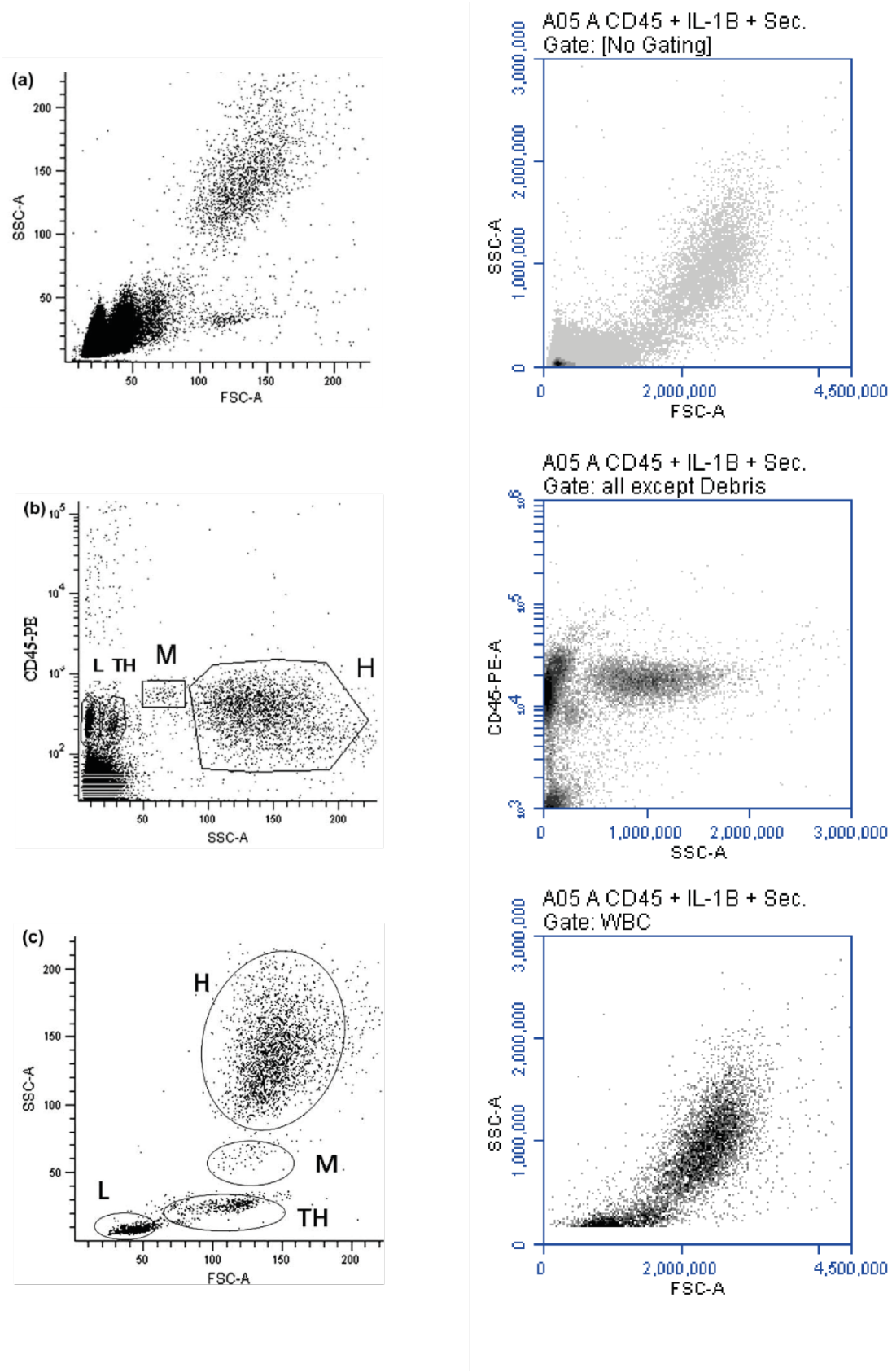


Figure 3.5: Comparison of flow cytometric phenotyping between De Boever et al. [317] (left) and the present study (right). Density plot of SSC-A versus FSC-A showing all events (a); density plot of CD45-PE versus SSC-A showing CD45+ events and gated WBC populations (b), and; density plot of SSC-A versus FSC-A showing only WBC populations (c).

However, the staining of heterophil granules was probably insignificant: firstly, the CF488A (molar mass ~910 g/mol) is a much larger molecule than fluorescein (molar mass 332 g/mol); and secondly, the CF488A fluorophore is minimally charged, unlike FITC and other fluorescein-based dyes, which have multiple negative charges. A longer incubation period could negate any concerns about granular staining, as LPS stimulation has been shown to cause almost complete degranulation after 16 hours [326].

3.3.4 Vitamin K_1 supplementation boosted blood vitamin K concentration

Despite the failure of the diet experiments to demonstrate an anti-inflammatory effect of vitamin K substances, there were some interesting and useful observations. Supplementation with QAQ produced a much higher blood concentration of phylloquinone (almost 20 ng mL^{-1}), compared to other diets (less than 10 ng mL^{-1}), as shown in Figure 3.6. Menaquinone-7 was detected only in the blood of the birds supplemented with MenaQ7, however the concentration was less than 5 ng mL^{-1}. Menadione did not appear in the blood from any diet supplement. These differences could be explained by the lower absorption of menaquinone-7 and the higher excretion rate of menadione compared to phylloquinone.

All four diets led to the appearance of some phylloquinone in the blood. This is unsurprising as the chicken feed is made with vegetable oils, which are known to contain appreciable amounts of phylloquinone [163]. The higher phylloquinone concentration in the control diet compared to the MNB and MenaQ7 diets is probably due to the intake of grass. Increased serum vitamin K concentrations are known to correspond to increased concentrations in tissues such as liver [320], [327], [328], so phylloquinone is likely to be the most effective treatment for improving vitamin K status.

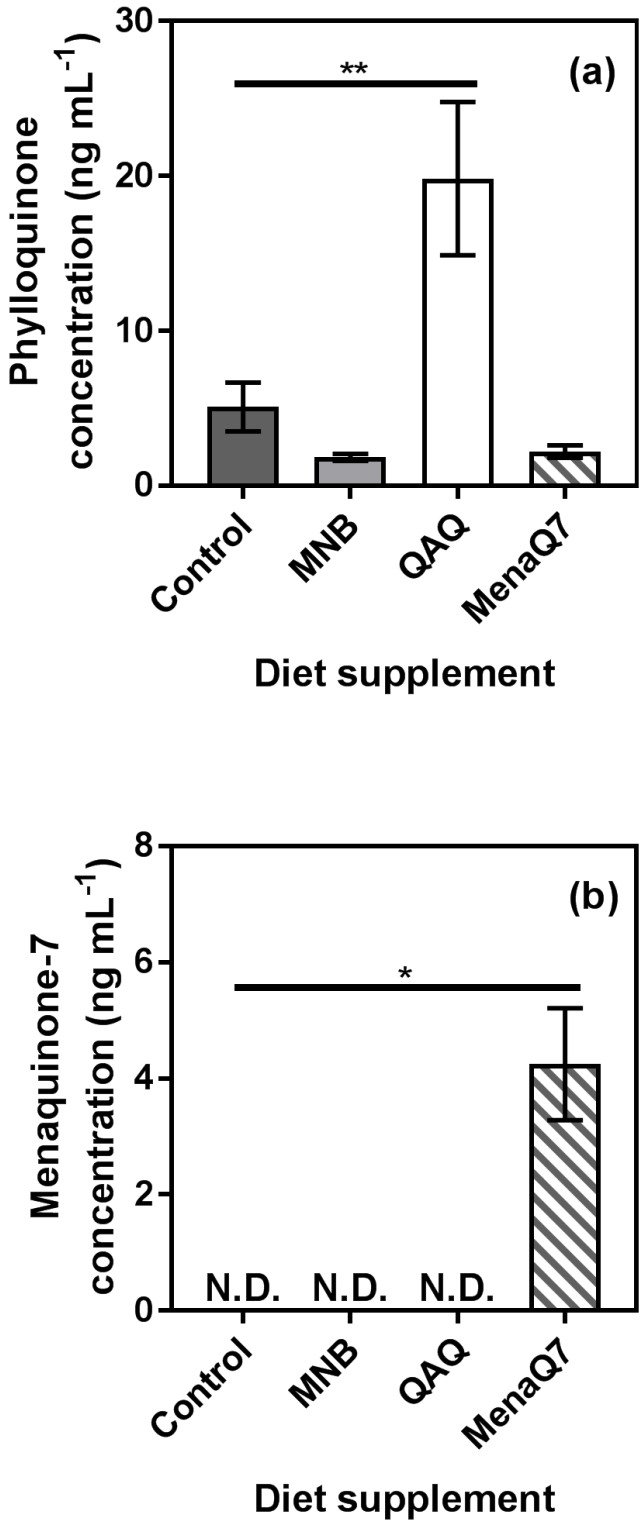


Figure 3.6: Concentration of (a) phylloquinone and (b) menaquinone-7 in blood plasma of birds fed diets supplemented with different vitamin K substances. Values are mean of 3 repeats, error bars are S.D. of repeats. Blank, QAQ and MenaQ7 compared using Welch's t-test, * $p < 0.05$, ** $p < 0.01$.

Research comparing the immune benefits of dietary vitamin K_1 and menadione in greenlip abalone (*Haliotis laevigata*) was also published recently [329]. Vitamin K was added as K_1 (QAQ®) or menadione at 0.5 mg kg^{-1} to the feed. Hemocyte count and phagocytic activity were measured as indicators of immune response. Viscera and muscle concentrations of menadione, vitamin K_1 and vitamin K_2 were measured as indicators of vitamin K status. No significant differences in hemocyte count or phagocytic activity were observed between the vitamin K_1 and menadione treatments at an addition rate of at 0.5 mg kg^{-1}. However, visceral and muscle vitamin K content in abalone fed vitamin K_1 was approximately double the concentration compared to those fed menadione. No menadione was detected in the viscera or muscle tissue. The effects of vitamin K_1 on tissue vitamin K concentrations were also tested at addition rates of 0, 1.0 and 5.0 mg kg^{-1}. The visceral vitamin K_1 concentration was approximately proportional to the vitamin K_1 feed rate. These results support the conclusion that, although it is difficult to detect immune benefits due to vitamin K_1, it is still the best form for boosting vitamin K status.

3.4 Summary

The effect of three different K vitamers (menadione, phylloquinone and menaquinone-7) on the inflammatory response of avian white blood cells was examined. No difference in pro-inflammatory cytokine levels was observed between the control and the three test groups; such results suggest that further work is needed to examine the mechanism by which vitamin K has an anti-inflammatory effect. Alternatively, a large observational study examining the effect of different vitamin K supplements on avian health and other metrics (e.g. egg production) may also be a useful approach. This study observed that supplementation of the diet with phylloquinone led to a significant increase in blood concentrations of phylloquinone. Such results suggest that future work should focus on the development of a vitamin K_1 product for incorporation into chicken feed.

4 Identification of a microalga for production of vitamin K_1

This chapter presents original research into microorganisms that could potentially be used to produce vitamin K_1. Some background is provided on organisms that are already known to contain high levels of vitamin K_1. Microalgae are identified as a suitable category of microorganisms and some examples of existing microalgal bioprocesses are given. Methods are specified for the analysis of vitamin K_1 content of different microalgal species, as well as for nutrition and toxicity testing. The results, identifying and characterizing an ideal microorganism, are discussed. Finally, the key points of the research presented in this chapter are summarized.

4.1 Background

Vitamin K_1 (phylloquinone) is found in a wide range of photosynthetic organisms including terrestrial plants [157], [330], [331], seaweed [157], [208] and microalgae [206], [208], [246], [332]. However, concentrations are highly variable [157], [208] and some photoautotrophs contain no vitamin K_1 whatsoever [86], [333], [334]. For the biosynthesis of vitamin K_1, microalgae may be favored over other photosynthetic organisms due to their higher growth rates, relative ease of screening, and genetic simplicity [209]. In comparison to the current chemical process, microalgal biosynthesis uses no organic solvents, requires little energy, and operates at ambient temperatures and pressures. Additional advantages of the biosynthesis are the fact that it only produces the active *E*-isomer of phylloquinone [195], [197], [199] and if a photoautotrophic process is used the primary feedstocks are light and CO_2. While there are no specific examples for the biotechnological production of phylloquinone, microalgae have been widely used for producing a range of compounds including carotenoid pigments [210], [264], [335]. Hence the aim of this chapter was to identify a suitable microalgal strain for biotechnological production of vitamin K_1.

4.2 Method and Materials

4.2.1 Flask cultures

Seven commonly used microalgal strains representing various phyla (summarized in Table 4.1) were grown in flasks and screened for phylloquinone content. All strains were obtained from the Australian National Algal Culture Collection (ANACC). Modified ASM-1 medium (MLA) was prepared as per Bolch and Blackburn [336], however B vitamins and selenium were omitted as they are not required by most strains [337], [338]. Guillard's and Ryther's *f* medium [339] was prepared at half strength (*f*/2) as per the ANACC modification [340]. A commercially available marine salts mixture was added at 35 g L^{-1} to the *f*/2 medium. Cultures were maintained in 50 mL Erlenmeyer flasks on a light pad at room temperature and passaged under aseptic conditions every 2-4 weeks.

To prepare experimental cultures, fresh medium (100 mL) was added to 250 mL Erlenmeyer flasks, then autoclaved and allowed to cool for 24 hours. The flasks were inoculated with 10 mL of culture in the exponential growth phase. The algae were grown on a LED light pad of 6000 K color temperature, with a 12-hour light to 12-hour dark cycle.

Table 4.1: Strains tested for phylloquinone content.

Phylum (common name)	Species	ANACC Strain	Growth medium
Cyanobacteria (blue-green algae)	*Spirulina* sp.	CS-785	50% MLA + 50% *f*/2
	Anabaena cylindrica	CS-172	MLA
Chlorophyta (green algae)	*Chlorella vulgaris*	CS-41	MLA
	Desmodesmus asymmetricus	CS-905	MLA
	Dunaliella salina	CS-265	*f*/2
Ochrophyta	*Nannochloropsis oculata*	CS-192	*f*/2
	Phaeodactylum tricornutum	CS-29	*f*/2

A Walz ULM-500 series light meter equipped with a US-SQS/L spherical sensor was used to measure the light intensity at 9 points over the light pad (corners, midpoints of each side, and center point), and the mean Photosynthetic Photon Flux Density (PPFD) was 70 ± 10 μmol m^{-2} s^{-1}. The optical density of the culture was measured daily. After two weeks, the remaining culture was harvested for vitamin K_1 analysis. Cultures were grown in triplicate; each replicate was analyzed once and the mean was calculated. Experiments were performed twice (n = 2).

4.2.2 Extraction and analysis of phylloquinone

Sample preparation was based upon the method of Breuer et al [341]. Culture (50 mL) was centrifuged (3000 RCF for 5 minutes), and the supernatant discarded. The residue was resuspended in deionized water, centrifuged and the supernatant discarded. This step was performed twice for strains grown in *f*/2 medium to ensure complete removal of the salts. The residue remaining after washing and centrifugation was freeze-dried. Approximately 10 mg of dry powder was weighed into a 1.8 mL bead-beater tube, pre-loaded with 0.5 mm diameter zirconia beads.

Menaquinone-4 (Sigma-Aldrich) was prepared at 10 μg mL^{-1} in *n*-hexane and 100 μL was added to each tube as an internal standard. Chromatography grade *n*-hexane was added to the tube (1.0 mL), which was subsequently beaten at 4000 rpm for 1 minute, followed by centrifugation and transfer of the supernatant to an amber glass vial. This step was performed three times such that the total volume of *n*-hexane extract in the amber vial was 3 mL. The *n*-hexane extract was diluted 10 times in mobile phase and syringe filtered (PTFE filter, 0.45 μm pore size) before analysis using HPLC.

Sample analysis was performed based upon the Association of Official Analytical Chemists (AOAC) method 999.15 [342] using a Shimadzu Prominence-i HPLC system. The mobile phase consisted of 1.37 g L^{-1} anhydrous zinc chloride (LR grade, Chem-Supply, Gillman, SA, Australia), 0.41 g L^{-1} anhydrous sodium acetate (AR grade, Sigma-Aldrich, St. Louis, MO, USA) and 0.30 g L^{-1} glacial acetic acid (AR

grade, Merck) dissolved in a solution of 90% (v/v) methanol and 10% (v/v) dichloromethane (both LiChrosolv for liquid chromatography, Merck).

The flow rate was 0.3 mL min^{-1} with a sample injection volume of 10 μL. The column assembly was maintained at 40 °C and consisted of three parts: C_{18} guard column (YMC-Guardpack ODS-AM, particle size 3 μm, pore size 120 Å, ID 4.0 mm × 10 mm); C_{18} analytical column (YMC-Pack ODS-AM, particle size 3 μm, pore size 120 Å, ID 2.0 mm × 150 mm), and; reducing column (YMC original empty column, ID 4.6 mm × 30 mm) packed with zinc dust (EMPLURA®, particle size < 63 μm). Fluorometric detection (FLD) of the reduced phylloquinone and menaquinone-4 was achieved using a Shimadzu RF-20Axs detector with excitation wavelength at 243 nm, emission wavelength at 430 nm and flow-cell temperature set to 37 °C. The retention times were approximately 5.8 minutes and 7.8 minutes for menaquinone-4 and phylloquinone, respectively (Figure 4.1).

To confirm the HPLC-FLD result of *A. cylindrica*, LC-ACPI-MS/MS analysis was performed based upon the method of Suhara et al. [343] using an Agilent 1100 HPLC system and a Bruker AmaZon SL with atmospheric-pressure chemical ionization (APCI). The mobile phase consisted of 9:1 methanol:2-propanol (both LiChrosolv grade for liquid chromatography, Merck). The flow rate was 0.3 mL min^{-1} with a sample injection volume of 50 μL. The analytical column (Sunfire C18 column, particle size 3.5 μm, pore size 100 Å, ID 2.1 mm × L 100 mm) was maintained at 25 °C. The APCI source temperature was 400 °C and the corona current was 5 μA. The scan range was 150-1000 m/z and all data were collected in positive ion mode. The precursor ion (m/z = 451.33) and product ion (m/z = 187.03) match the values found by Suhara et al. [343], corresponding to protonated phylloquinone and 2,3-dimethyl-1,4-naphthoquinone, respectively (Figure 4.2). The extract from *A. cylindrica* had the same retention time and mass-to-charge spectrum as the analytical standard (Figure 4.1), which confirmed beyond reasonable doubt that the compound from *A. cylindrica* was phylloquinone.

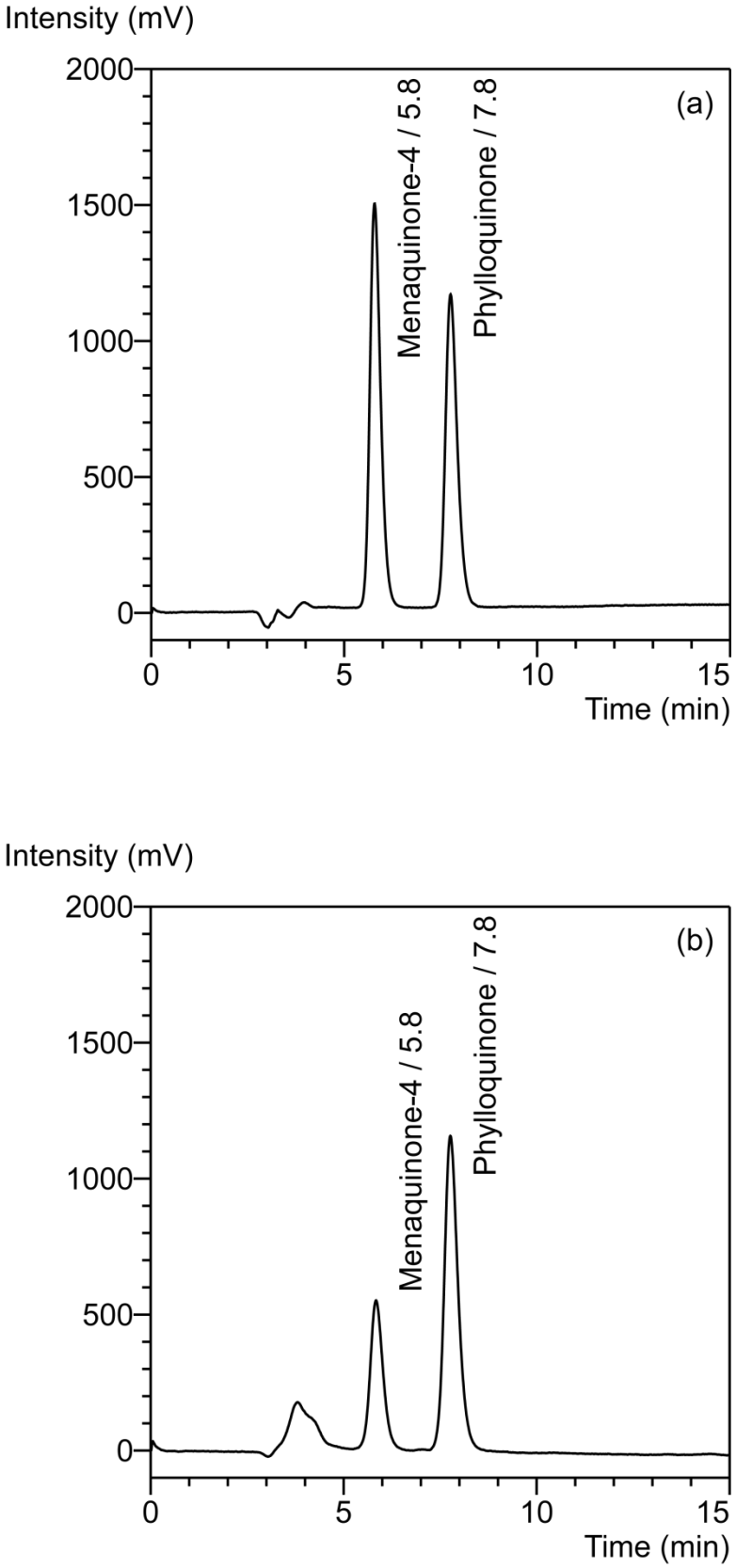


Figure 4.1: Chromatograms from fluorometric analysis of: (a) phylloquinone and menaquinone-4 standards; (b) hexane extract of *A. cylindrica*, spiked with MK-4.

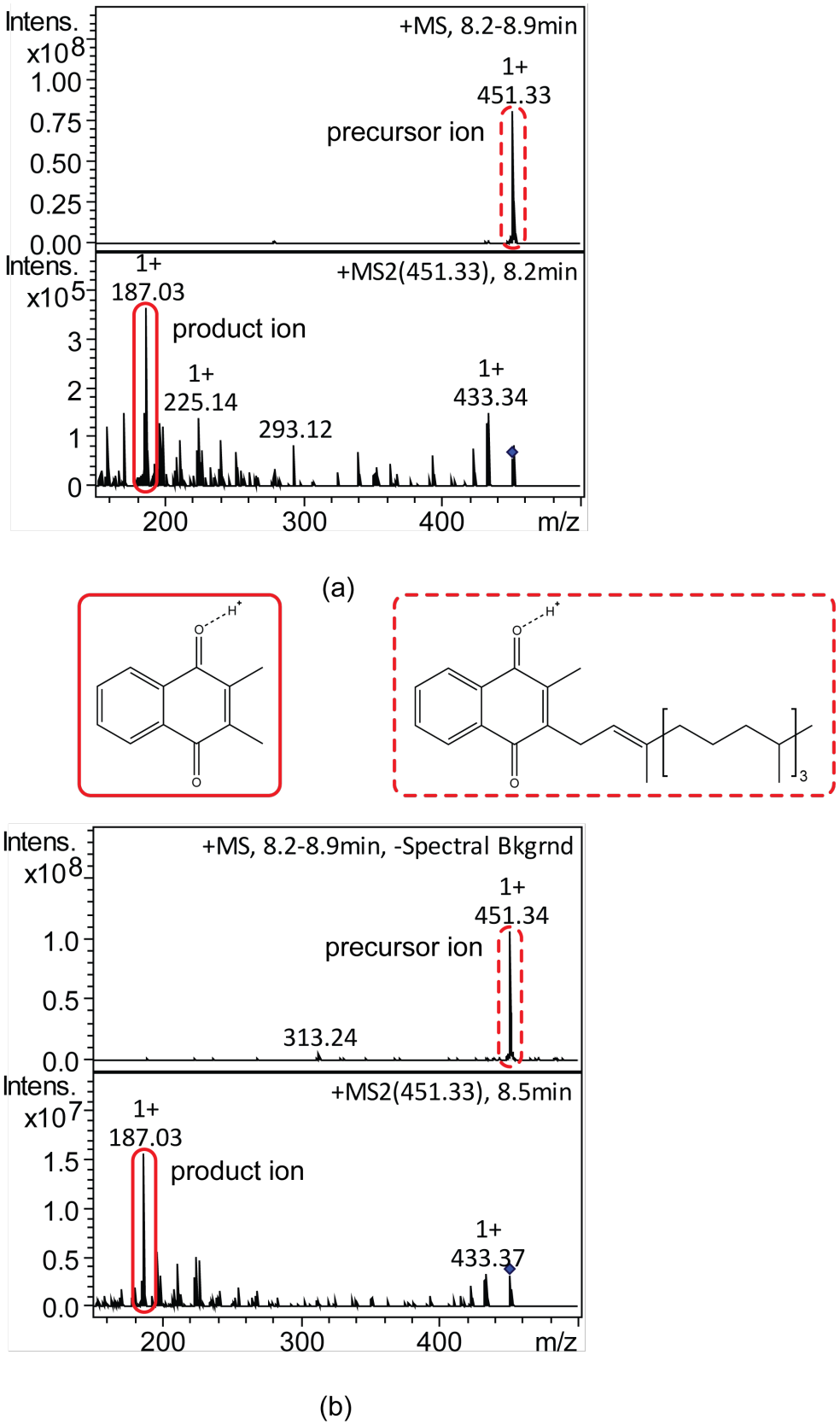


Figure 4.2: Mass-to-charge spectra from (a) an analytical standard and (b) a hexane extract of *A. cylindrica*. Dashed line indicates precursor ion, solid line indicates product ion.

4.2.3 Nutritional panel analysis

The cyanobacterium *A. cylindrica* was grown in a 50 L bubble-column PBR, with 2× MLA medium concentration at a constant temperature of 28 °C. Illumination was provided by LED lights of 6000 K color temperature and PPFD of approximately 300 μmol m^{-2} s^{-1} on a 12 hour:12 hour light:dark cycle. The culture was harvested at a density of approximately 0.8 g L^{-1} DCW, and dewatered using a WVO Raw Power centrifuge with power booster (WVO Designs, North Charleston, SC USA) running at maximum speed. The resultant slurry of approximately 100 g L^{-1} DCW was transferred to a sealed container, packed in ice and shipped overnight to the National Measurement Institute Food Analysis Laboratory (Port Melbourne, VIC Australia). Proximates, amino acids, fatty acids, vitamins (β-carotene, thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folate, cobalamin, ergocalciferol, α-tocopherol, phylloquinone and biotin) and minerals (calcium, iron, magnesium, potassium, sodium and zinc) were analyzed.

4.2.4 Animal study

The cyanobacterium *A. cylindrica* has not been used for food and is not Generally Recognized as Safe (GRAS). As many cyanobacteria are known to produce potent toxins (cyanotoxins) [344], an animal bioassay was used to demonstrate safety and a lack of cyanotoxins. The AOAC Official Method 959.08 for paralytic shellfish poisoning [345] has been used to determine the food safety of novel cyanobacterial species [346], however the method only tests for water soluble toxins. Here, the AOAC method has been adapted to administer whole biomass by the oral route, rather than a water soluble extract by the intraperitoneal route. Most cyanotoxins produce biological effects after a matter of hours at concentrations in the range of several hundred μg per kg bodyweight [344], [347].

Intracellular cyanotoxin concentrations are usually in the order of several thousand μg per g dry mass [348], which means that 1 g dry mass per kg bodyweight should produce a severe, acute response within one day. A 20 g mouse

will consume approximately 4 g of food per day, which equates to 200 g dry mass of food per kg bodyweight per day. To ensure that all diets would elicit a response in the event that *A. cylindrica* was acutely toxic, ten times the calculated toxic dose (10 g dry mass per kg bodyweight per day) was set as the minimum daily intake. This equated to a 5% (10/200) addition rate. On the other hand, feed studies have shown that non-toxic algae have detrimental effects at addition rates above 10-20% [349]–[351], so the inclusion rates of 10% and 15% were tested in order to estimate the detrimental addition rate.

Animal experiments were approved by the University of Sydney Animal Ethics Committee under protocol 2017/1243 (see approval letter in Appendix B). Twelve female mice of strain C57BL/6JAusb (Australian BioResources, Moss Vale, NSW Australia) were divided into 4 cages (3 mice per cage) at 8 weeks of age. The mice were coded with an ID number (#1, #2 and #3) for each diet group (control, 5%, 10% and 15%). Feeding of the experimental diets was begun at 10 weeks of age. The diets incorporated 0% (control), 5%, 10% and 15% *A. cylindrica* respectively. Every morning for 7 days, food residue from the previous day was removed and replaced with fresh food. Approximately 20 g of each diet was weighed onto a petri dish and placed in the bottom of the cage. The mice were also weighed daily.

The diet base was supplied as a powder (SF14-156, Specialty Feeds, Glen Forrest, WA Australia; see Appendix C), to which the freeze-dried *A. cylindrica* was added. The powder-algae mix was autoclaved with a 15 minute sterilization (121 °C and 1.1 bar) and 30 minute vacuum-drying (−0.9 bar) cycle. The autoclaved diets were mixed with water (5 parts diet to 2 parts water by mass) to make a dough mixture. The dough was rolled into ~20 g balls and stored at −30°C until needed. Frozen dough balls were thawed over night at 4°C before use.

4.2.5 Animal blood biochemistry and tissue histopathology

After 7 days, the mice were anesthetized with isoflurane and blood was taken by cardiac puncture. The mice were then euthanized by cervical dislocation and the brain, heart, lungs, kidneys, liver, and spleen were harvested. Blood samples were centrifuged at 10,000 RCF for 10 minutes. The serum was transferred to a clean 1.5 mL tube and frozen at −80 °C until time of analysis. Organs were fixed in neutral buffered formalin solution (cat. # HT5012, Sigma-Aldrich) and refrigerated at 4 °C for approximately 24 hours. Organs were then transferred to 70% ethanol solution and refrigerated at 4 °C until the time of tissue processing.

Serum biochemistry was analyzed by Veterinary Pathology Diagnostic Services (VPDS, University of Sydney, NSW Australia). Serum biochemistry tests included: serum total protein, albumin and globulins for general liver, kidney and immune function; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) for hepatocyte integrity; alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) for cholestatic function, and; urea and creatinine for kidney function [352].

Tissue histology was performed by the histology department at the Westmead Institute for Medical Research (Westmead, NSW Australia). After processing, embedding and sectioning, the specimens were stained with hematoxylin and eosin. Tissues were examined from n = 3 mice. The tissues examined included heart, kidney, lung liver, spleen, and stomach. The slides were scanned at high resolution (40 × objective) using an Aperio ScanScope CS slide scanner (Aperio, Vista, CA, USA).

4.3 Results and Discussion

4.3.1 Strain screening

The concentrations of phylloquinone showed great variability, from undetectable levels in *P. tricornutum* to around 200 μg g^{-1} in *A. cylindrica* on a dry weight basis (as shown in Figure 4.3). Fresh parsley, which is known to be a rich dietary source of phylloquinone [34], [159], was analyzed for comparison. The value plotted for parsley in Figure 4.3 is the mean of our wet-weight value (7.6 μg g^{-1}) and previously reported values (3.6 μg g^{-1} [159] and 5.5 μg g^{-1} [34]), where dry weight was determined to be 15% of the wet weight. The concentration of phylloquinone in *A. cylindrica* is almost six times higher than the 37 μg g^{-1} found in parsley. The concentration of phylloquinone found in *A. cylindrica* is higher than any previously recorded dietary source of phylloquinone [34], [157]–[161], as well as the other species of microalgae examined. *A. cylindrica* is therefore the most promising strain for the biotechnological production of phylloquinone.

The cyanobacteria tested here were observed to have higher concentrations of phylloquinone than strains from the other phyla. As previously discussed, the role of phylloquinone in these organisms is electron transport in photosystem I. While it is well known that the ratio of PSII/PSI in photosynthetic organisms is highly variable [353], generally speaking cyanobacteria have a much higher relative concentration (PSII/PSI < 0.5) of PSI compared to terrestrial plants and other eukaryotic photoautotrophs (PSII/PSI > 2) [353], [354]. This may explain our observations regarding the phylloquinone content of the species examined. No phylloquinone was detected in the diatom *P. tricornutum*; previous studies have shown that some species of diatom from genus *Chaetoceros* also do not contain phylloquinone [208], [355]. Overall the results from this screening analysis indicate that cyanobacteria, particularly *A. cylindrica*, have the highest potential for production of phylloquinone.

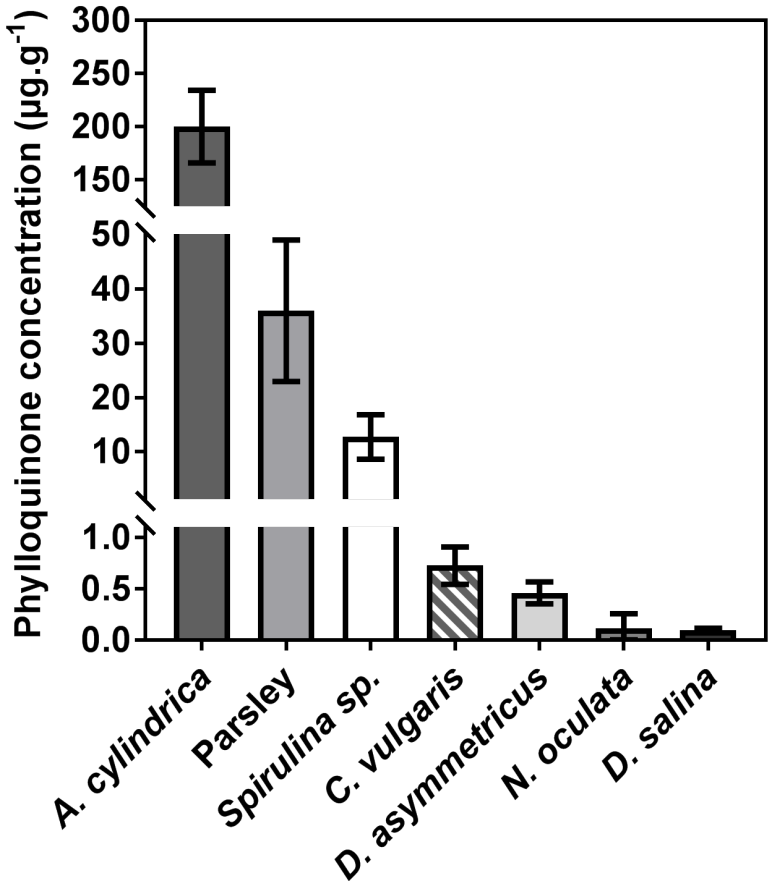


Figure 4.3: Phylloquinone concentrations determined for some common microalgae. Phylloquinone was not detected in *P. tricornutum* so no value is shown.

4.3.2 Nutritional analysis

The nutritional data for *A. cylindrica* were provided by NMI in reports RN1190636 and RN1193054 (see Appendix D). The values were reported on an "as-received" (wet-weight) basis and subsequently converted to a dry-weight basis. Typical nutrient values for Spirulina, taken from the USDA food database [356], are also reported here for the purpose of comparison. The proximate analysis revealed that *A. cylindrica* is primarily composed of protein with appreciable amounts of carbohydrates and fiber (Table 4.2). The quantity of fat is extremely low (3.5%) so the fatty acid profile is not reproduced here.

Table 4.2: Proximate analysis of *A. cylindrica*.

Proximate	Dry mass fraction % (w/w)	
	A. cylindrica	Spirulina [356]
Protein	68.6	57.5
Carbohydrates	11.6	23.9
Total dietary fiber	10.5	3.6
Ash	5.8	6.2
Fat	3.5	7.7

Table 4.3: Amino acid profile of *A. cylindrica*.

Amino acid	Concentration in dry mass (mg g^{-1})	
	A. cylindrica	Spirulina [356]
Glutamic acid	88.4	83.9
Aspartic acid	81.4	57.9
Leucine	60.5	49.5
Alanine	55.8	45.2
Arginine	48.8	41.5
Threonine	45.3	29.7
Glycine	41.9	31.0
Serine	39.5	30.0
Valine	38.4	35.1
Tyrosine	34.9	25.8
Isoleucine	33.7	32.1
Phenylalanine	30.2	27.8
Proline	24.4	23.8
Lysine	17.4	30.3
Methionine	14.0	11.5
Histidine	11.2	10.9
Tryptophan	7.4	9.3
Cysteine	3.6	6.6

Generally speaking, the Kjeldahl method and adjustment factor used to determine the protein content overestimates the true protein content by around 10% in microalgae [170]. Nevertheless, this means that around two-thirds of the dry mass of *A. cylindrica* is composed of protein. Approximately half of the protein content is composed the amino acids glutamic acid, aspartic acid, leucine, alanine and arginine (Table 4.3). Compared to the FAO/WHO/UNU reference pattern for essential amino acids, *A. cylindrica* exceeds the amino acids content for all but lysine (Table 4.4), and is therefore a good quality protein source. The calculated Essential Amino Acid Index (EAAI) [357] is 1.58, which is much higher than the reference pattern. However, the commonly used Protein Digestibility Corrected Amino Acid Score (PDCAAS) [358], [359] only considers the limiting amino acid (in this case lysine). Assuming a digestibility of 80% [360], *A. cylindrica* has a PDCAAS of 0.46, which is far below the reference score of 1.00.

The concentrations of the most nutritionally relevant minerals, as well as some vitamins, are shown in Table 4.5. The mineral content of microalgae usually reflects the concentration in the growth medium, which explains why Spirulina (grown in brackish, alkaline water) is almost five times higher in sodium than *A. cylindrica*. Nevertheless, 10 grams of *A. cylindrica* grown in 2× concentration MLA medium contains almost 600 $\mu g\ g^{-1}$ of iron. This is around 33% of the adult female iron requirement, according to the Australian guidelines [361]. The vitamin content of microalgae is less influenced by medium mineral content. Ten grams of *A. cylindrica* contains more than 30% of the adult iron requirement, 118% of the adult vitamin A requirement (as β-carotene), 640% of the adult vitamin B_{12} requirement and 3300% of the adult vitamin K_1 requirement [361].

Table 4.4: Indispensable Amino Acid (IAA) profile of *A. cylindrica* versus the FAO/WHO/UNU adult reference pattern [355].

Amino acid	Reference Pattern	*A. cylindrica*	Ratio
Leucine	55	89.3	1.62
Lysine	45	25.8	0.57
Valine	39	56.7	1.45
Phenylalanine + Tyrosine	38	96.2	2.53
Isoleucine	30	49.8	1.66
Threonine	23	67.0	2.91
Methionine + Cysteine	22	25.9	1.18
Histidine	15	16.5	1.10
Tryptophan	6	11.0	1.83

Table 4.5: Vitamin & mineral analysis of *A. cylindrica*.

Micronutrient	Concentration in dry mass ($\mu g\ g^{-1}$)	
	A. cylindrica	Spirulina [356]
Minerals		
Potassium	9,530	13,630
Magnesium	3,840	1,950
Calcium	3,140	1,200
Sodium	2,330	10,480
Iron	593	285
Zinc	16.3	20.0
Vitamins		
B-carotene	1,279	1,400
Phylloquinone (K_1)	233	0.3
Riboflavin (B_2)	11.6	36.7
Thiamine (B_1)	5.8	23.8
Cobalamin (B_{12})	1.5	6.6
Biotin	0.18	-

4.3.3 Animal study

The mass of the mice on each diet increased by 1-2% over the course of the experiment (Figure 4.4). No significant differences were found between initial mass, final mass or mass change when comparing test groups to the control group using unpaired *t*-tests (all $p \gg 0.05$). Blood biochemistry values for alkaline phosphatase (ALP) (Figure 4.6), alanine aminotransferase (ALT), aspartate aminotransferase (AST) (Figure 4.7), total protein, albumin, globulin (Figure 4.5), creatinine and urea (Figure 4.8) were within the normal ranges for female C57BL6 mice at around 2 months of age [362]–[365]. Values for γ-glutamyl transpeptidase (GGT) (Figure 4.6) were lower than the reference values [363], however this is not cause for alarm as only elevated levels are associated with liver or kidney disease [366]. Data for each biochemical marker were analyzed by Kruskal-Wallis one-way ANOVA on ranks and Dunn's post test. No significant difference was observed between any test group and the control group.

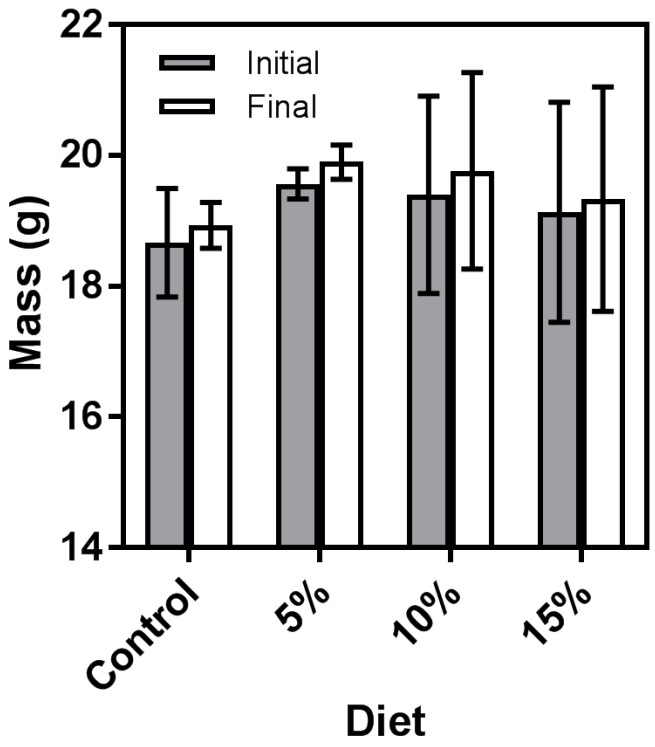


Figure 4.4: Mass of mice on each diet at day 0 (initial) and day 7 (final). Values are mean, error bars are SD.

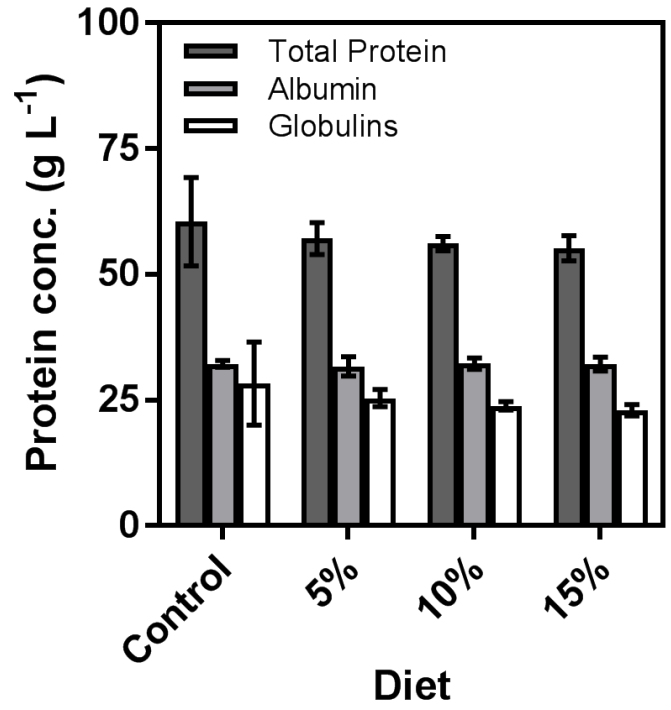


Figure 4.5: Albumin and globulins protein concentration in plasma (indicators of general liver, kidney and immune function) taken from mice on four different diets.

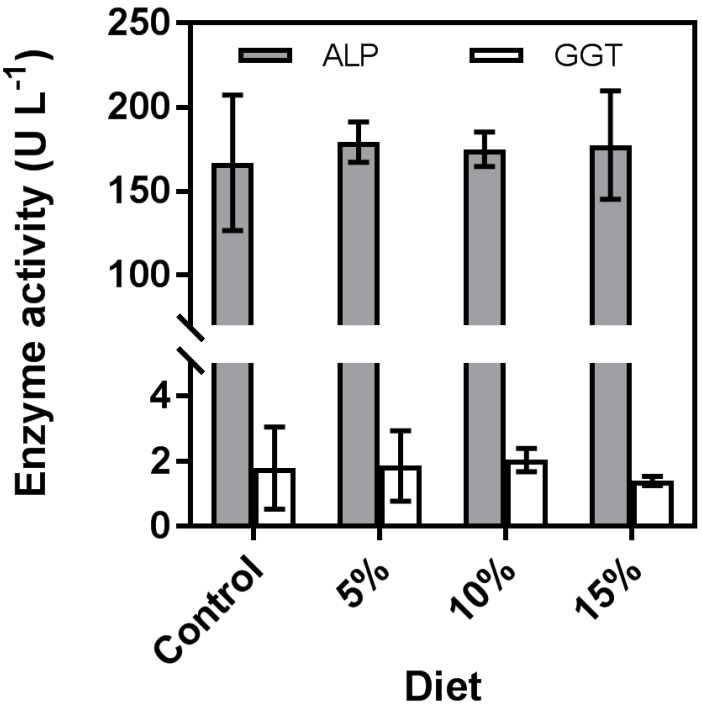


Figure 4.6: Alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) enzyme activity in plasma (indicators of cholestatic function) taken from mice on four different diets.

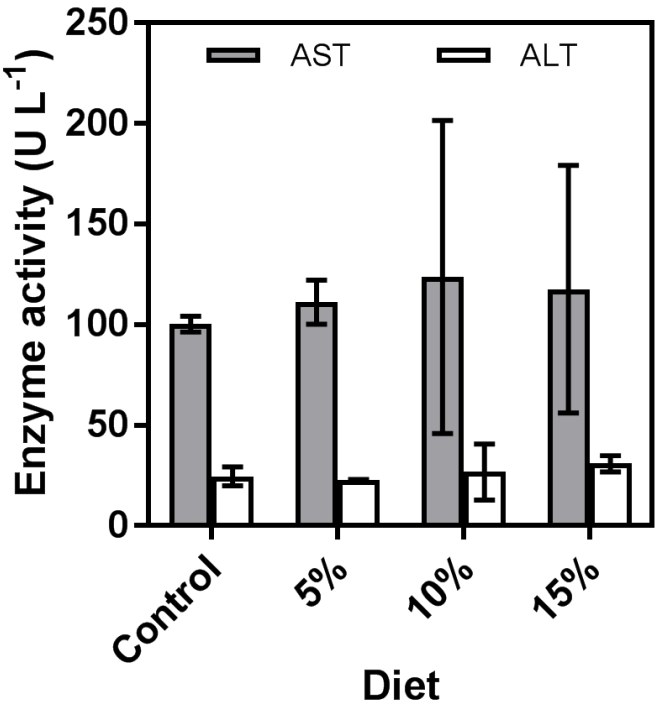


Figure 4.7: Aspartate transaminase (AST) and alanine transaminase (ALT) enzyme activity in plasma (indicators of hepatocyte integrity) taken from mice on four different diets.

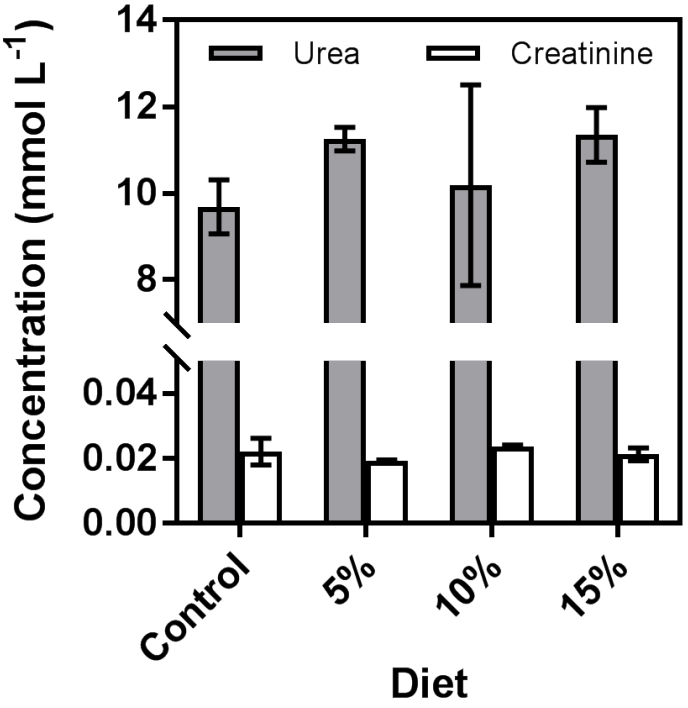


Figure 4.8: Urea and creatinine concentration in plasma (indicators of kidney function) taken from mice on four different diets.

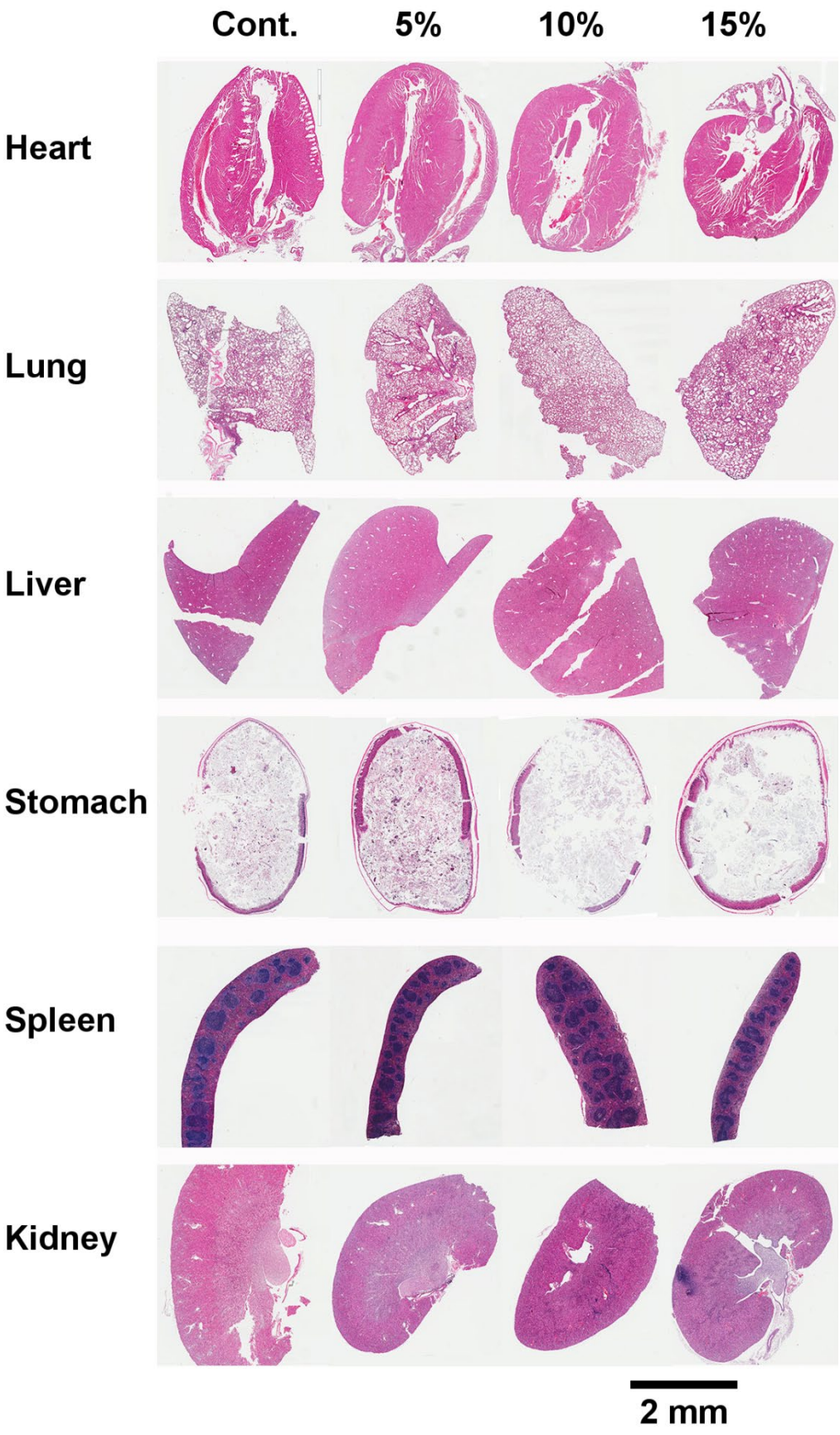


Figure 4.9: Histological micrographs of organs from mice fed control, 5% algae, 10% algae and 15% algae diets.

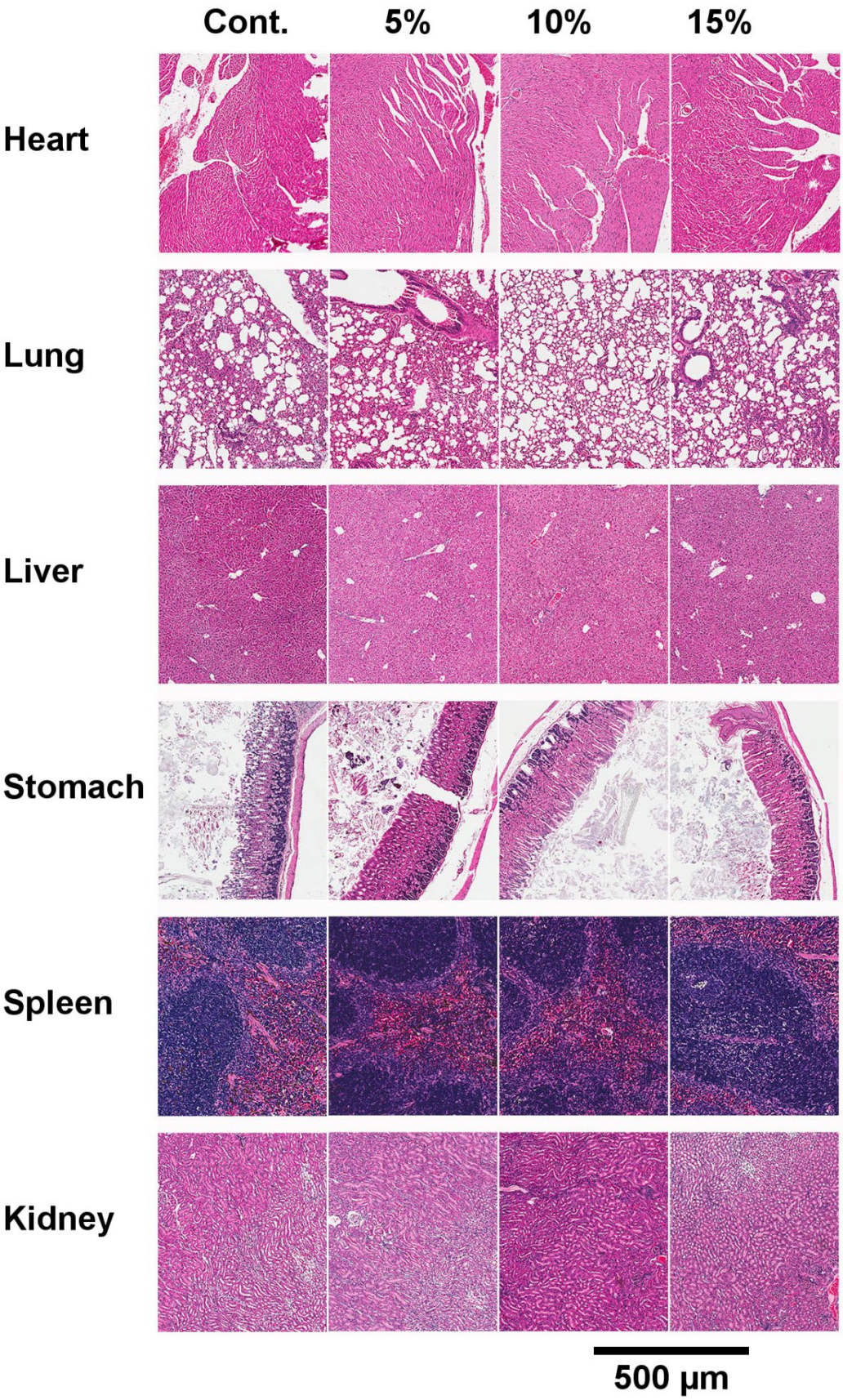


Figure 4.10: Histological micrographs of organ tissues from mice fed control, 5% algae, 10% algae and 15% algae diets.

Histopathological examination revealed no abnormalities in diet-fed mouse tissues. Representative slides from each tissue and diet group are presented for whole organ (Figure 4.9) and cellular (Figure 4.10) analyses. In the hearts, there was no evidence of cardiomyopathy or abnormal cardiac muscle tissue in any group. In the kidneys, there were no abnormalities in the renal capsule or in the renal cortex, and cell structures appear normal in all groups. In the lungs, alveoli appear normal in all groups. In the liver, there were no signs of liver damage or toxicity, and cell appearance is normal in all groups. In the spleen, no change in white/red matter and no overall enlargement of the spleen were observed in any group. In the stomach, no signs of damage to the villi of the stomach lining were observed. Notably, there are regions of imperfect histology thus there may be small ulcerations that remain undetected. However, based on the $n = 3$ sections which contain the majority of the stomach this was deemed to be unlikely.

Due to the acute toxicity of very low concentrations (parts per million) of cyanotoxins, even the mice on the 5% algae diet probably would have experienced toxic effects after only one day. It is unlikely that the diet processing and preparation method eliminated these toxins prior to feeding, as neither freeze-drying [367], [368] nor boiling [369]–[372] have been demonstrated to eliminate cyanotoxins. There were no differences in weight gain, blood biochemistry and tissue histology between the treatment and control groups. Therefore, these results demonstrate the absence of cyanotoxins in this strain of *A. cylindrica* beyond reasonable doubt. Both whole cell and cell extracts could potentially be used in human and animal health products.

4.4 Summary

The aim of this Chapter was to identify a suitable species of microalgae for the biotechnological production of phylloquinone. A range of microalgal strains were screened for phylloquinone content. The cyanobacterium *A. cylindrica* (ANACC strain CS-172) was identified as an extremely rich source of phylloquinone at 200 μg g^{-1} on a dry mass basis, which is approximately three times the Australian guideline for adult daily intake and six times higher than rich dietary sources such as parsley. Further detailed analysis of the biomass composition revealed that this strain was also a rich source of protein, iron and vitamin B_{12}.

One potential concern with the use of cyanobacteria in food products is the presence of cyanotoxins. To evaluate this, a preliminary study was performed using nine mice fed diets supplemented with *A. cylindrica*. No signs of acute toxicity were observed in any of the mice examined. Demonstrating the safety of the whole biomass is important because it means that purified extracts would also be safe.

A. cylindrica is a suitable organism for biotechnological production of phylloquinone. The biomass of *A. cylindrica* may also be used directly as a nutritional supplement. These results demonstrate the promise of whole-cell and cell-extract products from *A. cylindrica* for human and animal health products. Chapters 5 and 6 will focus on optimal culture conditions for *A. cylindrica* to maximize phylloquinone productivity as well as process scale-up.

5 Optimizing nutrient supply for production of vitamin K_1

In this chapter, original research is presented on the optimization of nutrient supply to *A. cylindrica* for the purpose of maximizing vitamin K_1 productivity. Some background information is provided on the key nutrients influencing microalgal growth. Methods are specified for the analysis of vitamin K_1 productivity in *A. cylindrica*. The results of the analyses are discussed in light of the key nutrients identified in the background. The optimal nutrient parameters are determined on the basis of main and interaction effects. Finally, the key points from the chapter are summarized and ideas for subsequent work are proposed.

5.1 Background

Microalgae are traditionally grown photoautotrophically – they use light to produce energy storage chemicals that power their metabolism and growth. Both the intensity and spectral distribution of light affect the uptake of nutrients and the rate at which metabolic processes occur [170]. Changes in light intensity and spectral distribution also induce specific metabolic and structural responses [220]. Therefore light is usually the most important input to the photoautotrophic microalgal biosynthesis of chemicals. Other limiting nutrients are usually carbon (primarily from the carbon dioxide-carbonate buffer system), nitrogen (from a variety of sources including atmospheric nitrogen, nitrates, ammonium and urea) and phosphorus (primarily as phosphate salts) [170]. Hence the aim of this chapter was to identify the influence of nutrient levels on the biotechnological production of phylloquinone using *A. cylindrica*. This is information is essential for maximizing the volumetric productivity of the culture vessel, which is normally the limiting step in microalgal biotechnological processes [170].

The primary role of phylloquinone is to transfer electrons from modified chlorophyll-*a* to the iron-sulfur complexes in photosystem I (PSI) [85], [216]. A role

has also been demonstrated for phylloquinone in the formation of protein disulfide bonds in the thylakoid lumen [218], [373]. Nevertheless, phylloquinone synthesis is primarily linked to PSI synthesis [246], [252], [374], [375]. There are exactly two phylloquinone molecules in every PSI [85], [216], so phylloquinone synthesis is proportional to PSI synthesis. The main controller of PSI number is light: decreasing the light intensity and/or shifting the spectrum towards blue wavelengths induces PSI synthesis [376]–[379]. In cyanobacteria, the *de novo* synthesis of chlorophyll is directed mainly towards PSI [380], [381]. This process appears to be regulated by possibly the most important gene in PSI biogenesis, pmgA [221], [376], [382]. Spectrophotometric analysis of cyanobacterial cultures has been used to probe changes in the PSI-related chlorophyll concentration in response to light intensity [383].

As PSI numbers cannot increase infinitely [354], [384], *de novo* synthesis of phylloquinone will occur mainly in new cells. Maximizing biomass growth is therefore also very important for phylloquinone production. Nitrogen and phosphorus are most frequently identified as the elemental nutrients that limit microalgal growth [235], [236], [385]. Besides carbon, they are the most abundant elements in microalgal cells at around 5-10% and 0.5-1% of dry mass, respectively [170]. Unlike carbon, which comes from atmospheric CO_2, nitrogen and phosphorus are usually required in the medium.

Nitrogen uptake and utilization is complicated, as photosynthetic organisms have the ability to degrade proteins and redistribute amino acids during nitrogen limitation [386], [387]. Although there is evidence that PSI is retained while PSII is degraded during nitrogen limitation [386], [388], ample nitrogen is required to maximize rate of photosynthesis [387]. Furthermore, utilization of nitrogen from the medium [389], [390] or atmosphere [237], [391] is governed by various feedback mechanisms from photosynthesis. Nitrogen utilization rate is also linked to carbon fixation rate [392]. The level of nitrogen in the medium would likely have significant effects on growth rate and interactions with light intensity.

Phosphorus is required at only 5-10% of the molar concentration of nitrogen [391], [393], [394]. However, phosphorus is an indispensable input to all cellular processes, including photosynthesis and carbon fixation. During photosynthesis, phosphate is incorporated into adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) [221]. During carbon fixation, the energy released by dephosphorylation of ATP and oxidation of NADPH is used to convert CO_2 into simple sugars [395]. The molecules ATP and NADPH are used to power nitrogen uptake [237], [389], [390], [396]. Phosphorus is also an essential element of DNA and phospholipids membranes [397]. Phosphorus level in the medium should also greatly impact the growth behavior.

Light, nitrogen and phosphorous supply have been identified as the nutrients that are most likely to impact phylloquinone productivity. From a practical perspective, intensity is the best aspect of light to vary as frequency and duration are normally dependent upon day:night cycles. In most microalgal culture media, nitrogen is supplied as nitrate and phosphorus as phosphate. Hence, the effects of light intensity, nitrate concentration and phosphate concentration were selected for testing.

5.2 Method and materials

5.2.1 Photo-bioreactor cultures

The effect of light intensity, nitrate and phosphate concentrations on phylloquinone productivity were investigated in *A. cylindrica*. The cyanobacterium was grown in custom-made flat panel PBRs, adapted from a design used previously [398] and constructed from clear acrylic sheet as shown in Figure 5.1. Deionized water, MLA nutrient concentrate and inoculum were added such that the liquid volume was 5 L, medium concentration was 1 × MLA, and the initial optical density (at a wavelength of 550 nm) was approximately 0.1. Sodium nitrate and/or dipotassium hydrogen phosphate were added at 10 × concentration in some experiments (see Table 5.1 for details). Light was provided by two or four cool white (6000 K) LED linkable aluminium strip lights (Jaycar). The light intensity was measured at 9 points across the front face of the reactor (Figure 5.2) and the mean PPFD was approximately 170 μmol m^{-2} s^{-2} for two strip-lights (1 × light) and 330 μmol m^{-2} s^{-2} for four strip-lights (2 × light). Measurements of light intensity were made with the PBRs full of water but without aeration.

Table 5.1: Medium ingredients and concentrations used in this experiment.

Ingredient	Concentration (mg L^{-1})			
	Normal	10 × N	10 × P	10 × N, 10 × P
$NaNO_3$	170	1700	170	1700
$MgSO_4.7H_2O$	49.2	49.2	49.2	49.2
K_2HPO_4	34.8	34.8	348	348
$CaCl_2.2H_2O$	29.4	29.4	29.4	29.4
$NaHCO_3$	16.8	16.8	16.8	16.8
H_3BO_3	2.40	2.40	2.40	2.40
Na_2EDTA	4.56	4.56	4.56	4.56
$FeCl_3.6H_2O$	1.58	1.58	1.58	1.58
$MnCl_2.4H_2O$	0.360	0.360	0.360	0.360
$ZnSO_4.7H_2O$	0.022	0.022	0.022	0.022
$CoCl_2.6H_2O$	0.010	0.010	0.010	0.010
$CuSO_4.5H_2O$	0.010	0.010	0.010	0.010
$Na_2MoO_4.2H_2O$	0.006	0.006	0.006	0.006

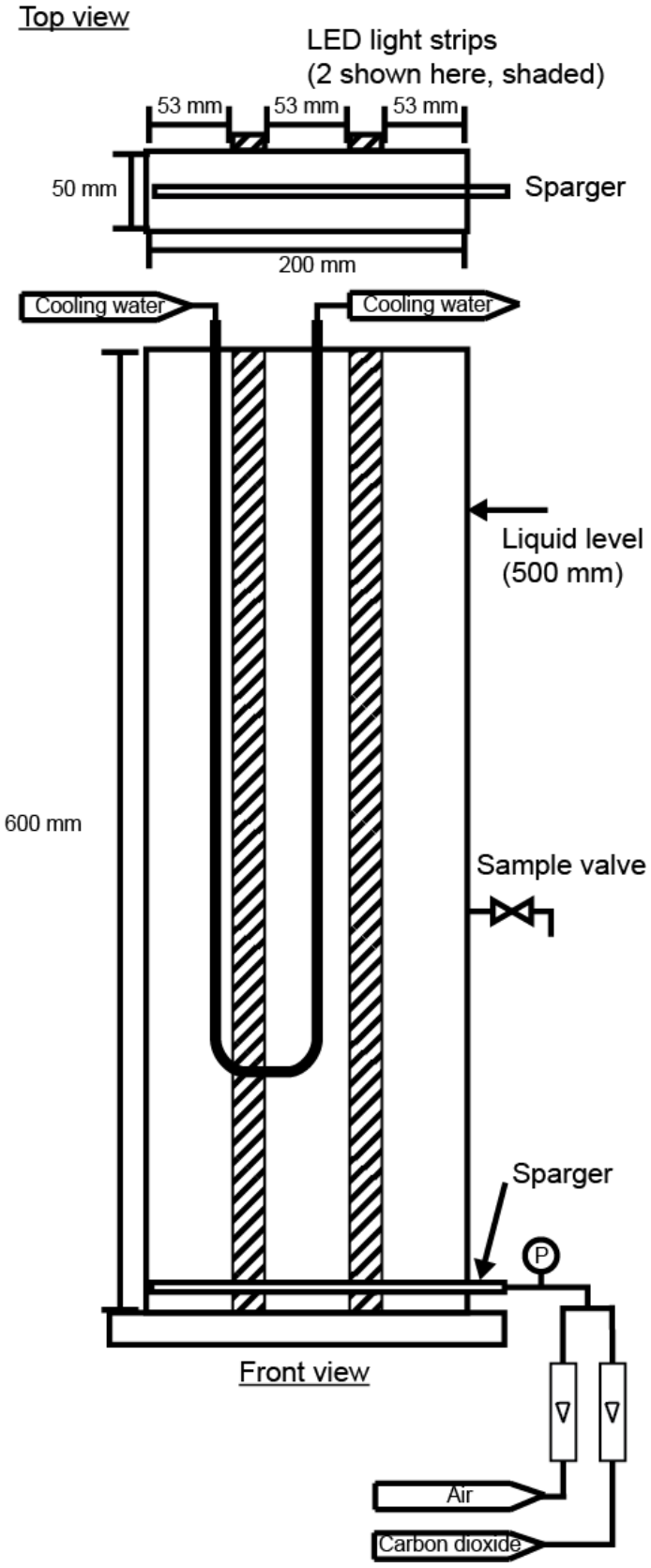


Figure 5.1: Schematic of the flat-panel PBR apparatus.

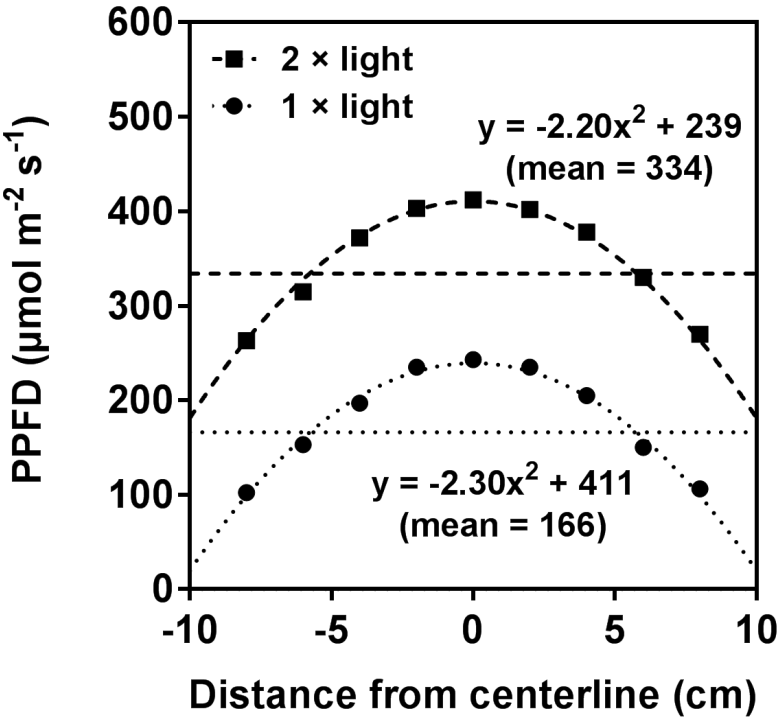


Figure 5.2: Light intensity distribution measured across the face opposite to the strip-lights. Tanks were filled with water but no air flow was supplied. Horizontal lines are the geometric means of the respective quadratic fits.

The temperature was regulated by 23 °C water passing through a 6 mm diameter U-shaped stainless-steel tube with an approximate surface area of 0.02 m^2. Air enriched with 1% CO_2 (food-grade, Coregas, Yennora, NSW, Australia) was introduced into the base of the PBR at 1 vvm (5 L min^{-1}) from a perforated, 150 mm-long, 6 mm diameter stainless-steel tube. Flow rates for both air and carbon dioxide were measured using RM series rotameters (Dwyer Instruments, Michigan City, IN). The optical density of the culture was measured daily. Minimum and maximum temperatures were measured daily using an inside/outside digital thermometer (Jaycar Electronics, Rydalmere, NSW, Australia). The pH was measured daily using MColorpHast™ pH test strips (Merck KGaA, Darmstadt, Germany). Samples were retained for Dry Cell Weight (DCW) and vitamin K_1 analysis. The total volume of medium was kept constant by the addition of deionized water into the PBR before sampling. Samples were retained for vitamin K_1 concentration and DCW analysis.

All photo-bioreactor experiments were performed 3 times (i.e. n = 3) to ensure reproducibility. Extraction and analysis of phylloquinone was conducted as per the method in Chapter 4. Sample preparation was based upon the method of Breuer et al [341], while the analysis method was adapted from AOAC method 999.15 [342].

5.2.2 Biomass growth analysis

Optical density of all cultures was measured by absorbance at 550 nm for estimation of biomass, as recommended by Myers et al. [399], using a Varian Cary 50 UV-vis spectrophotometer. Absorbance in the red portion of the spectrum was also measured as a potential proxy for phylloquinone content: 680 nm (typical algal red-absorption maximum [400]); 664 nm (chlorphyll-a absorption maximum [401]); and, 647 nm (chlorophyll-*b* absorption maximum [401]). The initial A_{550} was approximately 0.1 in all experiments. The method used to determine DCW was based upon that of Zhu and Lee [402]. Quantitative-grade glass filter papers (GA-55, Advantec Toyo Roshi Kaisha Ltd., Tokyo, Japan), were placed on a watch-glass and weighed using an analytical balance. Samples of culture of known volume (10-50 mL) were vacuum filtered through the filter papers and washed with 100 mL of 0.5 M ammonium bicarbonate solution. The filter papers were returned to the watch glass and dried overnight at 105 °C, allowed to cool in a desiccator, then weighed again. The DCW was calculated as the difference in final and initial weights divided by the volume of filtered culture.

5.2.3 Statistical analyses

Productivity metrics were determined for each of the 24 PBR test runs (8 test conditions, 3 repeats for each condition). These included specific growth rate (μ), DCW productivity, mean specific phylloquinone content and phylloquinone volumetric productivity. Statistical analyses were performed using GraphPad Prism 7 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Linear regression of the A_{550} and DCW results pooled from all 24 samples (184 data points) revealed a strong positive correlation (Figure 5.3, R^2 = 0.97). Values of A_{550}, which exhibited less

variation than values of DCW, were therefore used to calculate the specific growth rates (μ) over days 2-7 according to the exponential growth equation:

$$\text{Specific growth rate, } \mu = \frac{ln\left(\frac{X_t}{X_0}\right)}{t} \tag{5.1}$$

DCW productivity was determined from the slope of the linear fit of DCW versus time over days 2-10:

$$\text{Biomass productivity} = \frac{DCW}{t} \tag{5.2}$$

Phylloquinone productivity was determined from the slope of the linear fit of DCW × phylloquinone concentration versus time over days 2-10:

$$\text{Phylloquinone productivity} = \frac{DCW \times [PK]}{t} \tag{5.3}$$

Three-way analysis of variance (ANOVA) was run on values of μ, biomass productivity, phylloquinone concentration and phylloquinone productivity to examine the effect of light intensity, nitrate and phosphate concentration.

5.3 Results and discussion

5.3.1 Correlations between absorbance, DCW and phylloquinone specific concentration

Spectrophotometric analysis is known to be useful for rapid estimation of parameters such as chlorophyll content [403], DCW and more [399]. Dry cell weight (DCW) was plotted against A550, and a strong correlation found; DCW was approximately equal to 4 × A_{550}. Based upon the strong relationship between DCW and A_{550} (Figure 5.3, R^2 = 0.971), A_{550} is an appropriate proxy for DCW in the calculation of growth rate.

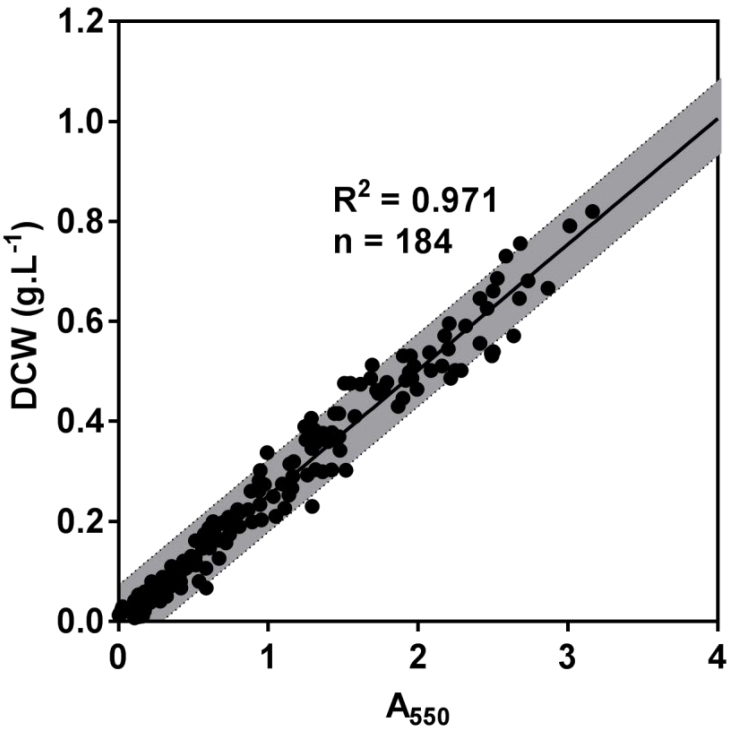


Figure 5.3: Dry Cell Weight (DCW) versus light Absorbance at 550 nm (A_{550}) for all samples collected during the course of the experiment.

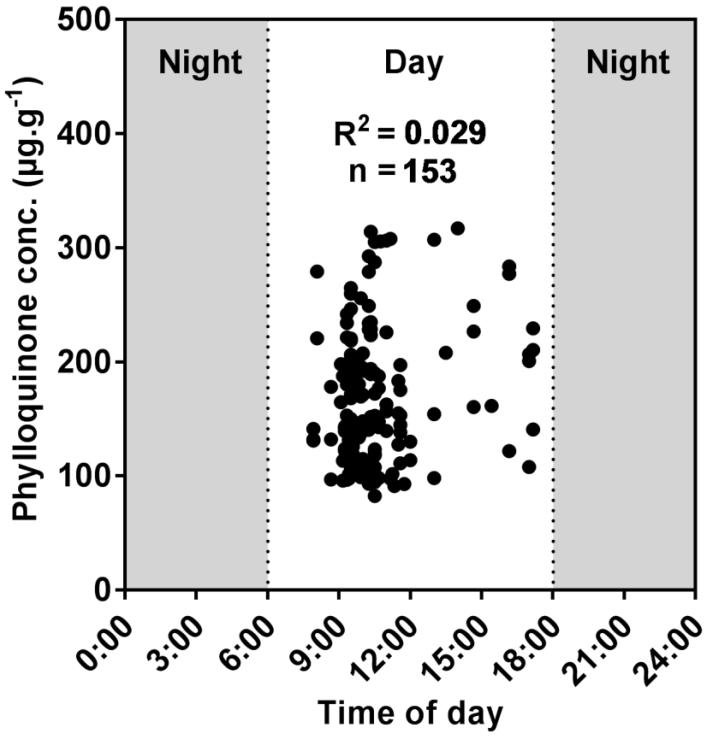


Figure 5.4: Phylloquinone specific concentration (based on dry mass) versus time of day.

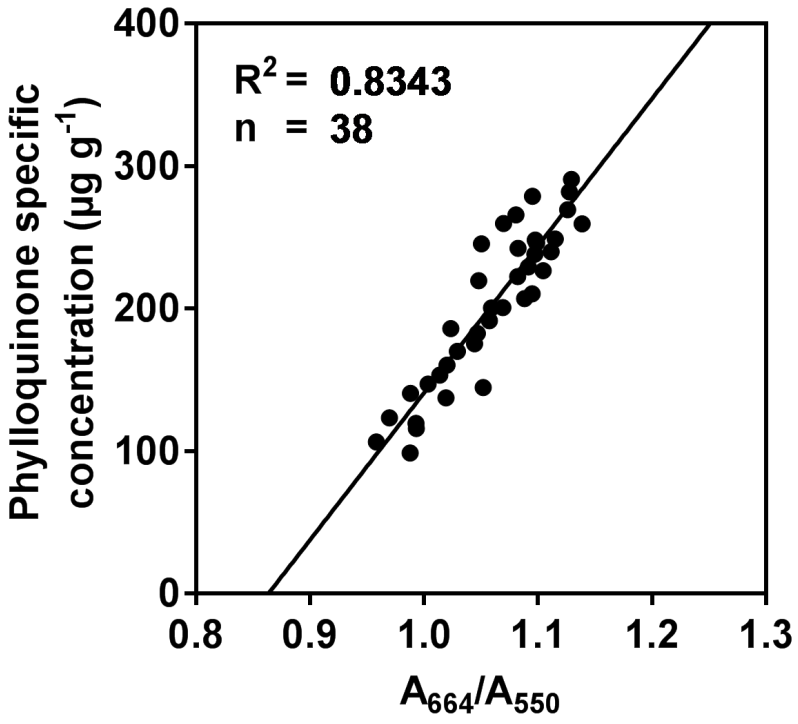


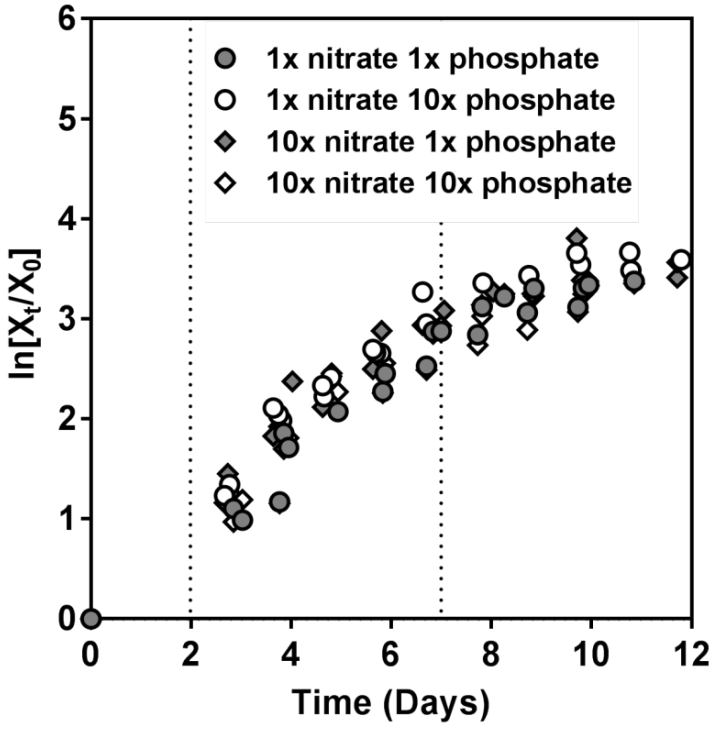
Figure 5.5: Correlation of chlorophyll-*a* absorbance (A_{664}) with specific phylloquinone concentration.

Prior to any calculation of correlations with phylloquinone, the phylloquinone specific concentration was plotted against time of day (Figure 5.4). No linear correlation was observed (R^2 = 0.029), so the time of sampling did not affect the results. This is consistent with the fact that PSI numbers change slowly over a period of days in the process of photoacclimation [223], [384], [404]. The variation in specific phylloquinone concentration therefore cannot be attributed to time of day effects.

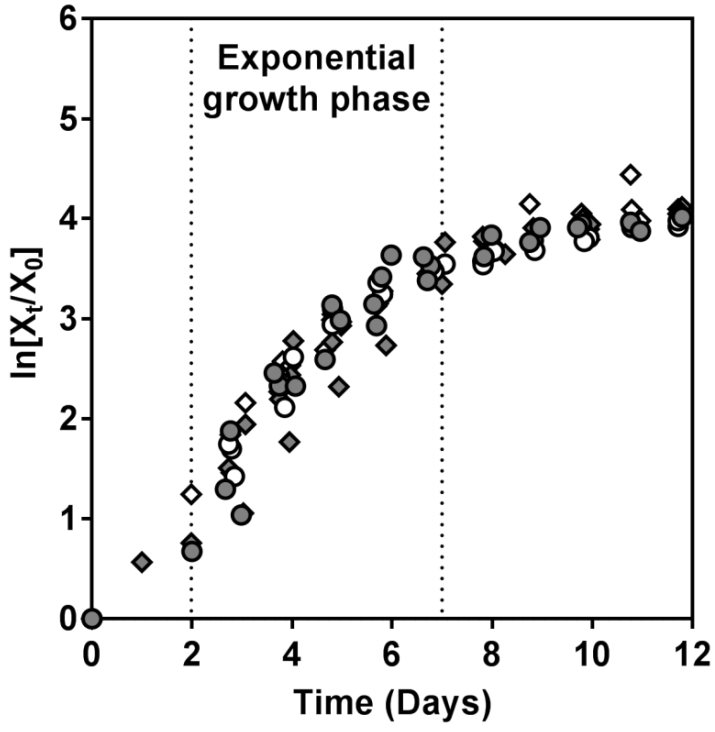
Absorbance of light at 647, 664 and 680 nm (normalized by the absorbance at 550 nm due to cell density) was plotted against specific phylloquinone concentration. The ratio A_{664}/A_{550} had the strongest linear relationship (Figure 5.5, R^2 = 0.834), followed by A_{647}/A_{550} (R^2 = 0.822). The linear fit of A_{680}/A_{550} (R^2 = 0.578) was poor. These results suggest that chlorophyll-*a* absorbance is a good proxy for phylloquinone concentration. In this case, phylloquinone specific concentration increased 103 μg g^{-1} for every 0.1 unit increase in A_{664}/A_{550}.

5.3.2 General trends in growth and phylloquinone productivity

The exponential growth phase and specific growth rates were determined from the data plotted in Figure 5.6. The slope (~0.5 d^{-1}), exponential growth phase (days 2-7) and linear growth phase (days 7-10) are especially clear in Figure 5.6b. The biomass productivity was determined from the data plotted in Figure 5.7. The day-on-day increase in DCW is clearly greater with 2 × light intensity (Figure 5.7b), though the effects of nitrate and phosphate are not so obvious. The phylloquinone volumetric productivity was determined from the data plotted in Figure 5.8. The effects of the nutrients are obvious, where 10 × nitrate is associated with the greatest day-on-day increase in both 1 × and 2 × light cases.

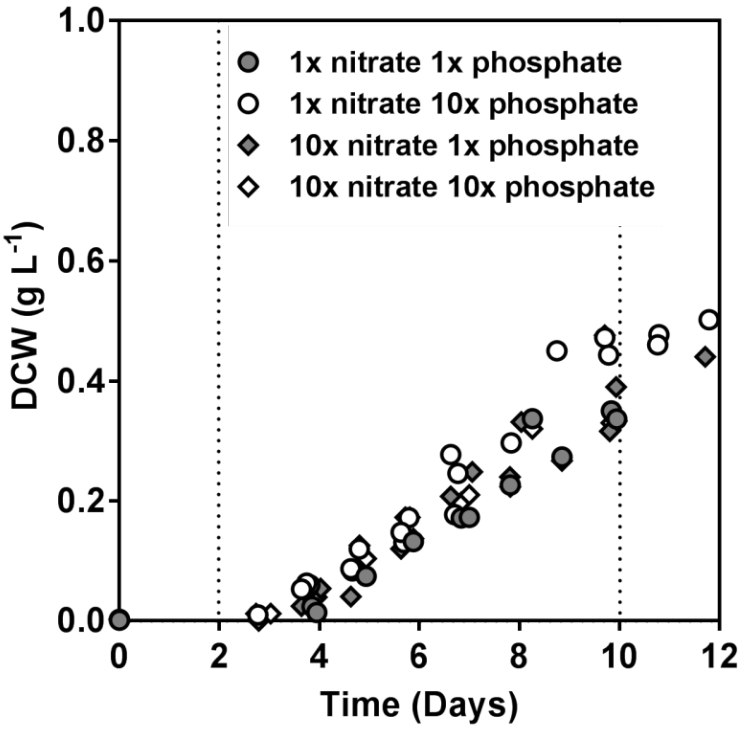


(a)

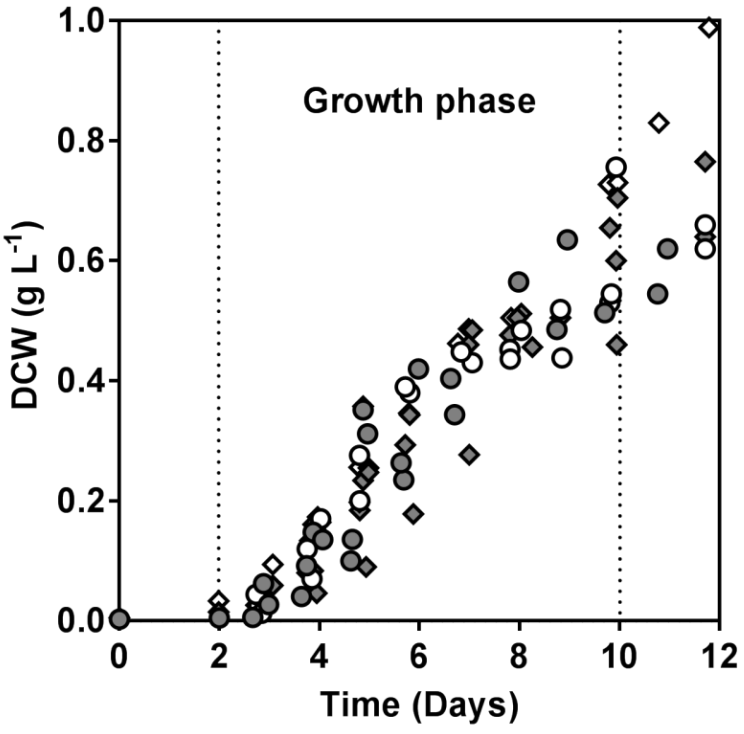


(b)

Figure 5.6: Log of culture density versus time for 1× lights (a) and 2× lights (b).

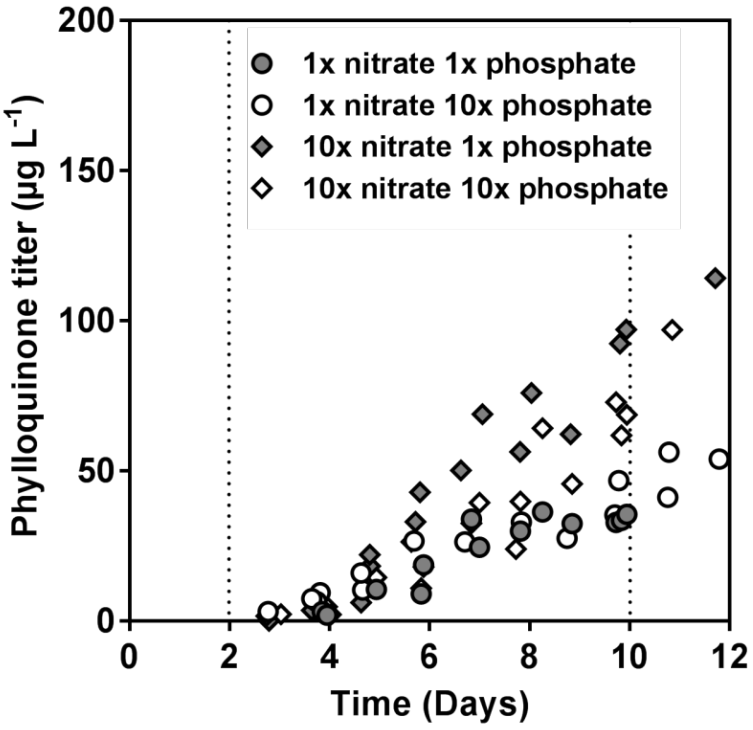


(a)

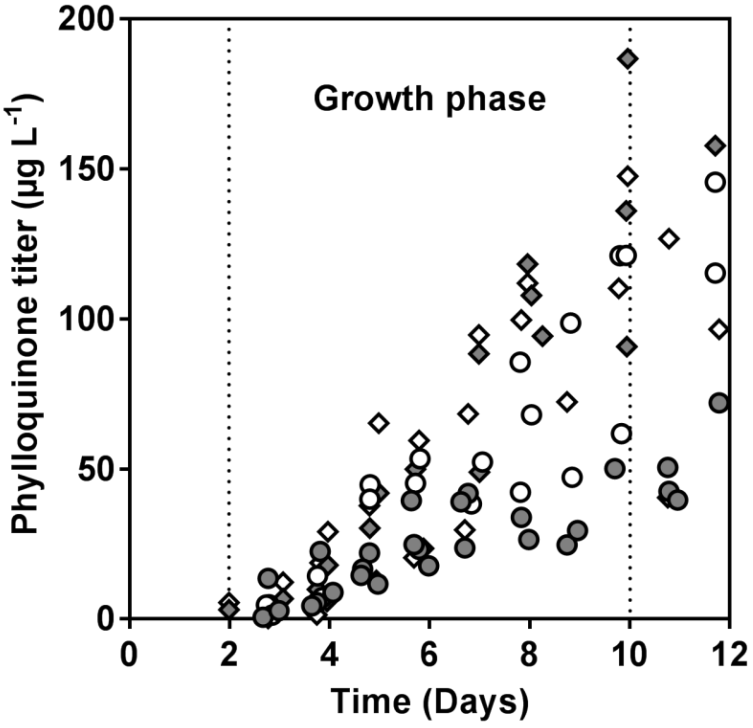


(b)

Figure 5.7: Dry cell weight versus time for 1× lights (a) and 2× lights (b); and phylloquinone titers versus time for 1× lights (e) and 2× lights (f).



(a)



(b)

Figure 5.8: Phylloquinone titers versus time for 1× lights (a) and 2× lights (b).

5.3.3 Optimal conditions

The effects of light intensity, nitrate and phosphate concentrations on growth and phylloquinone productivity in *A. cylindrica* are summarized in Table 5.2. No significant three-way interaction effects were observed. Generally, increased light intensity was correlated with faster growth rate and lower phylloquinone specific concentration. Increased nitrate concentration was correlated with higher phylloquinone specific concentration. Overall, increased light intensity and nitrate concentration were correlated with increased biomass and phylloquinone productivity.

The highest growth rate (0.56 d^{-1}) and phylloquinone productivity (22 μg L^{-1} d^{-1}) were at 2 × light (PPFD 330 μmol m^{-2} s^{-1}), 10 × nitrate and 1 × phosphate. The specific growth rates observed here were in the range 0.4 - 0.6 day^{-1}, which is typical for cyanobacteria at room temperature [405]–[407]. Biomass productivity was in the range 0.05 – 0.1 g L^{-1} d^{-1}, such values being comparable with previously reported values for the batch culture of cyanobacteria [407].

Table 5.2: Summary of test conditions and results for the effect of light, nitrate and phosphate on phylloquinone productivity in 5 L PBR cultures. Aggregate daily mean (S.D.) values for pH and temperature: pH 7.14 (0.65); minimum temperature 22.2 °C (0.75 °C); maximum temperature 23.7 °C (0.67 °C). Optimum values highlighted in bold.

PPFD (μmol m^{-2} s^{-1})	$NaNO_3$ (mg L^{-1})	K_2HPO_4 (mg L^{-1})	Specific growth rate, μ (d^{-1})	DCW at day 10 (g L^{-1})	Productivity (μg L^{-1} d^{-1})	Titer at day 10 (μg L^{-1})
170	170	34.8	0.44 (0.02)	0.34 (0.01)	4.9 (1.5)	34 (1.4)
170	170	348.0	0.39 (0.07)	0.46 (0.01)	5.7 (1.1)	41 (8.1)
170	1,700	34.8	0.45 (0.06)	0.39 (0.08)	15.0 (2.9)	95 (3.3)
170	1,700	348.0	0.44 (0.01)	0.33 (0.00)	12.1 (3.9)	68 (5.6)
330	170	34.8	0.52 (0.07)	0.56 (0.06)	5.0 (0.4)	47 (6.2)
330	170	348.0	0.52 (0.02)	0.61 (0.13)	11.8 (2.9)	101 (34.2)
330	1,700	34.8	0.56 (0.03)	0.65 (0.05)	22.0 (2.8)	161 (35.9)
330	1,700	348.0	0.44 (0.06)	0.73 (0.00)	16.0 (2.6)	129 (26.4)

5.3.4 Light intensity

The 3-way ANOVA of specific growth rates showed a significant main effect of light intensity ($F(1, 16) = 15.04$, $p = 0.0013$). The mean growth rate for 1 × light (i.e. an intensity of 170 μmol m^{-2} s^{-2}) was 0.43 day^{-1} while for 2 × light (an intensity of 330 μmol m^{-2} s^{-2}) it was 0.51 day^{-1}, indicating that the 1 × light cultures were mildly light-limited during the exponential growth phase (as shown in Figure 5.6). Such results are in line with the literature, where cyanobacteria were reported to generally grow fastest at light intensities > 200 $\mu mol.m^{-2}.s^{-1}$ [406], [408].

There was a significant main effect of light intensity on DCW ($F(1, 16) = 266.8$, $p < 0.0001$). Values for DCW productivity at 2 × light (mean 0.103 g L^{-1} d^{-1}) were approximately double those at 1 × light (mean 0.052 g L^{-1} d^{-1}). These results support the conclusion that light was the growth limiting factor in these experiments. Interestingly, it was observed that increased light intensity led to a decrease in the specific phylloquinone concentration (Figure 5.11). We hypothesize that this is due to a reduction in the PSI/PSII ratio with increased light intensity [220], [376]. Both the phylloquinone titer and productivity were observed to be significantly greater at higher light intensity, with the increase in cell growth more than compensating for the reduced concentration of phylloquinone.

5.3.5 Nitrate Concentration

The 3-way ANOVA of phylloquinone concentration showed that there was a significant main effect of nitrate concentration, $F(1, 16) = 40.15$, $p < 0.0001$. As shown in Figure 5.11, the addition of nitrate to the medium had a significant effect on the mean specific phylloquinone concentration (134 μg g^{-1} for 1× nitrate and 180 μg g^{-1} for 10 × nitrate). Volumetric productivity (Figure 5.12) also showed a significant interaction ($F(1, 16) = 83.73$, $p < 0.0001$), which can be explained by the increase in the specific phylloquinone concentration as the DCW (Figure 5.10) and specific growth rate (Figure 5.9) were comparable for experiments with both low and high levels of

nitrate. PSI is required for the photoreduction of nitrate and nitrite, and is therefore involved in the utilization of nitrate [389], [390], [409]–[411]. Nitrogen is an essential and often limiting ingredient for synthesis of proteins [387] in both PSI [221], [386], [387] and the phylloquinone biosynthetic pathway [412], [413]. It is possible that increased nitrogen leads to increased protein synthesis. Alternatively, it could be that nitrogen limitation is occurring in the 1 × nitrate cultures, and that this is limiting PSI and phylloquinone concentrations. It may be that both of these phenomena contribute to increased specific phylloquinone concentrations.

5.3.6 Phosphate concentration

Addition of phosphate to the medium did not lead to significant increases in the DCW productivity (Figure 5.10) or phylloquinone concentration (Figure 5.11). There was a significant two-way interaction of nitrate and phosphate on phylloquinone volumetric productivity ($F(1,16) = 16.18$, $P = 0.0010$). Addition of phosphate led to a decrease in the mean volumetric productivity from 18.48 μg L^{-1} d^{-1} (for medium with 10 × nitrate and 1 × phosphate) to 8.76 μg L^{-1} d^{-1} (for medium with 1 × nitrate 10 × phosphate).

Optimal atomic N:P ratios from the literature can range from 7:1 to 45:1 based upon genetic differences and growth stage, with a typical value being 15:1 [393], [414]. The standard ratio in 1 × MLA is 10:1 with 2 mmol L^{-1} nitrogen (28 mg L^{-1}) and 0.2 mmol L^{-1} phosphorus (6.2 mg L^{-1}). Although lower than average, a N:P ratio of 10:1 has been found to favor cyanobacterial growth [391], [393]. In freshwater, typical growth limiting values of phosphorus are 0.1 mg L^{-1} (5 μmol L^{-1}) or less [394], [415], and increasing the N:P ratio does not appear to negatively affect growth when phosphorus is adequate [394]. However, addition of large amounts of phosphorus alone at low N:P ratios does not improve the growth rate [236], [385], [394], and may even be inhibitory [281].

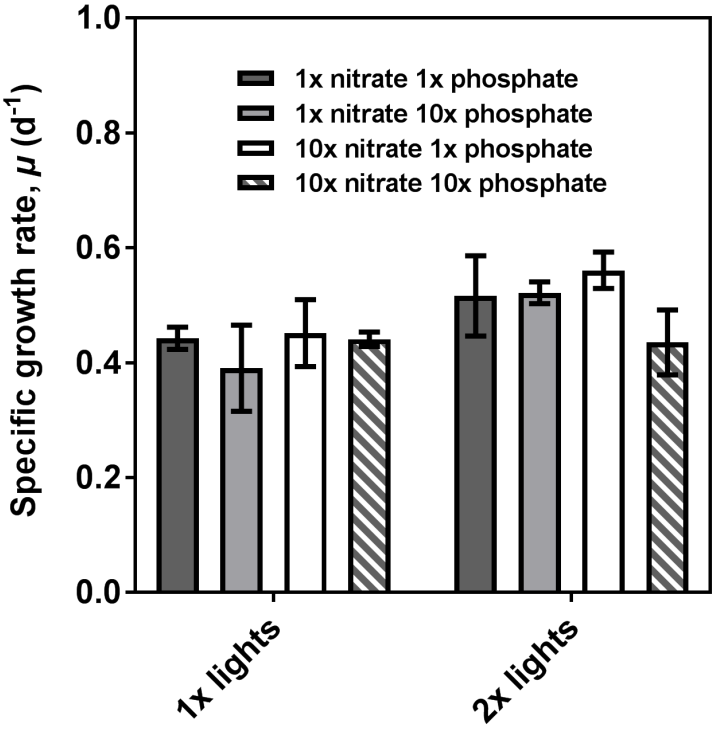


Figure 5.9: Plot showing specific growth rates versus light intensity for the different growth media used. Reported results are averages while error bars denote one standard deviation about the mean (n = 3).

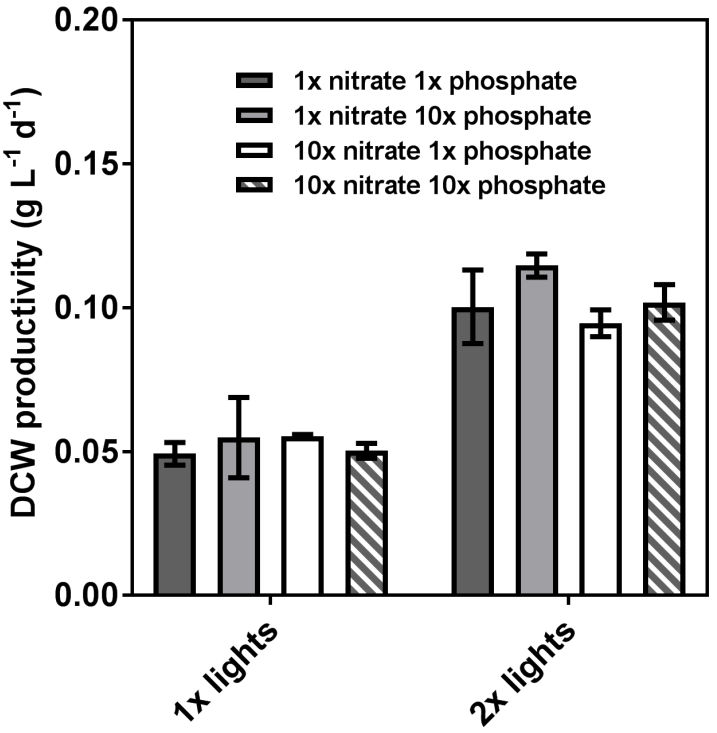


Figure 5.10: Plot showing DCW productivities versus light intensity for the different growth media used. Reported results are averages while error bars denote one standard deviation about the mean (n = 3).

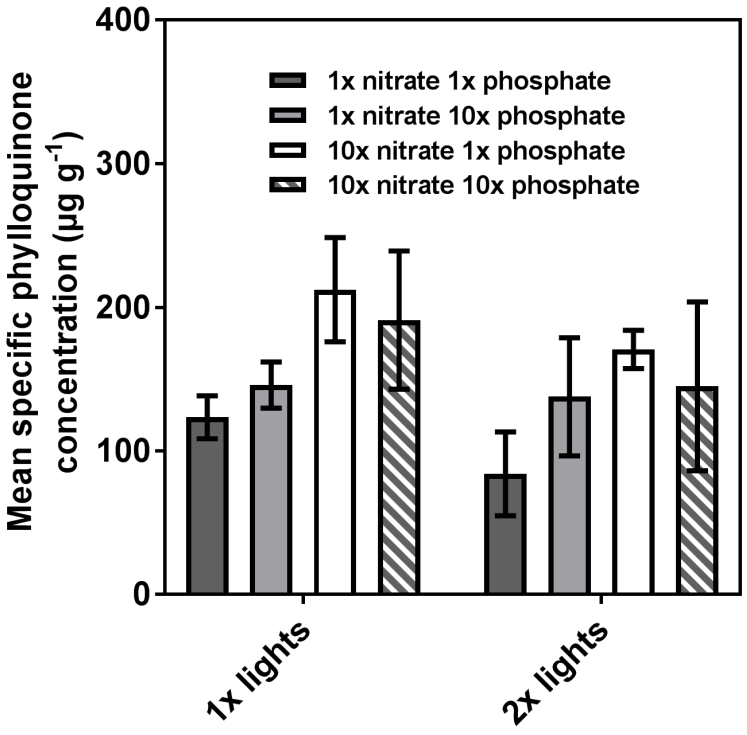


Figure 5.11: Plot showing mean specific phylloquinone concentrations over 10-days versus light intensity for the different growth media used. Reported results are averages while error bars denote one standard deviation about the mean (*n* = 3).

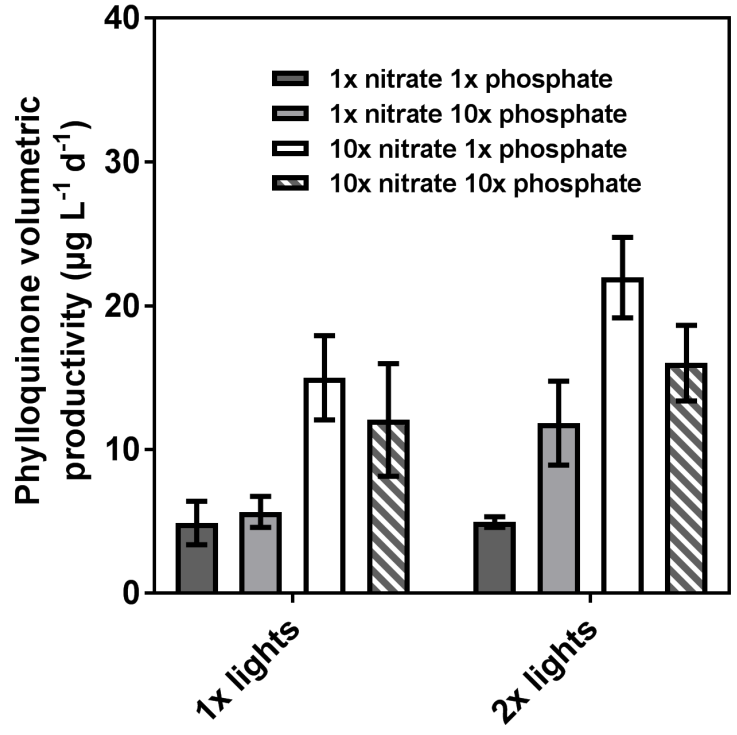


Figure 5.12: Plot showing phylloquinone productivities versus light intensity for the different growth media used. Reported results are averages while error bars denote one standard deviation about the mean (*n* = 3).

5.4 Conclusions

The aim of this work was to determine the effect of key nutrient levels on the biotechnological production of phylloquinone by *A. cylindrica*. The effects of light, and the concentrations of nitrogen and phosphorus, were examined. These variables were chosen because they are widely regarded as the most important for PSI synthesis and microalgal growth.

Generally, specific phylloquinone concentration increased with lower light and higher nitrate concentration. It was not correlated with time of day, though it was correlated with chlorophyll absorbance at 647 and 664 nm. Phylloquinone titer was most strongly influenced by biomass DCW. Biomass DCW was strongly correlated with absorbance at 550 nm. Furthermore, biomass productivity could be increased by boosting the nitrate concentration and light intensity. By increasing the nitrate concentration ten times and doubling the light intensity, it was possible to increase the phylloquinone productivity and final titer fourfold, to 22 $\mu g\ L^{-1}\ day^{-1}$ and 161 $\mu g\ L^{-1}$ respectively. On the other hand, increased phosphate concentration did not improve the phylloquinone productivity.

In addition to determining the effects of different levels of light, nitrate and phosphate, spectrophotometric indices were established for the rapid analysis of DCW and phylloquinone specific concentration. The DCW was proportional to A_{550}, while the specific phylloquinone concentration showed a linear relationship with A_{664}/A_{550}. Direct spectrophotometric analysis of the culture can be used to simply and rapidly estimate productivity metrics.

The ability to manipulate the biomass productivity and phylloquinone content of *A. cylindrica* by nutrient variation was successfully achieved. Such results demonstrate the potential for biotechnological production of phylloquinone from *A. cylindrica* grown in closed PBRs; future work will focus on both process optimization and scale-up.

6 Scale-up of *Anabaena cylindrica* culture in photo-bioreactors for vitamin K_1 production

This chapter presents original research on the scale-up of vitamin K_1 production using *A. cylindrica*; from the 5 L reactors in the previous chapter to 50 L. A comprehensive background on scale-up in bubble column PBRs is provided and the most important operating parameters are identified. Methods are given for the analysis of the performance of the 50 L PBR and the productivity of vitamin K_1. The results are discussed in light of the parameters tested. Finally, the key points and conclusions of the chapter are summarized.

6.1 Background

In order to produce any microalgal product at an industrial scale, the culture system must be carefully designed to maximize productivity while minimizing capital and operating costs. Key reactor design criteria for maximal productivity include high surface to volume ratio, low oxygen accumulation, good mixing, consistent temperature, adequate supply of CO_2, resistance to contamination, and ease of cleaning [170]. Industrial culture systems are generally known as Photo-Bioreactors (PBR), however the term PBR is used mainly to refer to closed systems rather than open ponds or raceways [170]. Bubble column PBRs are a promising class of culture systems as their areal and volumetric productivities are much better than ponds or raceways, and similar to flat-panel or tubular designs [275]. They are also estimated to be cheaper to build and operate than most other closed PBR designs [275].

The column of rising bubbles in bubble column PBRs can simultaneously remove excess oxygen and supply CO_2; these characteristics are largely independent of scale. However, achieving a high surface-to-volume ratio, maximizing mixing and minimizing cost of construction in large-scale systems requires a trade-off. Larger columns generally have a lower reactor cost per volume [275]; however, increasing the column diameter is known to reduce productivity due to increased light

limitation near the center of the reactor. The rate of growth is strongly influenced by the rate at which photosynthesis can proceed, which is in turn dependent upon the intensity of light. For example, it has been shown that the productivity of *Chlorella* sp. declines from 87 mg L^{-1} h^{-1} in a 16 mm diameter tube, to 51 mg L^{-1} h^{-1} in a 50 mm glass tube [416]. It is important to note that the maximum rate of photosynthesis is also dependent upon temperature [226], [227], [406], [417], so it is important to ensure that microalgae are grown at the optimal temperature [284], [418].

To ensure that all cells in a bubble column reactor experience a similar level of light, temperature, CO_2 and O_2, it is essential that mixing is good. Temperature gradients can develop in poorly mixed columns. Furthermore, the rate of photosynthesis may be negligible in the central "dark zone", leading to lower CO_2 consumption and O_2 generation. Mixing between the dark zone and the outer layer can be improved by increasing turbulence in the bubble flow regime [291]. The "churn-turbulent" flow regime is characterized by large bubbles (>4 mm diameter for air-in-water systems) flowing unsteadily (i.e. with appreciable side-to-side movement) as they rise [274], [290], [291]. However, the onset of churn-turbulent flow is generally observed at or above 0.2 m column diameter and 5.0 cm s^{-1} superficial gas velocity [291], [419]. A column diameter of 0.2 m is therefore a good trade-off in order to optimize mixing and surface-to-volume ratio.

Pioneering work led by Yusuf Chisti and Emilio Molina Grima at the University of Almeria, Spain, has demonstrated that BC-PBRs of 0.2 m diameter and 2 m height achieve comparable productivities to tubular PBRs; however, their reactor was operated at superficial gas velocities below 5 cm s^{-1} [275], [420]. They hypothesized that increasing bubble size in the dark zone would improve mixing without increasing the energy consumption or destructive mechanical stresses [275]. In the homogeneous (bubbly) flow regime of air-water systems, increased sparger hole diameters (in the 0-2.5 mm range) generate larger bubbles [274]. Increased sparger hole diameters [421] and larger bubbles [422], [423] also lead to reduced gas holdup. These observations support evidence that directly relates larger holes to the

generation of larger bubbles [424]. Larger bubbles are known to reduce the superficial gas velocity required to transition to a churn-turbulent bubble flow regime [291].

As previously mentioned, the main limitation in large-diameter bubble column PBRs is mixing between the dark zone and the surface of the reactor. The mixing can be quantified by the radial dispersion coefficient (D_r, $cm^2\ s^{-1}$), which represents the rate at which a concentration of matter is dispersed by advection and diffusion throughout the reactor volume. Very little research has been published on radial mixing, however increasing the superficial gas velocity (U_g, calculated by dividing the volumetric gas flow rate by the cross sectional area of the column) over the range 0-5.5 cm s^{-1} generally improves mixing [425]. The group of Chisti and Molina measured D_r in their system using an acid tracer method [426]. Increasing D_r was shown to increase biomass productivity; however D_r was reasonably constant at superficial gas velocities up to 5 cm s^{-1} [426]. This is unsurprising, as the turbulent bubble flow regime (characterized by high radial mixing) usually does not occur at superficial velocities below 5 cm s^{-1}. It is likely that superficial gas velocities of at least 5 cm s^{-1} are required in order to improve the radial dispersion coefficient. In a 20 cm diameter column, this requires an air flow rate of at least 85 L/min, which is much higher than the values reported in the literature [278], [283], [420].

Another means by which productivity can be improved in PBRs is by using continuous (24 hours per day) artificial illumination. This is usually implemented indoors, so artificially illuminating BC-PBRs is only feasible when the product is of high value and a high degree of cleanliness is required. This method has been successfully used to boost the productivity of astaxanthin (an antioxidant pigment) from *Haematococcus pluvialis* [427]–[429]. Commercially, continuous illumination is used to boost the astaxanthin productivity of *H. pluvialis* grown in bubble column reactors, such as at the New Zealand-based company Supreme Health [430]. Continuous illumination is not appropriate for all algae as it has been found to inhibit growth in some strains [431], [432].

In the present work, *A. cylindrica* was grown in a 50 L bubble column PBR very similar to the system used by the group of Chisti and Molina. The primary aim was to determine the feasibility of producing phylloquinone from *A. cylindrica* at a scale reflective of commercial reality. It was predicted that superficial gas velocity above 5 cm s^{-1}, larger bubble size and longer illumination duration would improve biomass (and thus phylloquinone) productivity. Therefore, the secondary aims were to investigate the effect of sparger design and air flow rate on biomass and phylloquinone productivity; and, the effect of duration of illumination on biomass and phylloquinone productivity.

6.2 Method and materials

6.2.1 Flask cultures

Anabaena cylindrica was grown in flasks to test the effects of different temperatures (23 °C, 28 °C, 33 °C, 38 °C) and pH (7.5 and 9.5) on growth rate. Modified ASM-1 medium (MLA) was prepared as per Bolch and Blackburn [336] without B vitamins and selenium. To prepare experimental cultures, fresh medium (100 mL) was added to 250 mL Erlenmeyer flasks, then autoclaved and allowed to cool for 24 hours. The pH was adjusted by addition of sterile-filtered 1 M sodium carbonate. The flasks were inoculated with 10 mL of culture in the exponential growth phase. The algae were grown in a temperature-controlled orbital incubator on a LED light pad of 6000 K color temperature, with a 12-hour light to 12-hour dark cycle. Cultures were grown and analyzed in triplicate. Experiments were performed twice (n = 2) to ensure reproducibility.

6.2.2 Photo-bioreactor cultures

To prepare the inoculum, fresh medium (200 mL) was added to 500 mL Erlenmeyer flasks, then autoclaved and allowed to cool for 24 hours. The flasks were inoculated with 20 mL of culture in the exponential growth phase. The algae were grown for 1-2 weeks on a LED light pad of 6000 K color temperature, with a 12-hour light to 12-hour dark cycle. Subsequently, flask cultures were pooled and 500 mL was used to inoculate a 5 L flat-panel PBR (see Section 5.2.1 for details), which was grown for another week with LED lights of 6000 K color temperature and approximately 330 μmol m^{-2} s^{-1} PPFD. The culture from the 5 L flat-panel reactor was used to inoculate the 50 L reactor.

6.2.3 Photobioreactor set-up

The effect of air flow rate and sparger design on productivity of *A. cylindrica* was investigated in a 50 L custom-made PBR, constructed from clear acrylic tube as shown in Figure 6.1 and Figure 6.2. Filtered tap water, MLA nutrient concentrate and inoculum were added such that the liquid volume was 50 L, medium concentration was 2 × MLA and the initial optical density (at a wavelength of 550 nm) was approximately 0.12. Tap water was filtered through a wound polypropylene depth cartridge with 1 μm pore size (Parker Hannifin Corporation, Cleveland, OH, USA) and a carbon filter cartridge with 0.5 μm pore size (Stefani Australasia Pty Ltd, Welshpool, WA, Australia). Light was provided by 27 × 9 W cool white (6000 K) LED linkable aluminium strip lights (Jaycar). The light intensity was measured along the center axis of the column using a Walz ULM-500 series light meter equipped with a US-SQS/L spherical sensor. Measurements were made with the PBR full of water but without aeration. The mean PPFD was approximately 300 μmol m^{-2} s^{-1} at the centerline of the reactor.

The temperature was regulated by 28 °C water passing through a 12.5 mm diameter U-shaped stainless-steel tube with an approximate surface area of 0.15 m^2. Air was introduced into the base of the PBR from one of two different types of spargers: a 100 mm long arm sparger with 3 rows of 10 × 2 mm diameter holes; or, a fused alumina sparger (pore size <0.3 mm [433]) of length 130 mm and diameter 30 mm (AquaOne cat. #10154, Kong's Pty Ltd, Ingleburn, NSW, Australia). In all runs, the air was enriched with CO_2 (food-grade, Coregas, Yennora, NSW, Australia) at 100 cc min^{-1}. Flow rates for both air and carbon dioxide were measured using RM series rotameters (Dwyer Instruments, Michigan City, IN).

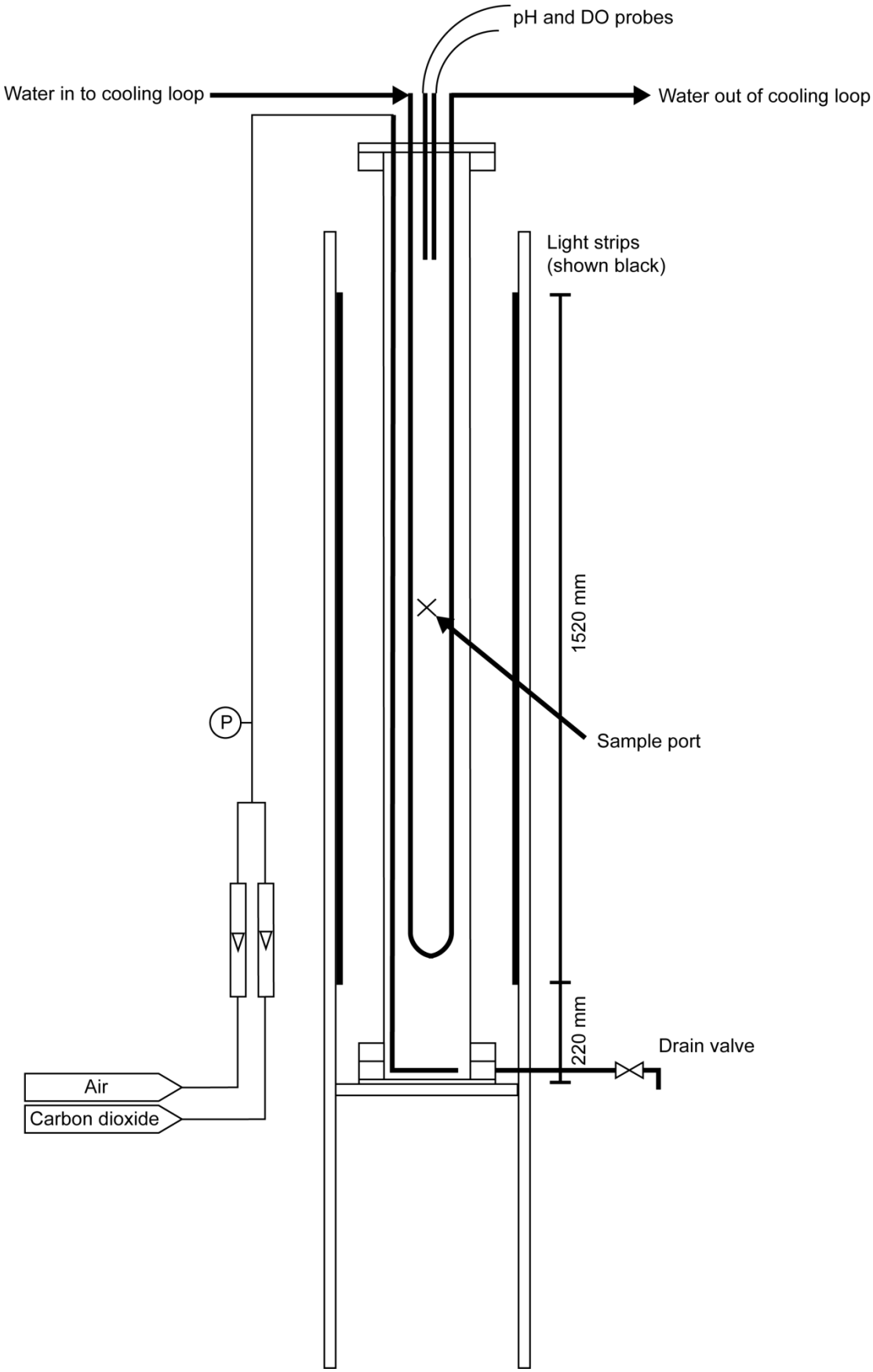


Figure 6.1: Schematic of the 50 L PBR set-up used in this work.

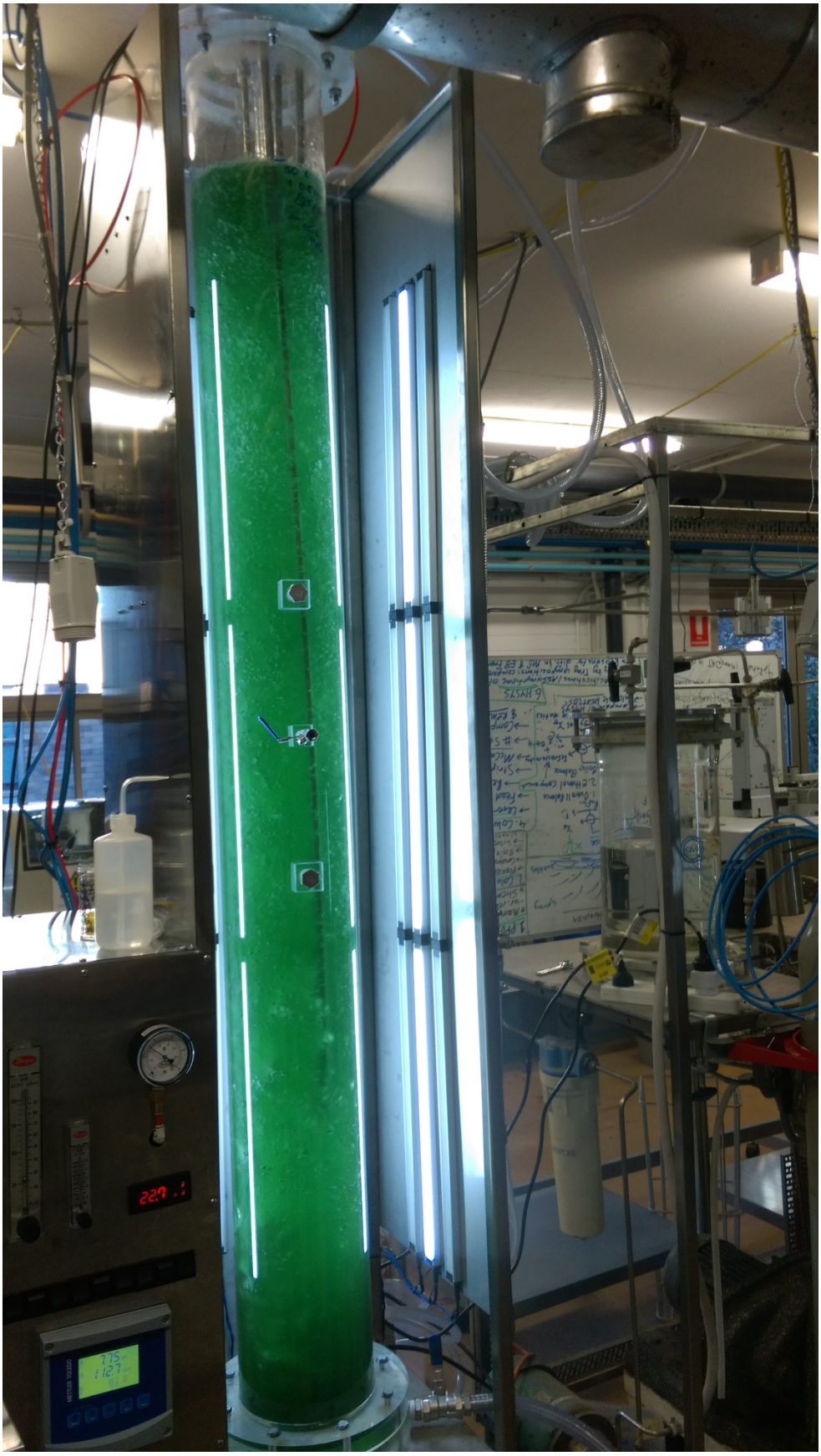


Figure 6.2: Photograph of the 50 L PBR set-up used in this work.

Optical density of all cultures was measured as per the method in Section 5.2.2. The temperature and pH were measured using an InPro 3250i pH probe (Mettler-Toledo, Columbus, OH, United States); dissolved oxygen (DO) was measured using a Mettler-Toledo InPro 6850i DO probe. Temperature, pH and DO measurements were logged using the M200 Transmitter Configuration Tool software via a Mettler-Toledo M200 2-channel transmitter. The total volume of medium was kept constant by the addition of filtered water into the PBR before sampling. Samples were retained for vitamin K_1 concentration analysis (as per method in Section 0) and DCW analysis (as per method in Section 5.2.2). All photo-bioreactor experiments were performed twice ($n = 2$).

6.2.4 Nitrate analysis

Analysis of dissolved nitrate in the culture medium was based upon the *Ultraviolet Spectrophotometric Screening Method 4500-NO^{3-} B* published by the American Public Health Association (APHA) [434]. Briefly, 250 μL of culture was diluted in 4.75 mL of reverse-osmosis (RO) water and then shaken vigorously with 100 μL of 1 M HCl. The mixture was filtered into a quartz cuvette and the absorption measured at 220 nm and 275 nm. Based on a calibration curve determined as per the method, the concentration of nitrate (in mg L^{-1}) was calculated using the following equation:

$$[NO_3^-] = 15.791 \times (A_{220} - 2A_{275}) \quad (6.1)$$

6.2.5 Gas holdup and superficial velocity determination

Assuming a constant cross-sectional area, the total volume (V_{total}) of the 3-phase mixture is proportional to the height of the sparged mixture ($h_{sparged}$), while the volume of gas (V_{gas}) is proportional to the height of the sparged culture mixture minus the height of the unsparged mixture ($h_{unsparged}$). Gas holdup for the mixture of culture and air was therefore calculated as follows:

$$\varepsilon_g = \frac{V_{gas}}{V_{total}} = \frac{h_{sparged} - h_{unsparged}}{h_{sparged}} \tag{6.2}$$

The actual volumetric flow rate of air through the rotameter (Q_2) at the calibrated temperature (T_2 = 294 K) and pressure (P_2 = 14.7 psia = 101 kPa) was calculated by adjusting the nominal reading (Q_1) for backpressure (P_1) and actual temperature (T_1) as per the manufacturer instructions using the following equation:

$$Q_2 = Q_1 \sqrt{\frac{P_1 T_2}{P_2 T_1}} \tag{6.3}$$

The superficial gas velocity (U_g) was calculated from the actual volumetric flow rate and the cross-sectional are of the reactor (A) using the following equation:

$$U_g = \frac{Q_2}{A} \tag{6.4}$$

6.3 Results and discussion

6.3.1 Optimal temperature and pH

The effect of culture temperature and initial medium pH on specific growth rate was analyzed by two-way ANOVA and Dunnett's multiple comparisons test. There was a significant main effect of temperature on growth rate ($F(3,8) = 123.1$, $p < 0.0001$), while the effect of pH was insignificant ($F(1,8) = 0.04118$, $p = 0.8443$). Dunnett's multiple comparisons between pooled values for 23 °C and the other temperatures revealed that all were significantly different. The pooled specific growth rates for 28 °C were greater than all others. Therefore this strain of *A. cylindrica* was cultured at 28 °C in the 50 L PBR. However, the growth rate of *A. cylindrica* was at least 80% of this value within the range 23-33 °C. Culture pH in the range 7.5-9.5 does not affect the growth rate; therefore, increases in pH over the course of 50 L culture experiments should not affect the results.

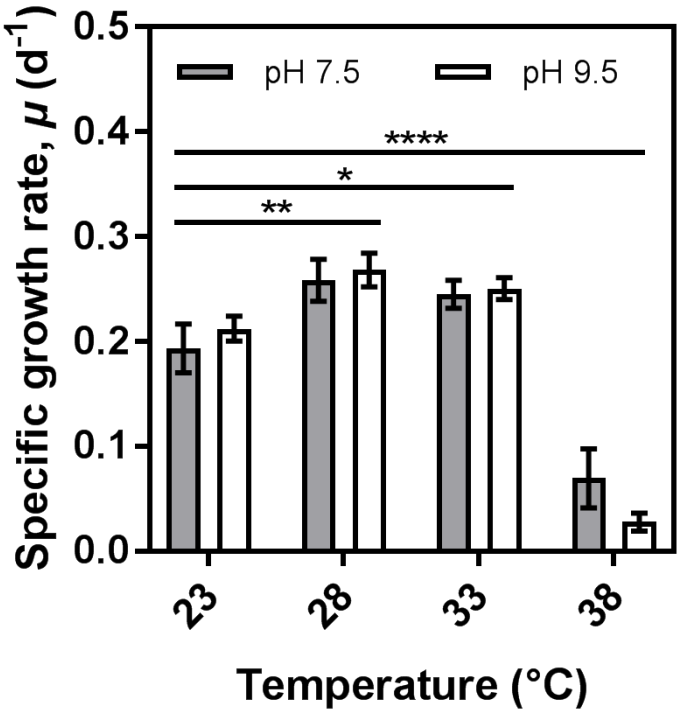


Figure 6.3: Effect of culture temperature and initial medium pH on specific growth rate of *A. cylindrica* grown in flasks. Values are means of 2 repeats, error bars are SD. Comparison performed using Dunnett's multiple comparisons test, * $p < 0.0332$, ** $p < 0.0021$, **** $p < 0.0001$.

6.3.2 Dissolved oxygen, temperature and pH

The DO, temperature and pH were remarkably stable for all conditions tested. This is consistent with the high heat-transfer and mass transfer rates observed in bubble columns compared to other reactor designs [274], [275], [290]. Their contributions to variation in the results would be negligible. Dissolved oxygen increased to around 105% when lights were switched on (Figure 6.4), returning to 100% when lights were switched off (Figure 6.4a). Temperature also increased slightly when lights were switched on, however it remained within ±0.5 °C of 28.0 °C. For cultures subjected to 24 hour light, temperature and DO were constant at 28 °C and 105 %; no circadian variations were observed (Figure 6.4b). In both cyclic and continuously illuminated cultures, the pH was constant at approximately 7.5 throughout the batch.

The phenomenon of increased DO during illumination is due to the photosynthetic production of oxygen. Increases in DO of around 20% during illumination ("day") compared to the un-illuminated state ("night") have been observed in similar bubble column systems [278], [283]. The present study supports previous data demonstrating that bubble column systems operate in the region of 100% DO, which is considerably below the inhibitory concentrations of 200-400% seen in other culture systems [275], [435].

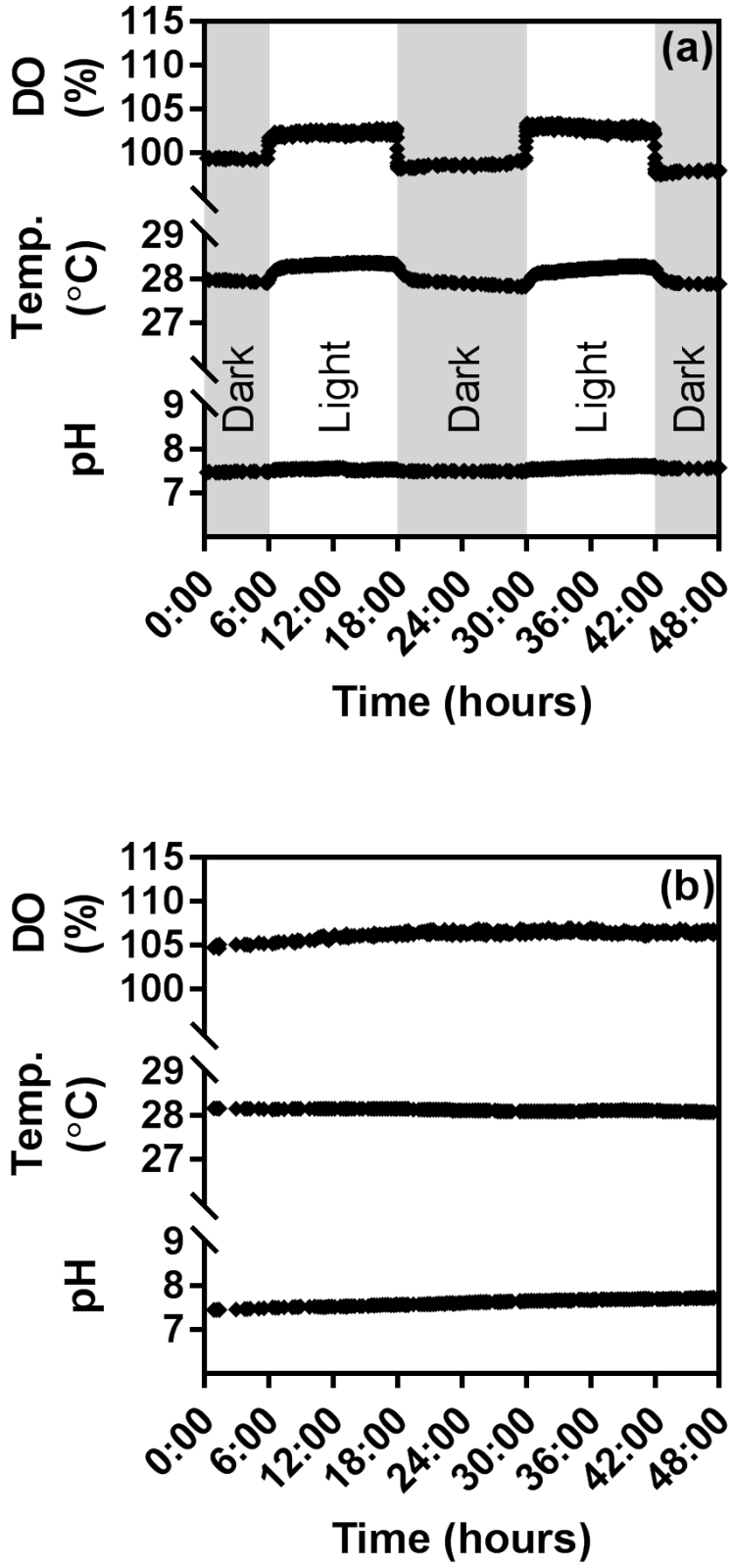


Figure 6.4: Variation of pH, temperature and dissolved oxygen over time for light:dark cycles of (a) 12:12 hours and (b) 24:00 hours.

6.3.3 Effects of air flow rate and sparger design

The effects of air flow rate and sparger design on phylloquinone productivity were tested. Initial batch cultures using the perforated steel tube were grown at nominal air flow rates of 0.2, 0.5, 1.0 and 2.0 vvm. The back-pressure varied between 25 and 35 kPa when changing the sparger design and air-flow rate. Approximately 18 kPa of back-pressure was due to the height of the culture medium in the reactor, the remainder being due to losses in the tubing and sparger. As Q_2 is quite insensitive to variations of ±5 kPa in P_1 (eqn. (6.3)), actual air flow rates were calculated for P_1 = 30 kPa (Table 6.1 and Figure 6.5).

Table 6.1: Actual and calculated air-flow values for perforated steel tube sparger. Flow rate adjusted based upon a back-pressure of 30 kPa as measured at the rotameters.

Flow rate reading (vvm)	Flow rate (L min^{-1})	Adjusted flow rate (L min^{-1})	Superficial gas velocity, U_g (cm s^{-1})	Gas holdup, ε_g
0.2	10	11.3	0.66	0.023
0.5	20	22.5	1.65	0.051
1.0	50	56.3	3.31	0.086
2.0	100	112.6	6.61	0.136

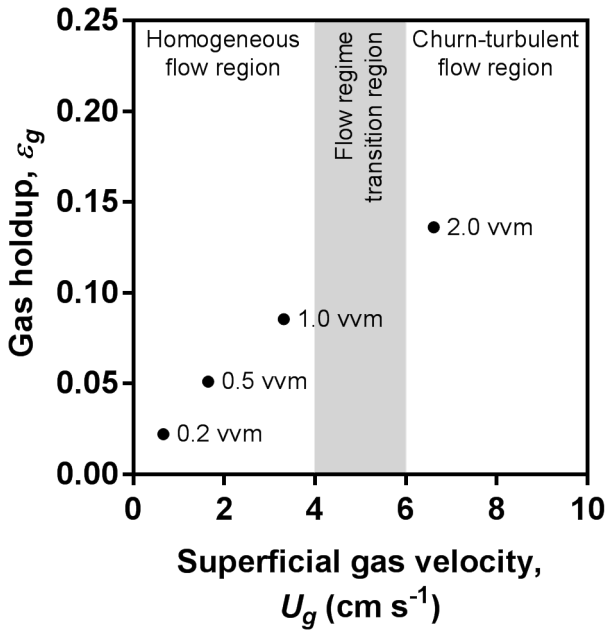


Figure 6.5: Gas holdup versus superficial velocity for various airflow rates through perforated steel tube sparger. Flow regimes based upon likely values [274], [290], [291].

The biomass productivity, nitrate consumption rate and phylloquinone volumetric productivity were determined from the slopes of the linear fits of the time-series data for DCW, nitrate concentration and phylloquinone titer, respectively. The *R*-square value exceeded 0.90 for all linear fits. Although evidence suggested that increasing air flow rate to over 5 cm s^{-1} (1.6 vvm in this system) should improve biomass productivity [426], [436], no significant differences in biomass productivity (Figure 6.6a), nitrate consumption (Figure 6.6b), phylloquinone specific concentration (Figure 6.7a) or phylloquinone titer (Figure 6.7b) were observed. This may be due to species-specific effects of shear-stress; for example, cells of *Dunaliella tertiolecta* were found to die at superficial gas velocities above 1 cm s^{-1} [418], while *Phaeodactylum tricornutum* was found to tolerate velocities up to 5.5 cm s^{-1} [436]. The absence of cell debris and short filaments upon microscopic examination (Figure 6.8) indicate that low productivities are not likely due to cell damage. Thus, flow rates of 0.2 vvm (U_g = 0.66 cm s^{-1}) and 0.5 vvm (U_g = 1.65 cm s^{-1}) were selected to compare the effects of aeration from the perforated steel tube (PST) and the ceramic airstone (CAS). These airflow rates reflect typical values from previous research, which are in the range 0.1-2.0 cm s^{-1} [278], [279], [283], [287].

Table 6.2: Summary of test conditions and results for effect of sparger type versus air flow rate.

Sparger type	Air flow rate (vvm)	Final DCW (g L^{-1})	Specific final phylloquinone conc. (μg g^{-1})	Final phylloquinone titer (μg L^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Phylloquinone productivity (μg L^{-1} d^{-1})
CAS	0.2	0.422-0.535	256-264	108-141	52.3-58.5	15.7-18.8
CAS	0.5	0.425-0.433	280-312	121-133	60.1-61.0	16.3-18.2
PST	0.2	0.437-0.469	294-319	138-139	61.2-61.9	18.8-19.5
PST	0.5	0.458-0.458	272-321	124-147	63.1-68.4	19.4-19.6

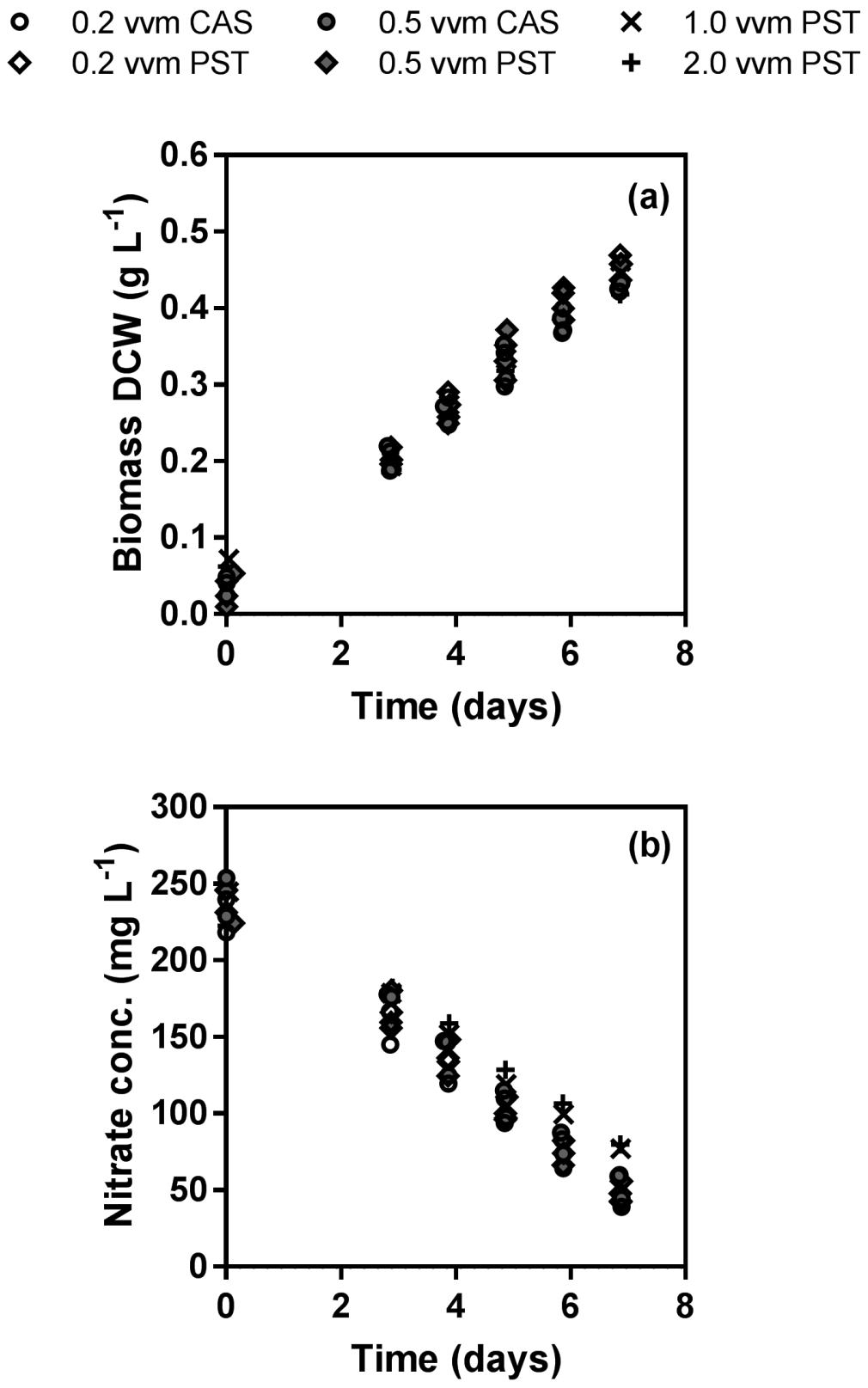


Figure 6.6: Effect of sparger type (ceramic airstone "CAS" and perforated steel tube "PST") and air flow (0.2, 0.5, 1.0 and 2.0 vvm) over time on (a) dry cell weight (DCW) and (b) nitrate concentration.

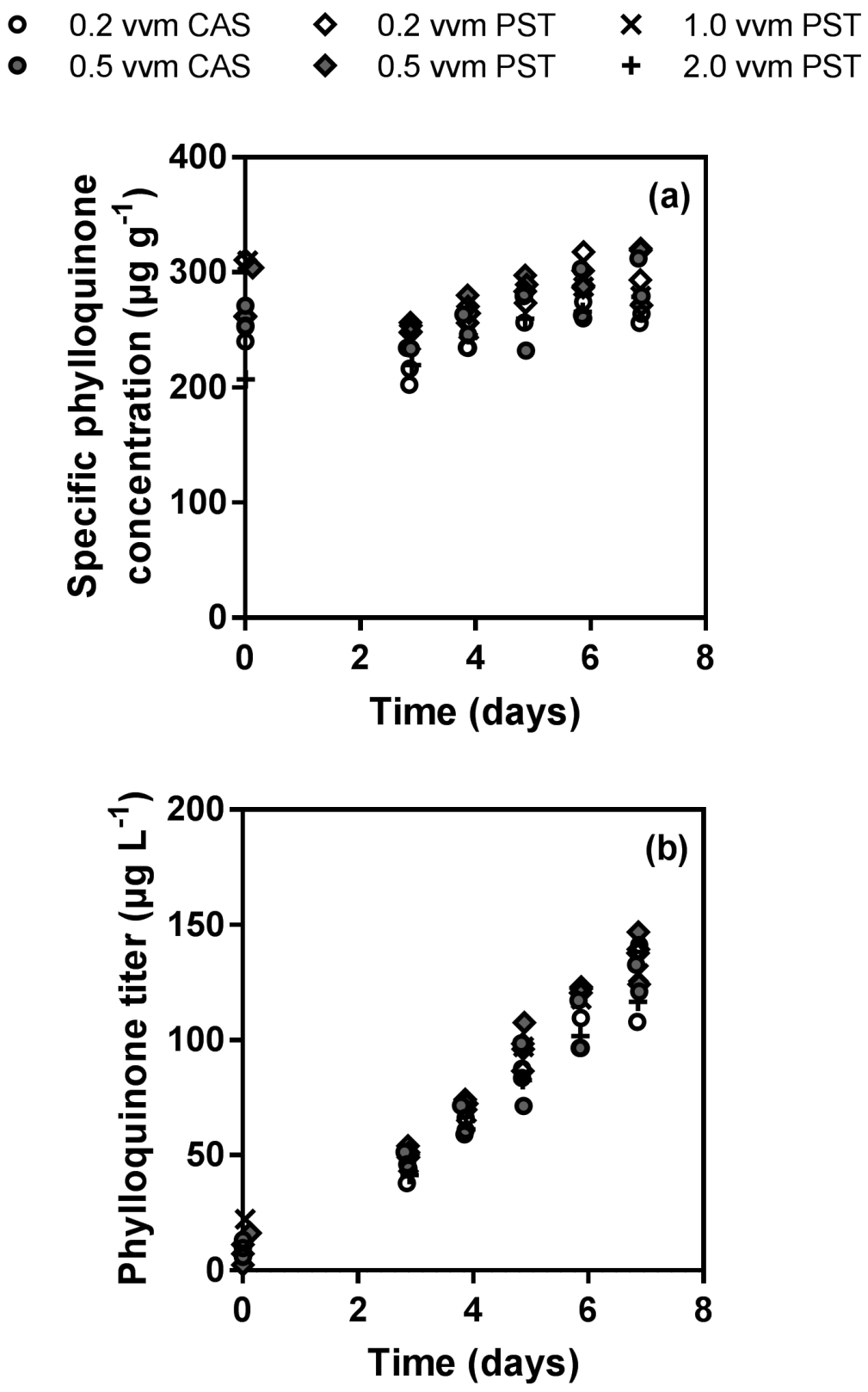


Figure 6.7: Effect of sparger type (ceramic airstone "CAS" and perforated steel tube "PST") and air flow (0.2, 0.5, 1.0 and 2.0 vvm) over time on phylloquinone (a) specific concentration and (b) titer.

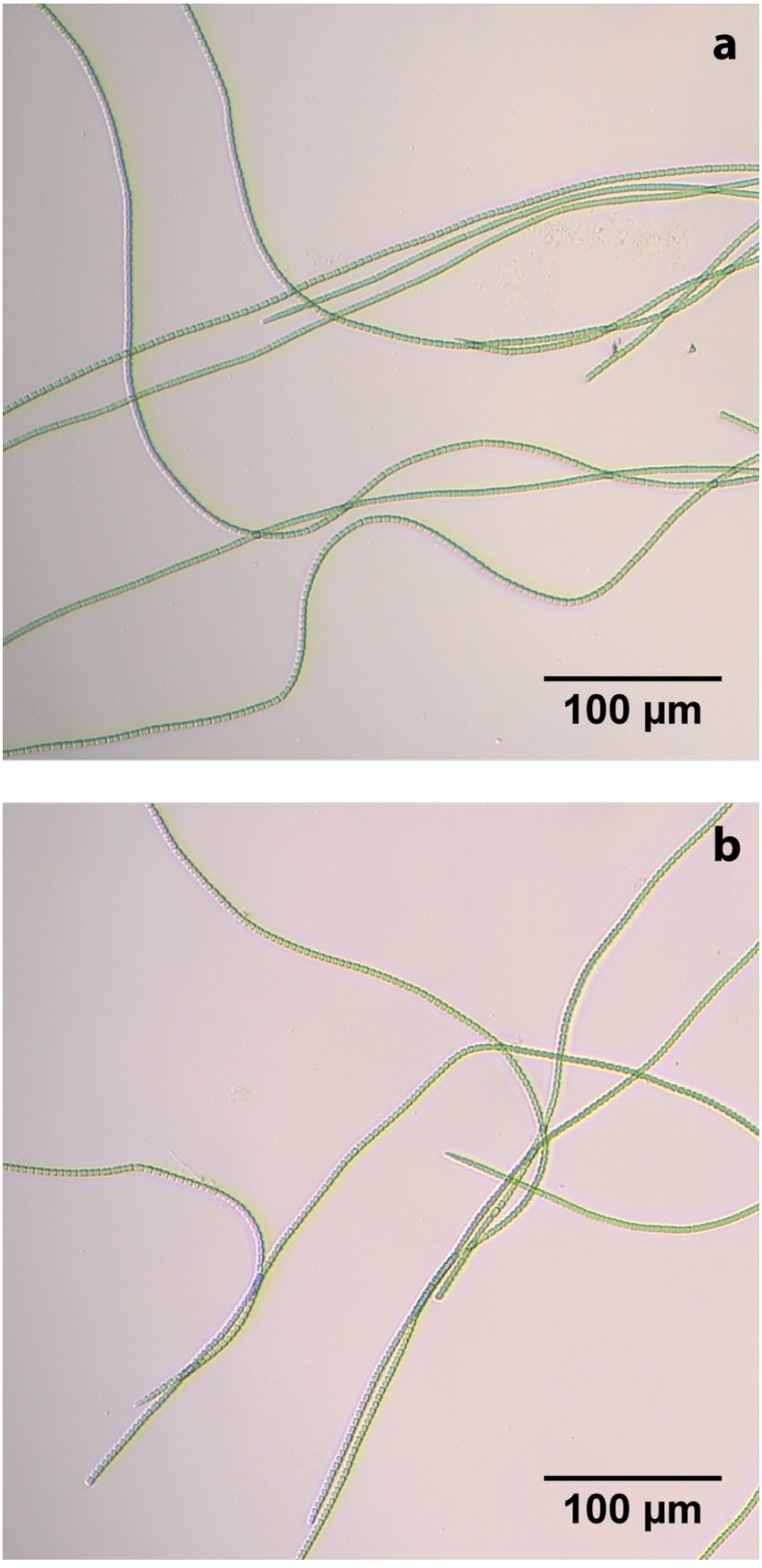


Figure 6.8: Micrographs at 10 × magnification of *A. cylindrica* grown at (a) 0.2 vvm and (b) 2.0 vvm aeration rates. Note long filaments of at least 400 µm (~80 cells) in both images.

The 2-way ANOVA of biomass productivity (Figure 6.9a), nitrate consumption rate (Figure 6.9b), final specific phylloquinone concentration (Figure 6.10a) and phylloquinone final titer (Figure 6.10b) revealed no significant main or interaction effects of air flow rate and sparger type. Generally, the DCW increased linearly from 0.025 to 0.45 g L^{-1} over 7 days. Medium nitrate concentration decreased linearly from approximately 230 to 50 mg L^{-1}. The phylloquinone titer increased linearly from approximately 15 to 125 μg L^{-1}. This linear growth behaviour is commonly seen during light limitation in large-scale PBR cultures [275], [278], [283], [420].

The specific phylloquinone concentration was approximately constant at around 260 μg g^{-1}; however, a slight drop was observed from day 0 to day 3. The concentration gradually increased to return to the initial value by day 7. This behaviour is consistent with photoacclimation in response to changing irradiance; PSI numbers (and thus phylloquinone concentration) are known to increase under low-light (or shaded) conditions. When the inoculum was added to the column at the start of each run, it was diluted 10-20 times. Thus, each cell suddenly received more light than in the inoculum tank; the numbers of PSI decreased in response. As the cells multiplied over time, the culture density gradually increased. Each cell received less and less light on average; therefore, the numbers of PSI were increased in response. This observation matches the effect of light intensity on phylloquinone seen in Chapter 5. The higher phylloquinone concentrations observed in this 50 L bubble column system (~260 μg g^{-1}) compared to the 5 L flat panels system (~150 μg g^{-1}) probably reflect the increased degree of light limitation in the 50 L bubble column.

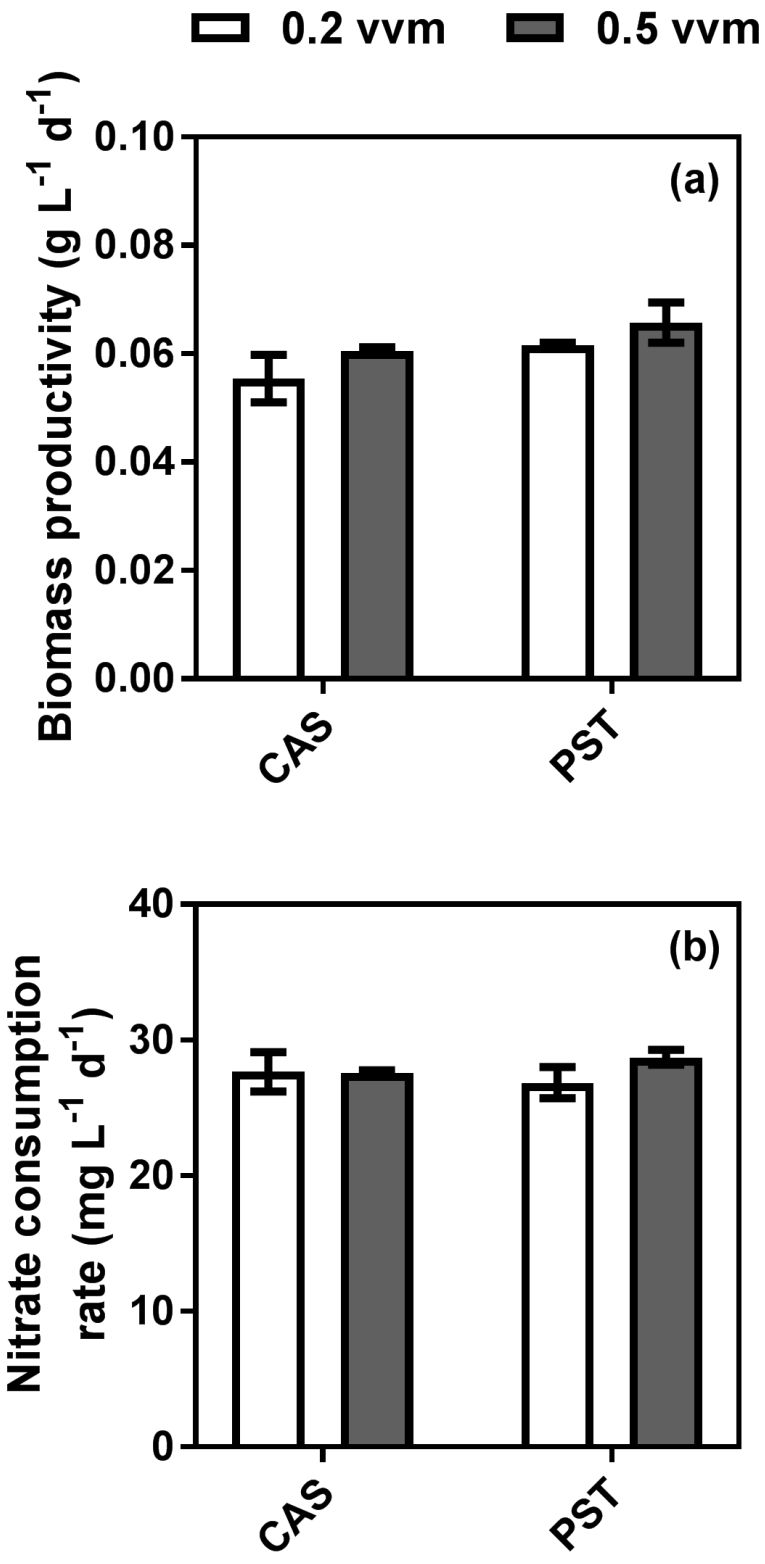


Figure 6.9: Effect of sparger type (ceramic airstone "CAS" and perforated steel tube "PST") and air flow (0.2 and 0.5 vvm) on (a) biomass productivity and (b) nitrate consumption rate. Values are mean of $n = 2$, error bars are SD.

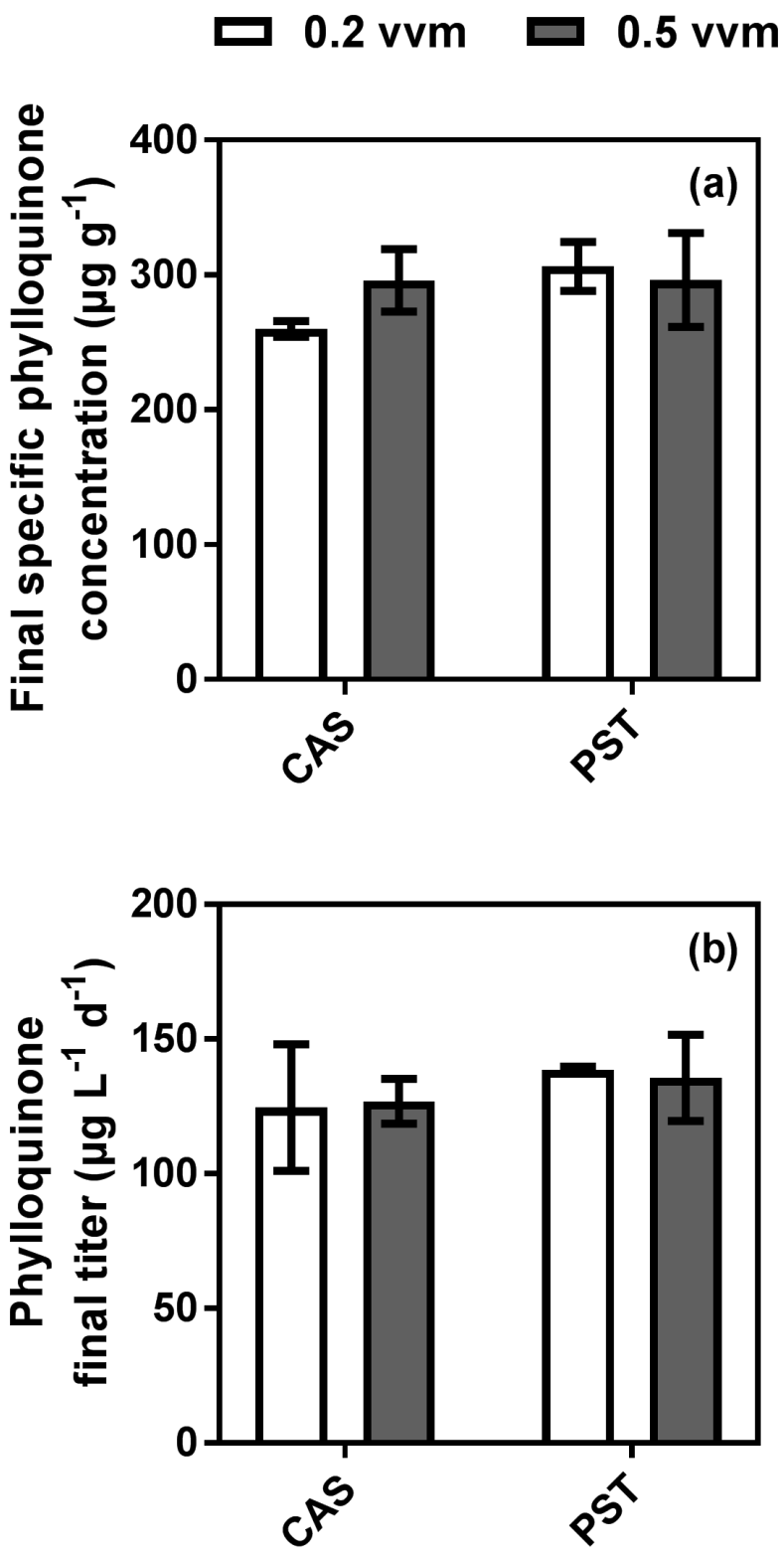


Figure 6.10: Effect of sparger type (ceramic airstone "CAS" and perforated steel tube "PST") and air flow (0.2 and 0.5 vvm) on phylloquinone final (a) specific concentration and (b) titer.

DCW productivity was constant at around 60 mg L^{-1} d^{-1}, which was 40% lower than the best productivity achieved in the 5 L flat-panel system in Chapter 5. These productivities are also 5-10 times lower than the 0.3 – 0.5 g L^{-1} d^{-1} achieved in a very similar 60 L system [275], [278], [283]. However, this 60 L system was used outdoors where the mean PPFD was 1150 μmol m^{-2} s^{-1}, almost four times the intensity used here. Given the known positive correlation between irradiance and productivity in such systems [275], it is likely that outdoor productivities of at least 0.25 g L^{-1} d^{-1} could be achieved in the system presented here. The absence of cell debris and short filaments upon microscopic examination (Figure 6.8) indicate that there was no cell damage.

Nitrate consumption was around 28 mg L^{-1} d^{-1}, which means that approximately 0.11 g of nitrogen was incorporated into every 1.0 g of biomass. Based on the Kjeldahl method, the biomass would be 69% (6.25 × 11%) protein, which agrees closely with the proximate analysis (68.6%) from Section 4.3.2.

The phylloquinone mean concentration (Figure 6.10a) and phylloquinone volumetric productivity (Figure 6.10b) were calculated for each test condition. There were no significant differences between the four different cases as determined by 2-way ANOVA. The phylloquinone volumetric productivity was 15-20 μg L^{-1} d^{-1}, which was very similar to the optimal value of 22 μg L^{-1} d^{-1} achieved in the 5 L flat-panel system. Despite the lower biomass productivity in the 50 L bubble column, the increased specific phylloquinone concentration led to a comparable volumetric productivity.

The sparger designs and airflow rates tested did not significantly affect biomass or phylloquinone productivity. In future, the stainless steel sparger should be used because it is cheaper and simpler to construct, operate and maintain. The volumetric flow rate of 0.2 vvm should also be used as less energy is consumed compared to higher flow rates.

6.3.4 Effects of light duration versus medium concentration

The effects of light duration and medium concentration on phylloquinone productivity were tested. Initial batch cultures using 2× MLA medium were grown with daily light:dark cycles of 12:12, 16:08, 20:04 and 24:00 hours. Increased light duration led to faster biomass growth (Figure 6.11a) and nitrate consumption (Figure 6.11b), however nitrate limitation may have caused a reduction in phylloquinone specific concentration in the fastest-growing cultures (Figure 6.12). Thus, medium concentrations of 2× and 5× MLA, and light:dark cycles of 12:12 and 24:00 hours, were selected to compare the effects of medium concentration and light duration on phylloquinone productivity. The results are summarized in Table 6.3.

Table 6.3: Summary of test conditions and results for effect of daylength versus medium concentration.

Duration of daylight (h)	Medium conc.	Final DCW (g L^{-1})	Final phylloquinone conc. (μg g^{-1})	Final phylloquinone titer (μg L^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Phylloquinone productivity (μg L^{-1} d^{-1})
12	2 ×	0.437-0.469	294-320	138-139	60.1-63.1	18.8-19.5
12	5 ×	0.462-0.510	291-307	134-141	62.6-63.2	17.7-18.6
24	2 ×	0.865-0.902	183-207	139-158	121.2-126.7	22.4-26.5
24	5 ×	0.813-0.876	319-341	270-299	118.5-118.8	39.2-42.0

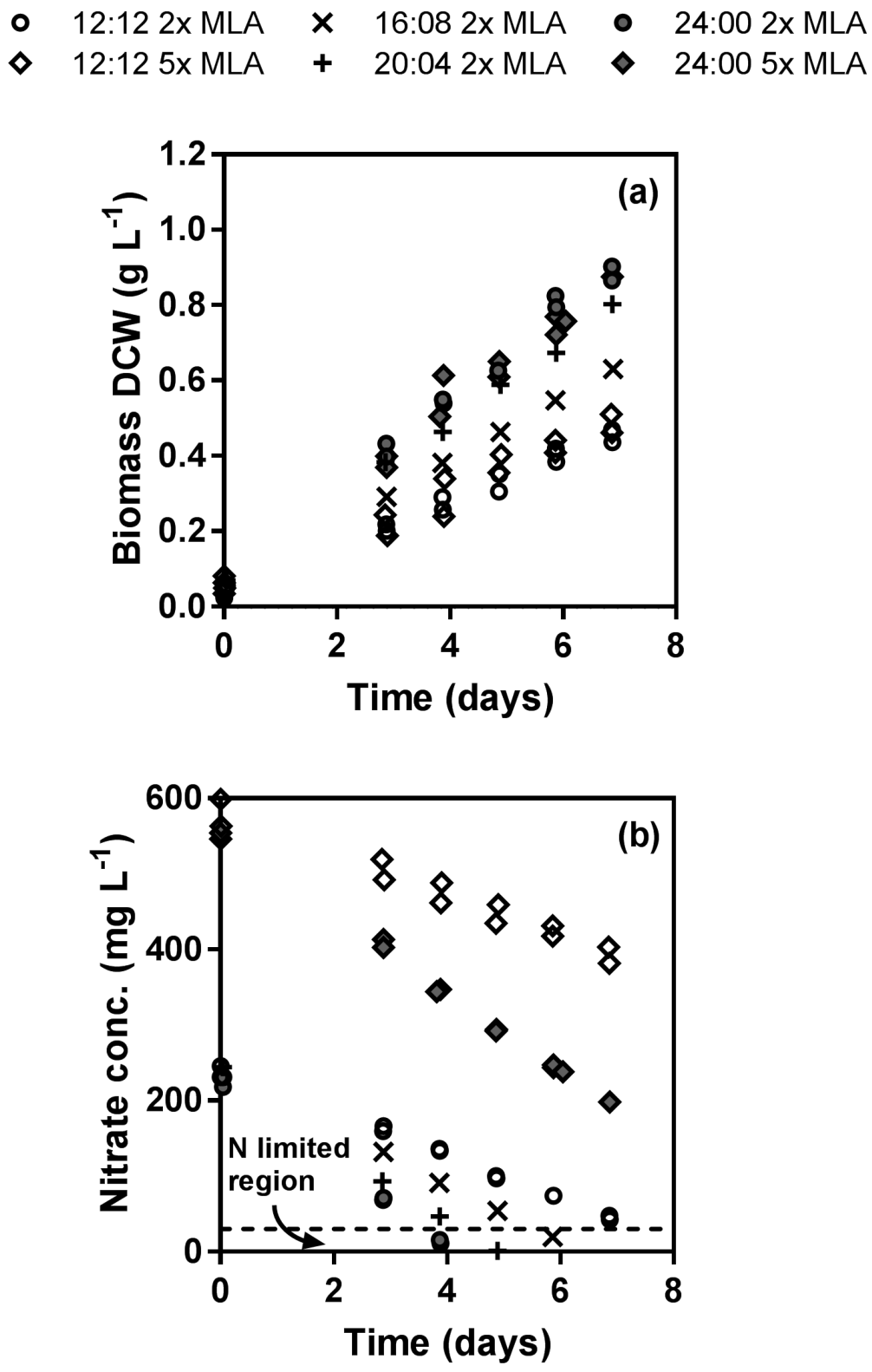


Figure 6.11: Effect of medium concentration (MLA 2× and 5×) and light:dark cycle (12:12, 16:08, 20:04 and 24:00 hours) over time on (a) dry cell weight (DCW) and (b) nitrate concentration.

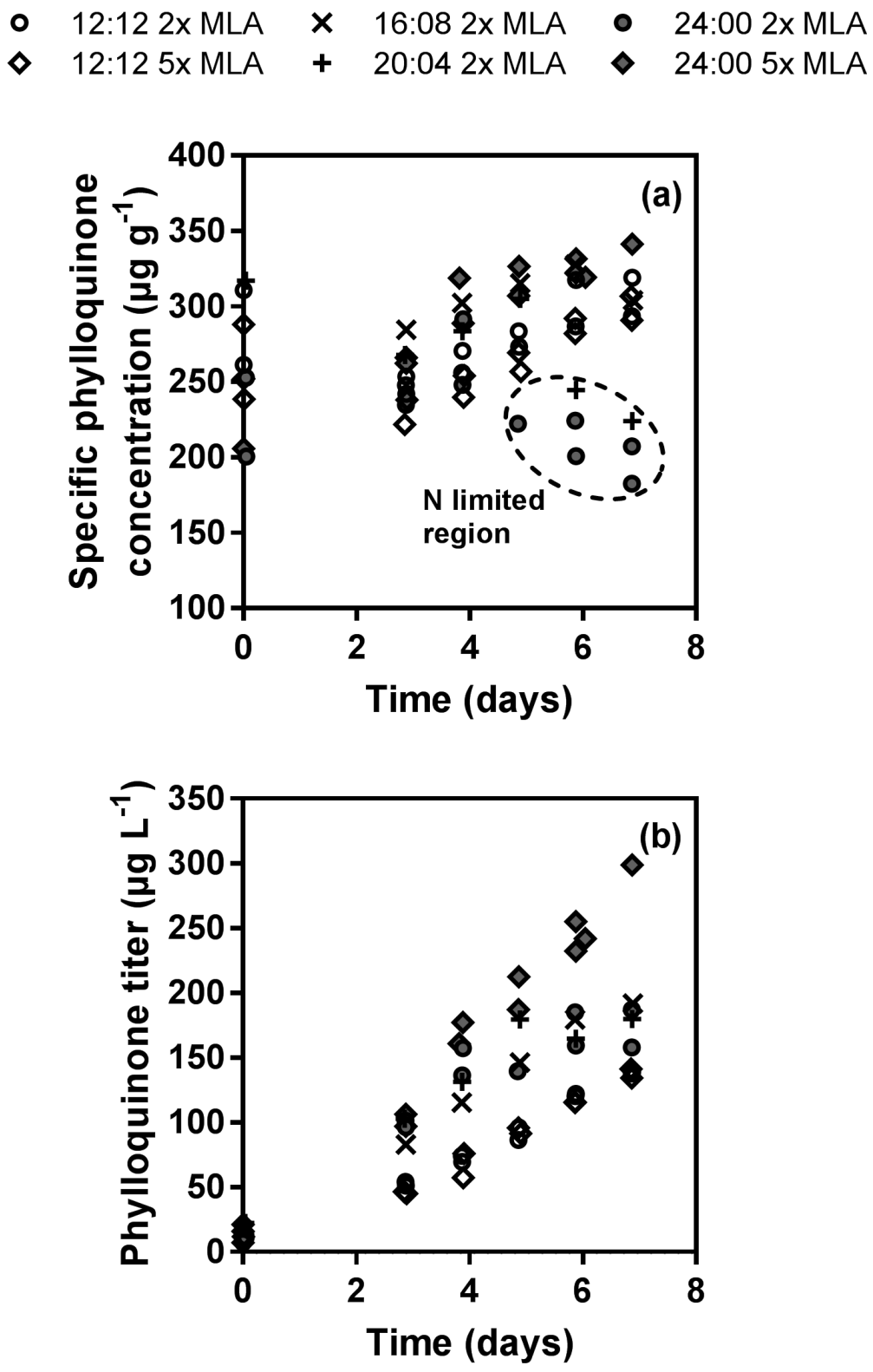


Figure 6.12: Effect of medium concentration (MLA 2× and 5×) and light:dark cycle (12:12, 16:08, 20:04 and 24:00 hours) over time on phylloquinone (a) specific concentration (b) titer.

The ordinary two-way ANOVA of biomass productivity (Figure 6.13a) showed a significant main effect of light duration ($F(1,4) = 1405$, $p < 0.0001$). This is a useful result, as 24-hour illumination is known to inhibit growth in some species [431], [432]. Responses to constant illumination vary considerably between microalgal species [437]–[445], even within the same genus or ecological niche, so it is difficult to predict the response of a specific strain. The nitrate consumption rates (Figure 6.13b) were also significantly affected by light duration ($F(1,4) = 647.9$, $p < 0.0001$).

The two-way ANOVA of final phylloquinone titer (Figure 6.14b) showed significant main effects of medium concentration ($F(1,4) = 28.2$, $p = 0.0060$) and light duration ($F(1,4) = 74.75$, $p = 0.0010$). There was also a significant interaction effect ($F(1,4) = 29$, $p = 0.0058$). Continuously illuminated cultures in both 2× and 5× MLA medium grew at the same rate (Figure 6.11a). However, the 2× MLA culture exhausted the nitrate content by day 4 (Figure 6.11b), and the specific phylloquinone concentration subsequently dropped (Figure 6.12a). This resulted in a final titer of approximately 150 μg L^{-1}, which was less than the titer of 300 μg L^{-1} achieved with 5× MLA and 24 h light, but slightly greater than the titer of 140 μg L^{-1} achieved in both 12 h light cases (Figure 6.12b).

Microscopic inspection on the final day of continuously illuminated cultures grown in 2× MLA revealed truncated filaments with the presence of heterocysts (Figure 6.15b), consistent with nitrogen limitation [446]. Heterocysts enable filamentous cyanobacteria (including *A. cylindrica*) to fix nitrogen from the atmosphere, and thereby continue to grow for a limited time [446]. Microalgae can also reallocate nitrogen by breaking down photosynthetic protein complexes during nitrogen limitation [386], [447]. Therefore nutrient (probably nitrate) limitation is likely responsible for the decrease in phylloquinone concentration despite the 2× MLA culture continuing to grow as fast as the 5× MLA culture. This confirms the importance of ample nitrogen supply as discussed in Section 5.3.5.

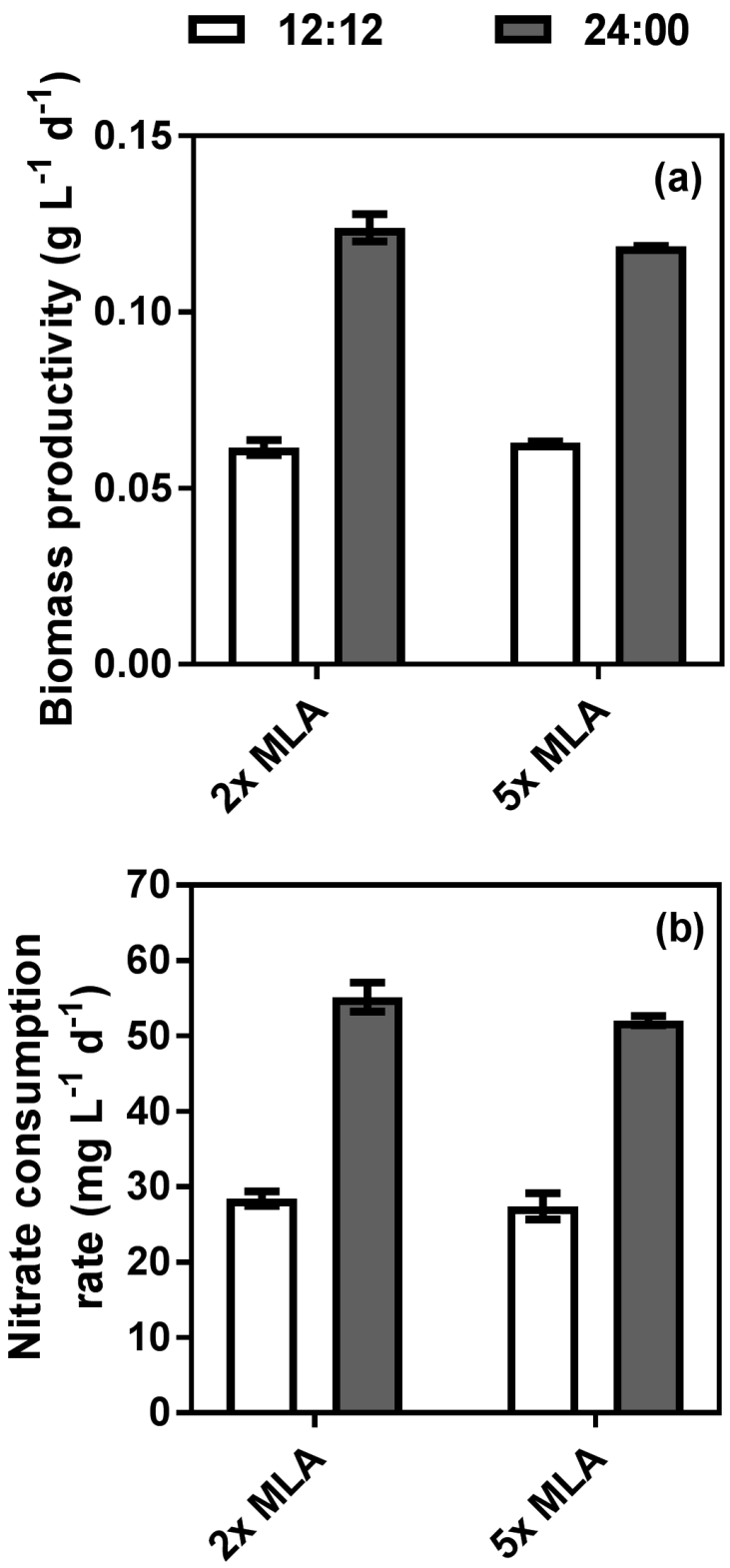


Figure 6.13: Effect of medium concentration (MLA 2× and 5×) and light:dark cycle (12:12 and 24:00 hours) on (a) biomass productivity and (b) nitrate consumption rate.

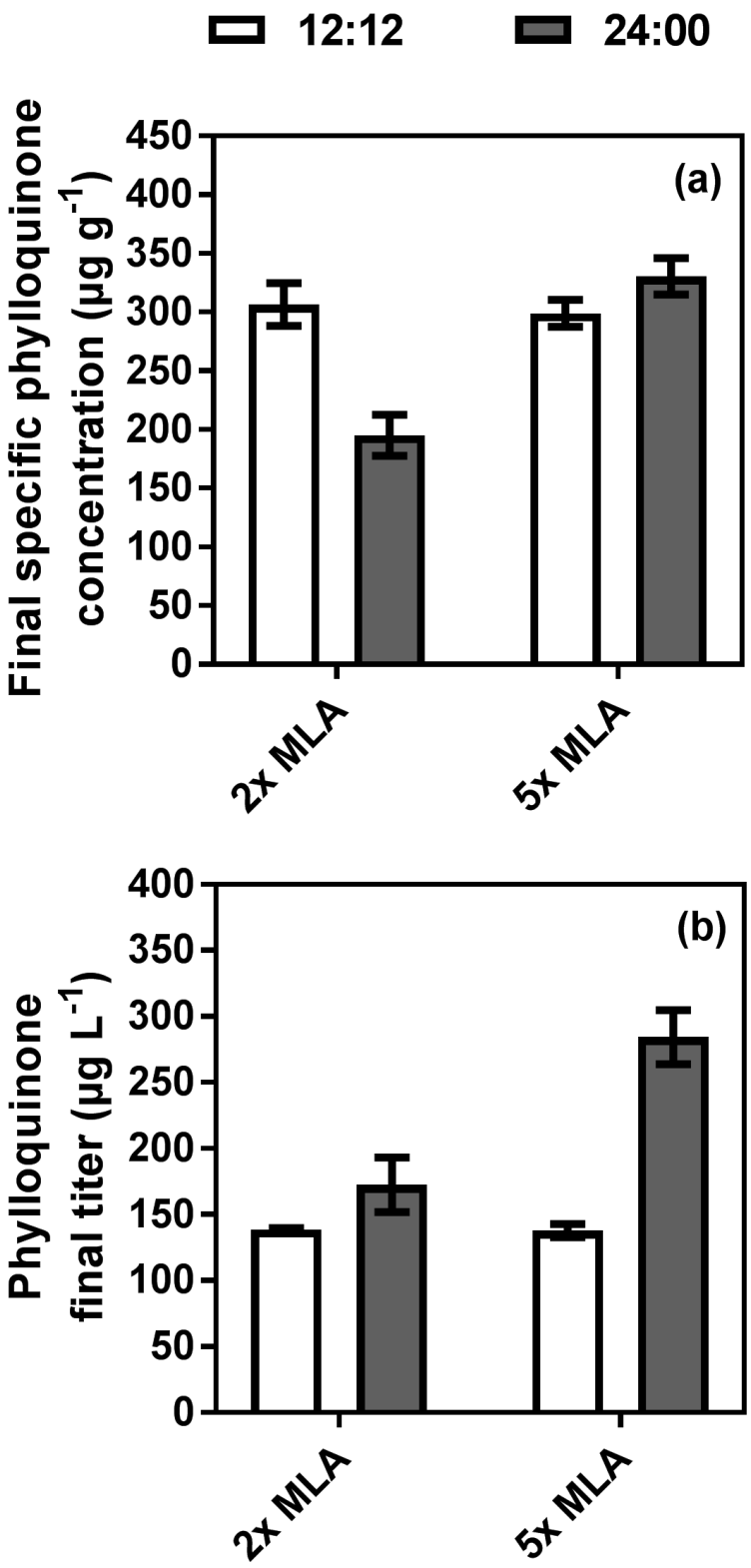


Figure 6.14: Effect of medium concentration (2× and 5× MLA) and light:dark cycle (12:12 and 24:00 hours) on phylloquinone final (a) specific concentration and (b) titer.

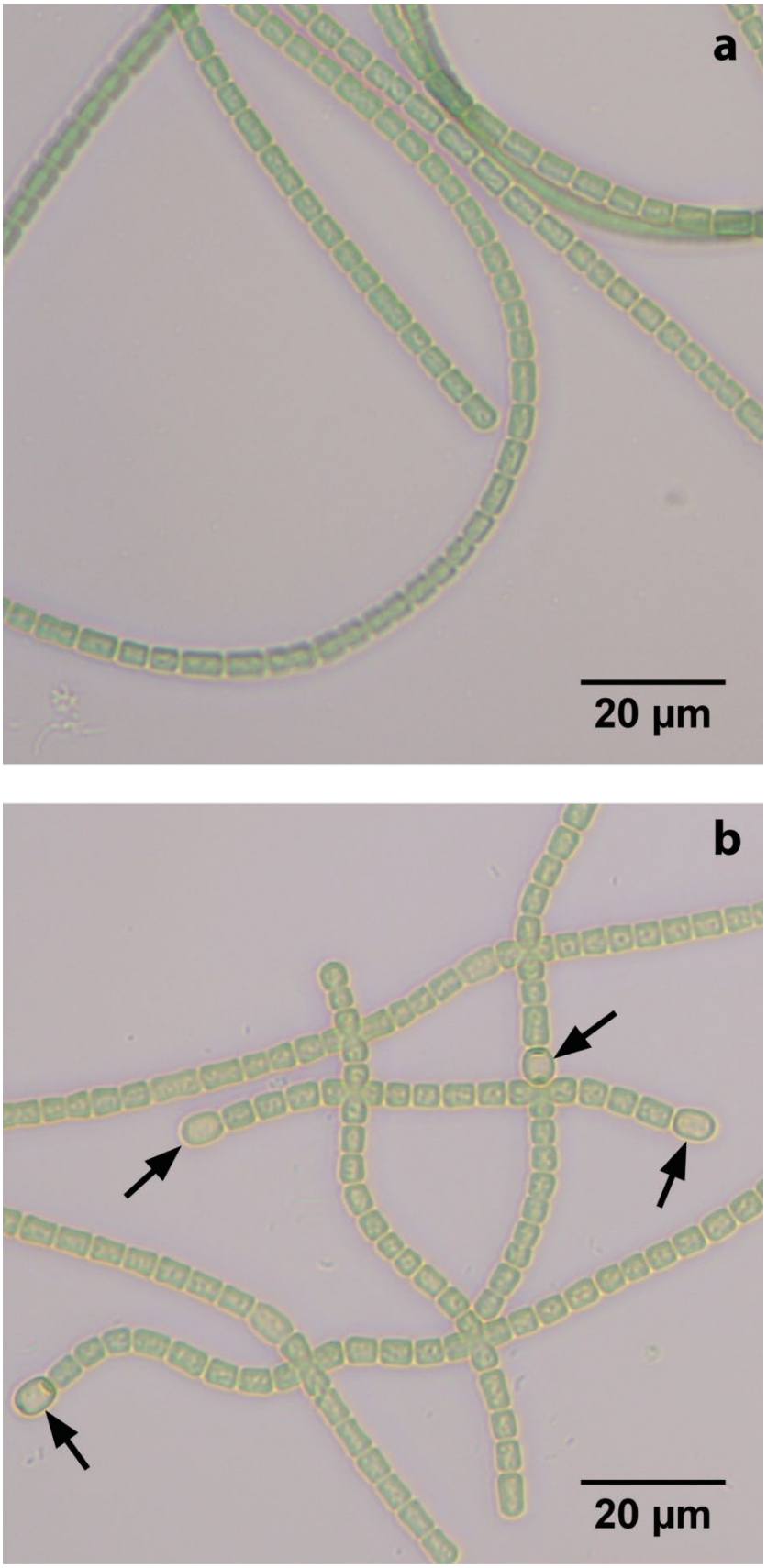


Figure 6.15: Light micrographs (40x magnification) of *A. cylindrica* after 7 days of growth in: (a) 5x MLA; and, (b) 2x MLA. Note the heterocysts (enlarged cells induced by nitrogen limitation) indicated by arrows in (b).

6.4 Conclusions

Anabaena cylindrica was grown in a 50 L bubble column PBR. The scale-up was successful when compared to the 5 L system from Chapter 5, where the best biomass productivity was around 100 mg d^{-1}, the best phylloquinone productivity was 22 μg L^{-1} d^{-1}, and the highest final titer was 161 μg L^{-1}. Here, the base case test conditions of 0.2 vvm aeration, 1% CO_2 enrichment, 2× MLA medium concentration, and a 12:12 hour light:dark cycle yielded a biomass productivity of approximately 60 mg L^{-1} d^{-1} and a phylloquinone productivity of approximately 18 μg L^{-1} d^{-1}, with a final titer of approximately 135 μg L^{-1}. Increasing the aeration rate and reducing the pore size of the sparger did not significantly influence the biomass and phylloquinone productivities of the system. By boosting the medium concentration and subjecting the culture to 24 hour light, the biomass productivity, phylloquinone productivity and final titer were doubled to approximately 120 mg L^{-1} d^{-1}, 40 μg L^{-1} d^{-1} and 285 μg L^{-1} respectively. However, phylloquinone specific concentration declined after nitrogen depletion, so an adequate supply of nitrogen in the medium is essential to ensure phylloquinone productivity.

In summary, the scale-up was successful. The biomass productivity of the system appears to be proportional to the cumulative irradiance received. In nitrogen replete cultures, phylloquinone concentration varied little and the phylloquinone productivity was directly proportional to the biomass productivity. Maximizing cumulative irradiance and ensuring nitrogen repletion are the most important factors in maximizing the phylloquinone productivity of this system.

7 Conclusions and future work

7.1 Conclusions

The objective of the research presented in this thesis was to develop a biotechnological process for the production of vitamin K_1 for human and animal health. Vitamin K_1 is the most common form of vitamin K in the diets of humans [34], [157]–[161]. It is also the only form used as a drug to correct vitamin K deficiency bleeding [197]. Natural food additives are becoming increasingly popular as consumer demand increases for "natural" products. For example, a type of vitamin K_2 (menaquinone-7) is commercially produced by fermentation and has become very popular in recent years [203]. Although vitamin K_1 is naturally occurring in food, currently it is only produced by chemical synthesis for use in supplements and drugs [197]. There is obvious demand for "natural" vitamin K_1, made by biotechnological means, that also has the potential to improve the sustainability of vitamin K_1 production.

Since the synthetic vitamin K analogs were banned due to toxicity in 1963 [18], vitamin K_1 has become the most widely used type of vitamin K in human nutrition and health. In the intervening years, numerous randomized controlled trials (RCTs) [60], [61] and cross-sectional studies [56]–[59] have demonstrated the health benefits of high vitamin K intake. Given the essentiality of vitamin K and the similarity between vitamin K-dependent proteins in vertebrates [113], it is probable that vitamin K_1 would also be superior in animal nutrition and health. However, vitamin K analogs such as menadione bisulfite are still the only forms used in animal nutrition [448]. Animal nutrition, especially the chicken industry, is the biggest user of vitamin K supplements [181]. Converting livestock industries to using vitamin K_1 has the potential to improve animal welfare and represents an enormous opportunity.

7.1.1 Effectiveness of vitamin K_1 for boosting vitamin K status in chickens

One of the key problems in the chicken industry is that there is no perceived benefit of natural vitamin K compared to analogs. However, there is ample evidence that demonstrates that vitamin K analogs are toxic [13], [14], [449]–[452] and ineffective [9], [10], [12], [77]. There is also some evidence to show that vitamin K_1 is the main form of vitamin K in the wild diets of *Gallus gallus* [39], [191]–[193]. However, there is little evidence to show that vitamin K, including vitamin K_1, is superior to analogs for the health of chickens. This was a gap worth investigating prior to the development of a new biotechnological process for the production of vitamin K_1.

Previous research by Talbot et al. has demonstrated the potential benefits of vitamin K_1 for improving bone density in chickens [453]. Bone fracture is a common problem in both egg laying and broiler (meat) chickens due to fast growth, lack of exercise and inadequate nutrition [454], [455]. For example, almost one-third of laying hens have fractures at the end of their laying life [456]. As chicken production intensifies [6], these problems will continue to plague the chicken industry. Another emerging area of vitamin K research is in the prevention of immune disorders [25], [26]. It is well established that nutrition is a powerful modulator of the immune system in chickens [7] but there is no evidence about the effects of any K vitamers or analogs on the immune responses of chickens. Nevertheless, analysis of the chicken blood confirmed that vitamin K_1 was the best form to achieve boosted vitamin K levels. This is known to translate to higher tissue and egg levels of vitamin K in chickens [453], [457]. Therefore, vitamin K_1 is the most effective nutritional form of vitamin K for improving vitamin K status in chickens.

7.1.2 Identification of a microorganism that is naturally rich in vitamin K_1

The first step in the development of the biotechnological process was to identify an organism that could produce phylloquinone in high concentrations. Phylloquinone is biosynthesized by photosynthetic organisms for use in electron transport [214], so plant leaves seem a logical place to begin. However, plant cell culture is very difficult and phylloquinone is found at low concentrations (<10 μg g^{-1}) in terrestrial plants. Many algae are known to contain much higher levels of phylloquinone (10-750 μg g^{-1}) [157], [208], and algal microorganisms ("microalgae") are much easier to culture than plant cells [238]. The identification and characterization of a strain of microalgae that is rich in phylloquinone was an essential step in the research.

A review of the literature and industry best-practice suggests that microalgae are the best organisms for biotechnological production of vitamin K_1. After screening seven common microalgae, one clearly superior strain was identified. At 200 μg g^{-1} dry mass, *A. cylindrica* (CS-172) was found to contain a significantly higher concentration of phylloquinone than the other strains, being six-times richer than good dietary sources such as parsley [34], [159]. Vitamin K_1 content was confirmed by APCI-LC-MS/MS and independent laboratory verification. A toxicology bioassay demonstrated the absence of acute toxicity, as would be expected from cyanotoxin-producing strains. The high concentration of authentic vitamin K_1 and the apparent absence of cyanotoxins demonstrates the potential of using *A. cylindrica* for the safe and efficient biotechnological production of phylloquinone.

7.1.3 Importance of light intensity and medium nitrogen for the production of vitamin K_1

The second step in the development of the biotechnological process was to determine the optimal culture conditions to maximize productivity of phylloquinone. They key inputs for microalgal growth are generally recognized as light, nitrogen and phosphorus [170]. Light is especially important, as it is known to

strongly affect growth rate and photosynthetic electron transport [220]. Nitrogen is important because it is essential for protein synthesis, and proteins make up all of the cellular machinery. Phosphorus is important because it is essential for DNA synthesis, building cell membranes, energy storage, and carbon fixation. Although carbon is required for all biological molecules, microalgae can capture it from atmospheric CO_2, so it is usually a cheap and plentiful input. Optimizing the levels of light, nitrogen and phosphorus was necessary to maximize phylloquinone productivity.

A. cylindrica was grown in 5 L flat panel PBRs, and the effects of key nutrient levels were tested. Low light (PPFD ~170 μmol m^{-2} s^{-1}) and high nitrogen (1700 mg L^{-1}) boosted phylloquinone specific concentration, while high light (PPFD ~330 μmol m^{-2} s^{-1}) and high nitrogen boosted biomass productivity. Overall, high light and nitrogen maximized phylloquinone productivity. It was also found that phylloquinone and biomass concentrations can be estimated by spectrophotometry, which is useful for industrial processes. These results are essential for the success of industrial scale cultures, as volumetric productivity is the main limitation of microalgal processes.

7.1.4 Robustness of vitamin K_1 production in *A. cylindrica* against variations due to scale-up

The third step in the development of the biotechnological process was to test the technical feasibility of producing phylloquinone using microalgae in a pilot-scale bubble-column PBR. Scale-up of any process to an industrial size almost always entails some loss of productivity. In the case of microalgal culture, this is usually due to light limitation [170]. As a rule of thumb, the culture depth should be no more than 5 cm thick in order to prevent self-shading [170]. Thinner cultures favor higher productivities. Conversely, it is much cheaper to scale-up a cylindrical culture vessel by increasing vessel diameter (volume is proportional to the square of diameter) than by increasing length (volume is proportional to length). This means that

industrial culture vessels will have a central "dark" zone where cells cannot photosynthesize. Therefore, it is important to develop strategies that improve the cumulative irradiance of cultures in large reactors. Ensuring fast and even mixing of the cells between the light and dark zones in large culture vessels is essential. In bubble column reactors, mixing is dependent upon the bubble flow regime, so this was the most important aspect of scale-up. If mixing cannot be further improved, increasing the cumulative irradiance is another possibility. Developing these strategies was essential for efficient scale-up.

A. cylindrica was grown in a 50 liter bubble-column PBR. The most important operating conditions (air flow rate, sparger design, cumulative irradiance, nutrient repletion) were tested. Air flow rate (U_g in the range 0.62 to 6.2 cm s^{-1}) and sparger design (2 mm holes versus <300 μm pores) had no significant effect on biomass or phylloquinone productivity. At a similar light intensity (PPFD ~300 μmol m^{-2} s^{-1}), the phylloquinone productivity was very similar in the 50 L PBR to the 5 L PBR (~20 μg L^{-1} d^{-1}). This was despite lower biomass productivity in the 50 L PBR. These phenomena are probably due to light-limitation, inducing PSI synthesis and slower growth. As lower light intensity appears to favor phylloquinone synthesis, the effect of self-shading has some benefit. An adequate concentration of nitrogen in the medium is essential for maximizing phylloquinone specific concentration; N-replete biomass contained more than 260 μg g^{-1}, compared to N-limited biomass with less than 200 μg g^{-1}. It appears that this strain of *A. cylindrica* is a very robust organism and that production of phylloquinone is not greatly affected by variations in temperature, pH and light availability due to self-shading.

7.2 Future work

The research presented here lays the foundation for development of a microalgal process for production of vitamin K_1. Naturally, there is much left to do in order to realize this opportunity. Furthermore, many questions have arisen from the research that bear further investigation. Ideally, these limitations and unanswered questions of the present study would be investigated in future work.

7.2.1 Benefits of vitamin K_1 for chicken welfare and productivity

Foremost among the unanswered is the question: does natural vitamin K, especially vitamin K_1, confer immune benefits upon chickens? This question is of both intense scientific and commercial interest. Our immunological assays did not reveal any differences between vitamin K_1, vitamin K_2 and menadione. Our experiment was powered to observe an expected effect size of approximately 50%. Similar effect sizes were observed in previous studies of the immunomodulatory effects of vitamin K [130], [309], [310], however these studies were *in vitro* and used doses that were much higher than the blood concentrations observed in our study. Therefore, it is likely that either higher doses or a more statistically powerful study design would be required in order to observe immune benefits. As the doses used here reflect the industry standard, a more statistically powerful experiment is the better option. A large (n > 1000 per group) observational study would satisfy this criterion. Furthermore, observational studies can use metrics that are more relevant to the poultry industry, such as feed conversion, laying efficiency and attrition rate. These metrics are easily interpreted by farmers and others in the industry, and form a more compelling case for the use of one treatment over another.

7.2.2 Safety of health products from cyanobacteria for repeated long-term use

An important aspect of human and animal nutrition is product safety. The strain of *A. cylindrica* used here was categorized by ANACC as non-toxic. The results

presented here support the absence of acute toxicity of *A. cylindrica*. However, these results provide no data on the long-term effects of repeated doses (e.g. the potential neurodegenerative effects of amino acids such as BMAA [458]). As *A. cylindrica* is a novel food organism, more comprehensive animal testing is required to demonstrate safety [459], [460]. The OECD Test no. 408 for oral toxicity [461] is typically used for testing potential sub-chronic toxicity of novel foods (including GM organisms) [462], [463]. This test standard stipulates that at least 20 animals (10 male and 10 female) should be used per diet group, and that the feed study should last at least 90 days. Such a study is an essential future step in the application of this strain to health products for human and animal consumption.

The strain of *A. cylindrica* used here may be only one of many that are rich in vitamin K_1; there are tens of thousands of microalgal species waiting to be tested and exploited for their valuable products [213], [464]. According to the literature, it is probable that other cyanobacteria and some rhodophytes (red algae) are also very rich sources of phylloquinone [354], [378], [384]. The fact that the cyanobacteria tested (*A. cylindrica* and *Spirulina* sp.) contained the highest and second-highest concentrations of vitamin K_1 lend credence to this hypothesis. Testing of other genera and families would potentially reveal alternative organisms for the biotechnological production of phylloquinone under industrially useful conditions, such as extreme pH (>9), salinity (>30 g L^{-1}) and temperature (>30 °C). Analysis of existing genomic data may be able to suggest non-toxic candidates with similar traits to *A. cylindrica*. Creation of super-productive and non-toxic strains may even be possible, thanks to the burgeoning field of metabolic engineering [256], [257], [465].

7.2.3 Optimization of nutrient inputs for an industrial scale process

Light was, as expected, identified as the most important input to the production of vitamin K_1. Nitrogen was also very important. Medium nitrogen is often supplied as urea or ammonium, which are cheaper than nitrate, and these are all utilized at different rates [170]. The effect of nitrogen source on cell growth should be tested. Iron may also have an appreciable effect on vitamin K_1 productivity. Iron is essential for PSI [221], so it would make sense to test the effects of different concentrations and oxidation states on productivity. The aim of these studies is to maximize productivity while minimizing the cost of mineral inputs, because laboratory culture medium is expensive to make. It would be of industrial relevance to test alternative, nutrient-rich water sources, such as waste water from hydroponics and aquaculture.

One particularly puzzling aspect of vitamin K_1 biosynthesis had to do with nitrogen limitation. There is a putative role for PSI in nitrogen fixation [221], and PSI function is usually preserved during nitrogen limitation [386]–[388], [447]. Why is it, then, that medium nitrogen limitation appeared to strongly decrease phylloquinone content? The ability to grow *A. cylindrica* in nitrogen-fixing mode is beneficial because it eliminates the cost of medium nitrogen (such as nitrate) and prevents fast-growing, non-nitrogen fixing microalgae (such as *Chlorella* sp.) from contaminating the culture. Genomic and proteomic analyses of *A. cylindrica* under different culture conditions could help to explain why it has such a high phylloquinone concentration, and further elucidate the mechanisms influencing phylloquinone concentration. Electron microscopy of the thylakoid membranes may also reveal useful information about the cellular influences on PSI and phylloquinone concentration. These results could be used to develop a method for producing vitamin K_1 by growing *A. cylindrica* in a nitrogen-free medium [466].

7.2.4 Optimization of irradiance in a pilot-scale photo-bioreactor

Maximizing the cumulative irradiance (the integral of light intensity over time) is clearly preferable. Whether this is best achieved by increasing irradiance duration or intensity (or a combination of both) is not immediately obvious. The effect of continuous light was to double the biomass and vitamin K_1 productivity of the system, compared to a 12:12 hour light:dark cycle. Therefore, it is hypothesized that any increase in cumulative irradiance will improve productivity. However, due to the constraints of the indoor system, light intensities tested were approximately one-quarter of actual mean daylight intensities. It remains to be seen whether: (a) maximum daylight intensities are actually inhibitory; and, (b) whether continuous light at maximum daylight intensity would also boost productivity. It may be that the effects of mixing become much more important at these high light intensities. In that case, methods should be tested for improving radial mixing in bubble-columns, such as baffles, draft tubes and large-bubble generators.

7.2.5 Advanced tools for monitoring of microalgal culture

Many aspects of microalgal growth, including nutrient uptake, toxin production and light response, can be monitored using so-called "omics" techniques [467]. The influence of genetic and environmental factors on microalgal behavior can be observed in changes to the proteome and metabolome [468]–[470]. Although research has focused mainly on the synthesis and storage of lipids for biofuel production [469], [470], these techniques can be applied to other aspects of algal metabolism. For example, the development of high-throughput mass-spectrometric and label-based methods has enabled the analysis of protein response to changes in light exposure [469]. Likewise, advances in chromatography and mass-spectrometry have enabled the analysis of the toxicity of a range of cyanobacterial metabolites [471]. These tools could be used to monitor proteins and other compounds that indicate positive (e.g. phylloquinone synthesis) and negative (e.g. toxin BMAA) characteristics of *A. cylindrica* cultures.

7.2.6 Optimization of downstream processing

The vitamin K-rich biomass of *A. cylindrica* can be incorporated into animal feed (as demonstrated in this thesis) and, possibly, cosmetics and nutritional products for human consumption. Mature dewatering technologies (gravity sedimentation, centrifugation and filtration) and drying technologies (freeze-drying or spray-drying) are adequate for the preparation of biomass for these purposes [264], [472]. However, to produce a pure vitamin K_1 product, it is necessary to perform downstream processing on the biomass.

Unit operations in the downstream processing of algal biomass would include cell disruption, debris removal and primary isolation, product enrichment and final isolation [45]. Although there are also mature technologies for each of these unit operations [45], substantial innovation is possible by combining or eliminating operations. For example, super-critical CO_2 extraction can replace traditional cell disruption (milling or homogenization), debris removal (filtration) and primary isolation (solvent extraction). Alternatively, disruption and primary isolation could be performed in the algae culture vessel by increasing the medium osmolarity and adding a non-polar solvent phase. Future research could investigate these or similar advances in downstream processing of algal biomass.

7.3 Final remarks

Biotechnological processes, such as the proposed microalgal process for vitamin K_1, have many benefits over chemical syntheses. Of course, there are also drawbacks to biotechnological processes. In the long-run, however, it is likely that the advantages will far outweigh the disadvantages.

Biotechnological processes currently face obstacles in the form of biology and ethics, and microalgal biotechnology is no exception. Wild-type organisms often do not make chemicals efficiently enough for industrial purposes. With genetic manipulation, it is often possible to make these organisms fit for industrial use. However, gene technologies are controversial and tightly regulated. Despite these obstacles, it is important to persevere with research in microalgal biotechnology.

Resource scarcity and environmental degradation are causing a host of social, economic and environmental problems. Ignoring these problems could be catastrophic. Microalgae do not consume scarce resources such as arable land and fresh food. Instead, they consume plentiful "resources" such as sunlight and carbon dioxide. The production of vitamin K_1 is just one example of the way in which microalgal biotechnology could change human industry for the better. Though it may take many years to realize this vision, it is a worthwhile and essential endeavor.

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Appendix A: AEC approval letter for project 2015/769

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY
Animal Ethics Committee

Wednesday, 11 February 2015

Prof David Raubenheimer
Vet Science Faculty; Faculty of Veterinary Science
The University of Sydney
Email: david.raubenheimer@sydney.edu.au

Dear Prof Raubenheimer

I am pleased to inform you that the University of Sydney Animal Ethics Committee (AEC) has approved your project entitled "**The effect of different K vitamers on the immune system of chickens**".

Details of the approval are as follows:

Project Number: **769**

Approval Period: **29/01/2015 – 29/01/2018**

Annual Report Due: **29/01/2016**

Authorised Personnel: **Raubenheimer David; Talbot Andrea; Regtop Hubert; Biffin Ray; Tarento Thomas**

Documents Approved:

Date	Document
13/01/2015	Chicken Monitoring Sheet
13/01/2015	Sample Size Calculations
13/01/2015	Sample Size Calculations
12/01/2015	Chicken Housing SOP
12/01/2015	Chicken Housing SOP
12/01/2015	Chicken Feeding and Cleaning SOP
12/01/2015	Chicken Feeding and Cleaning SOP
12/01/2015	Chicken Blood Collection SOP
12/01/2015	Chicken Blood Collection SOP

Animals Approved:
Please refer to the document at the end of this letter, which details your approved animal usage.

The project is approved for an initial period of **12 months** with approval for up to **(3) years** following receipt of the appropriate report under clauses 2.2.24, 2.2.32 and 2.4.34 of the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013).

Conditions of Approval

Approval of this project is conditional upon your adherence to the conditions outlined in this letter and your continuing compliance with the Animal Research Act (1985 – Animal Research Regulation 2010) and the 8th Edition of the Australian code for the care and use of animals for scientific purposes (NHMRC 2013).

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CRICOS 00026A

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1. The Animal Ethics Committee (AEC) reviews and approves protocols for their compliance with the NSW Animal Research Act (and associated regulations) and the 8th Edition of the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013).

2. This approval is in accordance with your original submission together with any additional information provided as part of the approval process.

3. Any changes to the protocol must be approved by the AEC before continuation of the study. This includes notifying the AEC of any changes to named personnel, source of animals, animal numbers, location of animals and experimental procedures.

4. Investigators should promptly notify the AEC of any unexpected **adverse events** that may impact on the wellbeing of an animal in their care, as per Clause 2.1.5 [v] [d] and 2.4.34 [ii] in the Australian code of practice (NHMRC, 2013). In the event that an unexpected adverse event occurs, please refer to the Animal Ethics website and log into IRMA to complete an Adverse Event form. For further information, please see the AEC Adverse Event Reporting Procedures (GL003) on the Animal Ethics website.

5. In the event an animal dies unexpectedly or requires euthanasia for welfare reasons, an autopsy should be performed by a person with appropriate qualifications and/or experience and the AEC should be notified promptly.

6. Animals must not be euthanised within sight or sound of other animals, in accordance with Clause 3.3.45 [vi] of the Australian code of practice (NHMRC, 2013).

7. Animals should not be housed singly unless otherwise approved by the AEC.

8. All animals must be provided with environmental enrichment appropriate for their species, unless otherwise approved by the AEC.

9. All pens, cages and containers used for holding animals must be clearly identified with chief investigator name, number of animals, DOB if provided and date of arrival, sex and strain.

10. A copy of this approval letter, together with all relevant monitoring records, must be kept in the facility where your animals are housed. These records must be updated regularly as breeding and husbandry events occur and current copies must be maintained in the animal house. Monitoring sheets must contain a section where expected post-operative effects are identified and observations recorded. Where relevant, the pens, cages and container number must be recorded on the monitoring sheet to ensure that affected animals can be easily located. Where electronic breeding records are kept instead of records on cage cards, printed copies of the records should be placed in a folder in the relevant animal house, where they can be inspected by the AEC.

11. Data should be accurately recorded in a durable, indexed and retrievable form that complies with relevant legislation, policy and guidelines. Following completion of the study all data including consent forms must be retained in a secure location, such as a locked filing cabinet, at the University of Sydney for a period of at least seven (7) years.

12. The AEC will make regular announced inspections of all animal facilities and/or specific research protocols. The Animal Welfare Veterinarian will be conducting unannounced inspections of all animal facilities and/or specific research protocols.

13. All new investigators must successfully complete the Introduction to Animal Research (ITAR) course.

Please do not hesitate to contact the Research Integrity (Animal Ethics) Office at animal.ethics@sydney.edu.au should you require further information or clarification.

THE UNIVERSITY OF SYDNEY

Yours sincerely

Professor David Allen
Chair
Animal Ethics Committee

The AEC is constituted and operates in accordance with the NSW Animal Research Act (1985) and its associated Regulations, the 8th Edition Australian code for the care and use of animals for scientific purposes (NHMRC, 2013) and the Australian Code for the Responsible Conduct of Research (2007). All personnel named on the protocol should be conversant with these documents.

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RESEARCH INTEGRITY
Animal Ethics Committee

Animals Approved:

Country	State	Invasiveness	Location	Classification one	Classification two	Common/strain name	Applied	Anticipated re-use
Australia	NSW	4. Minor surgery with recovery	Other	Birds	Poultry	ISA Brown	12	0

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Appendix B: AEC approval letter for project 2017/1243

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY & ETHICS ADMINISTRATION

Animal Research Authority

A copy of this approval letter must be kept in the facility where your animals are housed.

Friday, 13 October 2017

Prof David Raubenheimer
School of Life and Environmental Sciences (SOLES); Faculty of Science
The University of Sydney

Email: david.raubenheimer@sydney.edu.au

Dear Prof Raubenheimer

I am pleased to inform you that the University of Sydney Animal Ethics Committee (AEC) has approved your project entitled **"Benefits of algae and plant waste as diet supplements"**.

Details of the approval are as follows:

Project Number:	2017/1243
Project Type:	Experimental (non-wildlife)
Project Duration:	13 October 2017 – 13 October 2020
Approval Period:	**13 October 2017 – 13 October 2018**
Annual Report Due:	13 October 2018

In compliance with Section 27 of the NSW *Animal Research Act 1985*, this Animal Research Authority (ARA) remains in force for a period of 12 months from the date of issue, unless cancelled sooner.

Renewal of the ARA is conditional upon submission of a satisfactory annual report to the AEC in accordance with the *Australian code for the care and use of animals for scientific purposes 8th Edition 2013*.

Authorised Personnel: Raubenheimer David (CI); Arab Marjan; Macia Laurence; Schindeler Aaron; Tarento Thomas; Vasiljevski Emily;

Project Description:
The primary aim of the study is to determine the suitability of algae and vegetable waste for dietary inclusion. The secondary aim is to determine whether or not these inclusions lead to additional health benefits. As the global climate changes and the population grows, alternative foods and reuse of waste will become more important for ensuring food security. Aquatic organisms and vegetable waste represent good future food sources because they do not compete with current food crops for resources. Furthermore, "plant-based" foods contain many compounds that have health benefits beyond nutrition.Anabaena cylindrica is a non-toxic species of blue-green algae (BGA) that is found across the globe in freshwater systems, including the Murray-Darling and Hawkesbury-Nepean river systems. Global warming and nutrient enrichment of fresh water due to human activity tend to favour the growth of blue-green algae, which makes them attractive organisms for ensuring food security. Tomato (Lycopersicon esculentum Mill.) is one of the most widely consumed vegetables in the world. During production, harvesting, and processing of tomato products, up to 40% of the plant ends up as waste. Both BGA and tomato leaf are very rich in vitamins (such as vitamin K), minerals (such as calcium) and bioactive compounds (such as pigments). They also contain appreciable levels of dietary fibre and protein.

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Documents Approved:

05/09/2017	Other	Calculation sheet - number of animals required
05/09/2017	Monitoring Sheet	Monitoring sheet - males housed individually
05/09/2017	Monitoring Sheet	Monitoring sheet - females housed together
08/09/2017	Other	Example diet formulation
21/09/2017	Other	SOP - Cardiac blood collection (Terminal bleed)
03/10/2017	Responses	Tomato leaf 50% diet formulation
03/10/2017	Responses	Phylloquinone and solanesol extract diet formulation
03/10/2017	Responses	Article on solanum in animal diets
03/10/2017	Responses	Article on spirulina in animal diets 2
03/10/2017	Responses	Article on solanum in animal diets 2
03/10/2017	Responses	Control diet formulation
03/10/2017	Responses	Tomato leaf 20% diet formulation
03/10/2017	Responses	Article on spirulina in animal diets

Animals Approved:
Please refer to the document at the end of this letter, which details your approved animal usage and location(s).

Conditions of Approval

1. This project must be conducted according to the approved project including continuing compliance with the conditions outlined in this ARA and with the *Animal Research Act 1985*, *Animal Research Regulation 2010*, the *Australian code for the care and use of animals for scientific purposes 8th edition 2013* (the Code) and all other relevant legislation.

2. Any changes to the project must be approved by the AEC prior to their implementation.
This includes notifying the AEC of any changes to named personnel, source of animals, animal numbers, location of animals and experimental procedures.

3. An annual progress report or completion report must be submitted on or before the anniversary of approval of the project.

4. All unexpected adverse events that may impact on the wellbeing of an animal must be reported to the AEC within 48 hours, as per Clause 2.1.5 [v] [d] and 2.4.34 [ii] in the Code. Please refer to the Animal Ethics website and log into IRMA to complete an Adverse Event form.

5. The AEC must be notified if rodents are required to be singly housed.

6. All animal enclosures (e.g. pens, cages and containers) must be clearly identified with chief investigator name, number of animals, DOB if provided and date of arrival, sex and strain.

7. The following documentation must be kept in the facility where your animals are housed or with you when undertaking fieldwork:
   - A copy of this ARA
   - Emergency contact details in case of an animal emergency
   - Approved monitoring records

8. Personnel working on this project must be sufficiently qualified by education, training and experience for their role, or adequately supervised.
All new investigators must successfully complete the Introduction to Animal Research (ITAR) course.

9. Data must be retained and stored in accordance with the relevant legislation and University guidelines.

THE UNIVERSITY OF SYDNEY

10. The AEC will make regular announced inspections of all animal facilities and/or specific research projects. The Animal Welfare Veterinarian will be conducting unannounced inspections of all animal facilities and/or specific research projects.

11. Any drugs to be used for procedures involving animals must within date (not expired) and stored appropriately as per the manufacturer's recommendations. It is the responsibility of the Chief Investigator to ensure that all relevant and current authority for the use of restricted drugs is obtained.

Please do not hesitate to contact the Ethics Office at animal.ethics@sydney.edu.au should you require further information or clarification.

Yours sincerely

Professor Michael D'Occhio
Chair
Animal Ethics Committee
On behalf of the University of Sydney

The AEC is constituted and operates in accordance with the NSW Animal Research Act 1985 and its associated Regulations, the Australian code for the care and use of animals for scientific purposes 8th Edition 2013 and the Australian Code for the Responsible Conduct of Research 2007. All personnel named on the project should be conversant with these documents.

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY &
ETHICS ADMINISTRATION

Animal Research Authority

A copy of this approval letter must be kept in the facility where your animals are housed.

Animals Approved:

Country	State	Invasiveness	Location	Classification one	Classification two	Common/strain name	Applied	Anticipated re-use
Australia	NSW	1. Observation involving no or minor interference	Charles Perkins Centre	Laboratory mammals	Mice	C57BL6	70	0

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Appendix C: Diet formulation sheet for dough premix SF14-156

Specialty Feeds
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Glen Forrest
Western Australia 6071
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Email: info@specialtyfeeds.com

Diet SF14-156 Trial Dough Form Recovery Diet

A fixed formulation diet for Laboratory Rats and Mice fortified with vitamins and minerals to meet the requirements of breeding animals after the diet is autoclaved or irradiated.

- Trial diet supplied as powder to be mixed with water to make dough by facility.
- Standard rat and mouse chow supplemented with peanuts, dextrose and sodium caseinate to make diet more palatable for animals in recovery.

Calculated Nutritional Parameters

Protein	24.60%
Total Fat	8.50%
Crude Fibre	4.60%
Acid Detergent Fibre	5.86%
Neutral Detergent Fibre	12.11%
Total Carbohydrate	52.60%
Digestible Energy	15.5 MJ / Kg
% Total Calculated Digestible Energy From Lipids	20.10%
% Total Calculated Digestible Energy From Protein	26.70%

Diet Form and Features

- Cereal grain base powder diet.
- Pack size 20g single serve. Larger pack size on request
- Diet suitable for irradiation and for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

Ingredients

Wheat, barley, Lupins, Soya meal, Fish meal, Mixed vegetable oils, Canola oil, Salt, Calcium carbonate, Dicalcium phosphate, Magnesium oxide, Peanuts, Dextrose, Sodium Caseinate and a Vitamin and trace mineral premix.

Added Vitamins

Vitamin A (Retinol)	10 000 IU/Kg
Vitamin D (Cholecalciferol)	2 000 IU/Kg
Vitamin E (a Tocopherol acetate)	100 mg/Kg
Vitamin K (Menadione)	20 mg/Kg
Vitamin B1 (Thiamine)	80 mg/Kg
Vitamin B2 (Riboflavin)	30 mg/Kg
Niacin (Nicotinic acid)	100 mg/Kg
Vitamin B6 (Pryridoxine)	25 mg/Kg
Calcium Pantothenate	50 mg/Kg
Biotin	300 ug/Kg
Folic Acid	5.0 mg/Kg
Vitamin B12 (Cyancobalamin)	150 ug/Kg

Added Trace Minerals	
Magnesium	100 mg/Kg
Iron	70 mg/Kg
Copper	16 mg/Kg
Iodine	0.5 mg/Kg
Manganese	70 mg/Kg
Zinc	60 mg/Kg
Molybdenum	0.5 mg/Kg
Selenium	0.1 mg/Kg

Calculated Amino Acids	
Valine	1.28%
Leucine	2.00%
Isoleucine	1.05%
Threonine	0.94%
Methionine	0.45%
Cystine	0.31%
Lysine	1.42%
Phenylalanine	1.21%
Tyrosine	1.06%
Tryptophan	0.30%
Histadine	0.70%

Calculated Total Minerals	
Calcium	0.64%
Phosphorous	0.62%
Magnesium	0.18%
Sodium	0.30%
Potassium	0.69%
Sulphur	0.19%
Iron	185 mg/Kg
Copper	23 mg/Kg
Iodine	0.5 mg/Kg
Manganese	107 mg/Kg
Cobalt	0.5 mg/Kg
Zinc	93 mg/Kg
Molybdenum	1.1 mg/Kg
Selenium	0.3 mg/Kg
Cadmium	0.03 mg/Kg

Calculated Total Vitamins	
Vitamin A (Retinol)	10 760 IU/Kg
Vitamin D (Cholecalciferol)	2 000 IU/Kg
Vitamin E (a Tocopherol acetate)	117 mg/Kg
Vitamin K (Menadione)	20 mg/Kg
Vitamin C (Ascorbic acid)	No data
Vitamin B1 (Thiamine)	83 mg/Kg
Vitamin B2 (Riboflavin)	31 mg/Kg
Niacin (Nicotinic acid)	149 mg/Kg
Vitamin B6 (Pryridoxine)	28 mg/Kg
Pantothenic Acid	59 mg/Kg
Biotin	391 ug/Kg
Folic Acid	5.5 mg/Kg
Inositol	No data
Vitamin B12 (Cyancobalamin)	150 ug/Kg
Choline	1 300 mg/Kg

Calculated Fatty Acid Composition	
Myristic Acid 14:0	0.03%
Palmitic Acid 16:0	0.87%
Stearic Acid 18:0	0.42%
Palmitoleic Acid 16:1	0.01%
Oleic Acid 18:1	3.60%
Gadoleic Acid 20:1	0.10%
Linoleic Acid 18:2 n6	2.68%
a Linolenic Acid 18:3 n3	0.24%
Arachadonic Acid 20:4 n6	Trace
EPA 20:5 n3	0.02%
DHA 22:6 n3	0.04%
Total n3	0.31%
Total n6	2.68%
Total Mono Unsaturated Fats	3.73%
Total Polyunsaturated Fats	2.99%
Total Saturated Fats	1.58%

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**.

We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

Appendix D: Reports of analysis for nutritional content of *A. cylindrica*

Australian Government
Department of Industry, Innovation and Science

National Measurement Institute

REPORT OF ANALYSIS

Report No. RN1188180

Client	: THE UNIVERSITY OF SYDNEY	**Job No.**	: UNIS49/180214
	FACULTY OF ENGINEERING & IT	**Quote No.**	: QT-02094
	SCHOOL OF CHEMICAL & BIOMOLECULAR ENGINEERING	**Order No.**	:
	ROOM 108, ENGINEERING LINK BUILDNG	**Date Sampled**	:
	SYDNEY NSW 2006	**Date Received**	: 14-FEB-2018
Attention	: THOMAS TARENTO	**Sampled By**	: CLIENT
Project Name	:		
Your Client Services Manager	: Tony Lattari	**Phone**	: 02 9449 0196

Lab Reg No.	Sample Ref	Sample Description
V18/003304	1	Green Slurry

Lab Reg No.		V18/003304				
Sample Reference	Units	1				Method
Trace Elements						
Calcium	mg/kg	270				VL247
Iron	mg/kg	51				VL247
Magnesium	mg/kg	330				VL247
Potassium	mg/kg	820				VL247
Sodium	mg/100g	20				VL247
Zinc	mg/kg	1.4				VL247

Paul Adorno, Section Manager
Inorganics - Vic

7-MAR-2018

Lab Reg No.		V18/003304				
Sample Reference	Units	1				Method
Proximates						
Fructose	g/100g	<0.2				VL295
Glucose	g/100g	<0.2				VL295
Sucrose	g/100g	<0.2				VL295
Maltose	g/100g	<0.2				VL295
Lactose	g/100g	<0.2				VL295
Total Sugars	g/100g	<1				VL295
Moisture	g/100g	91.4				VL298
Fat (Mojonnier extraction)	g/100g	0.3				VL302
Saturated Fat	g/100g	0.2				VL289
Protein (N x 6.25)	g/100g	5.9				VL299

REPORT OF ANALYSIS

Report No. RN1188180

Lab Reg No.		V18/003304				
Sample Reference	Units	1				Method
Proximates						
Ash	g/100g	0.5				VL286
Carbohydrates	g/100g	1				VL412
Energy (kj)	kJ/100g	130				VL412
Mono trans fats	g/100g	<0.1				VL289
Mono-unsaturated fat	g/100g	<0.1				VL289
Omega 3 fats	g/100g	<0.1				VL289
Omega 6 fats	g/100g	<0.1				VL289
Poly trans fats	g/100g	<0.1				VL289
Poly-unsaturated fat	g/100g	<0.1				VL289
Trans fats	g/100g	<0.1				VL289
Vitamins						
Thiamin	mg/100g	0.05				VL290
Riboflavin (Vitamin B2)	mg/100g	0.10				VL290
Niacin (Vitamin B3)	mg/100g	<1.0				VL293
Retinol (Vitamin A)	ug/100g	<5				VL287
alpha-tocopherol	mg/100g	<0.1				VL291
Ergocalciferol (Vit D2)	ug/100g	<0.5				VL294
Pyridoxine (Vitamin B6)	mg/100g	<0.02				VL320
Vitamin K1	ug/100g	2000				VL406
Saturated Fatty Acids						
C4:0 Butyric	%	<0.1				VL289
C6:0 Caproic	%	0.3				VL289
C8:0 Caprylic	%	<0.1				VL289
C10:0 Capric	%	0.8				VL289
C12:0 Lauric	%	0.7				VL289
C14:0 Myristic	%	1.5				VL289
C15:0 Pentadecanoic	%	<0.1				VL289
C16:0 Palmitic	%	65.3				VL289
C17:0 Margaric	%	<0.1				VL289
C18:0 Stearic	%	2.1				VL289
C20:0 Arachidic	%	<0.1				VL289
C22:0 Behenic	%	0.5				VL289
C24:0 Lignoceric	%	2.3				VL289
Total Saturated	%	73.5				VL289
Mono-unsaturated Fatty Acids						
C14:1 Myristoleic	%	<0.1				VL289
C16:1 Palmitoleic	%	7.8				VL289
C17:1 Heptadecenoic	%	<0.1				VL289
C18:1 Oleic	%	13.4				VL289
C18:1 Vaccenic	%	1.2				VL289
C20:1 Eicosenic	%	<0.1				VL289

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National Measurement Institute

REPORT OF ANALYSIS

Lab Reg No.		V18/003304				
Sample Reference	Units	1				Method
Mono-unsaturated Fatty Acids						
C22:1 Cetoleic	%	0.6				VL289
C22:1 Docosenoic (Erucic)	%	<0.1				VL289
C24:1 Nervonic	%	<0.1				VL289
Total Mono-unsaturated	%	23.0				VL289
Poly-unsaturated Fatty Acids						
C16:4 Hexadecatetraenoic	%	<0.1				VL289
C18:4 Moroctic	%	<0.1				VL289
C18:2w6 Linoleic	%	2.4				VL289
C18:3w6 gamma-Linolenic	%	<0.1				VL289
C18:3w3 alpha-Linolenic	%	0.7				VL289
C20:2w6 Eicosadienoic	%	<0.1				VL289
C20:3w6 Eicosatrienoic	%	<0.1				VL289
C20:3w3 Eicosatrienoic	%	<0.1				VL289
C20:4w6 Arachidonic	%	<0.1				VL289
C20:5w3 Eicosapentaenoic	%	<0.1				VL289
C22:2w6 Docosadienoic	%	0.4				VL289
Omega 3 Fatty Acids	%	0.7				VL289
Omega 6 Fatty Acids	%	2.8				VL289
C22:4w6 Docosatetraenoic	%	<0.1				VL289
C22:5w3 Docosapentaenoic	%	<0.1				VL289
C22:6w3 Docosahexaenoic	%	<0.1				VL289
Total Poly-unsaturated	%	3.5				VL289
Total Mono Trans Fatty Acids	%	<0.1				VL289
Total Poly Trans Fatty Acids	%	<0.1				VL289
P:M:S Ratio		0:0.3:1				VL289
Amino Acids						
Aspartic Acid	mg/kg	7000				VL450
Serine	mg/kg	3400				VL450
Glutamic Acid	mg/kg	7600				VL450
Glycine	mg/kg	3600				VL450
Histidine	mg/kg	960				VL450
Arginine	mg/kg	4200				VL450
Threonine	mg/kg	3900				VL450
Alanine	mg/kg	4800				VL450
Proline	mg/kg	2100				VL450
Tyrosine	mg/kg	3000				VL450
Valine	mg/kg	3300				VL450
Lysine	mg/kg	1500				VL450
Isoleucine	mg/kg	2900				VL450
Leucine	mg/kg	5200				VL450
Phenylalanine	mg/kg	2600				VL450

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National Measurement Institute

REPORT OF ANALYSIS

Report No. RN1188180

Lab Reg No.		V18/003304				
Sample Reference	Units	1				Method
Amino Acids						
Methionine	mg/kg	1200				VL450
Hydroxyproline	mg/kg	<50				VL450
Taurine	mg/kg	<50				VL450

George Dabos, Analyst
Food Composition - Vic

Paul Adorno, Section Manager
Food Composition - Vic

Norbert Strobel, Analyst
Food Composition - Vic

Leo Demel, Analyst
Food Composition - Vic

Sam Barone, Chemist
Organics - Vic

7-MAR-2018

Lab Reg No.		V18/003304				
Sample Reference	Units	1				Method
Proximates						
Total Dietary Fibre	g/100g	0.9				
Vitamins						
Pantothenic Acid (Vitamin B5)	mg/100g	<0.04				
Total Folates	ug/100g	<3.0				
Vitamin B12 (Cobalamin)	ug/100g	13.2				
Miscellaneous						
Biotin	ug/100g	0.18				
Subcontracted						
Folic Acid	ug/100g	<3.0				

V18/003304
Pantothenic Acid (Vitamin B5) determined by Dairy Technical Services (DTS), Melbourne. DTS Report No:2542395
Fibre & Biotin determined by Dairy Technical Services (DTS), Melbourne. NATA Accred. 345.
DTS Report No: 2542395
Folate determined by Path West Laboratories, Perth. NATA Accred. 2858.
Path West Report No: 649721 Path West Method: RPH01161
Folic Acid determined by Path West Laboratories, Perth. NATA Accred. 2858.
Path West Report No: 649722 Path West Method: RPH01161
Vitamin B12 (Cobalamin) determined by Path West Laboratories, Perth. NATA Accred. 2858.
Path West Report No: 650274 Path West Method: RPH01157

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REPORT OF ANALYSIS

Report No. RN1188180

Lab Reg No.		V18/003304				
Sample Reference	Units	1				Method

Jereka Thavakumar
Laboratory Services Unit - Vic

Ruby Paul
Laboratory Services Unit - Vic

7-MAR-2018

Results relate only to the sample(s) tested.
This Report shall not be reproduced except in full.

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Australian Government
Department of Industry, Innovation and Science

National Measurement Institute

REPORT OF ANALYSIS

Report No. RN1193054

Client	: THE UNIVERSITY OF SYDNEY	**Job No.**	: UNIS49/180214/1
	FACULTY OF ENGINEERING & IT	**Quote No.**	: QT-02094
	SCHOOL OF CHEMICAL & BIOMOLECULAR ENGINEERING	**Order No.**	:
	CHEMICAL ENGINEERING BUILDING (J01)	**Date Sampled**	:
	UNIVERSITY OF SYDNEY NSW 2006	**Date Received**	: 3-APR-2018
Attention	: THOMAS TARENTO	**Sampled By**	: CLIENT
Project Name	:		
Your Client Services Manager	: Tony Lattari	**Phone**	: 02 9449 0196

Lab Reg No.	Sample Ref	Sample Description
V18/003304/1	1	Green Slurry

Lab Reg No.		V18/003304/1		
Sample Reference	Units	1		Method
Vitamins				
alpha-Carotene	ug/100g	<5.0		VL292
beta-Carotene	ug/100g	11000		VL292
Amino Acids				
Tryptophan	mg/kg	640		VL450

V18/003304/1

Leo Demel, Analyst
Food Composition - Vic

Sam Barone, Chemist
Organics - Vic

23-APR-2018

Lab Reg No.		V18/003304/1		
Sample Reference	Units	1		Method
Miscellaneous				
Cysteine	mg/g	0.31		

V18/003304/1

Cysteine determined by Australian Proteome Analysis Facility (APAF), Macquarie University Sydney.
Calculation based on amino acid residue mass in protein (molecular weight minus water)
APAF Project Number: 21572

1/153 Bertie Street, Port Melbourne Vic 3207 Tel: +61 3 9644 4888 Fax: +61 3 9644 4999 www.measurement.gov.au

REPORT OF ANALYSIS

Lab Reg No.		V18/003304/1		
Sample Reference	Units	1		Method

Robyn Ferguson
Laboratory Services Unit - Vic

23-APR-2018

Results relate only to the sample(s) tested.
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National Measurement Institute