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JAMES COOK UNIVERSITY
AUSTRALIA

Addressing quality deficits in farmed barramundi

Optimising flavour and quality through pre-harvest practices

Thesis submitted by

Ben C Jones

April 2016

For the degree of Doctor of Philosophy

In the College of Marine and Environmental Science

James Cook University

Townsville, Australia

ACKNOWLEDGEMENTS

This research project was made possible by the contributions of colleagues and friends who have offered broad ranging assistance throughout my PhD candidature. I wish to express my gratitude to those who have contributed their time and effort to assist me in experiments and in the preparation of written and oral presentations. I further wish to thank those who have offered their guidance and support throughout the project.

I would like to thank my supervisors Dr Guy Carton and Dr Igor Pirozzi for assistance in planning research activities and preparing written and oral presentations.

I would also like to thank Sue Poole, Crop & Food Science, Department of Agriculture and Fisheries (DAF), for her assistance in coordinating experiments, analytical procedures and sensory analysis and for her generous support of the project.

Dr Richard Smullen, Ridley Agriproducts Pty Ltd., also made a significant contribution to the planning and coordination of this project and his assistance and guidance is greatly appreciated.

I also acknowledge Marty Phillips, Australian Barramundi Farmers Association. Marty’s interest helped to drive the project and generated stronger industry links.

I further acknowledge Pejo Enterprises and Good Fortune Bay Fisheries barramundi farms for: access to these commercial aquaculture facilities; the supply of fish used in experiments; the use of equipment; the supply of feed; and access to farm records.

I am also grateful to Dr Heather Smyth and Paul Exley, Crop & Food Science, DAF. Their assistance and general support of the project was extremely valuable.

Steve Fuller, Crop & Food Science, DAF, also made a significant contribution to this project by developing methods and performing analysis of samples for geosmin, dimethylsulfide and bromophenols and I acknowledge his input and assistance.

I also acknowledge Dr Kirsten Heimann for her valuable contributions to the project. Kirsten, along with Stan Hudson and the team at NQAIF also provided valuable assistance in the culturing of cyanobacteria and I thank them for their contribution.

I also acknowledge the assistance of Ben Lawes, Andrew Thompson and Simon Weaver from the Marine and Aquaculture Research Facilities Unit whos assistance and support was valuable and appreciated.

Finally I would like to express my enormous gratitude to my wife who supported me continuously on this journey, during which we also welcomed our third child into the world, shifted our family of three children interstate, built a new house and managed our small business. I congratulate her for maintaining her sanity!

Statement on the Contributions of Others

Every reasonable effort has been made to gain permission and acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

This PhD project was supervised by Dr Guy Carton, JCU, who provided academic, scientific and editorial support.

Sue Poole, DAF, and Dr Richard Smullen, Ridley Agriproducts Pty Ltd., also provided mentoring roles and research assistance throughout the project.

Steve Fuller, DAF, developed methods and performed analysis of samples for geosmin, dimethylsulfide and bromophenols.

Heather Smyth, DAF, provided assistance in the planning of sensory assessment procedures.

Ben Lawes, Andrew Thompson and Simon Weaver, Marine and Aquaculture Research Facilities Unit (MARFU), assisted with the procurement and maintenance of fish housing facilities and husbandry equipment.

This research was supported by a Research Incentive Grant from James Cook University. Financial assistance was also provided by the Australian Barramundi Farmers Association and Ridley Agriproducts Pty Ltd..

Components of the research program were performed at Pejo Enterprises barramundi farm in Innisfail, Queensland. Pejo Enterprises also supplied fish for use in experiments.

Good Fortune Bay Fisheries also contributed fish for use in experiments.

The specific contributions of co-authors to each chapter are outlined below.

Chapter	Reference of publication or title of the submitted paper on which each chapter is based	Nature and extent of the intellectual input of each author, including the candidate, and their affiliations
2	Jones, B., Fuller, S. & Carton, A. G. (2013). Earthy-muddy tainting of cultured barramundi linked to geosmin in tropical northern Australia. *Aquaculture Environment Interactions*, *3*, 117-124.	**Ben Jones**[1] (Candidate): • Conceptualised research study • Designed and carried out experiments • Analysed data • Wrote the paper **Guy Carton**[1] (Principal supervisor): • Provided mentoring and support • Edited the paper **Steve Fuller**[2]: • Developed methods and undertook analysis for geosmin in water and flesh

3	Under review - submitted to Aquaculture International: Controlled off-flavour tainting of cultured fish using the geosmin-producing cyanobacterium, *Anabaena circinalis*	**Ben Jones**[1] (Candidate): • Conceptualised research study • Designed and carried out experiments (70%) • Analysed data (70%) • Wrote the paper (70%) **Guy Carton**[1] (Principal supervisor): • Provided mentoring and support • Edited the paper **Samuel Cirés**[1]: • Provided mentoring and support • Edited the paper **Lena Geitung**[1]: • Carried out experiments (20%) • Analysed data (20%) • Wrote sections of the paper (30%) **Kirsten Heimann**[1]: • Provided mentoring and support • Edited the paper **Matt Jago**[1]: • Carried out experiments (10%) • Analysed data (10%)
4	Under review - submitted to Aquaculture: Uptake, depuration and spatial distribution of the off-flavour tainting compound geosmin in farmed barramundi, *Lates calcarifer*	**Ben Jones**[1] (Candidate): • Conceptualised research study • Designed and carried out experiments • Analysed data • Wrote the paper **Guy Carton**[1] (Principal supervisor): • Provided mentoring and support • Edited the paper
5	Jones, B., Smullen, R. & Carton, A.G. (2016). Flavour enhancement of freshwater farmed barramundi (*Lates calcarifer*), through dietary enrichment with cultivated sea lettuce, *Ulva ohnoi*. *Aquaculture, 454,* 192-198.	**Ben Jones**[1] (Candidate): • Conceptualised research study • Designed and carried out experiments • Analysed data • Wrote the paper **Guy Carton**[1] (Principal supervisor): • Provided mentoring and support • Edited the paper **Richard Smullen**[3]: • Provided mentoring and support

6	Jones, B. C. & Carton, A. G. (2015). Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi (*Lates calcarifer*). *Aquaculture, 448*, 280-287.	**Ben Jones**[1] (Candidate): • Conceptualised research study • Designed and carried out experiments • Analysed data • Wrote the paper **Guy Carton**[1] (Principal supervisor): • Provided mentoring and support • Edited the paper

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ABSTRACT

This thesis identifies factors resulting in quality deficits in Australian farmed barramundi and generates data on which to develop management practices that optimise flavour and quality. Five interrelated studies were undertaken focussing respectively on:

- The occurrence of earthy-muddy tainting of cultured barramundi linked to geosmin in tropical northern Australia.
- The controlled off-flavour tainting of cultured fish using the geosmin-producing cyanobacterium, *Anabaena circinalis*.
- The uptake, depuration and spatial distribution of the off-flavour tainting compound geosmin in farmed barramundi, *Lates calcarifer*.
- The flavour enhancement of freshwater farmed barramundi, *Lates calcarifer*, through dietary enrichment with cultivated sea lettuce, *Ulva ohnoi*.
- The effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi *Lates calcarifer*.

Three of these studies have been published in the scientific literature and two have been submitted for publication. These scientific papers are presented in chapters 2-6. Chapter 1 reviews the scientific literature on flavour and quality of aquaculture fish and explains the rationale for the studies undertaken. Chapter 7 summarises research findings and reviews the significance of the body of research for: aquaculture research; aquaculture production and product quality globally; and the Australian barramundi aquaculture industry.

The findings from this body of research relate primarily to three topics: off-flavour tainting; a lack of flavour complexity in farmed fish; and deterioration in product quality during the post-harvest storage period.

Water samples from outdoor barramundi rearing ponds were analysed for the presence of geosmin (GSM) and 2-methylisoborneol (MIB). GSM was deemed to be the compound responsible for off-flavour tainting in pond-reared barramundi, persisting at moderate (~2.00 $\mu g\ L^{-1}$) to extreme levels (~14.36 $\mu g\ L^{-1}$), while MIB was never detected during the study. The accumulation of GSM in the flesh of barramundi was directly related to GSM levels of the holding water. Elevated levels

of GSM in fish-flesh resulted in increases in the intensity of off- flavour tainting. The uptake of GSM by barramundi exposed to an extreme concentration of GSM (15.1μg L^{-1}) was extremely rapid; a significant increase in flesh GSM was observed after three minutes of exposure, with GSM concentration reaching 0.98±0.54μg kg^{-1}. GSM continued to accumulate in flesh reaching a maximum concentration of 8.8μg±1.88μg kg^{-1} after 3 hours of exposure. GSM deposition within the fillet was spatially variable with the ventral belly region containing approximately three times more GSM than either the dorsal shoulder or posterior tail regions. When returned to untainted water, the concentration of GSM in flesh declined exponentially, with a half-life of 99 hours at 27°C although GSM was still present (0.77±0.32μg kg^{-1}) in muscle tissue after 14 days of depuration. The potential to recover flavour quality was assessed for fish exposed to a moderate level of GSM (2.15μg L^{-1}) by depurating them in untainted water. Human sensory assessment revealed that off-flavour tainting was eliminated after 8 days of depuration.

As part of the investigation of off-flavour tainting in farmed barramundi, a new technique for producing natural GSM, for the purpose of intentionally imparting off-flavour tainting in fish, was developed. This technique is more precise and lower cost than existing methods, ensures authenticity of the organoleptic nature of off-flavour taint and is suitable for use on large cohorts of fish.

The potential to add flavour complexity to farmed barramundi was assessed by feeding the marine macroalgae *Ulva ohnoi* to cultured freshwater barramundi (1800-2000 g). When barramundi were fed diets containing ≥20% inclusion level of *U. ohnoi* for 7-21 days fish developed stronger crab-like/seafood flavour, cooked crab aroma and sweetness resulting in increased desirability and flavour complexity compared to fish fed a standard rearing diet. The potent flavour compound dimethylsulfide (DMS) was found to be more elevated (~8 fold) in fish fed *U. ohnoi* and appears to be a key flavour compound in this instance.

Pre-harvest dietary enrichment with α-tocopherol acetate in combination with different post-harvest processing techniques was investigated with regard to quality deterioration of farmed barramundi, during chilled storage. Fish were fed commercial rearing diets supplemented with two levels of α-tocopherol acetate (standard level 192mg kg^{-1} and enriched level 628 mg kg^{-1}) for a period of 5 months. Dietary α-tocopherol enrichment in combination with storing fish whole and ungutted constrained lipid oxidation over 14 days of chilled storage when compared to fish fed

the standard diet and filleted prior to storage. Filleting also resulted in significant colour changes, with reddening and yellowing of the flesh.

These findings have considerable significance for aquaculture research. Researchers should be aware that GSM can persist at moderate to extreme levels, for prolonged periods in tropical aquaculture ponds and that uptake by fish can occur extremely rapidly. The spatial distribution of GSM in fish fillets should also be considered as this has serious implications for the design of future research and sampling procedures. The potential to enhance flavour through the short-term application of diets that incorporate marine algae is critical information for researchers and further studies should be undertaken to build on this finding. This thesis also provides the first report of the potential association between the flavour of farmed fish and DMS. The observation that flavour and quality can be optimised during the post-harvest period by feeding diets enriched with α-tocopherol acetate and ensuring that fish are stored whole and ungutted also has implications for aquaculture research. Future studies should consider not only the benefits of pre-harvest and post-harvest strategies to optimise storage stability but also possible interactive effects between the two.

The findings also have practical significance for aquaculture producers, including in the Australian barramundi aquaculture industry. Aquaculture producers, especially those in tropical locations, should be aware of the potential for GSM to persist in pond water for extended periods of time and reach exceptionally high concentrations. This thesis also reveals the potential to recover flavour quality by depurating fish in clean water prior to slaughter. The spatial distribution of GSM within the fillet has further implications and may enable producers to more accurately determine the presence of off-flavour taint during sensory assessment or may provide an opportunity to reduce off-flavour tainting by removing the most heavily tainted fillet regions prior to human consumption. Diets that include a significant fraction of marine algae may additionally be used by producers to optimise flavour and quality. Aquaculture producers can also benefit from the use of diets enriched with α-tocopherol acetate. This can limit lipid oxidation during storage. Products can be further fortified against quality deterioration by storing fish whole and ungutted.

JOURNAL ARTICLES AND CONFERENCE PRESENTATIONS

Peer-reviewed journal articles

- Jones, B., Fuller, S. & Carton, A. G. (2013). Earthy-muddy tainting of cultured barramundi linked to geosmin in tropical northern Australia. *Aquaculture Environment Interactions*, *3*, 117-124.
- Jones, B. C. & Carton, A. G. (2015). Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi (*Lates calcarifer*). *Aquaculture*, *448*, 280-287.
- Jones, B., Smullen, R. & Carton, A. G. (2016). Flavour enhancement of freshwater farmed barramundi (*Lates calcarifer*), through dietary enrichment with cultivated sea lettuce (*Ulva ohnoi)*. *Aquaculture*, *454*, 192-198.

Articles submitted to peer-reviewed journals

- Jones, B. C., Cirés, S., Geitung, L., Heimann, K., Jago, M. & Carton, A. G. (under review). Controlled off-flavour tainting of cultured fish using the geosmin-producing cyanobacterium, *Anabaena circinalis. Aquaculture International* (submitted).
- Jones, B. C. & Carton, A. G. (under review). Uptake, depuration and spatial distribution of the off-flavour tainting compound geosmin in farmed barramundi, *Lates calcarifer. Aquaculture* (submitted).

Conference presentations

- Jones, B. (2010). Improving the quality of farmed barramundi: Developing best practice for depuration. Australian Prawn Farmers Association & Australian Barramundi Farmers Association Conference 2010, Gold Coast, Queensland.
- Jones, B. (2011). Optimising the quality of farmed barramundi: Pre-harvest strategies. 2011 Ridley Aqua-Feed Prawn & Barramundi Conference, Sydney, NSW.

- Jones, B. (2012). Producing Australia's finest finfish - enhancing product quality. 2012 Ridley Aqua-Feed Prawn & Barramundi Conference, Palm Cove, Qld.
- Jones, B. (2013). Quality enhancement of farmed barramundi - it's all about flavour and freshness. 2013 Ridley Aqua-Feed Prawn & Barramundi Conference, Palm Cove, Qld.
- Jones, B. (2014). Optimising Barramundi Flavour: Where we've been and where we're going. 2014 Ridley Aqua Feed Australian Prawn & Barramundi Farmers Symposium, Gold Coast, Qld.
- Jones, B., Pirozzi, I., Carton, G. (2014). Adding Flavour Complexity to Farmed Barramundi through Dietary Manipulation. 16th International Symposium on Fish Nutrition and Feeding, Cairns, Australia.
- Jones, B., Pirozzi, I., Carton, A.G. (2014). Adding Flavour Complexity to Freshwater Farmed Barramundi, *Lates calcarifer*, Through Dietary Manipulation. World Aquaculture 2014, Adelaide, SA.
- Jones, B., Jago, M. (2015). Flavour enhancing finishing diet, now and into the future. 2015 Ridley Australian Prawn & Barramundi Farmers Symposium, Gold Coast, Qld.

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Table 1.5 Contribution of co-authors to the published manuscript: Jones, B. C. & Carton, A. G. (2015). Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi (Lates calcarifer). *Aquaculture*, *448*, 280-287. 36

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CHAPTER 1

Introduction and literature review

1.1 Introduction

Flavour and quality of the post-harvest product are critical factors in determining the financial success of aquaculture farms in developed nations. In general, quality refers to those characteristics of an aquaculture product that are used to measure its standard against other similar products. This includes a range of physical, sensory, chemical and microbial attributes such as visual appearance, flavour, aroma and texture that greatly affect consumer perception (Gill, 1990; Huss, 1994; Carton & Jones, 2013). Optimising these characteristics can generate a competitive marketing advantage over similar products. Product differentiation can be used to market these distinctive, premium products and has the potential to increase financial returns to producers by increasing market share and/or sale price.

While aquaculture production has maintained strong growth across many developing nations over the past three decades, the rate of growth in developed nations has decreased markedly from 5.5% per annum during the 1980s to less than 2% per annum during the first decade of this century (FAO, 2012a). During this period, global food-fish (finfish, crustaceans, molluscs and other aquatic animals) production more than doubled, increasing from 71.9 million tonnes in 1980 to 148.1 million tonnes in 2010 (FAO, 2012a). Aquaculture accounted for a large proportion of this increase and now contributes almost half of the global production of food-fish. Approximately 67 million tonnes of food-fish was produced by aquaculture in 2012. The majority of production occurred in developing nations with China (41.1 million tonnes), India (4.2 million tonnes), Vietnam (3.1 million tonnes), Indonesia (3.1 million tonnes) and Bangladesh (1.7 million tonnes) making up the top 5 aquaculture

producing nations. The majority of fish produced in these developing nations was exported from the country of origin and destined for developed nations that collectively account for 73% of global imports of food-fish (FAO, 2012a).

The recent increase in global fish production has had a profound effect on the way aquaculture farms are managed in developed countries. In the past, the focus of many aquaculture industries was to maximise production capacity and output while minimising the cost of production. However, as the global supply of wild-caught and aquaculture fish has expanded, increased competition and supply has reduced the market value of some aquaculture products. In response, many aquaculture industries have increasingly focused on enhancing product quality in order to achieve a competitive advantage in the market and optimise financial returns. This has been especially important for aquaculture products that compete directly with wild-caught species. In some cases, aquaculture products are perceived as being inferior in flavour and quality to wild-caught species which are considered to be a premium product.

1.2 Product quality in aquaculture

Flavour and quality are arguably the most important factors affecting the profitability of modern aquaculture enterprises. Genetic improvements, enhanced nutrition, disease management and refined husbandry practices can optimise the efficiency of aquaculture production. However, consumer demand and sale price are largely driven by product quality and this factor has a major bearing on the profitability of aquaculture farms. High-quality products can stimulate greater demand in the market place, lead to increased sales price and enhance financial returns to aquaculture farmers. Conversely, placing poor-quality fish in the marketplace typically lowers consumer confidence and ultimately reduces commercial returns. This is exemplified

by the supply of poor-quality catfish into the American market during the 1990s which resulted in a 30% reduction in sales of cultured catfish (Engle et al., 1995).

Quality aspects of aquaculture products include a wide range of physical, sensory, chemical and microbial attributes (Gill, 1990; Huss, 1994; Carton & Jones, 2013). Visual appearance, flavour, aroma and textural characteristics are used by consumers to assess the standard of fish products (Gill, 1990; Anderson & Anderson, 1991; Gram, 1992; Huss, 1994; Lindsay, 1994; Gram & Huss, 1996; Olafsdóttir et al., 1997; Bonilla et al., 2007). Based on this assessment, consumer behaviours such as purchase and re-purchase decisions can be influenced while sale price is also heavily affected.

1.3 Scientific research pertinent to quality issues farmed fish

Aquaculture systems afford producers a high level of control over product quality. This sets aquaculture apart from wild-capture fisheries that are restricted in their ability to control many pre-harvest and harvesting factors. Environmental, nutritional, harvesting and processing factors impact heavily on quality and these can be actively manipulated in aquaculture systems. The culture environment, including water quality, can be accurately monitored and controlled (Stewart, 1967; Cooke & Kenedy, 1981; Torrans & Lowell, 1987, Schrader, 1998; Tucker, 2000; Tucker, 2006). The composition of the diet can be manipulated (Frigg et al., 1990; Baker & Davies, 1996; Harare et al., 1998; Ruff et al., 2003; Ma et al., 2005; Kim, et al., 2007; Chen et al., 2008; Fuller et al., 2008). Harvesting, slaughter and storage methods can be designed to optimise the quality of the final product (Khayat & Schwall, 1983; Undeland et al., 1998; Bosworth et al., 2007, Wilkinson et al., 2008; Carton & Jones 2013).

It is well known that the culture environment plays an important role in determining the flavour quality of fish (Tucker, 2000; Howgate, 2004). Many water-borne chemicals are known to cause unfavourable flavours and odours in fish (Persson,

1984; Tucker 2000). This is referred to as off-flavour tainting and episodes arising from water-borne chemicals are widely reported in the literature (Yurkowski & Tabachek, 1974; Persson, 1979; Lovell, 1983; Lovell & Broce, 1985; Zhang, et. al., 2000; Robertson, et. al., 2005; Schrader, 2005; Vallod, et. al. 2007; Guttman & Rijn, 2008; Percival, et. al. 2008). Aquaculture systems are designed such that water quality conditions can be actively manipulated. This ability to control the culture environment enables aquaculture farmers to prevent off-flavour events by precluding off-flavour chemicals from the culture environment either throughout the growing period or in the final stages of production, prior to harvest (Cooke & Kenedy, 1981; Torrans & Lowell, 1987, Schrader, 1998; Tucker, 2000; Yamprayoon & Noomhorn, 2000; Robertson, 2005; Tucker, 2006).

Dietary manipulation also has the potential to enhance the flavour quality of farmed fish (Ma et al., 2005). The flavour of fish is directly affected by diet in the immediate pre-harvest period (Ackman et al., 1972; Levasseur et al., 1994; Whitfield et al., 1998; Ma et al., 2005;). Critical flavour compounds are produced by certain species of marine algae which, when consumed, can significantly alter the flavour of fish (Ackman et al., 1972; Levasseur et al., 1994; Whitfield et al., 1998). The total exclusion, or reduction, of these compounds can result in fish products that lack flavour complexity and can be considered bland (Ma et al., 2005; Fuller et al., 2008; Frank et al., 2009). The ability to manipulate the diet of captive fish has the potential to precisely control the abundance of flavour-affecting chemicals in the diet thereby optimising the flavour quality of end-products (Whitfield et al., 2002; Ma et al., 2005; Kim et al., 2007).

Harvesting and processing methods are also known to affect the flavour and quality of fish products across a range of species (Fletcher & Hogdson, 1988; Frigg et al.,

1990; Undeland et al., 1998; Huidobro et al., 2001; Ruff et al., 2003). Subjecting fish to excessive stress, physical activity and rough handling at the point of harvest has been shown to cause deteriorations in product quality (Wilkinson et al., 2008; Erikson et al., 2011; Howieson et al., 2013a, Carton & Jones, 2013). As cultured fish are confined within distinct culture units such as tanks, ponds or cages, harvesting methods can be developed that minimise stress and limit physical activity. For example, the use of suitable anaesthetics at the point of harvest has been shown to improve the flavour and quality of cultured fish and mitigate quality deterioration during the post-harvest storage period (Fletcher et al., 2003; Bosworth et al., 2007; Wilkinson et al., 2008). Furthermore, enriching fish flesh with antioxidants such as α-tocopherol acetate during the pre-harvest period, which is readily achieved by dietary manipulation, has been shown to limit lipid oxidation during the post-harvest storage period thereby improving quality at the point of consumption (Frigg et al., 1990; Baker & Davies, 1996; Harare et al., 1998; Ruff et al., 2003; Chen et al., 2008).

While flavour and quality are the terms that are widely used to describe the relative standard of fish and fish products, from a research standpoint the flavour and quality of aquaculture products can be categorised into three main topics of concern: (1) off-flavour tainting, (2) lack of flavour complexity and (3) quality deterioration during the post-harvest storage period.

Off-flavour tainting of farmed fish is extensively researched and some excellent reviews are available that detail the causes of, and management responses to, off-flavour tainting in a number of culture species (Tucker, 2000; Howgate, 2004). A growing body of scientific literature investigating the underlying factors affecting flavour complexity is also emerging as researchers explore the possibility of manipulating the flavour of cultured fish (Whitfield et al., 2002; Ma et al., 2005; Kim

et al., 2007; Fuller et al., 2008). Methods that limit deterioration in product quality during the post-harvest period are also extensively studied for a number of important aquaculture species with concepts developed in other meat industries showing good potential in farmed fish (Frigg et al., 1990; Baker & Davies, 1996; Harare et al., 1998; Ruff et al., 2003; Chen et al., 2008).

Typically, aspects of product quality are investigated in isolation from each other. However it may be advantageous to take a holistic approach to product quality to ensure that the most significant aspects of quality are not overlooked, resulting in sub-optimum quality outcomes. Scientific research designed to benefit the Australian barramundi aquaculture industry must strategically investigate all of the most significant factors impacting on flavour and quality. To fully address the flavour and quality of aquaculture products, an integrated approach is required that explores off-flavour tainting, lack of flavour complexity and quality preservation during the post-harvest period (Fig. 1.1).

The following sections of this chapter will provide a review of previously reported studies that have investigated the various aspects of flavour and quality in farmed fish. This review provides the scientific foundation for the research studies reported in the papers that comprise this thesis (chapters 2-6), which are all concerned with improving the flavour and quality of cultured barramundi in the Australian industry.

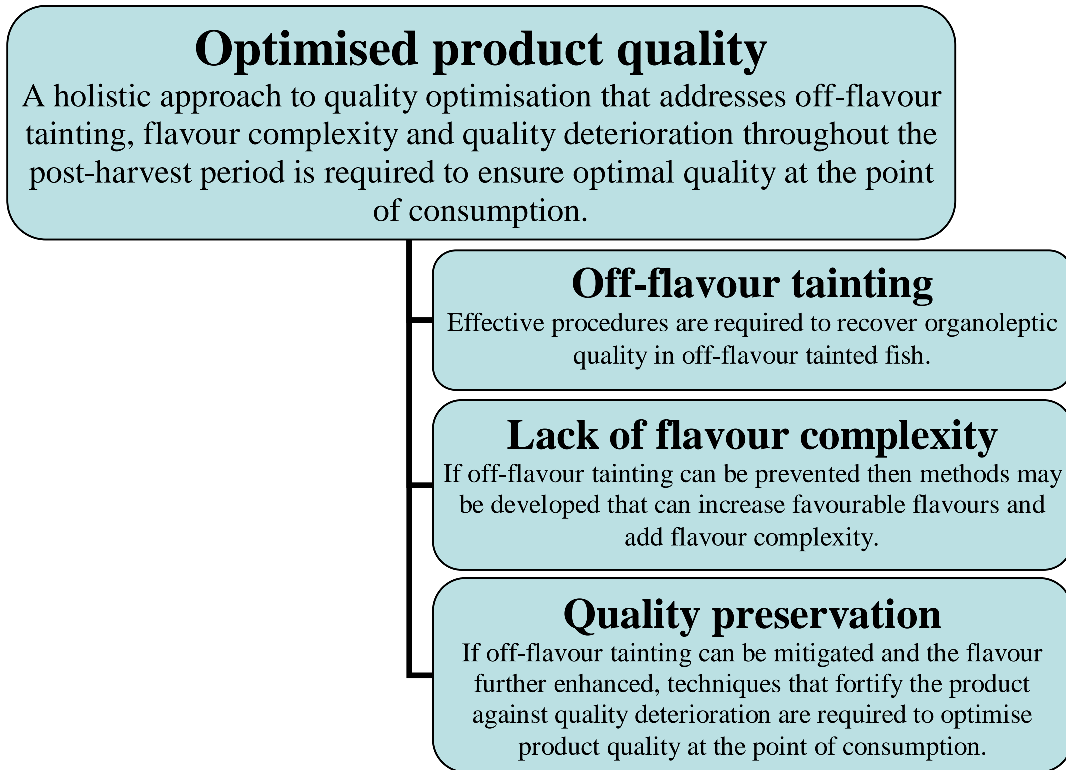

Fig. 1.1 An integrated approach to research and industry practice designed to optimise flavour and quality of farmed fish.

1.3.1. Off-flavour Tainting

Off-flavour tainting refers to any flavours or odours that occur in the end product that are considered objectionable by consumers (Tucker, 2000). Those observed most frequently in cultured fish are muddy, earthy or weedy flavours and aromas that are often noted in water bodies and in freshwater fish (Tucker, 2000). Muddy-earthy type off-flavour tainting is reported globally and is widely documented in numerous wild-capture (e.g., Farmer et al., 1995) and cultured species across a range of geographic locations. For example, off-flavour tainting of cultured fish has been reported in carp grown in France (Vallod, et. al. 2007) and China (Zhang, et. al., 2000); rainbow trout cultivated in Canada (Yurkowski & Tabachek, 1974) and the United Kingdom (Robertson, et. al., 2005); channel catfish, large-mouth bass, white sturgeon, and Atlantic salmon in the United States (Lovell, 1983; Schrader, 2005; Davidson et al., 2014); bream in Finland (Persson, 1978); barramundi in Australia (Percival, et. al. 2008); shrimp in Ecuador (Lovell & Broce, 1985) and tilapia in Israel (Guttman & Rijn, 2008).

Off-flavour tainting events are typically associated with the presence of one or both of two critically important flavour impairing chemicals, geosmin (GSM) and 2-methylisoborneol (MIB) (Tucker, 2000; Howgate, 2004). Numerous studies have confirmed the causative effects of these compounds on the flavour quality of fish (Yurkowski & Tabachek, 1974; Persson, 1978; Lovell, 1983; Lovell & Broce, 1985; Zhang, et. al., 2000; Robertson, et. al., 2005; Schrader, 2005; Vallod, et. al. 2007; Guttman & Rijn, 2008; Percival, et. al. 2008).

Muddy-earthy off-flavour tainting of freshwater reservoirs was first reported in 1891 (Berthelot & Andre, 1891 cited in Gerber, 1965) and in fish as early as 1936 (Thayson, 1936). However it was not until the 1960s and the development of gas

chromatography – mass spectrometry (GC-MS) techniques that GSM and MIB were first described and observed to possess muddy-earthy-musty aromas. At this time, several species of soil bacteria (actinomycetes) were observed to produce GSM and MIB and these compounds were subsequently implicated in off-flavour events in water reservoirs and harvested fish. Since these first observations, many species of cyanobacteria have also been identified as producers of GSM and MIB (e.g. Izaguirre & Taylor, 2004). GSM and MIB, arising predominantly from actinomycetes and cyanobacteria, are now generally acknowledged as the predominant causatives for muddy-earthy off-flavour tainting of freshwater bodies and fishes (Persson, 1979; Van der Ploeg et al., 1992; Persson, 1996).

Muddy-earthy type off-flavour events are not reported from marine waters or fishes. Marine species of bacteria have not been observed to produce significant quantities of GSM or MIB (Tucker, 2000). However, Lovell & Broce (1985) observed that when salinity was reduced to very low levels, off-flavour tainting occurred in ponds that were normally saline and free from off-flavours. This was presumably due to the occurrence of freshwater microbes which were able to establish in the low-salinity water or due to surface water runoff entering the ponds that contained GSM or MIB.

GSM and MIB occur naturally within the environment and often reach perceptible levels in water courses and wild fish (Persson 1980, Tucker, 2000). Both compounds are produced by microbes and are usually found at their highest levels where conditions are most favourable for microbial growth (Paerl & Tucker, 1995). High nutrient loading and high temperatures provide an ideal environment for microbial growth. This has significant implications for aquaculture farms, especially those located in warm climates, where high nutrient loading results from the supply of nutrient-dense aquaculture diets. While much of the diet is assimilated by the fish as

tissue growth, significant nutrient loading occurs in water as dissolved nutrients are excreted into the water as faeces and waste metabolites. Natural temperature fluctuations and surface water run-off can also cause seasonal taint issues to arise (Paerl & Tucker, 1995).

When fish are exposed to a chemical contaminant in the water, such as GSM or MIB, it passively passes into the fish (Streit, 1998) and accumulates in the tissues. Uptake can occur through the gills, skin and gut with the relative importance of these pathways being determined by the compound's octanol/water partition coefficient. Movement of the chemical into/out of the fish is reversible and when fish are exposed to a chemical it will pass into the fish until fluxes into and out of the fish become balanced and there is no net flow of the chemical in either direction (Howgate, 2004). At this point, the fugacities of the chemical in the water and the fish, as a whole, are equal. However, fish tissues are comprised of solid, water and lipid phases and chemicals are differentially deposited into these phases. It can be assumed that the chemical will not be deposited into the solid phase while deposition into the lipid and water phases is dependent on the relative solubility of the compound in water and lipid.

The uptake of GSM has been demonstrated to be overwhelmingly through the gills (From & Hørlyck, 1984) and this is also assumed to be the case for MIB (Howgate 2004). After uptake, GSM and MIB will partition into the water and lipid phases of fish tissues with the concentration in the water phase being equal to exposure water. However as these chemicals are far more soluble in lipid (lipophilic), they become concentrated in the lipid phase of tissues above the concentration of exposure water. The concentration of a chemical in the lipid phase of tissues can be determined by its lipid/water partition coefficient which is a measure of the difference in solubility of

the compound in lipid and water. While the lipid/water partition coefficient of GSM and MIB have not been measured, it is adequately represented by octanol/water partition coefficients (K_{ow}), expressed as Log K_{ow}. These can be estimated by fragmental constant methods (Mannhold et al., 1998), revealing Log K_{ow} values of 3.57 for GSM and 3.31 for MIB (Howgate, 2004). Consequently, the concentration of GSM and MIB in fish tissue is a function of its octanol/water coefficient and the proportion of lipid in the tissue. Since octanol/water coefficients of GSM and MIB are relatively high, and if it assumed that muscle tissue is more than slightly fatty, the concentration in fish flesh following exposure to these chemicals should exceed that of exposure water. This relationship is described as bio-concentration.

Following exposure to GSM and MIB fish develop unpleasant muddy, musty, weedy and/or earthy flavours and aromas (Yurkowski & Tabachek, 1974; Persson, 1980; Tucker, 2000; Howgate, 2004; Percival et al., 2008). Experimental studies have revealed that uptake of these chemicals occurs rapidly (within minutes) following exposure (Lovell, 1979; Yamprayoon & Noomhorm, 2000; Howgate, 2004; Robertson 2005). However, when fish are returned to clean water that is devoid of off-flavour contaminants, GSM and MIB are depurated from the tissues. Depuration occurs more slowly than uptake and, after exposure to GSM and MIB, off-flavour tainting can persist for several weeks (Lovell, 1979; Tucker, 2000; Yamprayoon & Noomhorm, 2000; Howgate, 2004; Robertson et al., 2005).

In order to minimise or prevent off flavour tainting in aquaculture animals, GSM and MIB must either be prevented from occurring in the culture water or be removed from the fish prior to harvest. Both approaches have been attempted and studied.

Management strategies to limit the occurrence of off flavour compounds in aquaculture ponds have had mixed levels of success and have been generally

disappointing. This is primarily due to the complex nature of aquatic systems and the persistent nature of the off-flavour tainting bacteria (Tucker, 2000).

The use of herbicides to control blooms of cyanobacteria has been attempted to control off-flavours. Some success has been had using this technique where small amounts of herbicides are added frequently during known problem periods (Schrader, 1998). However when used on existing cultures of cyanobacteria, the use of herbicides can increase the level of off-flavour tainting in farmed fish due to an increase in the level of off-flavour compounds that are available for uptake. For example, as much as 95-99% of GSM in cyanobacterial cultures is contained intracellularly (Li et al., 2012). Cell death and lysis occurring from the application of herbicide can release intracellular off-flavour compounds and lead to a dramatic increase in uptake by fish (Peterson, et al., 1995).

As well as having the potential to increase off-flavour tainting, the application of herbicides to culture water has a number of other potential drawbacks. The use of potentially harmful chemicals, relating to human health or environment effects, has the potential to provoke a negative consumer reaction. Synthetic herbicides also have considerable impacts on pond dynamics. For example, many herbicides are non-specific and affect all phototrophs in the water body. This can lead to increased levels of dissolved nutrients as fish waste products are not synthesised by the phytoplankton (Paerl & Tucker, 1995). The technique also relies on being able to predict when problem periods may occur in order to prevent problem species from becoming established. In many instances this is impossible as off-flavour tainting may occur only sporadically and be unpredictable.

Many species of cyanobacteria are known to be able to fix nitrogen from the water. The relative abundance of phosphorous (P) to nitrogen (N) influences the growth of

nitrogen fixing species (Stewart, 1967). When available N is limiting, N-fixing species dominate pond waters. However the relative abundance of N:P can be manipulated, with the intention of preventing the proliferation of off-flavour tainting cyanobacteria. This may be achieved by selecting aquaculture diets with a high N:P ratio (Sarker et al., 2014) or by reducing dissolved P in the water through the addition of aluminium or calcium which bind with available P (Cooke & Kenedy, 1981). Dissolved N can also be added directly to the water to stimulate growth of non-nitrogen fixing species of phytoplankton. While these methods can change the ratios of dissolved nutrients in pond water, not all species of off-flavour tainting cyanobacteria are able to fix nitrogen and such processes may result in these species becoming dominant (Barica et al., 1980; Tucker, 2000).

Stocking ponds with planktivorous fish has also been attempted in order to graze out off-flavour tainting cyanobacteria. Many cyanobacteria form large, filamentous colonies and are usually the first to be grazed by planktivores (Smith, 1989). This technique has been successful under certain conditions (Torrans & Lowell, 1987). However, results have varied with Tucker (2006) showing no response in algal bloom or off-flavour occurrence to grazing by silver carp.

Controlling the abundance of actinomycetes in aquaculture ponds is difficult. Actinomycetes have a characteristically complex life-cycle and are able to persist in sediments for an extended period of time (Cross, 1981). Furthermore, actinomyctes are abundant soil microbes and GSM and MIB produced in mud and soil adjacent to pond waters can enter ponds and cause off-flavour tainting (Tucker, 2000). Actinomycetes thrive in nutrient-rich environments and limiting nutrient availability is likely to have an impact on the overall abundance of these organisms. However, methods of controlling the abundance of actinomycetes are largely unexplored.

Since it has proven very difficult to reliably prevent GSM and MIB from occurring in aquaculture ponds, aquaculture producers often seek to identify and exclude off-flavour tainted cohorts of fish prior to harvest (Johnsen & Kelly, 1990; Van der Ploeg, 1991; Tucker, 1999). This can be an effective means of preventing the harvest and sale of off-flavour tainted fish although the method has some limitations.

To accurately identify and exclude off-flavour tainted cohorts of fish from harvest requires controlled and standardised methods of organoleptic assessment. Off-flavour tainting can exhibit distinctive patterns of spatial variability within fish fillets (Percival et al, 2008; Grimm et al., 2015; Zimba & Grimm, 2015). Percival et al. (2008) noted that off-flavour tainting was more intense in the belly region of farmed barramundi fillets. Grimm et al. (2015) found that GSM and MIB concentrations were highest in skin sections of channel catfish fillets, while Zimba & Grimm (2015) observed that off-flavour compounds were most elevated in the section of the channel catfish fillets nearest the head, with concentrations declining successively in the mid-section and the tail-end of the fillet. The spatial distribution of off-flavour taint is poorly understood for most culture species but must be further explored and taken into consideration if organoleptic assessment is to be used to identify and exclude off-flavour tainted fish prior to harvest.

As it is often difficult to prevent the occurrence of off-flavour taint, aquaculture researchers have explored the alternative strategy of depurating off-flavour tainting from fish prior to harvest. It has been shown that off-flavour tainting can be reliably eliminated from rainbow trout, channel catfish and tilapia by depurating fish in clean water, devoid of off-flavour compounds, for a sufficient period of time prior to harvest (Thayson, 1936; Yamprayoon & Noomhorm, 2000; Percival et al., 2004; Robertson et al., 2005). This technique also appears to be promising for barramundi

with Percival et al. (2004) observing a significantly less noticeable muddy odour in depurated fish compared to lake-farmed fish. As the concentration of GSM and MIB in fish flesh is reduced, the intensity of off-flavour tainting is also reduced. If sufficient time is allowed to elapse, the concentration of off-flavour compounds will fall below the threshold of human sensory detection. The threshold of human sensory detection of GSM and MIB are somewhat divergent between species but are usually in the range of 0.5 to 0.9 $\mu g\ kg^{-1}$ (Persson 1980; Grimm et al 2004; Robertson et al. 2006,).

1.3.2. Lack of flavour complexity

In the absence of off-flavour tainting, farmed fish generally possess favourable organoleptic attributes. However, some reports highlight an obvious difference between the flavour of cultivated and wild-caught seafood (Whitfield et al., 1997; Grigorakis et al., 2003; Ma et al., 2005; Grigorakis, 2007; Frank et al., 2009; Carton & Jones, 2013). The organoleptic quality of some aquaculture products has been reported as being less complex and lacking in ocean or 'sea-fresh' characteristics and is perceived as bland when compared to wild-caught marine products (Whitfield et al., 1997; Ma et al., 2005).

The organoleptic quality of wild caught seafood and aquaculture products is profoundly influenced by dietary factors during the pre-harvest period (Ackman et al., 1972; Levasseur et al., 1994; Whitfield et al., 2002; Ma et al., 2005). The diet of wild-caught seafood can be diverse, comprising a wide range of marine algae, vertebrates and invertebrates which often possess high concentrations of critical flavour compounds. Often this contributes to the complex and distinctive flavour of seafood products (Boyle et al., 1992; Whitfield et al., 1997; Whitfield et al., 1998). However, aquaculture systems typically utilise manufactured feeds which may lack the variety

or abundance of natural flavour compounds found in ecological diets. Accordingly, aquaculture products may be considered bland or lacking in organoleptic complexity.

A wide array of compounds is implicated in the flavour quality of fish and seafood (Kawai et al., 1996; Mansur et al., 2003; Frank et al., 2009). The aroma volatiles present in edible tissues vary widely between fish species, contributing to variations in species-specific flavour profiles. These species-specific variations also show trends with respect to the environment from which fish are collected. For example Kawai et al. (1996) present a detailed review of the volatile aroma compounds of fish from freshwater, estuarine and marine environments. This review characterises freshwater fish as having high concentrations of pyrrolidine and muddy-earthy off-flavour chemicals derived from the environment. Estuarine fish were characterised as having high concentrations of unsaturated carbonyls and alcohols derived from the breakdown of polyunsaturated fatty acids. Marine fish were presumed to be nearly odourless due to a low abundance of aroma volatiles. However, Whitfield et al. (1997; 1998) and Boyle et al. (1992) have shown that the concentration of critical aroma compounds in marine fishes can vary widely and are strongly influenced by diet. Whitfield (1998) observed that benthic carnivores, diverse omnivores and restricted omnivores contained a greater abundance of critical flavour compounds compared to pelagic, carnivorous fish, presumably due to dietary variations.

Sea fish are generally characterised as possessing sweet, melony and plant-like aromas, although these may be accompanied by distinctive iodine and sea-like aromas (Maarse, 1991; Lindsay, 1994). These aromas are often attributed to the presence of bromophenol compounds (Boyle et al., 1992; Whitfield et al., 1997; 1998). Dimethylsulphide is also known to have a potent aroma, and has been attributed with

giving the sea its distinctive smell (White, 1982 cited in Van Alstyne et al., 2009; Wolfe, 2014).

Bromophenols (BPs) are common secondary metabolites found in marine algae (Liu et al., 2011) and are known to impart the desirable marine, sea salt and iodine-like flavours that characterise marine seafoods (Boyle et al. 1992, 1993a, b, Lindsay 1994). Although the ecological function of BPs in marine algae is not yet clear, some forms are thought to play a role in chemical defence and deterrence of herbivores (Woodin et al. 1987; Kicklighter et al., 2004).

At high concentrations, BPs can impart potent medicinal/iodine flavours (Boyle, et al., 1993; Whitfield et al., 1998) rendering fish unpalatable. However, at lower concentrations these compounds have been attributed responsibility for producing a distinctive sea aroma (Boyle, et al., 1993) and are associated with positive flavour attributes in seafood (Boyle et al., 1992; Whitfield et al., 1997; Ma et al., 2005). In particular, BPs are often attributed with adding complexity to the flavour of seafood with shellfish flavours reported (Boyle et al., 1993; Whitfield et al., 1997; Ma et al., 2005). In contrast, freshwater fish are devoid of bromophenols (Lindsay 1994), most likely as a result of freshwater environments lacking bromine and the flora/fauna capable of producing BPs through bromination (Fenical, 1982).

Dimethylsulfide (DMS) is one of the most abundant volatile compounds in the marine environment and is produced by algae as a result of the enzyme regulated dimethylsulphoniopropionate (DMSP) cleavage reaction which also yields acrylate and acrylic acid (Van Alstyne, 2008). DMS in marine alga has been hypothesised to function as an antioxidant (Ross & Van Alstyne, 2007; Sunda, et al., 2008) as well as acting as a herbivore feeding deterrent (Wolfe, et al., 1997; Van Alstyne et al., 2001b; Van Alstyne & Houser, 2003).

Like BPs, DMS is often reported in off-flavour events in wild-caught seafood (e.g. Ackman et al., 1972; Levaseaur et al., 1994). At excessive concentrations, DMS imparts repulsive sulphur, chemical and/or rotting shellfish aroma into fish (Brooke et al., 1968; Ackman et al., 1972; Levaseaur et al., 1994). However DMS is also attributed with giving the sea its pleasant and distinctive aroma (White, 1982 cited in Van Alstyne et al., 2009). DMS is also associated with desirable shellfish flavours (Brooke et al., 1968; Haard, 2002) and is attributed as being the predominant aroma compound in fresh soft-shell clams (Brooke et al., 1968). Low concentrations of DMS are also a recognised contributor to the desirable flavour characteristics of shellfish (Ackman & Hingley 1968; Iida & Tokunaga, 1986; Hill, et al., 2000).

Diet is generally understood to be a source of critical flavour compounds in marine fishes. Whitfield et al. (1998) observed elevated concentrations of BP in the gut of fish and suggested diet to be the most likely source of BPs in fish flesh. Other studies have also drawn the link between diet and the BP content of fish with Boyle et al. (1992) observing elevated bromophenol levels in salt water salmon while bromophenols were virtually absent in freshwater specimens. This was hypothesised to be a result of diet with analysis of potential marine prey species revealing notable concentrations of bromophenols in all salt water samples assessed, while only sporadic, low concentrations were observed in freshwater fish.

There also appears to be great potential to actively manipulate the BP content of fish flesh. Kim et al. (2007) successfully increased the BP content of cultured green grouper by enriching diets with a considerable fraction of marine algae and Ma et al. (2005) increased the intensity of seafood-like flavour in cultured silver sea bream, again by enriching diets with marine algae rich in bromophenols.

Flavour enhancement through dietary enrichment with DMS and DMSP remains unexplored. However, in an investigation into the source of off-flavour in Atlantic cod, Ackman et al. (1966) observed that DMSP levels in cod tissue could be manipulated by incorporating DMSP into the diet. Other studies have identified diet to be a critical factor in determining the DMS/DMSP concentration of fish flesh and the flavour of such fish (Sipos & Ackman, 1964; Ackman et al., 1972; Levasseur et al., 1994).

BPs and DMS appear to have many similar characteristics. Both are highly odorous, natural compounds produced by marine algae. They are readily passed up the food web and affect the flavour of fishes. Both also possess powerful and repulsive odours at high concentrations which become favourable within a certain concentration range.

Many commonly collected and cultivated marine algae are known to be rich in critical flavour compounds. *Ulva* is a genus of coastal marine green algae distributed throughout temperate and tropical regions (Kirkendale et al., 2013, Lawton et al., 2013). Species within this genus are known to synthesise a large number of olfactory compounds including BPs, DMSP and DMS (Sugisawa et al., 1990; Flodin et al., 1999; Whitfield et al., 1999 b; Jago et al., 2014). *Ulva ohnoi* is a species of the genus *Ulva* which is successfully cultivated in aquaculture systems (Mata et al., 2015) and is considered an ideal species to target bioremediation in land based aquaculture farms (Lawton et al., 2013). *U. ohnoi* is also used as a bioremediation tool in abalone waste water tanks with the produced biomass being used to feed the cultured animals (Bolton et al., 2009). Diets incorporating a relatively high inclusion level of *Ulva* are readily consumed by finfish (Pereira et al., 2012; Marinho et al., 2013; Wassef et al., 2013; Jago et al., 2014) and may have the potential to manipulate organoleptic properties of cultured species.

1.3.3. Post-harvest quality deterioration

Although off-flavour tainting of farmed fish may be prevented prior to harvest and the flavour of cultured animals further enhanced by dietary manipulation, quality can be gradually lost during the post-harvest transport and storage period. Quality often deteriorates during post-harvest storage as a result of oxidative, enzymatic and/or bacterial processes. These processes degrade flavour, taste and aroma qualities and serve to promote changes to texture, colour, pH and nutritional status, which can ultimately render fish products unmarketable and unfit for consumption (Gram, 1992; Gram & Huss, 1996; Olafsdóttir et al., 1997; Bonilla et al., 2007). However, it is often possible to limit or prevent quality deterioration during the post-harvest period thereby optimising product quality at the point of consumption.

The high quality of aquaculture products can be maintained for prolonged periods if post-harvest handling and storage conditions are optimal. For example early and rapid chilling of fish is well known to extend the shelf-life of the post-harvest product (Fletcher & Hodgson, 1988; Olafsdóttir et al., 2004; Bao et al., 2007; Zakhariya et al., 2015). This slows the rate of undesirable biochemical and chemical reactions in fish flesh and impedes the growth and spoilage activity of microorganisms (Sikorski & Sun Pan, 1994).

Product quality is also affected by a range of important processes occurring at the time of harvest. Excessive physical activity and stress of fish prior to harvest is known to decrease muscle pH in the post-harvest product which results in a rapid onset of rigor mortis and may also increase muscle gaping, blood spotting and flesh texture alterations while reducing water holding capacity of the muscle (Jerrett et al., 1996; Robb & Kestin, 2002; Wilkinson et al., 2008). Furthermore, bleeding fish prior to slaughter can extend shelf life by reducing the abundance of haem proteins, iron and

white blood cells in flesh. This can reduce the development of lipid oxidation and off-odours during post-harvest storage (Maqsood & Benjakul 2011).

Pre-harvest factors can also have a considerable bearing on the shelf-life of aquaculture products. For example, enriching the diet of cultured fish with powerful antioxidants can limit the rate of lipid oxidation occurring during the post-harvest period (Frigg et al., 1990; Baker & Davies, 1996; Harare et al., 1998; Ruff et al., 2003; Chen et al., 2008). The oxidation of lipid in fish and meat products during the post-harvest period can result in a loss of product quality through the development of off-flavours, potent odorants, and a loss of colour and texture (Ladikos & Lougovois, 1990; Liu et al., 1995; Gray et al., 1996). This is especially important in cultured fish where the lipid content of the flesh can often be considerably higher than in wild counterparts.

Lipid oxidation is a major cause of quality deterioration in meat products (Ladikos & Lougovois, 1990; Kanner, 1994; Liu et al., 1995; Gray et al., 1996). Fish flesh is acutely prone to lipid oxidation due to the abundance of highly unstable polyunsaturated fatty acids (PUFAs) (Hultin, 1994; Undeland, 2001) which limits the storage life of fish products. For post-harvest lipid oxidation to progress, two substrates are required, molecular oxygen and lipid. As fatty acids in the flesh react with oxygen, hydroperoxide is formed; this decomposes to produce aldehydes, ketones and alcohols (Hultin, 1992; Undeland 2001). These volatile secondary products lead to the development of unpleasant aromas and flavours, and can affect the colour, texture and nutritional quality of flesh (Carton & Jones, 2013).

The rate of lipid oxidation in tissue is affected by the balance between pro-oxidants, which promote oxidation, and anti-oxidants, which restrain oxidation (Hultin, 1992; Undeland, 2001). The most well understood pro-oxidants are haem proteins such as

haemoglobin and myoglobin which are potent catalysts during the initiation and propagation steps of lipid oxidation (Hultin, 1992; Kanner, 1994; Undeland 2001). These pigments are abundant in blood and have catalytic properties, a result of their ability to break down hydroperoxide to volatile secondary products (Ladikos & Lougovois 1990). Cultured fish are generally not bled during routine harvest procedures. As a consequence, haemoglobin is not removed and is considered to be a major contributor to lipid oxidation in fish products (Richards & Hultin, 2002). As well as containing haemoglobin, fish blood contains white blood cells which can also generate superoxide, hydrogen peroxide and hydroxyl radical (Gabig & Babior, 1981), and lipoxygenase products (Pettitt et al., 1989) which can promote lipid oxidation (Maqsood & Benjakul, 2011). Thus, bleeding cultured fish at the point of harvest is considered to be a potential means of constraining lipid oxidation during post-harvest storage.

As well as constraining lipid oxidation, bleeding fish has been shown to be an effective means of preventing the development of off-odours in farmed fish (Maqsood & Benjakul, 2011). However the bleeding of live fish is often impractical during routine harvest procedures and has the potential to raise concerns over the humane treatment of animals, unless suitable techniques can be developed to overcome such concerns.

Lipid oxidation can also be constrained by enriching edible tissues with antioxidants (Frigg et al., 1990; Baker & Davies, 1996; Harare et al., 1998; Ruff et al., 2003; Chen et al., 2008). Antioxidants such as ubiquinol (Petillo et al. 1998) and the carotenoid pigments important in flesh colouration in salmonids (Christophersen et al. 1992) have the potential to constrain lipid oxidation. However the most widely known anti-oxidant in fish is α-tocopherol which is found in the lipid interior of membranes

(Hultin, 1994; Undeland, 2001). This compound has the highest biological activity of the vitamin E homologues and is also retained in fish tissues to a greater extent than other tocopherols in fish (Harare & Lie, 1997).

Enriching tissues with anti-oxidants such as α-tocopherol acetate, added to the diet at a supra-nutritional level, has been shown to slow the rate of lipid oxidation in various meat products (Liu et al., 1995), including fish (Frigg et al., 1990; Baker & Davies, 1996; Harare et al., 1998; Ruff et al., 2003; Chen et al., 2008). The anti-oxidant α-tocopherol acetate is effective in scavenging free radicals in both the initiation and propagation steps of autoxidation (St. Angelo, 1996) thereby restraining lipid oxidation and post-harvest deteriorations in quality. This has been shown to improve the quality and storage life of the product (Frigg et al., 1990).

Enriching fish flesh with α-tocopherol by dietary manipulation has distinct advantages over alternative anti-lipid oxidation strategies. Most significantly, the storage life of the product is improved through the application of a diet, which is very similar in composition to existing diets. As a consequence, it does not require additional infrastructure or significant changes to management or harvesting procedures which may additionally be cost-prohibitive.

Slowing the rate of lipid oxidation can also be achieved by limiting exposure to or the complete exclusion of molecular oxygen. This is often accomplished through vacuum or modified atmosphere packaging (Khayat & Schwall, 1983; Siah & Ariff, 2002). However any post-harvest processing that results in the disruption of tissues, such as filleting and skinning has the potential to accelerate lipid oxidation. To this extent filleted fish have been shown to have low lipid oxidation stability (Hutlin, 1994). Maintaining the fish whole and/or the skin intact following harvest has been

suggested to limit the availability of molecular oxygen, and has been demonstrated to reduce the rate of lipid oxidation in herring fillets (Undeland et al., 1998).

1.4 Product quality in barramundi aquaculture

In Australia and throughout South East Asia, *Lates calcarifer*, known locally as barramundi or Asian sea bass is an economically important aquaculture species. The species is widely harvested by commercial fisheries throughout its entire geographical distribution and it is also an important aquaculture species in tropical regions with a total aquaculture production of ~65,000t per annum (FAO, 2012b). Australia is currently a relatively small producer, with a total production of approximately ~4000t per annum (Savage & Hobsbawn, 2015). While the quality of barramundi is generally well accepted, some consumers have commented on an obvious difference between aquaculture and wild-caught products (Frank et al., 2009; Carton & Jones, 2013).

A number of specific quality issues have been identified in Australian farmed barramundi and are thought to impact heavily on consumer perception. The President of the Australian Barramundi Farmers Association has stated that the perception of inferior quality is the most significant factor affecting the future growth and viability of the Australian barramundi aquaculture industry (Phillips, 2010). Off-flavour tainting, reported as a potent muddy/earthy flavour and aroma, is periodically observed in farmed barramundi (Percival et al. 2008; Carton & Jones 2013; Exley 2014). This off-flavour tainting can render affected fish unpalatable or unfit for sale. It has also been observed that some farmed barramundi may lack the flavour complexity of wild-caught barramundi, lacking distinctive sea-like characteristics (Fuller et al., 2008; Frank et al, 2009; Carton & Jones, 2013). Fillet colour is also a quality concern in farmed barramundi with these fish having a distinctively different appearance to wild-caught specimens (Howieson et al., 2013b). The quality of fish products is also

known to deteriorate during the post-harvest storage period, prior to human consumption (Undeland, et al., 1998). Despite this, the storage stability of Australian farmed barramundi is unknown and pre-harvest practices designed to optimise shelf-life are unexamined.

Although product quality is acknowledged as a serious concern in the Australian barramundi aquaculture industry, as farm gate prices have declined (Brown et al., 1997; Savage & Hobsbawn, 2015), many aspects remain unexplored. Targeted scientific research is required to better understand the factors affecting product quality and to develop practices that can optimise the quality of farmed barramundi.

The declining farm gate prices achieved for farmed barramundi are thought to have occurred as a result of two main factors. Firstly, competition in the market place has increased due to higher volumes of local produce and a significant increase in the volume of imported barramundi and other white fish (Phillips, 2010). Secondly, inconsistencies in product quality have also reduced farm gate prices by precipitating a perception that farmed barramundi is inferior to other fish products (Howieson et al., 2013a). As a result of these factors, farm gate prices have gradually declined over the past 20 years falling from ~$11.00 kg^{-1} in 1994/95 to ~$9 kg^{-1} in 2014/15 (Brown et al., 1997; Savage & Hobsbawn, 2015). Specifically, quality concerns have arisen in the three key areas discussed above: (1) off-flavour tainting, (2) lack of flavour complexity and (3) quality deterioration during the post-harvest storage period.

In tropical northern Australia episodes of muddy-earthy tainting of freshwater outdoor pond reared barramundi (*Lates calcarifer*) are frequently reported (Exley, 2014; Phillips, 2010). This issue, has been highlighted among the primary causes of an escalation in negative consumer perceptions of Australian cultured barramundi and a growing resistance to future purchases (Howieson et al. 2013a; Exley, 2014).

Similar issues have previously been reported in barramundi cultured in floating cages in Lake Argyle, Australia's largest (~1,000km^2) man-made lake (Percival et al. 2008). In this instance, MIB was identified as the primary compound responsible for off-flavour tainting. However, off-flavour tainting of barramundi cultured in outdoor intensive freshwater systems has not been addressed. This lack of information is constraining efforts to understand the mechanisms responsible for off-flavour tainting and the development of practices aimed at recovering flavour quality and consumer confidence.

Australian farmed barramundi has also been observed to lack the marine-like flavour notes that characterise wild caught marine or estuarine barramundi (Frank, 2009; Carton & Jones, 2013). Frank et al. (2009) observed that wild-caught, marine barramundi had a stronger prawn-like flavour compared to farmed barramundi and identified a wide range of organoleptically important compounds in flesh samples. Fuller et al. (2008) attempted to increase the BP content of farmed barramundi by enhancing the diet with critical flavour compounds. This was achieved by spiking the diet with synthetic BPs. However, the organoleptic effects were not reported (pers com., S. Fuller). The flavour of captive barramundi is also known to be affected by diet with fish that were fed baitfish diets having a greater intensity of fishy flavour compared to fish fed compound extruded diets (Glencross et al., 2008).

As feed manufacturers seek to replace marine-sourced ingredients (e.g., fish meal and fish oil) with more sustainable terrestrial alternatives, flavour and taste are likely to become more prevalent issues in aquaculture. This is especially important for cultured barramundi as artificial diets for this species comprising a very low fraction of marine sourced ingredients are currently being produced by commercial feed manufacturers (Glencross, 2015).

Although off-flavour tainting and a lack of flavour complexity are significant concerns for the Australian barramundi farming industry, it is also critical that quality is maintained during the post-harvest handling and storage period. The storage life of Australian farmed barramundi is unknown as no data currently exist as to the extent of quality deterioration during the post-harvest period. This is a significant issue as production typically occurs in sparsely populated regions in the tropical north of Australia, a considerable distance from major metropolitan markets. This geographical isolation results in protracted transportation and storage periods, with product often reaching consumers as many as nine days after harvest (Carton & Jones, 2013). Additionally, during the transportation period as many as 4-5 transfers through the supply chain can occur, during which time fish can be exposed to sub-optimal storage conditions.

Barramundi grown in Australia is sold chilled and unfrozen, either as a whole ungutted fish or as skinned fillets (Harrison et al., 2013). Due to considerable geographic isolation and storage concerns during transit, the Australian barramundi aquaculture industry has developed harvest methods assumed to limit lipid oxidation and quality degradation. Farmed barramundi are chilled rapidly at the point of harvest by ice emersion and thermal conditions during handling and packing are controlled to limit product spoilage (Carton & Jones, 2013). However, there is a paucity of information relating to quality changes following packing, during transport and the extended storage period. There is a need to understand and thereby improve the susceptibility of barramundi products to lipid oxidation and changes in quality over the post-harvest storage and transportation period.

Post-harvest quality of farmed barramundi has been explored with respect to harvest and processing methods. Investigations into the effects of different harvest methods

found that significant improvements could be achieved by minimising stress and physical activity at the point of harvest (Wilkinson et al., 2008; Howieson et al., 2013b). Howieson et al. (2013b) also investigated the cause of grey colouration in the fillets of farmed barramundi but was unable to determine causes or preventative strategies to mitigate fillet greying. The effects of bleeding on quality deterioration during the post-harvest period have also been investigated and it was observed that lipid oxidation, microbial growth and the development of unfavourable odours could be constrained by this practice (Maqsood & Benjakul, 2011). Several studies have also assessed post-harvest quality changes in barramundi with respect to modified atmosphere packaging, storage temperature, freezing and processing (Siah & Ariff, 2002; 2003; Bakar et al., 2010; Maqsood & Benjakul, 2011; Zakhariya et al., 2014; Truong et al., 2015; Zakhariya et al., 2015).

Both pre- and post-harvest management practices have potential to impact on the quality of farmed barramundi. Although a number of studies have investigated the potential to optimise the shelf life of barramundi through post-harvest processing and storage practices, pre-harvest management practices that prevent lipid oxidation and maintain high quality are currently unexplored.

1.5 Summary of research pertinent to quality issues in farmed barramundi

In general, a significant body of evidence pertinent to quality issues in farmed fish has been developed. It is well understood that quality is largely affected by three critical aspects; off-flavour tainting, a lack of flavour complexity and quality deterioration during the post-harvest storage period. Much of this research has been undertaken on a largely *ad hoc* basis, in isolation from related studies on product quality and across a broad range of aquaculture species. However, the accumulated

data provides a sound basis to guide further research designed to optimise the quality of farmed barramundi.

To build on existing research findings and to improve industry practices, it is necessary to identify research topics that appear to have the potential to significantly improve flavour and quality in farmed barramundi. In the planning stage of this thesis, three strategically important research areas pertinent to product quality were identified: off-flavour tainting, a lack of flavour complexity and quality deterioration during the post-harvest storage period.

Firstly, as already discussed, a significant body of research has investigated off-flavour tainting across a range of culture species (Tucker, 2000). In particular, a large number of studies have investigated various aspects of off-flavour tainting in cold and temperate water species such as channel catfish and rainbow trout (e.g. Lovell, 1983; Robertson et al., 2005). Despite this, the causatives for off-flavour tainting in tropical pond-reared barramundi remain unclear and management responses to prevent off-flavour tainting in farmed barramundi are largely unexplored. This was identified as the first research topic for this thesis.

Secondly, while it is generally understood that a lack of flavour complexity in farmed fish is likely to result from a deficiency of critical flavour compounds in the diet (Whitfield et al., 2002; Frank et al., 2009), relatively few studies have explored the flavour enhancement of farmed fish by dietary manipulation. It has, however, been demonstrated that there is great potential to manipulate the abundance of critical flavour compounds in the tissues of farmed fish by feeding diets that incorporate a high fraction of marine algae (Kim et al., 2007). It has also been demonstrated that this has the potential to alter the flavour characteristics of farmed fish (Ma et al., 2005). While previous studies have investigated the use of algal species rich in BPs

(Ma et al., 2005; Kim et al., 2007), using algae rich in DMS for flavour enhancement is unexplored. The potential to enhance the flavour of farmed barramundi through such dietary manipulation was identified as a second topic for thesis research.

Thirdly, it has been demonstrated in a number of cultured species that lipid oxidation and the associated reductions in product quality can be mitigated by enriching fish tissues with α-tocopherol and/or by preventing tissue disruption prior to storage (Fletcher & Hogdson, 1988; Frigg et al., 1990; Undeland et al., 1998; Huidobro et al., 2001; Ruff et al., 2003). However it is currently unknown if the benefits achieved for these species also apply for barramundi. Furthermore, it is unclear if the beneficial effects of dietary α-tocopherol enrichment are affected by post-harvest processing and storage methods. For example, it may be advantageous to enrich edible tissues with α-tocopherol while also preventing tissue disruption prior to storage. These questions provided the third research focus for the thesis.

A research program was designed to investigate each of these strategically important aspects of product quality in pond-reared barramundi. This holistic program of research investigated factors impacting on flavour and quality of cultivated barramundi during the pre and post-harvest stages of production.

1.6 The program of research

Chapters 2-6 of this thesis will present the findings of targeted research that investigated specific aspects of quality optimisation for the Australian barramundi aquaculture industry. In Chapters 2-6, three published papers (chapters 2, 5 and 6) and two papers submitted for publication to peer-reviewed, scientific journals (chapters 3 and 4) are reproduced in published or submitted form. The only changes made to these papers are: the numbering of figures and tables to correspond with chapter numbers; the addition of references to the other chapters of the thesis where required;

standardisation of reference style; and minor editorial changes. In addition, a brief preface to each chapter provides linkages amongst the chapters as well as the full reference or title of the submitted paper and the name of the scientific journal currently reviewing the paper. The full list of references contained in the published or submitted paper is provided at the end of each of these chapters, as these comprise part of the published/submitted paper. A full list of references including all citations in all chapters of the thesis, including chapters 1 and 7, is provided at the end of the thesis.

Chapter 2, *Occurrence of earthy-muddy tainting of cultured barramundi linked to geosmin in tropical northern Australia,* details an investigation of the underlying factors leading to off-flavour tainting in tropical, pond-reared barramundi. The objectives of this study were to: quantify levels of GSM and MIB in freshwater barramundi growout ponds; determine the relationship between levels of off-flavour tainting compounds in the flesh of market sized barramundi and levels in the culturing water; quantify the impact of off-flavour tainting compounds on sensory attributes of barramundi fillets; and resolve the relationship between levels of off-flavour compounds in the flesh and the sensory properties of cultured barramundi. This information is critical to understanding the mechanisms of off-flavour tainting in pond-reared barramundi. This information was previously unknown, even though most Australian farmed barramundi are produced in tropical pond-culture systems. This chapter has been published in *Aquaculture Environment Interactions*. The contributions of co-authors is shown in Table 1.1.

Table 1.1 Contribution of co-authors to Chapter 2. The full citation to this published paper is:
Jones, B., Fuller, S. & Carton, A. G. (2013). Earthy-muddy tainting of cultured barramundi linked to geosmin in tropical northern Australia. *Aquaculture Environment Interactions*, *3*, 117-124.

Contributor	Statement of contribution
Ben Jones (Candidate)	Conceptualised research study Designed and carried out experiments Analysed data Wrote the paper
Steve Fuller	Developed and undertook chemical analysis procedures
Guy Carton (Principal supervisor)	Provided mentoring and support Edited the paper

Research findings revealed that the concentration of off-flavour contaminants in culture water fluctuated widely. These fluctuations were impossible to predict, complicating the development of logical, well planned experimental designs to further investigate various aspects of off-flavour tainting. This was a significant barrier to the investigation of several critical factors affecting off-flavour tainting in farmed barramundi, including the rate and pattern of uptake and depuration of off-flavour tainting in barramundi, the potential to recover flavour quality in tainted fish and the spatial distribution of tainting compounds in barramundi fillets. In order to investigate such factors, a reliable method of generating off-flavour tainted fish 'on demand' was developed and is presented in Chapter 3.

Chapter 3, *Controlled off-flavour tainting of cultured fish using the geosmin-producing cyanobacterium, Anabaena circinalis* reports on the development of a low-cost, reliable method capable of generating GSM on-demand and imparting natural off-flavour taint into barramundi. The method reported is capable of controlling the concentration of GSM in holding tanks in order to manipulate the degree of GSM contamination in fish flesh and imparting varying intensities of off-flavour. This chapter has been submitted to *Aquaculture International* and is currently under

review. The contribution of each co-author to the submitted manuscript is presented in Table 1.2.

Table 1.2 Contribution of co-authors to the manuscript submitted to *Aquaculture International*: Controlled off-flavour tainting of cultured fish using the geosmin-producing cyanobacterium, *Anabaena circinalis.*

Contributor	Statement of contribution
Ben Jones (Candidate)	Conceptualised research study Designed and carried out experiments (70%) Analysed data (70%) Wrote the paper (70%)
Guy Carton (Principal supervisor)	Provided mentoring and support Edited the paper
Samuel Cirés	Provided mentoring and support Edited the paper
Lena Geitung	Carried out experiments (20%) Analysed data (20%) Wrote sections of the paper (30%)
Kirsten Heimann	Provided mentoring and support Edited the paper
Matt Jago	Carried out experiments (10%) Analysed data (10%)

Chapter 4, *Uptake, depuration and spatial distribution of the off-flavour tainting compound geosmin in farmed barramundi, Lates calcarifer* presents an investigation of practical management responses to off-flavour tainting in farmed barramundi. This research study draws on findings presented in Chapters 2 and 3 that revealed the underlying factors leading to off-flavour tainting in pond-reared barramundi and developed a highly effective method to reliably generate off-flavour taint. The potential to recover flavour quality was investigated by depurating fish in clean, untainted water. Patterns of uptake and depuration of off-flavour compounds in farmed barramundi were also investigated to assist in the development of depuration procedures. The spatial distribution of off-flavour compounds in barramundi fillets was also explored with respect to the various regions of the fillet and the abundance of crude fat. This chapter has been submitted to *Aquaculture* and is currently under

review. The relative contribution of the co-author in the submitted manuscript is presented in Table 1.3.

Table 1.3 Contribution of the co-author to the manuscript submitted to *Aquaculture*: Uptake, depuration and spatial distribution of the off-flavour tainting compound geosmin in farmed barramundi, *Lates calcarifer*.

Contributor	Statement of contribution
Ben Jones (Candidate)	Conceptualised research study Designed and carried out experiments Analysed data Wrote the paper
Guy Carton (Principal supervisor)	Provided mentoring and support Edited the paper

In the absence of off-flavour tainting, farmed barramundi may lack flavour complexity compared to wild-caught specimens and other marine seafood. In order to address this, a research project was designed to investigate the potential to enhance flavour quality by dietary manipulation.

Chapter 5, *Flavour enhancement of freshwater farmed barramundi, (Lates calcarifer), through dietary enrichment with cultivated sea lettuce, Ulva ohnoi* details an investigation into the use of *U. ohnoi*, in the diet, to enhance the organoleptic attributes of farmed fish. Initially, the palatability of *Ulva* in aquaculture diets was investigated to determine its suitability for use. The effects of this dietary ingredient on the aroma and flavour of harvested fish was explored. The optimal inclusion level of *Ulva* in the diet was then assessed with respect to organoleptic changes while the temporal response of key flavour attributes to dietary manipulation was also explored. Finally, key flavour compounds in the muscle tissue of *Ulva* fed barramundi were investigated to explore likely flavour-affecting compounds. This chapter is published in *Aquaculture*. The contribution of all co-authors in presented in Table 1.4.

Table 1.4 Contribution of co-authors to the published manuscript. The full citation to this published paper is:
Jones, B., Smullen, R. & Carton, A.G. (2016) Flavour enhancement of freshwater farmed barramundi *(Lates calcarifer),* through dietary enrichment with cultivated sea lettuce, *Ulva ohnoi. Aquaculture*, 454. pp. 192-198.

Contributor	Statement of contribution
Ben Jones (Candidate)	Conceptualised research study Designed and carried out experiments Analysed data Wrote the paper
Guy Carton (Principal supervisor)	Provided mentoring and support Edited the paper
Richard Smullen	Provided mentoring and support

While the negative effects of off-flavour tainting may be addressed by pre-harvest management practices, and the flavour further enhanced by dietary manipulation, it is critical that flavour and quality be maintained throughout the post-harvest storage period. This aspect of barramundi quality is poorly understood.

Chapter 6: *Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi, Lates calcarifer* presents an investigation into lipid oxidation and quality changes that occur during the post-harvest storage of farmed barramundi. The ability to constrain lipid oxidation through dietary enrichment with α-tocopherol acetate and by limiting tissue disruption was explored. Impacts on fillet colour, pH and bacterial spoilage were also investigated. This greatly increases our awareness of the storage stability of culture barramundi while presenting practical management responses that optimise quality at the point of consumption. This chapter is published in *Aquaculture*. The contribution of the co-author is presented in Table 1.5.

Table 1.5 Contribution of co-authors to the published manuscript. The full citation to this published paper is:
Jones, B. C. & Carton, A. G. (2015). Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi (*Lates calcarifer*). *Aquaculture*, *448*, 280-287.

Contributor	Statement of contribution
Ben Jones (Candidate)	Conceptualised research study Designed and carried out experiments Analysed data Wrote the paper
Guy Carton (Principal supervisor)	Provided mentoring and support Edited the paper

The final chapter of this thesis, *General Discussion*, provides a review of the accumulated research presented in Chapters 2-6. This chapter discusses the most significant findings of the research program with respect to: advances in the field of aquaculture research; aquaculture production and product quality globally, across a diverse range of species and geographic locations; and applications for Australian barramundi aquaculture. Recommended areas of future research are also discussed.

The scientific knowledge accumulated through this research program provides critical information to underpin the development of strategies that optimise the quality of farmed barramundi and farmed fish globally. For this to be effective, knowledge must be transferred to industry members to facilitate the adaptation of scientific data into farm practices. The grounding for this knowledge transfer has already been established through close collaboration with industry members and industry bodies during the course of the research program. Funding for this research was provided by the Australian Barramundi Farmers Association (ABFA) and Ridley Agriproducts Pty Ltd.. Research plans and findings have been presented at consecutive annual conferences of the ABFA between 2010 and 2015. Findings have also been presented to the International Symposium on Fish Nutrition and Feeding (2014) and at World Aquaculture Adelaide (2014). A full list of conference presentations to industry and academic conferences can be earlier in this theis. The importance and high standard of

this research has been recognised with conference presentations being awarded Most Innovative Research Paper at the 2010 Prawn and Barramundi Farmers Conference and Outstanding Presentation at the 2014 International Symposium of Fish Nutrition and Feeding. Strategies that translate the research findings of this thesis into good industry practice will be further discussed in the final chapter of this thesis.

1.7 Summary

Factors affecting the flavour and quality of farmed barramundi have been relatively unexplored. Furthermore, there has been a general paucity of information detailing strategies to optimise the quality of farmed barramundi. A number of studies have previously investigated various flavour and quality aspects in a diverse range of farmed fish species. However, it has been unknown if observations in these species are applicable to farmed barramundi. This thesis addresses these deficits in industry-related research and provides a thorough investigation into the flavour and quality of Australian farmed barramundi, the causes of quality deficiencies and potential management strategies to optimise product quality.

CHAPTER 2

Occurrence of earthy-muddy tainting of cultured barramundi linked to geosmin in tropical northern Australia.

Preface

Muddy-earthy off-flavour tainting is anecdotally reported in Australian farmed barramundi and represents a significant barrier to the future growth and viability of this nascent industry. Despite this, there is a general paucity of information regarding causative factors that affect the occurrence and intensity of off-flavour tainting events. This chapter reports an investigation of these factors and provides the first scientific observation of the levels of off-flavour tainting compounds in freshwater barramundi aquaculture ponds. The relationship between levels of off-flavour tainting compounds in the culturing water and in the flesh of barramundi is revealed, while the impact of off-flavour tainting compounds on the sensory attributes of barramundi fillets is reported. A novel method of quantitative instrumental analysis was used to resolve the relationship between levels of off-flavour tainting compounds in flesh and the sensory properties of cultured barramundi.

This chapter has been published in *Aquaculture Environment Interactions.* The full citation is:

Jones, B., Fuller, S. & Carton, A. G. (2013). Earthy-muddy tainting of cultured barramundi linked to geosmin in tropical northern Australia. *Aquaculture Environment Interactions*, *3*, 117-124.

The contribution of each of the co-authors is shown in Table 1.1.

2.1 Introduction

Muddy-earthy-musty type flavours are generally regarded as a natural characteristic of wild caught freshwater fish (Tucker, 2000; Howgate, 2004) although the occurrence of such flavours has also been reported for a diverse range of freshwater aquaculture species (Lovell, 1983; Yamprayoon & Noonhorm, 2000; Robertson et al., 2005; Peterson et al., 2011). Fish presenting with these flavour characteristics are often referred to as being 'off-flavour' or 'tainted' and are commonly considered to be spoiled or of low quality. Placing tainted fish in the marketplace typically lowers consumer confidence in the cultured product and ultimately results in significantly lower commercial returns, for example it has been estimated that off-flavour tainting can cause a 30% reduction in the sales of cultured catfish (Engle et al., 1995).

The source of muddy-earthy-musty flavours in freshwater fish is commonly acknowledged as originating from two compounds, geosmin (GSM) and/or 2-methylisoborneol (MIB). GSM and MIB are metabolites of certain groups of actinomycetes and cyanobacteria (Tucker, 2000) and are found in various water sources such as lakes, reservoirs, and running waters (Jüttner & Watson, 2007).

These compounds are known to be particularly problematic in intensive aquaculture systems due to persistent and elevated nutrient loading. Brief exposure to even low concentrations of GSM and/or MIB are known to impart an intense muddy-earthy type flavour in cultured fish, most notably American channel catfish (Persson, 1980; Martin et al., 1980) and rainbow trout (Robertson et al., 2006; Selli et al., 2009). Uptake of these tainting compounds is primarily via the gills (From & Hørlyck, 1984) and accumulation in the flesh is influenced by the concentration of the compound(s) in the holding water, water temperature, and the physiology and lipid content of the fish (Neely, 1979; Clark et al., 1990; Streit, 1998; Howgate, 2004).

Barramundi is the dominant aquaculture finfish species in tropical northern Australia with a current production volume of ~4000t per annum (Savage & Hobsbawn, 2015), although global production is considerably higher at ~164,000t per annum (FAO, 2012b). The most common growout system in Australia is freshwater outdoor earthen ponds, with small fish typically maintained in floating cages and larger fish (>2kg) being 'free-ranged' (Schipp, 1996).

In tropical northern Australia episodes of muddy-earthy tainting of freshwater outdoor pond reared barramundi (*Lates calcarifer*) are frequently reported (Phillips, 2010). Recently this issue has been highlighted as the primary cause of an escalation in negative consumer perceptions of Australian aquacultured barramundi and a growing resistance to future purchases. Episodes of off-flavour tainting are ultimately eroding the market value of end products and returns to producers.

Similar issues have previously been reported in barramundi cultured in floating cages in Lake Argyle, Australia's largest (~1,000km^2) man-made lake (Percival et al., 2008). In this instance MIB was identified as the primary compound responsible for tainting. Off-flavour tainting of barramundi cultured in outdoor intensive freshwater systems however has not been addressed, this lack of information is constraining efforts to understand the mechanisms of off-tainting tainting and the implementation of practices aimed at regaining product quality and consumer confidence.

The objectives of this study were to; 1) quantify levels of GSM and MIB in freshwater barramundi rearing ponds, 2) determine the relationship between levels of off-flavour tainting compounds in the flesh of market sized barramundi and levels in the rearing ponds, 3) quantify the impact of off-flavour tainting compounds on the sensory attributes of barramundi fillets, and 4) employ effective extraction and

instrument analysis to resolve the relationship between levels of off-flavour taint in the flesh and the sensory properties of cultured barramundi post-harvest.

2.2 Materials and Methods

2.2.1 Water sampling from rearing ponds

Three outdoor freshwater barramundi culturing ponds (~5 million litres per pond) located in North Queensland, Australia (17°42'4.88"S, 146° 2'3.18"E), were sampled weekly over a period of three months. Triplicate 50 ml water samples were collected from each pond, immediately placed on ice and frozen ($^{-}$18°C) within 25 mins of collection. Samples were collected at a fixed location in each pond (adjacent to the water outlet) from approximately 20 cm below the water surface. Samples were analysed for geosmin (GSM) and 2-methylisoborneol (MIB), using solid phase micro extraction (SPME) and gas chromatograph mass spectrometry (GC-MS) techniques (see below).

2.2.2 Preparation of water samples

At the time of analysis water samples were removed from -18°C storage and allowed to thaw at room temperature. Samples were then shaken vigorously to mix and suspend any particulates and a 10 ml aliquot taken and dispensed into a 20 ml headspace vial. A 10 uL aqueous solution (10,000 ug L^{-1}) of tetramethylpyrazine (TMP) was then added as an internal standard and the vial contents mixed for 20 sec. Finally 2.0 g of sodium chloride was added to the vial and the contents mixed until dissolved. Samples were prepared in duplicate. Calibration standards were prepared using 10 ml of deionised water known to be free of GSM and MIB. For each calibration level the TMP concentration was maintained at 10ug L^{-1} while GSM and MIB ranged from 0 to 20ug L^{-1}.

2.2.3 Uptake of off-flavour tainting compounds

To produce fish with dissimilar off-flavour tainting intensities an uptake experiment was established using forty eight market sized fish (~2.5 kg), obtained from a single rearing pond that gave no perceivable sensory indication of the presence of GSM or MIB. Fish were randomly allocated across four 5000 L indoor holding tanks located on the same site and maintained at ambient temperature (~26°C) and photoperiod. There were no significant differences in the mean weight between the four groups. Four levels of tainting intensities were established (0.0 ug L^{-1}, 1.16 ug L^{-1}, 2.49 ug L^{-1}, 3.98 ug L^{-1}) across the four holding tanks. This was achieved by varying the proportions of taint free bore-water and water sourced directly from culturing ponds presenting with an intense level off-flavour taint. Duplicate water samples were taken from all holding tanks at the conclusion (24 h) of the uptake period and stored at -18°C until analysis. Plastic floating cages were used to establish three groups of four individuals (n = 4) within each 5000 L holding tank. Stocking holding tanks with replicate groups ensured that all individuals within a single treatment were exposed to identical holding conditions and concentrations of off-flavour compounds over the duration of the uptake period. After 24 h exposure cages were removed from the holding tanks and fish euthanized using standard commercial methods (ice emersion), and stored at 2°C. After 48 h storage fish were filleted and then frozen at -18°C. One fillet from each fish was used for sensory evaluation and the other fillet consigned for the determination of GSM and MIB levels using SPME and GC-MS techniques (see below).

2.2.4 Preparation of fish samples

Barramundi fish muscle (100 g) was minced using a blender then 5 g accurately weighed into each of two ball mill cups. A 10 ml volume of 10.0 ug L^{-1} TMP in water as the internal standard solution was added to each cup. The cups were then sealed and attached to the mill which was then run at 30 cycles sec^{-1} for 60 sec to homogenize the fish muscle. Preparation of the calibration homogenates was identical to that of the sample homogenates except the fish muscle was sourced from wild marine caught barramundi known not to contain GSM and MIB. Additionally, the 10 ml aliquot of the solution containing the internal standard also contained GSM and MIB at concentrations ranging from 0-20 ug L^{-1}.

2.2.5 Extraction of fish homogenate

Approximately 20 g of homogenised fish muscle was transferred to a Markham still together with 10 ml of deionized water and 1 ml of 1M NaOH. Steam was then metered into the extraction chamber of the Markham still until approximately 8 ml of condensate was collected in a 20 ml headspace vial. Deionized water was added to the vial to bring the total volume to 10 ml. Sodium chloride (2 g) was then added, the vial capped and the salt dissolved using a vortex mixer. The extracts were stored at -18°C until time of analysis.

2.2.6 Analysis of geosmin and 2-methylisoborneol by GC-MS

At the time of analysis, water samples, fish extracts and their corresponding calibration extracts were removed from -18°C storage, thawed at room temperature and mixed thoroughly by vortex stirring. Sample analysis was undertaken by static headspace sampling of the extracts by SPME coupled with GC-MS. A 50/30µm carboxen/divinylbenzene/polydimethylsiloxane (Car-DVB-PDMS StableFlex, Supelco) SPME fibre was used for all analysis. The GC was fitted with a 50 metre

capillary column and the inlet programmed to splitless injection. The mass spectrometer was set to electron ionization mode and programmed for selective ion monitoring. The ion source was set at 70 eV and the electron multiplier at 1350 V. Identification of GSM, MIB and TMP was on the basis of the correct retention times and the correct ion ratios of the selected qualifier ions for each compound. A 10 point internal standard calibration was made by the addition of GSM and MIB to barramundi muscle known to be free of these compounds. The concentration of the two target compounds (GSM and MIB) was determined using a calibration curve based on the ratios of the selected quantifying ions for the target compounds and quantifying ion of the internal standard (TMP).

2.2.7 Sensory assessment of barramundi fillets

The sensory characteristics of fish from each uptake treatment were assessed using human sensory evaluation following the methods previously outlined by Percival et al. (2008). Six panellists were selected from an initial group of 22 and specifically trained in the sensory assessment of barramundi. Initial training sessions used both wild caught and aquacultured barramundi. Sensory participants were trained to identify and describe the most significant sensory properties (flavour, odour and aftertaste) present in each sample. Following the training period assessors then evaluated the sensory properties of randomly selected portions of fish sourced from each replicate of each treatment group from the uptake experiment. Samples were randomly assigned to assessors and only identifiable using a blind randomly generated three digit code, at no time were participants aware of the research objectives of the trial. Each sensory descriptor was evaluated along a 150 mm ungraded line ranging from 0 (absent) to 150 (intense) and a percentage score was then derived. Distilled water and flat bread were used to clean the palate between samples.

2.2.8 Comparison of sensory and chemical analysis of barramundi

Sensory assessment scores were plotted against flesh concentrations of off-flavour tainting compounds and subjected to least squares regression analysis to resolve the correlation between sensory attributes and instrument measurement. This is seen as important in the development of a rapid quantitative method to forecast expected sensory attributes over a wide range of flesh taint concentrations.

2.3 Results

Geosmin (GSM) was consistently detected in water samples from freshwater rearing ponds (Fig. 2.1), being present in ~88 % of all samples (n = 42). In contrast 2-methylisoborneol (MIB) was not detected at any time over the sampling period. Levels of GSM in water samples ranged from below detectable limits (<1 ng L^{-1}) to an extreme 14.37 ug L^{-1}. GSM levels differed between rearing ponds and were highly variable within individual ponds, with mean GSM levels showing coefficients of variation (*CV*) ranging from 74.9 to 125.2 %. Despite this high variability GSM levels most often (~70%) ranged between 0.2 µg L^{-1} to 1.75 µg L^{-1}. These results indicate that GSM is the dominant tainting compound in barramundi culturing ponds, and is present at levels that have the potential to impart off-flavour taint in farmed barramundi. Water temperatures over the sampling period averaged 27.5°C (± 2.1 S.D), while total solar energy averaged 20.0 MJ m^{-2} (± 3.34 S.D), both failed to show any clear relationship to GSM levels in culturing ponds.

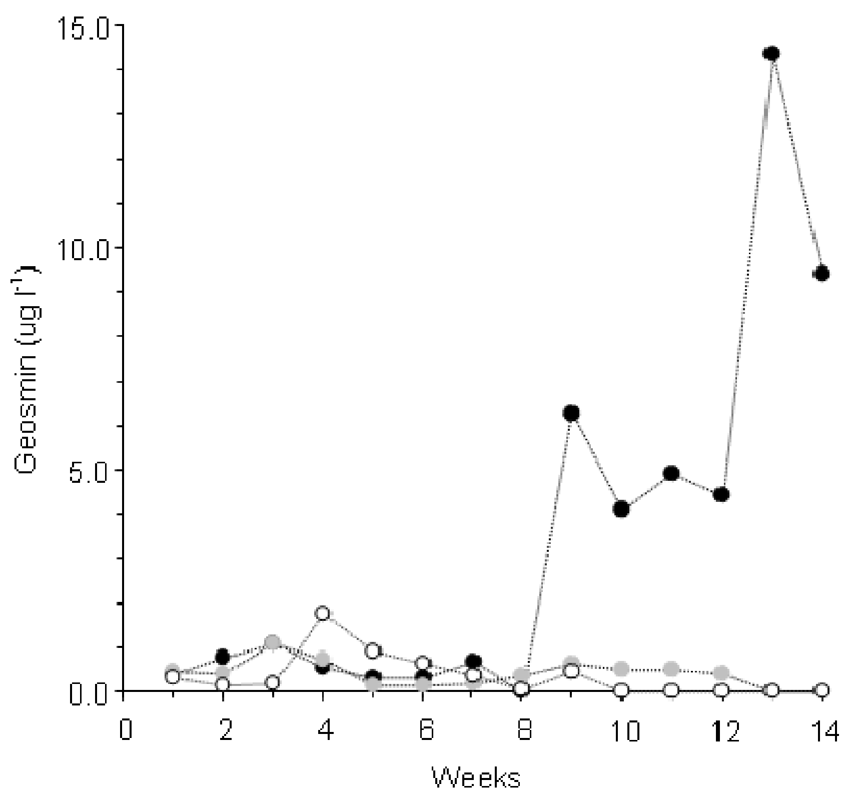

Fig. 2.1 Geosmin concentration in three freshwater barramundi growout ponds.

Market sized barramundi exposed to known levels of water borne GSM for 24 h showed a strong positive correlation ($r^2 = 0.97$) between the level of GSM in the holding water and concentrations measured in the flesh (Fig. 2.2). The uptake and accumulation of GSM by barramundi is clearly dependent on the concentration of GSM in the holding water. Chemical analysis of fish held in pure bore water (0 ug L^{-1} GSM) did however show low levels (0.74 ug kg^{-1}) of GSM in the flesh after 24 h, this is most likely a result of fish having some residual GSM in the flesh when originally sourced from the rearing pond, despite water and fish having no perceivable flavour taint when originally sourced.

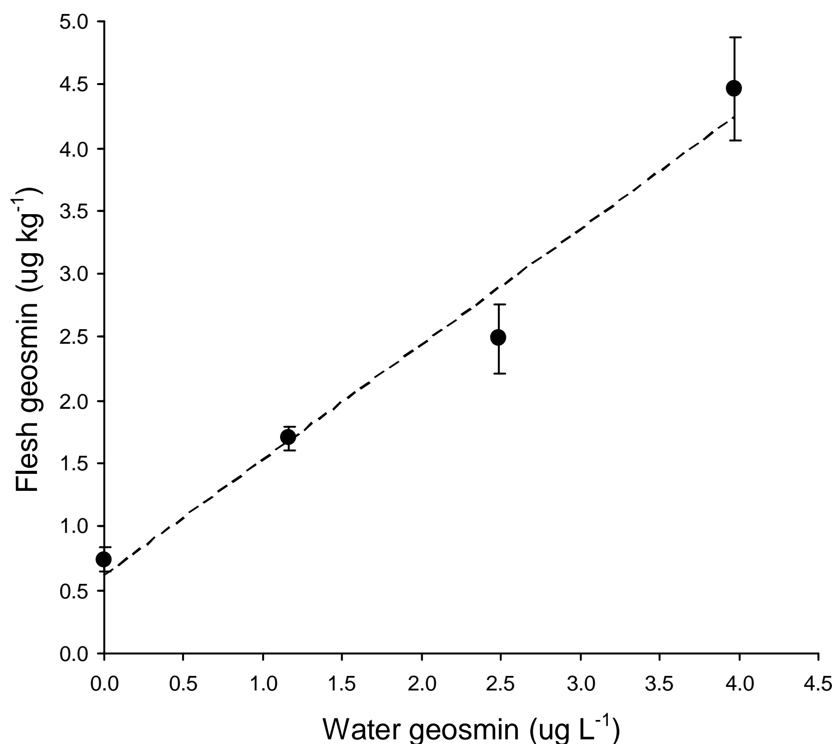

Fig. 2.2. Relationship between the concentration of geosmin in water (0.0 ug L^{-1}, 1.16 ug L^{-1}, 2.49 ug L^{-1}, 3.98 ug L^{-1}) and flesh (n = 3), for 2.5 kg barramundi held in static water conditions for 24h. Dashed line represents least squares regression ($r^2 = 0.97$; $y = 0.91x+0.62$). Bars represent standard error of the mean.

Clear differences were observed over the 12 descriptive terms used to define the sensory attributes of barramundi fillets across the various GSM concentrations measured in the flesh (see Appendix 1). Sensory evaluation profiles (Fig. 2.3) revealed striking differences in several key attributes and were most divergent between the lowest (0.74 ug kg^{-1}) and highest (4.47 ug kg^{-1}) flesh GSM values tested.

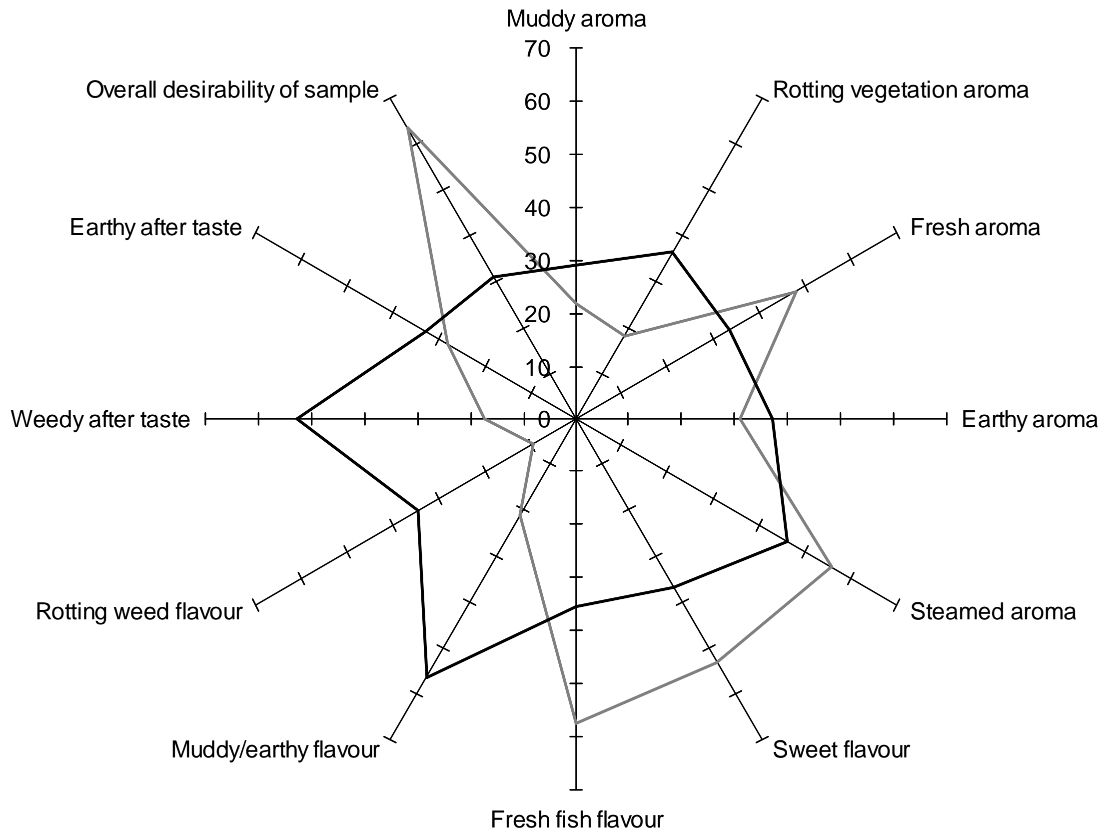

Fig. 2.3. Sensory taste and aroma attributes of barramundi at two concentrations of flesh geosmin. Black line denotes barramundi with a flesh GSM concentration of 4.47 ug kg^{-1}, the grey line represents barramundi with a flesh GSM concentration of 0.74 ug kg^{-1}.

In general sensory assessment scores for individual descriptive terms displayed a graded type response, with scores strongly correlated to GSM levels in the flesh. The sensory attributes that possessed the strongest positive correlations with GSM flesh concentration (Fig. 2.4) were muddy/earthy flavour ($r^2 = 0.99$) and weedy after taste ($r^2 = 0.94$). In contrast the strongest negative correlations with GSM flesh concentration were the sensory attributes of fresh fish flavour ($r^2 = 0.98$) and overall desirability ($r^2 = 0.91$).

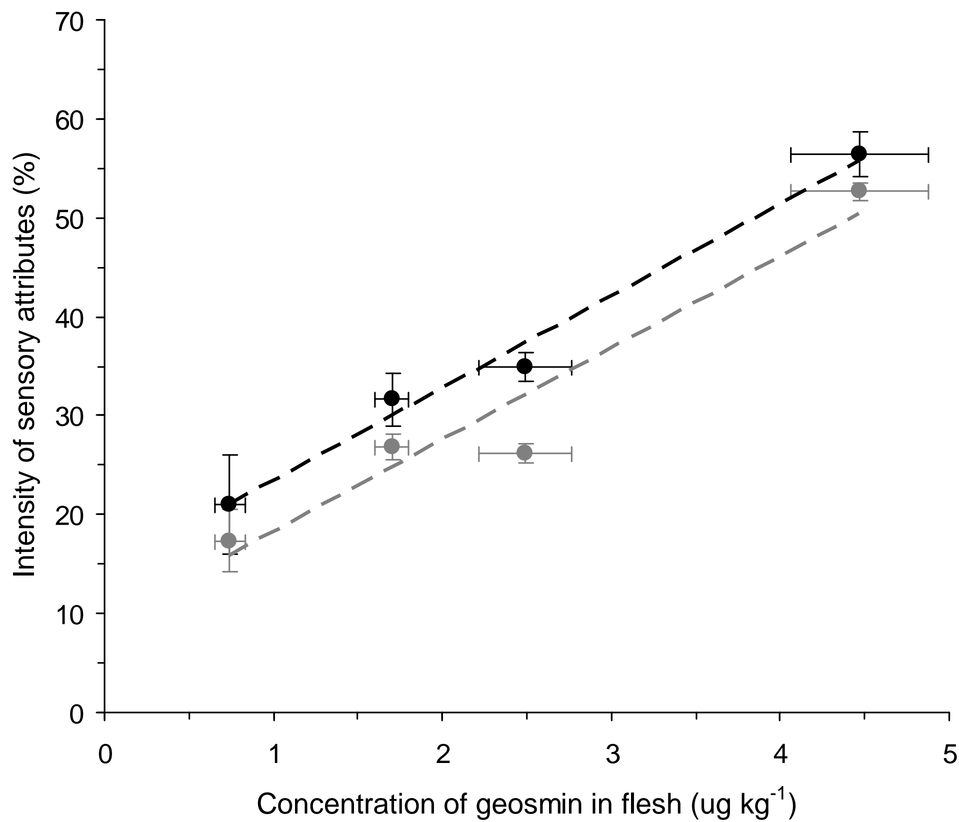

Fig. 2.4. Relationship between the intensity of two negative sensory attributes, muddy/earthy flavour (black circles) and weedy aftertaste (grey circles) and the concentration of geosmin as measured in barramundi flesh. Dashed lines represent least squares regression (muddy/earthy flavour, $r^2 = 0.99$; $y = 9.32x+14.1$ and weedy aftertaste, $r^2 = 0.94$; $y = 9.29x+8.94$). Bars represent standard error of the mean.

Instrumental analysis of flesh GSM levels were strongly correlated with the scores obtained from the evaluation panel across a number of key sensory attributes. This confirms that members of the sensory evaluation panel were capable of clearly differentiating between fish with differing taint intensities. This finding suggests that instrumental analysis has the potential to be employed as a forecasting tool with which to predict the impact of GSM levels on the flavour and taste attributes of pond reared barramundi.

2.4 Discussion

This study has identified the compound geosmin (GSM) as the primary contributor to off-flavour tainting of tropical, freshwater pond-reared barramundi. GSM was found to persist at moderate to extreme levels during the sampling period, with water borne levels of GSM directly related to the presence and intensity of off-flavour tainting. Another compound, 2-methylisoborneol (MIB), often associated with off-flavour tainting episodes (Tucker, 2000) was not detected in culturing ponds at any time. The impact of MIB on pond cultured barramundi is cautiously assumed to be negligible. This finding is in contrast to those of Percival et al. (2008), in which MIB was identified as the primary compound responsible for episodes of muddy-flavour taint of cage reared barramundi at Lake Argyle. This production system is vastly different from the smaller (<5000m^2) earthen ponds most commonly used in the Australian barramundi aquaculture industry. Differences in system design may account in part for the different results observed, although further sampling is required to confirm the impacts of GSM and MIB on pon-reared barramundi.

Barramundi growout ponds showed wide variations in water borne GSM levels. Concentrations varied within individual ponds at different sampling times as well as between different ponds. Such variations in the concentration off flavour compounds between rearing ponds has been well documented (Lovell, 1983; Lovell et al., 1986; Martin et al., 1988; Van der Ploeg & Boyd, 1991; Van der Ploeg et al., 1992). Periods of elevated (>0.5 ug L^{-1}) GSM levels typically persisted for 2-4 weeks, although GSM was undetectable in only 12% of all samples, this illustrates that GSM was consistently present in culturing ponds. GSM levels above 4.0μg L^{-1} were observed in Pond 1. Such high levels of off flavour taint are generally regarded as being exceptionally high, although similar levels have been observed in channel catfish

(*Ictalurus punctatus*) rearing ponds (Martin et al., 1988; Van der Ploeg & Boyd, 1991; Van der Ploeg et al., 1992).

In temperate localities the production of off-flavour taint is somewhat seasonal with GSM/MIB production often suppressed during winter periods (Lovell et al., 1986; Robertson et al., 2006; Robin et al., 2006). Lower temperatures and decreased solar radiation appear to be the cause as the microbes responsible for GSM/MIB production experience suboptimal conditions. Hurlburt et al. (2009) for example have demonstrated that soil temperature and rainfall can be risk factors promoting episodes of off-flavour tainting. Although the rearing ponds in this study were not sampled over a 12 month period, the general persistence of GSM observed highlights that tainting episodes in the Australian barramundi industry have the potential to be severe and prolonged. This finding is supported by the findings of Exley (2014) who assessed GSM in barramundi culture waters over a three year period, throughout the geographic range of production. The persistent and extreme nature of off-flavour tainting in tropical barramundi ponds is not surprising given that tropical localities are characterised by factors that would clearly favour the growth of taint producing microorganisms, such as high temperatures and prolonged periods of solar radiation. Despite these considerations there were no obvious relationships between GSM levels in barramundi growout ponds and water temperature or total solar energy.

Nutrient availability is also known to impact on the development of taint producing microbes. Although nutrient levels were not measured in this study, rearing ponds were in full production and therefore nutrients would not be expected to be limiting at anytime. Robin et al. (2006) have shown that an increased ratio of phosphorus relative to nitrogen and total suspended solids in aquaculture ponds promote a shift in the structure of the phytoplankton community towards taint producing cyanobacteria.

Numerous other factors are also known to impact on the development of off-flavour episodes. The complexity of factors influencing the development of GSM producing microbes may help to explain the variability in GSM levels observed in rearing ponds.

The uptake and accumulation of GSM in the flesh by barramundi is highly dependent on water-borne GSM levels. This is in agreement with previous findings for channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) (Johnsen and Lloyd, 1992; Robertson et al., 2005; Peterson et al., 2011). Accumulation of tainting compounds in flesh is extremely rapid (Perkins & Schlenk, 1997; Robertson et al., 2005) with uptake occurring passively, predominantly across the gills (Streit, 1998; Howgate, 2004).

Levels of GSM in barramundi fillets were only marginally higher (1.3x) than GSM levels in the holding water following 24 hours of exposure under static conditions. Previous studies have demonstrated higher levels of bio-concentration in the flesh following GSM exposure, with values ranging from ~20x for Arctic charr (Houle et al., 2011), ~30x for rainbow trout (Robertson et al., 2005), and between 1 and 45x for channel catfish (Martin et al., 1988). As GSM is more soluble in lipid than water it becomes sequestered and concentrated in the lipid of tissues (Howgate, 2004). It has been proposed that due to the relationship between GSM uptake and lipid content that the production of leaner fish could potentially lower the concentration of taint compounds in farmed fish (Johnsen & Lloyd, 1992; Dionigi et al., 1998; Robertson et al., 2005). Although lipid was not measured in this study, total body lipid has been shown to be ~10% (wet weight) in barramundi fed artificial diets (Glencross et al., 2008) compared to ~24-44% (dry weight) in farmed channel catfish (Andrews & Stickney, 1972). Farmed barramundi also show strong regionalisation of body lipid, with the lowest lipid levels (~1.3%) occurring in the anterior dorsal area and highest

levels (~30%) occurring in the belly area (Percival et al., 2008). This regionalised distribution of lipid may underlie spatial differences in GSM levels throughout the fillet. This would agree with the findings of Percival et al. (2008), reporting that off-flavour caused by MIB was most perceptible in the high lipid belly region. In channel catfish MIB has been shown to be related to muscle lipid; fish with > 2.5% muscle lipid accumulated ~3 times more MIB than fish with < 2% lipid (Grimm et al., 2004). In the present study flesh samples for chemical analysis and sensory evaluation were taken from the anterior dorsal region of the fish, this area is most likely to have the lowest low lipid and GSM level within the fillet and may partly explain the low concentration of GSM in barramundi flesh relative to that of holding water. Further assessment of lipid and GSM concentration in various regions of barramundi fillets is required to quantify this relationship.

Whilst GSM concentrations in barramundi flesh are predictable under static and controlled conditions, such predictions are more difficult in the field. The relationship between levels of water borne GSM and flesh GSM is complex under pond aquaculture conditions due to the dynamic nature of GSM production (Howgate, 2004).

This study has for the first time paired direct assessment of GSM levels in barramundi flesh using chemical analysis, to sensory assessment of flavour quality attributes, thereby providing a better understanding of the impact of GSM on barramundi flavour quality. The sensory evaluation panel detected clear differences over a broad range of taste and flavour attributes across the various GSM concentrations as measured in the flesh. Striking differences were observed between the lowest (0.74 ug kg^{-1}) and highest (4.47 ug kg^{-1}) flesh GSM levels, with increasing GSM levels resulting in increases in negative sensory attributes, with muddy/earthy flavour showing the

highest increases. Positive sensory attributes such as fresh fish flavour, overall desirability and sweet flavour progressively improved with reducing GSM flesh levels. The impact of GSM on the flavour quality of pond reared barramundi is comparable to that previously reported by Percival et al. (2008) for barramundi tainted by MIB although the levels of GSM experienced and the intensity of off-flavour tainting were much higher in the current trial.

Sensory levels are often categorized relative to the degree of tainting. Previous studies of rainbow trout have categorized “on flavour” as containing <0.25 to 1.12 μg kg^{-1} GSM and “strongly tainted” as containing 2.05 to 4.18 μg kg^{-1}. Similar categories could easily be applied to farmed barramundi. In order to achieve this, consumer assessment would be required to determine how various levels of GSM tainting are perceived by the consumer.

Although this study did not undertake any direct assessment of threshold level of detection for GSM in barramundi flesh some inferences can be made. At the lowest GSM flesh concentration of 0.74 ug kg^{-1}, muddy/earthy flavour scored 21%, while overall desirability of the sample scored 63%. This suggests that the threshold level for detection of GSM in barramundi is likely to be <0.74 μg kg^{-1}. This estimate agrees well with previous data for detection thresholds of GSM in other species. Robertson et al. (2006) determined the sensory threshold of GSM in rainbow trout flesh to be 0.9 μg kg^{-1}, Grimm et al (2004) reported odor thresholds between 0.25 and 0.5 μg kg^{-1} for GSM in channel catfish, while Persson (1980) indicated a sensory threshold of 0.90 and 0.59 μg kg^{-1} for bream and pike, respectively. The sensory threshold of GSM will also be influenced by variations in sensory evaluation panels, the sensory characteristics of different species, and/or the presence or intensity of other flavours that may serve to mask GSM detection.

The use of instrumental analysis for quantifying GSM levels in fish flesh has increasingly become widespread (Grimm et al., 2004; Robertson et al., 2005; Peterson et al., 2011). In the present study SPME-GCMS analysis of GSM levels in barramundi flesh were significantly correlated with the scores obtained from the sensory evaluation panel across a number of key attributes that are often ascribed to GSM tainting. This finding agrees well with previous comparisons between human sensory scores and instrumental analysis of GSM for channel catfish (Grimm et al., 2004), and rainbow trout (Robertson et al., 2005; Peterson et al., 2011). The high correlation between these two methods suggests that instrumental analysis has the potential for use as a forecasting tool with which to predict changes in the flavour and taste profile of barramundi across a broad range of taint intensities. As such, instrumental analysis is highly advantageous as it offers rapid and reliable assessment of GSM levels in flesh and overcomes the major limitations of sensory evaluation panels, as it does not succumb to sensory overload (Grimm et al., 2004) and has the capacity for high sample through-put.

2.5 Conclusions and implications

The findings of this study have implications for the production of farmed barramundi in tropical systems. Geosmin was identified as the primary compound associated with off-flavour tainting of pond reared barramundi; concentrations were found to be elevated and persistent. Levels of geosmin in barramundi flesh were highly dependent on levels in the holding water. Knowledge of the relationship between the geosmin concentration in the water and expected content in the fish will allow farmers to act on critical geosmin concentrations and expected levels of off-flavour taint. Sensory evaluation of taste and flavour attributes of pond reared barramundi clearly

demonstrates that the intensity of tainting is highly correlated with the concentration of geosmin present in flesh. The threshold level of detection for geosmin in farmed barramundi is below 0.74 ug kg^{-1} and this is comparable to the sensory threshold of GSM in trout (0.9 μg kg^{-1}) and MIB in catfish (0.7 μg kg^{-1}).

This information is of critical importance if producers and researchers are to understand the underlying mechanisms of off-flavour tainting of freshwater pond reared barramundi. Furthermore, the findings of this study can be used to establish protocols and practices that serve to mitigate off-flavour tainting and improve the flavour quality of barramundi cultured in freshwater earthen ponds and other land based tropical aquaculture systems in general.

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CHAPTER 3

Controlled off-flavour tainting of cultured fish using the geosmin-producing cyanobacterium, *Anabaena circinalis*.

Preface

In Chapter 2, an investigation of the causative factors that affect the occurrence and intensity of off-flavour tainting events in farmed barramundi was reported.

This provides the first scientific observation of the levels of off-flavour tainting compounds in freshwater barramundi aquaculture ponds. GSM was identified as the primary compound associated with off-flavour tainting of pond reared barramundi with concentrations found to be elevated and persistent. The level of GSM in the flesh of farmed barramundi was observed to be dependant on the level of GSM in exposure water while the intensity of off-flavour tainting showed a graded response, becoming more intense as the level in the flesh increased. This knowledge provides valuable insights into the off-flavour tainting of farmed barramundi. However, if off-flavour tainting is to be mitigated, strategies must be developed that can eliminate off-flavour tainting from fish prior to slaughter.

Chapter 2 also revealed that the level of GSM in pond water was highly variable, erratic and unpredictable. This is a significant barrier to the development of logical and well-planned experimental designs that investigate strategies for mitigating the detrimental effects of off-flavour tainting. In order to develop such strategies, a reliable supply of off-flavour tainted fish is required. The following chapter presents an accurate, reliable and reproducible method of generating off-flavour tainted fish on-demand, at pre-determined levels of taint. This will facilitate an investigation into a range of aspects of off-flavour tainting and the development of management strategies that can mitigate off-flavour tainting in farmed fish.

This paper presented in this chapter was submitted to *Aquaculture International* on 29/1/16 under the title:

Controlled off-flavour tainting of cultured fish using the geosmin-producing cyanobacterium, *Anabaena circinalis.*

The contributions of co-authors to the submitted manuscript are presented in Table 1.2.

3.1 Introduction

Off-flavour tainting, the occurrence of muddy-earthy flavours and aromas, is a frequent problem in freshwater aquaculture worldwide (Tucker, 2000; Howgate, 2004). Off-flavour tainting is most commonly caused by the bioaccumulation of two compounds, geosmin (GSM) and/or 2-methylisoborneol (MIB) (Tucker, 2000; Howgate, 2004; Carton & Jones, 2013). Uptake of these compounds by fish is rapid occurring primarily via the gills (From & Hørlyck, 1984). Fish tainted with these muddy-earthy off-flavours are considered low quality, which significantly reduces commercial returns (Engle et al., 1995).

Although a wide array of environmental compounds have been implicated in off-flavour tainting of farmed fish, GSM and MIB appear to be especially problematic and are most frequently implicated in muddy-earthy off-flavour events (Tucker, 2000; Howgate, 2004; Percival et al., 2008; Carton & Jones, 2013). GSM and MIB are secondary metabolites synthesized by certain groups of bacteria (actinomycetes, myxobacteria and cyanobacteria) (Blevins et al., 1995; Wood et al., 2001; Schöller et al., 2002; Smith et al. 2008), with cyanobacteria frequently cited as the cause of freshwater odour problems.

Although the generalised effects of GSM and MIB exposure and off-flavour tainting are known, several important questions remain concerning sensory detection thresholds, relationships between flesh concentrations and the intensity of the taint, the species-specific distribution of taint compounds in various tissues and the effect of factors such as fish size, water holding temperature, physical activity levels and lipid content on rates of uptake and loss of GSM/MIB. Such information is critical for industries aiming to prevent the occurrence and impact of off-flavour tainting.

The ability to explore these questions requires access to reliable supplies of off-flavour tainted fish and/or tainting compounds. However off-flavour tainting episodes

occurring in captive rearing facilities are most often sporadic and unpredictable (Dionigi et al., 1998; Robin et al., 2006; Percival et al., 2008; Houle et al., 2011), and additionally fluctuate in intensity, as detailed in Chapter 2 (also Lovell, 1983; Lovell et al., 1986; Martin et al., 1988; Van der Ploeg & Boyd, 1991; Van der Ploeg et al., 1992; Carton & Jones, 2013). It is also impossible to control the timing and intensity of tainting episodes while assessing the concentration of taint compounds in water involves costly and time consuming gas chromatography–mass spectrometry (GC-MS) (Grimm et al., 2004; Robertson et al., 2005). These factors are significant impediments to logical and well-planned experiments that address issues of off-flavour tainting.

To overcome the above limitations, synthetic off-flavour compounds have been used (Johnsen & Lloyd, 1992; Johnsen et al., 1996; Robertson et al., 2005). However the use of synthetic off-flavour compounds can be cost-prohibitive in large-scale experiments. It also remains unexplored as to whether uptake and accumulation kinetics and/or the organoleptic characteristics of synthetic tainting compounds differ from naturally produced compounds. Further to this, the use of synthetic compounds in human sensory assessment can be problematic in some countries, such that participants must be fully informed on the use and nature of the synthetic compound being used, which may lead to experimental bias during sensory evaluation sessions.

Agar plating techniques (Safferman et al., 1967) have previously been employed to naturally produce off-flavour tainting compounds, however practical use is limited to fine-scale uptake experiments on individual fish (From & Hørlyck, 1984).

To overcome these obstacles, the cyanobacterium *Anabaena circinalis*, a known GSM producer (Ho et al., 2009; Giglio et al., 2011; Li et al., 2012) commonly found in freshwater systems throughout Australia (Fabbro & Duivenvoorden, 1996; Preite,

2008; Ho et al., 2009) was cultivated. While some strains of *A. circinalis* are known to produce toxins, the strain selected for this study (CS-139) from the CSIRO Microalgae Supply Service, Australian National Algae Culture Collection (Hobart, Australia) is confirmed as non-cyanotoxin producer.

Here a novel method for the production of natural GSM, using *A. circinalis* cultures, for the purpose of intentionally imparting off-flavour taint into farmed fish is reported. Although *A. circinalis* is known to produce GSM and is readily cultured in the laboratory, its use for the controlled tainting of captive fish requires rapid estimation of the level of GSM in the culture. This would enable direct control over the level of GSM to which fish are exposed and the manipulation of off-flavour tainting. To facilitate this, the suitability of several low-cost, simple methods to rapidly and accurately estimate concentrations of GSM in *A. circinalis* cultures was assessed. Human sensory analyses were then used to determine the organoleptic characteristics of the widely cultivated tropical fin fish *Lates calcarifer* (Percival et al., 2008; Carton & Jones, 2013) exposed to cultures of *A. circinalis,* as detailed in Chapter 2. The potential to manipulate GSM concentrations and off-flavour intensity in the tissue of farmed fish was assessed by manipulating the level of GSM, produced by *A. circinalis*, in exposure water.

3.2 Materials and Methods

3.2.1 Growth and geosmin production in *Anabaena circinalis* cultures

The cyanobacterial strain *Anabaena circinalis* (CS-139) was obtained from the CSIRO Microalgae Supply Service, Australian National Algae Culture Collection (Hobart, Australia) and mother cultures were maintained as continuously aerated 2 L

batch cultures in BG11 culture medium (Rippka et al., 1979) at 24 °C under continuous illumination of 15 µmol photons m^{-2} s^{-1}.

Four-week growth experiments were inoculated with stationary-phase mother cultures of *A. circinalis* to an initial biomass concentration of approximately 0.1 g dry weight L^{-1}. Culture conditions were as above, except that a 12h:12h photoperiod was applied.

Samples for the determination of optical density at 750 nm (O.D. $_{750nm}$), cell concentrations, ash-free dry weight (AFDW) and chlorophyll *a* (Chl *a*) were taken every second day until day 6 and every fourth day until day 22 with the final sample being taken on day 28. For total GSM and MIB determination, 50 mL samples of *Anabaena circinalis* were collected on days 0, 4, 14, 24 and 28 and stored (-20°C) prior to GC-MS analysis as outlined below (3.2.2.2. Instrumental analysis for GSM and MIB by GC-MS).

O.D.$_{750nm}$, the absorbance of the culture at 750 nm, was determined spectrophotometrically in 96-well plates (Greiner) on a spectrophotometer Spectramax plus 384 (Molecular Devices, USA). AFDW (g L^{-1}) was obtained by filtering 15 mL of culture under gentle vacuum through pre-combusted, pre-weighed Whatman GF/F glass fibre filters (47mm). For determination of AFDW (g L^{-1}), filters were desiccated at 80 °C until reaching constant weight (dry weight, DW) followed by combustion at 500 °C for 4 h and subtracting the ash content from DW. For determination of cell concentrations (cells mL^{-1}), 1 mL culture samples were fixed with acidic Lugol solution and stored in darkness at 4°C (Cires et al., 2013) until analysed. Cell counts were performed using a Neubauer improved haemocytometer under 400x magnification on an Olympus CX21 light microscope.

Chlorophyll *a* (Chl *a*) concentration was determined spectrophotometrically, filtering 15 mL culture samples through 47 mm-GF75 glass fibre filters (Advantec MFS Inc., Japan) under gentle vacuum. Filters were stored in darkness at -20 °C until analysis. Chl *a* was extracted into 8 mL of 90% (v/v) cold methanol at 4 °C and absorbances of the clarified extracts, obtained by centrifugation (10,000 x *g* for 5 min) to remove cell debris and filter material, were measured at 665 nm (chl *a* absorbance peak) and 750 nm (turbidity) on a SPECTRAmax PLUS 384 spectrophotometer. Chl *a* concentration (µg L^{-1}) was calculated using equations derived from Marker et al. (1980).

3.2.2 Using *Anabaena circinalis* cultures to manipulate off-flavour taint in captive fish

3.2.2.1 Experimental fish

A series of experiments was conducted to determine the suitability of using *A. circinalis* cultures to manipulate off-flavour tainting in farmed fish. For each experiment, farm-reared barramundi (Good Fortune Bay Fisheries, Kelso, Australia) were initially held in a single 10,000 L (~27 °C) holding tank, supplied with continuous flow-through water known to be devoid of off-flavour compounds, for a period of 4 weeks. Following this period, fish were exposed to varying levels of GSM achieved by diluting cultures of *A. circinalis* with charcoal filtered municipal water known to be free from GSM and MIB. To ensure that fish in each experiment were exposed to identical conditions (temperature, water flow, physical activity, GSM concentration, etc.) over the duration of the uptake period, fish were held in a single 2000 L tank containing diluted cultures of *A. circinalis* at ambient temperature (24-27°C) and photoperiod. Following this exposure period fish were euthanized in an ice slurry using standard commercial techniques (Carton & Jones, 2013). Whole fish were then stored in an ice slurry prior to being filleted and frozen at −18°C for later human

sensory evaluation and/or GC-MS analysis (see below). Water samples (50 mL) were also collected from fish holding tanks (n=2) and stored in glass jars at -20 °C until analysed by GC-MS as outlined below.

3.2.2.2 Instrumental analysis for GSM and MIB by GC-MS

Water samples and fish samples for the determination of GSM and MIB concentration were prepared and analysed according to the methods presented in Chapter 2 except that menthol was used in place of tetramethylpyrazine as the internal standard for the analysis of fish samples.

Sample analyses were performed by Innovative Food Technologies, Department of Agriculture and Fisheries, Brisbane, Australia.

3.2.2.3 Assessing organoleptic profile of off-flavour tainted fish

To validate the organoleptic characteristics of the off-flavour taint produced by cultures of *A. circinalis*, human sensory analyses were performed on barramundi sourced from three different treatments; (1) held in water sourced from a local commercial freshwater aquaculture facility experiencing an episode of off-flavour tainting (1.98 µg L^{-1} GSM), (2) held in water containing diluted laboratory cultures of *A. circinalis* (2.79 µg L^{-1} GSM), and (3) held in de-chlorinated, charcoal-filtered municipal water, known to be free of off-flavour tainting compounds.

For each treatment, fish (~2.0 kg) were removed from the holding tank (see 3.2.2.1. Experimental fish) and held in a single 2,000 L tank at ambient temperature (~27 °C) and photoperiod for a period of 24 h. Within each holding tank, plastic cages (60cm x 60cm x 80cm) were used to establish 3 groups of 2 individual fish (n=6 for each treatment). After 24 h all fish were removed, euthenised and processed, as previously detailed. Water samples (50 mL) were collected from fish holding tanks at the conclusion of the 24 h holding period as previously described.

3.2.2.4 Organoleptic profiling of barramundi fillets

Prior to human sensory assessment, the anterior dorsal (shoulder) portion was removed from each fillet. The four shoulder portions from each replicate cage were then divided into 20 g portions and randomly assigned to sensory assessors. The organoleptic attributes of fish from each of the three treatments were assessed using human sensory evaluation following the methods detailed in Chapter 2. Briefly, six panellists were selected from an initial group of 22 and exclusively trained in the sensory assessment of barramundi. Sensory participants were trained to identify and describe the 10 most significant sensory properties related to off-flavour tainting in farmed barramundi (Chapter 2). The sensory attributes assessed by the panel were: muddy aroma, muddy/earthy flavour, earthy aroma, rotting vegetation aroma, rotting weed flavour, weedy aftertaste, fresh aroma, fresh fish flavour, steamed aroma and sweet flavour) (See Appendix 2 for definitions of these sensory descriptors). Following this initial training period, assessors were then asked to evaluate the sensory properties of randomly selected 20 g portions of fish from each treatment detailed above. Randomly assigned samples were identifiable using a blind randomly generated three digit code. Assessors evaluated each sensory descriptor along a 150 mm ungraded line ranging from 0 (absent) to 150 (uppermost intensity). Distilled water and flat bread were used by assessors to clean the palate between samples.

3.2.2.5 Manipulation of GSM in fish tissue

To confirm the uptake of GSM from diluted cultures of *A. circinalis*, GC-MS analysis was used to assess tissue accumulation at two exposure levels; 2.15 $\mu g\ L^{-1}$ and 15.1 $\mu g\ L^{-1}$ GSM, these representing frequently occuring and extreme exposure levels respectively (see Chapter 2). To provide a comparison with untainted fish, three farm-reared barramundi (~1.75 kg), were collected from the holding tank (see 3.2.2.1.

Experimental fish) prior to GSM exposure. The fillets of these fish were removed from the right and left sides and frozen at −18°C for later GC-MS analysis as previously detailed. For each GSM exposure level, three fish (~1.75 kg) were randomly selected from the holding tank (3.2.2.1. Experimental fish) and transferred to a single 2,000 L fibreglass tank containing diluted cultures of *A. circinalis*. After 48 hour-exposure, all fish were removed, filleted and the fillets frozen (− 18°C) for later GC-MS analysis as previously detailed.

3.2.2.6 Manipulation of off-flavour intensity using *A. circinalis* cultures

To determine if the intensity of off-flavour tainting could be manipulated by varying the level of exposure to *A. circinalis* cultures, human sensory analyses were used. The intensity of off-flavour taint was assessed in fish from three different sources; (1) held in water containing *A. circinalis* cultures and a GSM concentration of 0.55 $\mu g\ L^{-1}$, (2) held in water containing *A. circinalis* cultures and a GSM concentration of 1.51 $\mu g\ L^{-1}$, and (3) held in de-chlorinated, charcoal-filtered municipal water, known to be free of off-flavour tainting compounds.

For each treatment, 5 fish were randomly selected from the holding tank (see 3.2.2.1. Experimental fish) and transferred to a single 100 L tank containing diluted cultures of *A. circinalis*. Cultures of *A. circinalis* were diluted into the holding tanks to achieve GSM concentrations of 0.55 $\mu g\ L^{-1}$ and 1.51 $\mu g\ L^{-1}$ respectively. A third group of 5 fish was held in an identical 100 L tank containing de-chlorinated, charcoal-filtered municipal water known to be free from GSM and MIB . Following the 24 hour holding period, fish and water samples were collected and processed as previously described prior to GC-MS analysis for GSM and MIB.

3.2.2.7 Sensory assessment of off-flavour intensity

Sensory assessment of off-flavour intensity was identical to the procedure used for organoleptic profiling (above) except that eight sensory participants were trained to identify and rate the most significant organoleptic properties of off-flavour tainted barramundi; muddy/earthy flavour, muddy aroma and earthy aroma. Following this initial training period, assessors were then asked to evaluate the intensity of each of these organoleptic attributes in randomly selected 20 g portions of cooked fish. Values obtained from the panel were converted to report intensities as a rating out of 10. A numerical system was developed to report the overall level of off-flavour taint for each treatment. This was calculated as the sum of human sensory scores for muddy/earthy flavour, muddy aroma and earthy aroma and is referred to as off-flavour score.

3.3 Results and Discussion

3.3.1 Manipulating taint in farmed fish

To validate the nature of the off-flavour taint imparted to barramundi by cultures of *A. circinalis*, the organoleptic characteristics of fish were characterised for (1) fish intentionally tainted with diluted cultures of *A. circinalis*, (2) fish sourced from an aquaculture facility experiencing an episode of off-flavour tainting, and (3) fish held in de-chlorinated, charcoal-filtered municipal water. GC-MS analysis confirmed GSM concentrations of 2.79 µg L^{-1} in diluted cultures of *A. circinalis* and 1.98 µg L^{-1} in water sourced from a commercial aquaculture facility while GSM was not detected in charcoal-filtered municipal water. MIB was not detected in water samples at any time.

Tainted fish from both sources exhibited identical flavour profiles across ten key organoleptic attributes (Fig. 3.1). The attributes described by sensory assessors for

fish exposed to GSM were: muddy aroma, muddy-earthy flavour, earthy aroma, rotting vegetation aroma, rotting weed flavour and weedy aftertaste (see Appendix 2) which are well recognised characteristics of off-flavour tainted fish (Howgate, 2004; Tucker, 2000), including barramundi exposed to GSM, as detailed in Chapter 2 (Carton & Jones, 2013). As expected, fish held in clean water free of GSM and MIB were noticeably divergent in organoleptic properties with fresh aroma, fresh fish flavour, steamed aroma and sweet aroma being the most dominant properties (Fig. 3.1.). These results confirm that laboratory cultures of the cyanobacterium *A. circinalis* can be used to impart off-flavour tainting into fish. Furthermore, the off-flavour attributes described were identical to those observed in fish tainted by commercial aquaculture pond water, across 10 key organoleptic attributes.

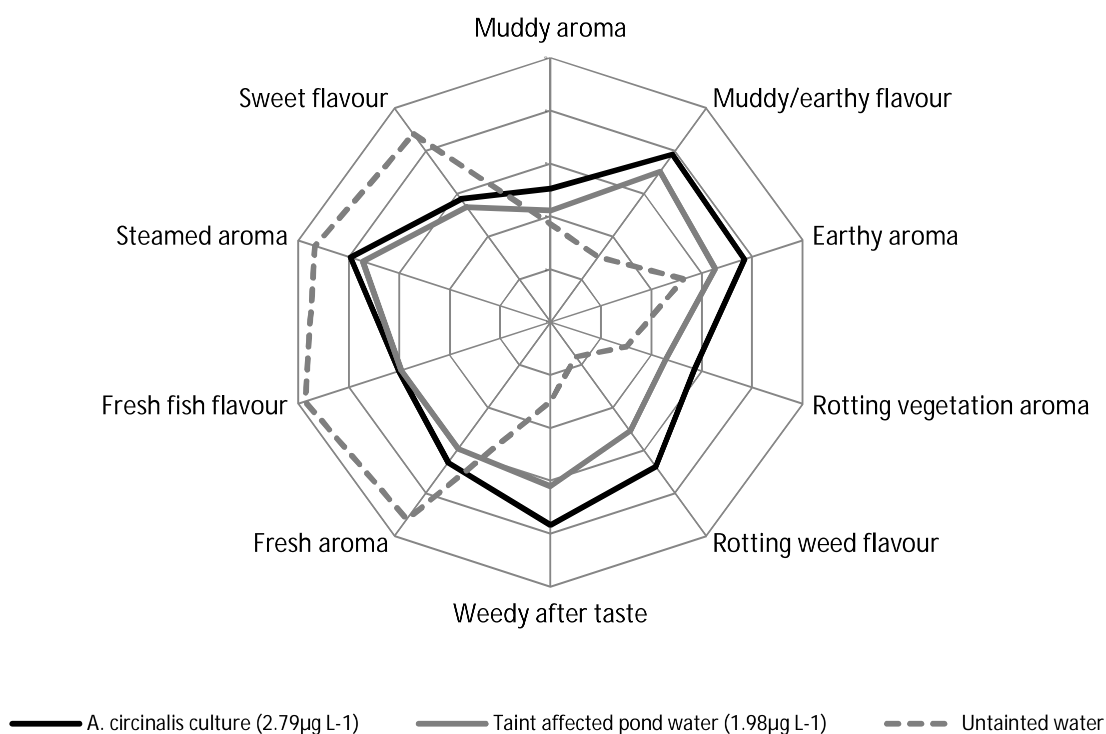

Fig. 3.1 Flavour profiles of untainted barramundi (dashed grey line), barramundi exposed to 1.98 µg L^{-1} geosmin sourced from a naturally occurring off-flavour episode (solid grey line) and barramundi exposed to 2.79 µg L^{-1} produced by laboratory cultures of *Anabaena circinalis* (solid black line).

To confirm that GSM produced by cultures of *A. circinalis* was taken up and deposited into white muscle tissue, fish were held in water containing diluted cultures of *A. circinalis*. GC-MS analysis confirmed that fish were exposed to 2.15 µg L^{-1} and 15.1 µg L^{-1} in water, respectively. At these exposure levels, flesh concentrations of 4.83µg kg^{-1} and 8.25µg kg^{-1} were observed, respectively. A GSM concentration of 0.13µg kg^{-1} was observed in fish held in untainted water (Table 3.1) and is assumed to be residual GSM remaining in farm-reared fish. MIB was not detected in any flesh samples. These results confirm that cultures of *A. circinalis* can be used to actively manipulate the GSM content of fish tissues, thus providing researchers with a reliable method to investigate the uptake of this critical off-flavour compound throughout a wide range of exposure levels.

Table 3.1 Accumulation and bio-concentration of geosmin by barramundi at differing levels of exposure to geosmin water concentrations generated by *A. circinalis cultures*. Values represent the mean ± the standard error of the mean.

	Water geosmin concentration (µg L^{-1})	Flesh geosmin concentration (µg kg^{-1})	Bioconcentration Factor (BCF)
Untainted water	**0.00**	**0.13±0.04**	**-**
Moderate GSM concentration	**2.15±0.024**	**4.83±0.33**	**2.25**
High GSM concentration	**15.1±0.55**	**8.25±0.62**	**0.55**

The accumulation of GSM from exposure water was somewhat divergent between fish exposed to 2.15 µg L^{-1} and 15.1 µg L^{-1} with bioconcentration factors (BCFs) of 2.25 and 0.55, respectively being observed (Table 3.1). This represents the ratio of GSM in tissue compared to that in exposure water. The cause of this variability is difficult to resolve, however considerable variability in BCFs for GSM accumulation have previously been observed. For example, BCFs for GSM of ~0.6-1.3 for barramundi (See Chapters 2 and 4), ~4.8-20 for tilapia (Yamprayoon & Noomhorn,

2000), ~20-30 for Arctic charr (Houle et al., 2011), ~26 for rainbow trout (Robertson et al., 2005) and ~0.06-~23 for channel catfish (Schrader et al., 2011) have previously been observed. The underlying cause of this variability is unclear and highlights the need for further research aimed at understanding the uptake of GSM from naturally occurring sources.

To determine if diluted cultures of *A. circinalis* could be used to manipulate the intensity of off-flavour tainting, fish were exposed to varying concentrations of GSM. The intensity of off-flavour taint was assessed for fish held in de-chlorinated, charcoal-filtered municipal water and for fish exposed to diluted cultures of *A. circinalis* at two GSM exposure levels. GSM was not detected in dechlorinated municipal water while concentrations of 0.55 µg L^{-1} and 1.51 µg L^{-1} respectively were observed in exposure tanks. Off-flavour tainting was most intense in fish exposed to 1.51 µg L^{-1} GSM, with an off-flavour score of 8.5. Fish exposed to 0.55 µg L^{-1} developed less intense off-flavour tainting with an off-flavour score of 5.07 and off-flavour tainting was lowest in fish held in untainted water with a reported off-flavour score of 2.2 (Fig. 3.2). This confirms the efficacy of this procedure for intentionally manipulating the intensity of off-flavour tainting in farmed fish.

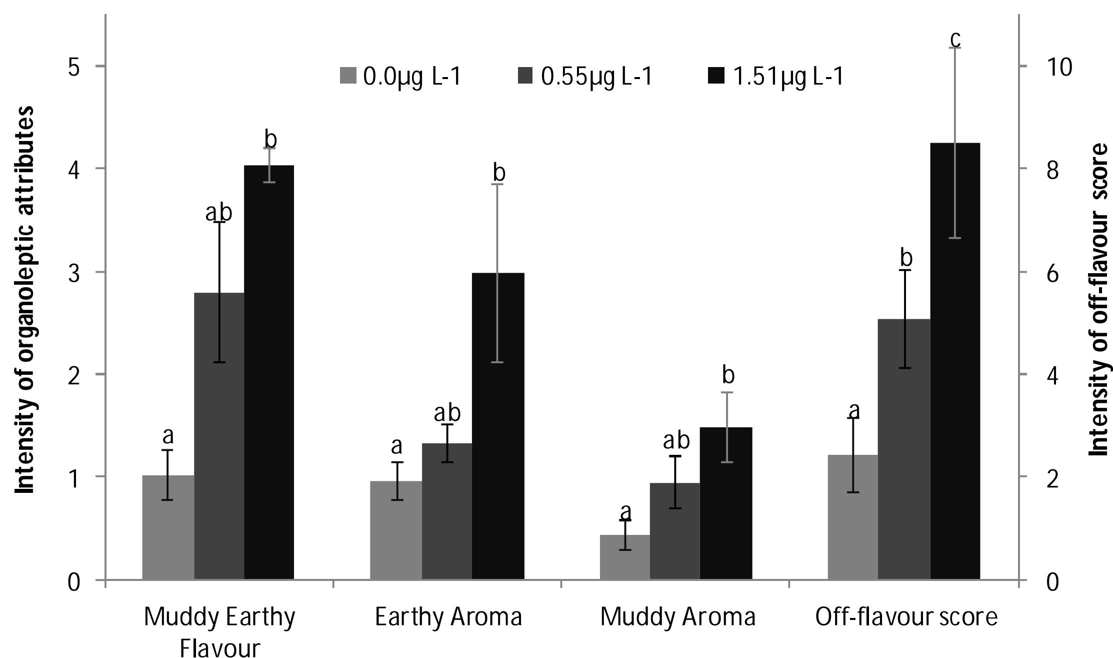

Fig. 3.2 Intensity of off-flavour attributes in barramundi exposed to 0.0 µg L^{-1} (black bars), 0.55 µg L^{-1} (light grey bars) and 1.51 µg L^{-1} (dark grey bars) GSM generated by cultures of *A. circinalis*. Values for muddy/earthy flavour, muddy aroma and earthy aroma are shown on the left axis and off-flavour score is shown on the right axis. Different letters above bars indicate significant differences in the intensity of off-flavour attributes between GSM exposure levels. Error bars represent standard error of the mean.

As this technique produces GSM 'on-demand' and the quantity produced is limited only by the volume of cultures prepared, it can be used to reliably impart GSM and off-flavour tainting to large numbers of fish at pre-determined tainting levels. This method therefore allows for fine-scale control regarding the timing of experiments but more importantly enables manipulation of the level of GSM to which fish are exposed and the intensity of off-flavour tainting imparted. This facilitates researchers to explore a range of aspects of off-flavour tainting such as the kinetics of uptake and loss of GSM into/out of fish across a range of water tainting concentrations, effects of variability in lipid content of fish on uptake and elimination of GSM, distribution of GSM among various tissues, variation in human threshold detections of GSM across

species and the impact of GSM exposure on the sensory attributes of specific farmed fish.

A significant advantage of this method is that the off-flavour tainting compound, in this instance GSM, is produced naturally by an organism already implicated in aquaculture tainting episodes, including Australian farmed barramundi (Tucker, 2000; Smith et al., 2008), and drinking water (Bowmer et al., 1992; Rosen et al., 1992; Izaguirre & Taylor, 2007; Ho et al., 2009) globally. This method relies on the natural production of off-flavour taint, rather than the use of synthetic alternatives. Consequently, any difficulties arising due to institutional ethical standards relating to the use of synthetic chemical standards in human sensory procedures are alleviated.

3.3.2 Prediction of GSM production in *A. circinalis* from biomass indicators

To use *A. circinalis* for the manipulation of off-flavour tainting in captive fish requires that laboratory cultures of this cyanobacterium have the capacity to generate high concentrations of GSM. It is also critical that GSM concentrations in culture water be rapidly predicted prior use in tainting experiments such that the exposure level can be accurately controlled. To facilitate this, GSM production was monitored during culture growth while various culture parameters were assessed as convenient indirect methods with which to rapidly estimate GSM concentration.

Laboratory cultures of *A. circinalis* produced GSM, while MIB was not detected at any time during the culturing period. Under the experimental conditions of this study, cultures of *A. circinalis* CS-139 generated total GSM concentrations ranging from 21.3 μg L^{-1} (on day 4) to 266.8 μg L^{-1} (on day 28). These concentrations represent the total GSM pool composed of intracellular, expected to represent 95-99% of the total GSM in *A. circinalis*, (see Li et al. 2012), and extracellular GSM. This GSM pool will be fully available to fish upon cell lysis mostly due to culture aging or other factors

(excessive light, temperature etc.). Given that GSM concentrations of 15.0 µg L^{-1} are typically not exceeded in Australian barramundi aquaculture ponds or in waters and ponds globally (Van der Ploeg et al., 1992; Zimba & Grimm, 2003; Exley, 2014), cultures of *A. circinalis* represent a sufficient natural source of GSM for large-scale tainting and depuration experiments, in line with concentrations applied in most studies on uptake and depuration (Dionigi et al., 2000; Yamprayoon & Noomhorm, 2000; Robertson et al., 2005).

To evaluate the utility of various culture parameters as convenient indirect methods with which to estimate GSM production, correlations between several biomass proxies (OD_{750nm}, cell concentration, AFDW and chl-*a*) and concentrations of GSM in *A. circinalis* cultures, measured using GC-MS (Fig. 3.2), were examined. All culture biomass parameters showed exponential positive correlations with GSM concentrations in *A. circinalis* cultures, which are in agreement with previous studies. For example, Su et al. (2013) demonstrated that cell density and total GSM concentration are tightly correlated over the initial growth phase of the culture. Similarly, quantitative PCR analysis has also showed a positive correlation between GSM concentration and the number of copies of the GSM synthase; one gene (GSG) required for GSM biosynthesis in *Anabaena* sp. (Su et al., 2013; Tsao et al., 2014).

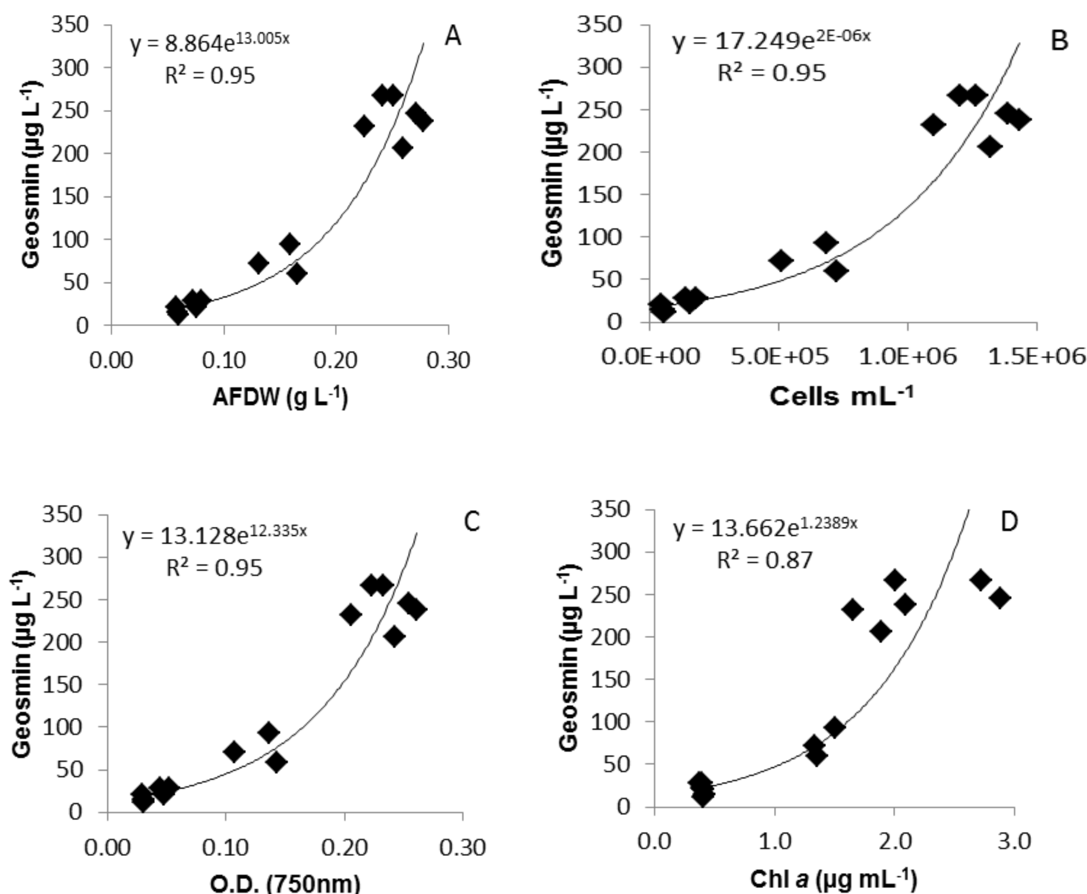

Fig. 3.3 Correlation between biomass proxies, ash-free dry weight (AFDW), cell concentrations, optical density at 750 nm (O.D. $_{750nm}$) and chlorophyll *a* (Chl *a*), and total geosmin concentration in *A. circinalis* cultures

While any of the direct and indirect biomass predictors used here could potentially be employed to estimate GSM concentrations of *A. circinalis* cultures with a sufficient degree of accuracy, Chl-*a* (μg mL $^{-1}$) was the least suitable (R^2=0.87), with correlation noticeably reduced at GSM concentrations exceeding 100 μg L^{-1}. Cell density (R^2=0.95) and AFDW (R^2=0.95) were satisfactory indirect measures of GSM concentration, however these measures are somewhat time-consuming with protracted turn-arounds of ~2 h (cell density) and ~24 h (AFDW). In contrast, turbidity (O.D.$_{750\ nm}$), often employed as a rapid measure of biomass in cultures of microorganisms (Shuler & Kargi 2005), was the best indirect measure of GSM concentration (R^2=0.95), due to its simplicity and capacity to provide almost real-time (minutes)

estimations of GSM concentration. This is an essential consideration, as GSM production is highly dynamic and concentrations of this off-flavour taint can be rapidly reduced as a result of volatilisation and/or biodegradation (Zhou et al., 2011; Li et al., 2012). OD_{750nm} also correlated strongly with the other biomass proxies (cell concentrations, AFDW) (Fig. 4), which could thus be easily inferred from simple OD_{750nm} measurements.

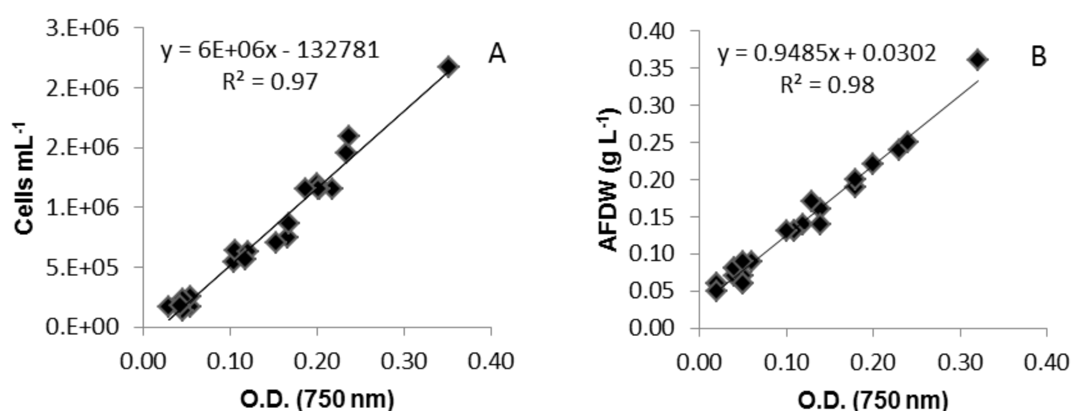

Fig. 3.4 Correlation between optical density (OD_{750nm}) and biomass parameters, cell concentrations and ash-free dry weight (AFDW), in *A. circinalis* cultures.

3.4 Conclusion

In summary, the results of this study represent a simple yet precise, reproducible and low-cost procedure for producing natural GSM that is readily taken up and accumulated by fish, imparting off-flavour tainting that is identical to that occurring in farming situations. This approach can now be used to manipulate the GSM content of fish tissues and the intensity of off-flavour tainting, assisting further research into the mechanisms of off-flavour tainting in farmed fish. This method, using natural GSM produced by *A. circinalis* to intentionally induce off-flavour tainting in fish, together with GC-MS analysis of GSM in flesh will aid in the development of accurate detection thresholds of GSM in different fish species, understanding the quantitative

relationships between perceived off-flavour intensity and the concentration of GSM in water and/or in fish tissues, and exploring various factors of fish size, species, holding temperature, activity levels and lipid content on rates of uptake and elimination of GSM induced off-flavour taints.

3.5 References

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CHAPTER 4

Uptake, depuration and spatial distribution of the off-flavour tainting compound geosmin in farmed barramundi, *Lates calcarifer*.

Preface

Drawing on the methods presented in Chapter 3, which detailed a reliable strategy for generating off-flavour tainted fish on demand, at pre-determined levels of taint, Chapter 4 presents an investigation of various aspects of off-flavour tainting in farmed barramundi. The pattern of uptake and loss of GSM in barramundi tissue is presented while the potential to eliminate off-flavour tainting by depurating fish in clean water is detailed. The spatial distribution of GSM within the white muscle fillet is also discussed with respect to mitigating the detrimental effects of off-flavour tainting.

The paper presented in this chapter was submitted to *Aquaculture* on 14/12/15 under the title:

Uptake, depuration and spatial distribution of the off-flavour tainting compound geosmin in farmed barramundi, *Lates calcarifer.*

The contributions of co-authors to the submitted manuscript are presented in Table 1.3.

4.1 Introduction

Off-flavour tainting of freshwater finfish is a global problem and can result in significant economic loss to both wild-harvest fisheries and aquaculture producers (Engle et al., 1995; Tucker, 2000). The most common off-flavour taint is the muddy-earthy flavour discussed in Chapters 2 and 3 of this thesis, which typically renders fish unpalatable and unfit for sale (Lovell, 1983; Yamprayoon & Noonhorm, 2000; Robertson et al., 2005; Percival et al., 2008; Petersen et al., 2011). Off-flavour tainting is recognised as a significant barrier to the growth and viability of the Australian barramundi aquaculture industry as well as several other freshwater finfish industries globally (Engle et al., 1995; Tucker, 2000 Robertson et al., 2006; Burr et al., 2012; Carton & Jones, 2013) and consequently remains a primary focus for many freshwater aquaculture sectors.

Muddy-earthy off-flavour tainting of cultured fish is acknowledged most frequently as originating from two compounds, geosmin (GSM) and/or 2-methylisoborneol (MIB), as previously discussed (Yurkowski & Tabachek, 1974; Persson, 1980; Lovell, 1983; Lovell & Broce, 1985; Tucker, 2000; Howgate, 2004; Robertson et al., 2005; Schrader, 2005; Vallod et. al., 2007; Guttman & van Rijn, 2008; Percival et al., 2008; Carton & Jones, 2013). Both GSM and MIB are produced by microbes in aquatic systems (Paerl & Tucker, 1995; Tucker, 2000; Smith et al., 2008). When fish are exposed to these compounds, they are passively absorbed, primarily across the gills, and enter the bloodstream (From & Hørlyck, 1984). Both compounds are acknowledged as being highly lipophilic and following uptake typically become concentrated in lipid rich tissues (Howgate, 2004).

Strategies to mitigate off-flavour tainting that have been assessed include pond management techniques aimed at preventing the occurrence of off-flavour

contaminants (Schrader, 2005; Tucker, 2006; Krishnani et al., 2008; Exley, 2014), the detection and exclusion of off-flavour tainted cohorts of fish prior to harvest (Johnsen & Kelly, 1990; Van der Ploeg, 1991; Tucker, 1999) and depurating off-flavours by removing tainted fish to clean, taint-free water prior to harvest (Dionigi et al., 2000; Yamprayoon & Noomhorn, 2000; Howgate, 2004; Robertson, 2005; Percival et al., 2008; Davidson et al., 2014).

Depuration is the practice of placing fish in clean water free of off-flavour compounds for a period of time, over which taint compounds are passively lost or purged from tissues. The efficacy of depuration for many aquaculture species, especially tropical species is however unclear (Tucker, 2000). Rates of uptake and depuration can be highly variable and influenced by the tainting compound responsible as well as a number of environmental, physiological and species-specific factors (Neely, 1979; Clarke et al., 1990; Streit, 1998; Howgate, 2004). As such, the efficacy of depuration practices must be assessed on a species-specific basis, for each given tainting compound and under environmental conditions that most often prevail during production.

Barramundi, (*Lates calcarifer*) is an important aquaculture species in tropical and sub-tropical regions with a total global aquaculture production of ~164,000t per annum (FAO, 2012b). Australia produces approximately ~4000t per annum (Savage & Hobsbawn, 2015), the majority of which is cultivated in land based earthen ponds. In tropical northern Australia pond-reared barramundi are periodically affected by episodes of off-flavour tainting as shown in Chapter 2 (Percival et al., 2008; Carton & Jones, 2013). In Chapter 2 it was demonstrated that moderate off-flavour tainting episodes occur frequently, with the majority of off-flavour episodes involving GSM concentrations of up to ~2.0μg L^{-1}. However, Chapter 2 also highlighted the

occurrence of extreme off-flavour tainting episodes during which GSM concentrations were shown to reach a remarkable 14.36µg L^{-1}. MIB has also been implicated in off-flavour tainting episodes of tropical barramundi reared in floating cages in Lake Argyle, north-western Australia (Percival et al., 2008). Despite this, MIB was not detected in pond water at any time in the current research program, a finding that is consistent with that of Exley (2014).

In Chapter 2, it was shown that off-flavour tainting of barramundi occurs within 24 hours following exposure to water containing GSM. However the uptake and deposition of GSM into muscle tissue over the period of exposure is currently unknown. It is also unknown if the level of GSM in muscle tissue continues to increase beyond 24 hours, while the time required for GSM in tissue to reach a steady state with exposure water is also unresolved. In addition, management tools designed to mitigate off-flavour tainting remain largely unexplored for tropical species. Exley (2014) has highlighted a number of pond management strategies aimed at eliminating GSM producing microbes from culture waters in northern Australia, however these procedures were not considered reliable.

The relative abundance of lipid in tissues is also known to influence the amount of GSM and MIB that is accumulated (Grimm et al., 2004; Grimm et al., 2015; Howgate, 2004; Martin et al., 1988), which in turn affects the intensity of off-flavour tainting (Howgate et al., 2004; Jones et al., 2013). Spatial differences in off-flavour tainting intensity of barramundi tissue has previously been reported for MIB, with the most intense off-flavour taint occurring in the lipid rich ventral belly region of the fillet (Percival et al., 2008). Spatial analysis of channel catfish (*Ictalurus punctatus*) fillets has demonstrated similar findings with the spatial distribution of off-flavour compounds being closely aligned with spatial variations in the lipid content of the

fillet (Grimm et al., 2015). The spatial distribution of tainting compounds in fillets is a critical consideration for producers seeking to identify off-flavour tainted fish prior to harvest. This also has implications for post-harvest processing of fish as removal of the most heavily tainted portions prior to sale would reduce the incidence of off-flavour tainting at the point of consumption. At present the spatial distribution of GSM deposition in barramundi remains unknown.

As GSM has been identified as the primary off-flavour tainting compound in pond-reared barramundi (Exley, 2014), it is therefore critical that uptake, depuration and the spatial distribution of GSM within barramundi fillets be quantified. Such knowledge is fundamental if researchers, farmers and processors are to develop strategies that mitigate the detrimental effects of off-flavour tainting in farming situations.

The objectives of this study were to; 1) determine the efficacy and time course of depuration to recover the flavour quality of barramundi following exposure to a moderate level (2.15µg L^{-1}) of GSM, this represents the level most frequently observed in earthen pond culture across tropical Australia, 2) investigate the uptake and loss of GSM in barramundi white muscle tissue following exposure to an extreme (15.1µg L^{-1}) concentration of GSM, and 3) identify the spatial distribution of GSM within barramundi fillets and determine the relationship between crude fat content and GSM deposition. These objectives were investigated by conducting two separate experiments. The first used organoleptic assessment to evaluate the efficacy of depuration to recover flavour quality in fish while the second experiment employed gas chromatography-mass spectrometry (GC-MS) analysis to investigate the uptake, loss and spatial distribution of GSM in muscle tissue.

4.2 Materials and Methods

4.2.1 Production of GSM

GSM was produced by preparing laboratory cultures of *Anabaena circinalis* as detailed in Chapter 3. Briefly, the cyanobacterial strain *A. circinalis* (CS-139), obtained from the CSIRO Microalgae Supply Service, Australian National Algae Culture Collection (Hobart, Australia) was kept in continuously aerated 2 L Schott bottles containing BG11 culture medium (Rippka et al., 1979), at 24 °C and under continuous illumination of 15 µmol photons m^{-2} s^{-1} until used for experiments. Cultures were then diluted into holding tanks until the desired concentration of GSM was achieved.

4.2.2 Experiment 1. Efficacy of depuration to recover flavour quality

4.2.2.1 Fish treatment groups

Market sized barramundi (1.5-2.0 kg, n=24) were sourced from a commercial aquaculture facility (Good Fortune Bay Fisheries, Kelso, Australia) and returned to the Marine and Aquaculture Research Facilities Unit (MARFU), James Cook University. Untainted barramundi were prepared by holding fish for 28 days in a single 10,000L fibreglass tank supplied with continuous flow-through water (27°C) known to be free of off-flavour tainting compounds. During this period fish were hand fed a commercial diet (10mm floating pellet, Ridley Agriproducts Pty Ltd. Aquafeed, Narangba, Australia) once daily to satiation. Following this holding period, 12 fish were randomly selected, euthanised by ice emersion, filleted and the anterior-dorsal section (Fig. 4.1) of the fillet removed, and stored at $^{-}$18°C prior to sensory assessment. To confirm that GSM and MIB were not present in holding water duplicate 50mL water samples were collected at the conclusion of the 28 day holding

period and stored at -18°C prior to GC-MS analysis as previous described in Chapter 2.

The remaining fish (n=12) were then intentionally tainted by exposing fish to a moderate level (2.15µg L^{-1}) of GSM, produced as outlined above (4.2.1 Production of GSM), by diluting laboratory cultures of *A. circinalis* directly into a single aerated 2000L fibreglass tank (27°C). This level of GSM represents the upper limit of the most frequently occurring GSM concentrations observed during water sampling (Chapter 2). All fish (n=12) were transferred to this tank and held for a period of 48 hours. Although the approach of using a single tank for the uptake of GSM could be regarded as pseudo-replication, this ensured that all fish within the group experienced identical environmental and behavioural conditions (temperature, water flow, physical activity, GSM concentration, etc.) over the duration of the uptake period (see Chapter 2). Water was not exchanged during this period so as to facilitate the uptake of GSM and impart off-flavour taint. As outlined above, duplicate water samples (50ml) for GC-MS analysis for GSM and MIB were collected from the GSM uptake tank after 0, 24 and 48 hours to determine the level of GSM/MIB to which fish were exposed. At the conclusion of the 48 hour uptake period, fish were randomly assigned to one of three 500L fibreglass depuration tanks (n=4 fish per tank). These tanks were supplied with continuous flow through charcoal filtered municipal water known to be free from off-flavour tainting compounds, tanks were also aerated by gentle air diffusion. During depuration water was maintained at 27°C, which is equivalent to water temperatures observed during normal barramundi production (Glencross & Bermudes, 2012). A single fish was removed, euthanised and processed as outlined above from each tank after 2, 4, 8 and 14 days of depuration. Feed was withheld during the depuration period.

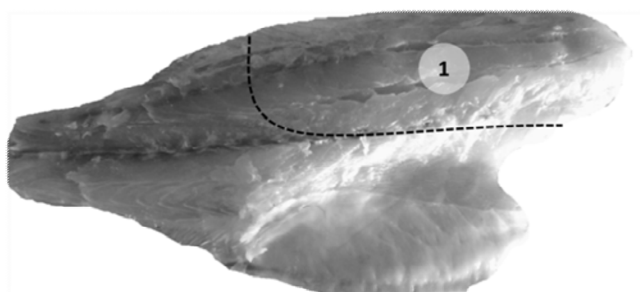

Fig. 4.1 (1) Anterior dorsal section of barramundi fillet used for GCMS analysis of GSM and MIB and for sensory assessment.

4.2.2.2 Organoleptic assessment of fish

Human assessors were used to assess the efficacy of each depuration period (2, 4, 8 and 14 days) in recovering flavour quality of off-flavour tainted fish (GSM 2.15 µg L^{-1}).

Initially, assessors experienced in the sensory assessment of farmed reared barramundi were used to confirm that fish were untainted prior to GSM exposure. Six panellists, initially chosen from a group of 21, were trained to distinguish muddy-earthy off-flavour tainting in farm-reared barramundi. Training consisted of presenting individuals with samples of off-flavour tainted barramundi, sourced from a local barramundi farm experiencing an episode of off-flavour tainting and wild barramundi caught from marine habitats, free of off-flavour tainting compounds. This allowed panellists to re-familiarise with the precise nature of muddy-earthy off-flavour tainting in farm-reared barramundi. Assessors were presented with sub-samples of tissue (20g) from fish (n=12) held in off-flavour taint free water for 28 days (as outlined above) and asked if muddy-earthy off-flavour was detectable. Each panellist assessed a single portion from each of the 12 fish from the taint-free group such that each panellist assessed a portion from each fish.

Triangle tests were then used to identify the time required to recover flavour quality following a moderate (GSM 2.15 µg L^{-1}) off-flavour tainting episode. Sequential triangle tests were performed on fish that had been depurated for 2, 4, 8 and 14 days. Tests were carried out according to the Australian standard for sensory analysis (AS 2542.2.2-2005 Sensory analysis - Specific methods - Triangle test; ISO 4120:2004). Each panellists was presented with a set of three 20 g portions removed from the anterior dorsal region of the fillet (Fig. 4.1). Each set contained either; 1) two untainted and one depurated portion, or 2) one untainted and two depurated portions.

Cooked barramundi portions were prepared by wrapping individual portions in aluminium foil and cooking in a fan-forced oven for 10 minutes at 200°C. Samples were only identifiable by a randomly generated three digit blind code.

4.2.3 Experiment 2. Uptake, loss and spatial distribution of GSM in muscle tissue

4.2.3.1 Uptake and loss of GSM in muscle tissue

The uptake and deposition of GSM into the muscle tissue of fish exposed to an extreme (15.1 µg L^{-1}) off-flavour tainting episode was investigated over a 48 hour period. Market sized barramundi (1.5-2.0 kg, n=48) were again sourced from a commercial aquaculture facility (Good Fortune Bay Fisheries, Kelso, Australia) and returned the Marine and Aquaculture Research Facilities Unit (MARFU), James Cook University and held for 28 days as described above (4.2.2.1 Fish treatment groups). At the conclusion of this period, 3 fish were collected from the holding tank and euthanized as outlined above, the remaining 45 fish were then transferred into a single 2,000L fibreglass tank containing diluted cultures of *A. circinalis* as described previously. Three fish were selected at random from the tank after 0.05, 0.5, 3, 12, 24 and 48 hours of exposure and euthanized as previously described. Fish were filleted

within 30 minutes of being euthanized and the anterior dorsal section (Fig. 4.1) was removed and frozen (-18°C) prior to GC-MS analysis for GSM and MIB. Duplicate water samples (50ml) were also collected as previously described at each sampling point during the 48 hour exposure period and frozen (-18°C) prior to GC-MS analysis.

At the conclusion of the 48 hour uptake period the remaining 27 fish were removed and randomly assigned to one of three 500L fibre glass tanks (n=9 fish per tank) to investigate the loss of GSM from tissue. Tanks were supplied with flow through dechlorinated municipal water known to be free of off-flavour tainting compounds. A single fish was removed from each tank after 6, 12, 24, 48, 96, 192 and 336 hours, euthanized by ice emersion and filleted. The anterior dorsal portion of each fillet was then removed, frozen and stored (-18°C) prior to GCMS analysis. Water samples were collected as detailed above, at each sampling time for GC-MS analysis of GSM and MIB levels.

4.2.3.2 Spatial distribution of GSM and crude fat within fillets

To quantify the spatial distribution of GSM and its association with crude fat in the fillet, instrumental analysis was used to measure crude fat and GSM in the fillets of fish that were collected from depuration tanks after 12 and 24 hours (n=3). Fish were euthanized by ice emersion, filleted and the fillets then separated to yield three distinct portions; dorsal shoulder, posterior tail and ventral belly (Fig. 4.2). These portions were selected to determine differences in GSM and crude fat levels in three spatially isolated regions of the fillet. Fillet portions were frozen and stored (-18°C) prior to instrumental analysis for GSM, MIB and crude fat.

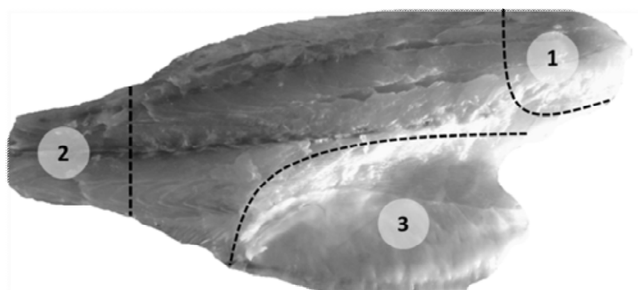

Fig. 4.2 Various fillet regions selected to assess the spatial distribution of GSM and MIB in fillet tissue. (1) Dorsal shoulder, (2) Posterior tail, (3) Ventral belly.

4.2.3.3 Instrumental Analysis for GSM and MIB by GC-MS

Water samples were prepared according to the methods presented in section 2.2.2 with menthol used in place of tetramethylpyrazine as the internal standard to improve analytical precision.

Fish samples were prepared according to the methods presented in section 2.2.4 with the exception that menthol was used as the internal standard.

Water samples and flesh extracts were analysed for GSM and MIB according to the methods detailed in section 2.2.6. Sample analyses were performed by Innovative Food Technologies, Department of Agriculture, Fisheries and Forestry, Brisbane, Australia.

4.2.3.4 Determination of crude fat in fillet regions

Samples were analysed for crude fat by the Lincoln Marine Science Centre (Port Lincoln, School of Biological Sciences, Flinders University) by ethyl acetate extraction - gravimetric determination based on the Norwegian Standard Method (NS 9402 E) (NSA 1994) (D'Antignana et al., 2012). Immediately prior to analysis, frozen, minced flesh samples were allowed to partially thaw at 19°C. A weighed sample of this homogenised flesh (~10 g), 40 g of anhydrous sodium sulphate and 80 mL of ethyl acetate were placed into a clean polypropylene bag which was agitated in

a stomacher mixer (IUL Instruments) for a period of three minutes. The resulting extract was filtered (Whatmans GF/C filter papers). A 40 mL volume of the filtrate was decanted into a pre-weighed plastic beaker and placed in a fume hood overnight. After this period, all the solvent had evaporated. The beaker, containing the filtrate, was placed in an oven set to 80°C for approximately 60 minutes. This removed any traces of moisture. The beaker was then weighed to determine the fat weight (± 0.001 g).This was expressed as a percentage of the muscle wet weight (g) using the following formula:

$$Crude\ fat\ content\,\% = \frac{\left(\frac{Extracted\ fat\ (g)}{Dilution\ Factor}\right)}{Extracted\ Muscle\ wet\ wt\ (g)}$$

4.2.4 Statistical analysis

One-way ANOVA was used to analyse the results obtained for GSM concentration and crude fat content of fish. Data homogeneity and normality were assessed graphically. The significance of the relationship between crude fat and GSM concentration was determined by ANOVA. The level of significance was defined at $p<0.05$. All results are reported as the mean ± the standard error of the mean (SEM).

4.3 Results

4.3.1 Experiment 1. Efficacy of depuration to recovery flavour quality

Human sensory assessment was used to validate that fish were initially free of muddy-earthy off-flavour taint prior to tainting treatments. Assessors experienced in the sensory evaluation of farmed barramundi were unable to detect muddy-earthy flavour in any of the samples assessed, thereby confirming that experimental fish were untainted prior to GSM exposure. GC-MS analysis of water samples taken from the

holding tank complemented this result, neither GSM nor MIB being detected in holding water.

Water samples from the uptake tank confirmed that fish were exposed to a GSM concentration of 2.15±0.02μg L^{-1} during the uptake period. MIB was not detected in water samples at any time.

Sensory assessment of fish by triangle testing revealed that the flavour of barramundi depurated for 2 (p=0.043) and 4 days (p=0.013) was different from untainted barramundi. Participants reported tainted samples as having muddy, earthy and weedy flavour characteristics. In contrast, participants reported that the flavour of barramundi after 8 (p=0.22) and 14 days (p=0.52) of depuration was not significantly different to the flavour of untainted barramundi (Table 4.1). This indicates that off-flavour tainting was eliminated from fish after 8 days of depuration following a moderate (2.15μg L^{-1}) tainting episode.

Table 4.1. Results of triangle testing performed on barramundi depurated for 2, 4, 8 and 14 days. p values were calculated using the formula: p = 1-BINOMDIST(x - 1,n,(1/3),True) where x=the number of correct responses and n=the total number of responses.

Days of depuration	Correct responses/ total responses	*p value*	Result	Interpretation
2	**10/18**	**0.043**	**Treatments are different**	**Fish are off-flavour tainted**
4	**16/29**	**0.013**	**Treatments are different**	**Fish are off-flavour tainted**
8	**8/18**	**0.22**	**Treatments are not different**	**Fish are not off-flavour tainted**
14	**7/20**	**0.52**	**Treatments are not different**	**Fish are not off-flavour tainted**

4.3.2.1 Experiment 2. Uptake and loss of GSM in tissue

The uptake and loss of GSM in tissue was measured by exposing fish to a GSM concentration approximately equal to the upper limit of concentrations known to

occur in a pond culture situation (See Chapter 2). Analysis of water samples from the uptake tank revealed that fish were exposed to a GSM concentration of 15.1±0.55µg L^{-1} during the uptake period.

The uptake of GSM into muscle tissue occurred rapidly with a concentration of 0.98±0.54µg kg^{-1} recorded after only three minutes of exposure. The concentration of GSM in tissue continued to increase over time, eventually reaching a maximum concentration of 8.80±1.88µg kg^{-1} after three hours exposure. Following this, no further increase in tissue GSM was observed for the duration of the exposure period. After 48 hours of exposure, a final tissue concentration of 8.25±0.62µg kg^{-1} was observed (Fig. 4.3), this was not significantly different (ANOVA, $p<0.05$) from the concentration recorded at 3 hours.

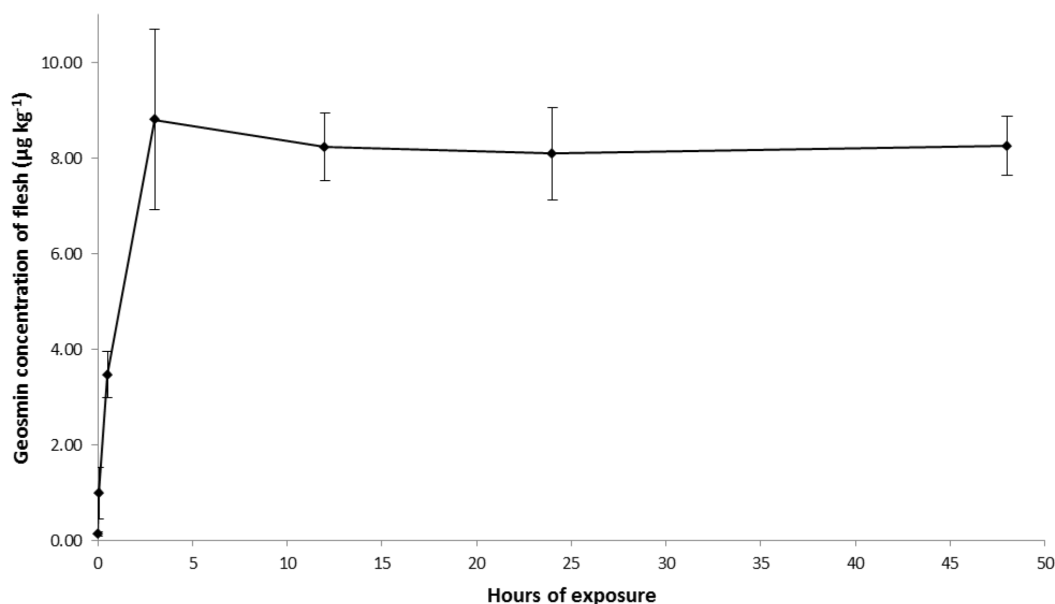

Fig. 4.3 Uptake of GSM in the flesh of barramundi (n = 3 fish at each sampling time) exposed to a GSM concentration of 15.1±0.55µg L^{-1}. Bars represent standard error of the mean.

When removed to GSM free water, depuration was considerably more protracted than uptake. Loss of GSM from muscle tissue was most rapid during the first 4 days of depuration, with tissue GSM reducing by approximately half the pre-depuration

value, from 8.25±0.62μg kg^{-1} to 3.44±0.49μg kg^{-1}. Following this, depuration slowed dramatically with 0.77±0.32μg kg^{-1} GSM remaining in the tissue after 14 days of depuration (Fig. 4.4). The loss of GSM during the depuration period was described by the equation:

$$y = 8.25e^{-0.007x}$$

where y is the concentration of GSM in the tissue, and x is the time in hours since fish were returned to clean water free of off-flavour taint. Using this equation, the half-life of GSM in tissue was approximately 4 days (99 hours). Analysis of water samples from depuration tanks confirmed that GSM was not present above trace levels (0.01±0.003μg L^{-1}).

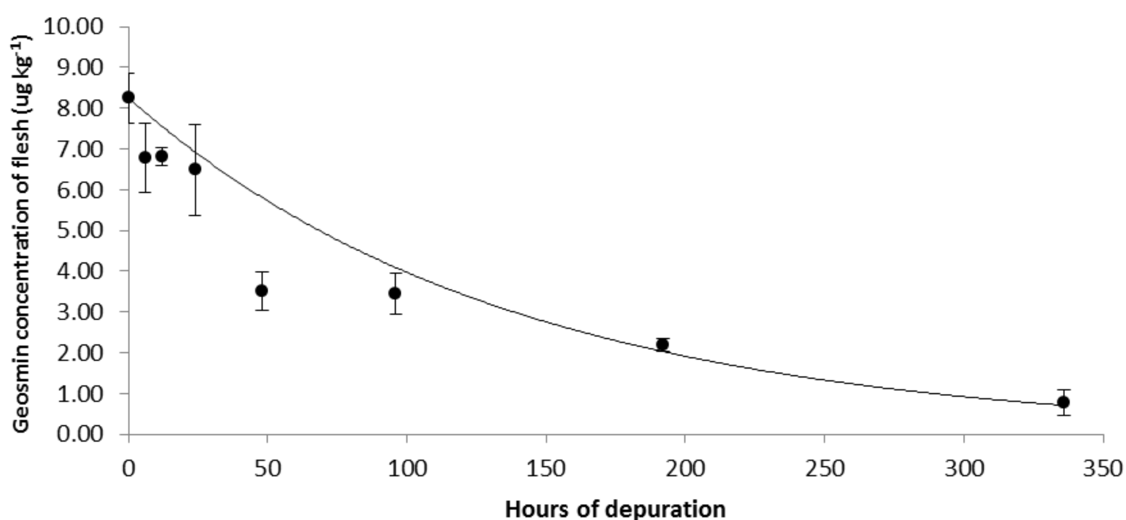

Fig. 4.4 Loss of GSM from barramundi flesh (n = 3 fish at each sampling time) following transfer to water known to be devoid of GSM. Line represents least squares regression (R^2 = 0.92; $y = 8.25e^{-0.007x}$). Bars represent standard error of the mean.

4.3.2.2 Spatial distribution of GSM and crude fat within fillets

A clear spatial pattern of crude fat distribution was observed within the fillet. The ventral belly region was significantly higher than either the dorsal shoulder or posterior tail regions (ANOVA $p<0.05$), comprising of 25.61±1.80% crude fat, in comparison to 3.26±0.74% and 4.61±1.09%, respectively (Fig. 4.5). A comparable

spatial distribution of GSM was also observed within the fillets of off-flavour tainted fish. The ventral belly region was significantly higher (ANOVA, $p<0.05$) than either the dorsal shoulder or posterior tail regions, containing 16.88±0.29µg kg^{-1} GSM, in contrast to 5.24±1.42µg kg^{-1} and 5.97±1.57µg kg^{-1}, in the dorsal shoulder and posterior tail regions (Fig. 4.5).

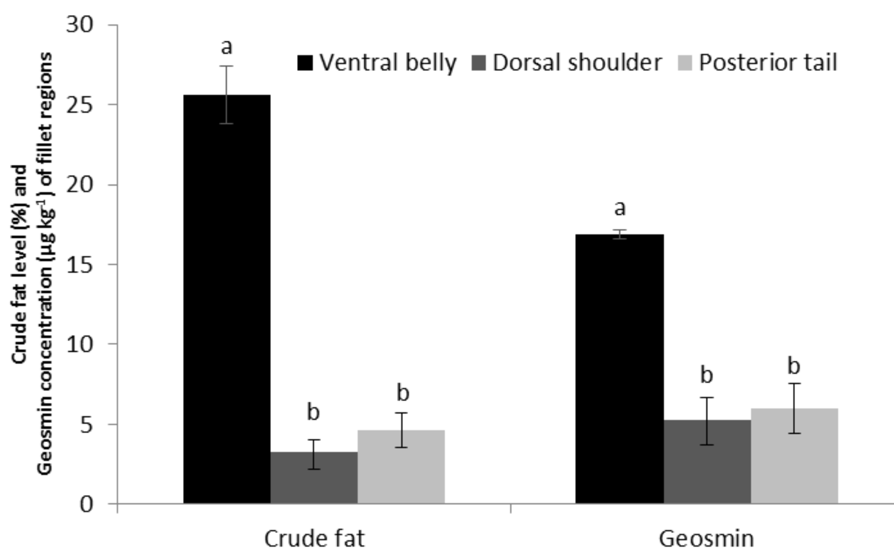

Fig. 4.5 Spatial distribution of crude fat and GSM in barramundi fillets (n = 3). Black bars represent crude fat and GSM concentration of ventral belly portion, dark grey represents dorsal shoulder region and light grey represents posterior tail region. Error bars represent standard error of the mean. Letters denote significant differences between the regions for crude fat or GSM respectively.

GSM concentration was positively related to crude fat such that a significant (ANOVA, $p<0.05$) relationship was observed between GSM tissue concentrations and crude fat levels across the three fillet regions examined (R^2 = 0.93) (Fig. 4.6).

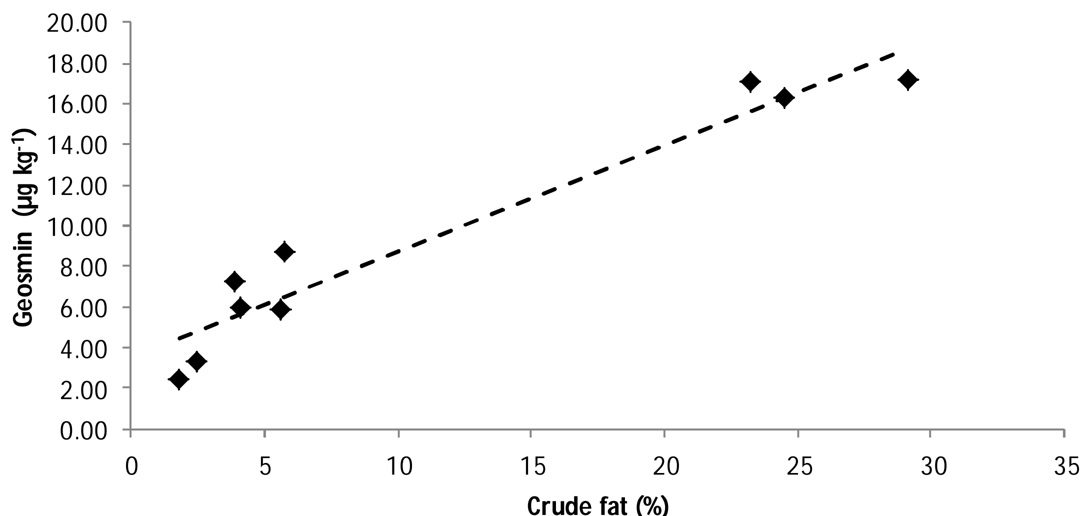

Fig. 4.6 Relationship between GSM concentration and crude fat level as measured in barramundi flesh. Dashed lines represent least squares regression (R^2 = 0.93; y = 0.52x + 3.55).

4.3.2.3 Effect of depuration time on GSM and crude fat within the fillet regions

After 24 hours of depuration, the concentration of GSM in the ventral belly region was significantly reduced (ANOVA, p<0.05) from the level observed after 12 hours of depuration, decreasing by 35% from 16.88±0.29µg kg^{-1} to 10.97±1.94µg kg^{-1}. A marginally higher loss of GSM was observed over the same period in the dorsal shoulder and posterior tail regions, declining by 40%, from 5.24±1.42µg kg^{-1} to 3.12±1.04 µg kg^{-1}, and 39%, from 5.97±1.57µg kg^{-1} to 3.69±0.76µg kg^{-1}, respectively (Fig. 4.7), although the loss of GSM in these regions was not statistically significant (ANOVA, p>0.05). Depuration time did not affect (ANOVA, p>0.05) crude fat levels with the ventral belly, dorsal shoulder and posterior tail regions being composed of 25.61±1.80%, 3.26±0.74% and 4.61±1.09% crude fat respectively after 12 hours depuration and 24.54±5.59%, 4.11±0.86% and 5.67±1.74% crude fat respectively following 24 hours depuration.

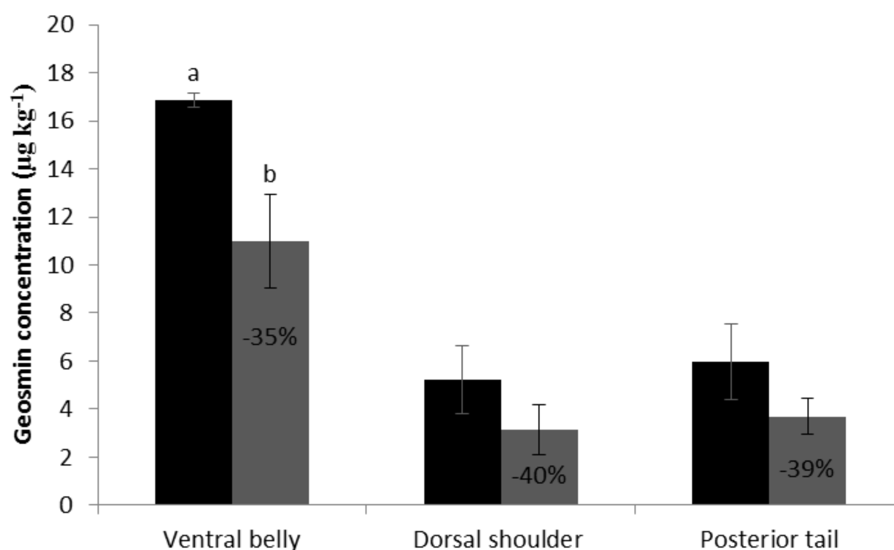

Fig. 4.7 GSM concentration in the various fillet regions during depuration (n = 3). Black bars represent the concentration of GSM present after 12 hours of depuration, grey bars represent the concentration of GSM present after 24 hours of depuration. Bars represent standard error of the mean. Letters denote significant differences in GSM concentration between 12 and 24 hours of depuration. Labels indicate the fraction of GSM lost from each fillet region between 12 and 24 hours of depuration.

4.4 Discussion

4.4.1 Organoleptic effects of depuration

To determine if depuration was effective in recovering flavour quality in tropical farmed barramundi, fish were exposed to 2.15µg L^{-1} GSM prior to depuration in clean water. This concentration broadly represents the most frequently occurring GSM concentrations in barramundi ponds in tropical northern Australia (Exley, 2014). Human sensory assessment revealed that off-flavour tainting persisted after 2 and 4 days of depuration ($p<0.05$) with muddy-earthy and weedy off-flavours still being present. However the panel was unable to differentiate fish that had been depurated for 8 or 14 days from untainted fish ($p>0.05$). This result clearly indicates that 8 days of depuration is sufficient to eliminate off-flavour taint from fish that have been exposed to a moderate level of GSM.

Despite this, the time required to eliminate off-flavour tainting in a farming situation will most likely be affected by a number of environmental and physiological factors. These include the initial concentration of GSM present in tissue, the lipid content of tissues, fish size, water temperature, metabolic rate and other species-specific factors such as gill structure and ventilation rate (Neely, 1979; Clarke et al., 1990; Streit, 1998; Howgate, 2004). These factors complicate attempts to accurately predict the depuration time required to eliminate off-flavour tainting. Howgate (2004) summarises a number of numerical models that can be used to predict the uptake and depuration of GSM and MIB by fish. Whilst uptake and depuration can be predicted relatively well from these models, Howgate (2004) highlights that, the data used to assemble such models is small and does not adequately explore a range of factors including fish size, species, holding temperature, activity and, importantly, lipid content on rates of uptake and elimination of tainting compounds. As such, the time period of 8 days observed to eliminate muddy-earthy off-flavour taint in this study should be carefully considered as it does not adequately account for many of the aforementioned factors. The results do however highlight that following exposure to a moderate and frequently encountered level of GSM (≤2.15μg L^{-1}), off-flavour tainting was eliminated from market sized (1.5-2kg) barramundi after 8 days of depuration at a constant temperature of 27°C.

4.4.2 Uptake and loss of GSM in tissue

Intense off-flavour tainting episodes in barramundi grow-out facilities in tropical northern Australia are reported in Chapter 2 and supported by the findings of Exley (2014) with water-GSM concentrations in grow-out ponds observed to reach 14.36μg L^{-1}(See Chapter 2). To explore the uptake and loss of GSM from tissues under such extreme conditions, fish were exposed to 15.1μg L^{-1} GSM. Under such conditions

uptake was exceedingly rapid with a significant increase in flesh GSM observed after only three minutes of exposure. The GSM concentration observed at this point was above the threshold of human sensory detection which has been estimated to be ~0.74μg kg^{-1} for barramundi (Chapter 2), and would likely impart a discernible off-flavour taint. Following this initial period, uptake continued, reaching a maximum concentration after 3 hours, with no further increase in GSM concentration observed. GSM in fish tissue reached a peak value of 8.8μg kg^{-1} after three hours. At this concentration, intense off-flavour tainting would clearly be expected given that muddy-earthy flavour and weedy aftertaste is known to be tightly correlated with the concentration of GSM in the tissue of farmed barramundi (Chapter 2). The uptake of GSM to above the threshold of detection in such a short period of time is a concern to producers and highlights the potential for off-flavour tainting to occur after only minutes of exposure.

GSM is lost from fish tissue upon returning off-flavour tainted fish to clean, taint free water. Understanding the dynamics of GSM loss from tissue would enable growers to have some predictive ability with regard to the depuration time required for fish to recover flavour. In the present study, depuration was most rapid during the first four days, with approximately half of the GSM being lost in this time. Beyond this, depuration slowed and after 14 days 0.77μg kg^{-1} GSM still remained, which is marginally above the assumed threshold of sensory detection in barramundi flesh (as discussed in Chapter 2). The loss of GSM from barramundi tissue during depuration was best defined by exponential decay (Fig. 4.4) which is typical for GSM depuration in fish (Howgate, 2004; Robertson et al., 2005). During depuration of market sized fish (1.5-2.0kg), at 27°C, a half-life for GSM in tissue of approximately 4 days was observed.

In general the uptake of GSM by barramundi was initially rapid and plateaued after 3 hours, in contrast, depuration was protracted, a trend consistent with other studies. The uptake and depuration of GSM by rainbow trout, observed by Robertson et al. (2005), was remarkably similar to that observed in this study. Similar patterns of rapid uptake and prolonged depuration of off-flavour compounds have also been observed in channel catfish (Lloyd & Johnsen, 1992; Martin et al., 1988) and tilapia (Yamprayoon & Noomhorn, 2000).

4.4.3 Spatial distribution of GSM and the importance of crude fat content in barramundi fillets

Following uptake, a distinctive pattern in the spatial distribution of GSM within fillets was observed. The shoulder and tail sections were similar in this respect, containing 5.24μg kg^{-1} and 5.97μg kg^{-1} GSM, respectively. The belly section however had a significantly higher concentration of GSM reaching 16.88μg kg^{-1} (Fig. 4.5). This would cause a dramatic reduction in flavour quality in this location with muddy-earthy and rotting weed flavours known to become more intense with increasing GSM concentration, as discussed in Chapter 2. This clarifies the previous findings of Percival et al. (2008) who observed that off-flavour tainting was most perceptible in the belly region of barramundi fillets. Crude fat analysis revealed a similar trend to that observed for GSM accumulation. Whilst the shoulder and tail sections were very similar (3.26% and 4.61 % respectively), the belly section was higher in crude fat (25.6%) (Fig. 4.5). GSM concentration was tightly correlated with crude fat level (R^2=0.93, Fig. 4.6). This is unsurprising given that GSM is lipophilic, however the effect of lipid content on spatial distribution of GSM within the fillet has only previously been investigated in channel catfish where GSM and MIB concentrations are known to be more elevated in the portion of the fillet nearest the head, with

concentrations declining towards the tail end of the fillet (Zimba & Grimm, 2015) and elevated in skin sections, associated with the presence of sub-cutaneous lipid (Grimm et al, 2015). MIB tainting has also been correlated with muscle lipid, with fish > 2.5% muscle lipid accumulating approximately ~3 times more MIB than fish with < 2% lipid (Grimm et al., 2004).

It has previously been suggested that due to the relationship between GSM accumulation and lipid content that the production of leaner fish could potentially lower the concentration of off-flavour tainting compounds (Lloyd & Johnsen, 1992; Dionigi et al., 1998; Robertson et al., 2005). While off-flavour may be reduced in this way, the high content of polyunsaturated fatty acids (PUFAs) found in fish lipid is often a desirable marketing attribute due to the associated human health benefits (Glencross et al., 2003), as such reducing lipid content of farmed barramundi may be undesirable. The investigation of lipid and GSM content during short-term (12-24 hour) depuration revealed that GSM was reduced by 35-40% without any adverse impact on lipid content. This finding supports depuration as a stand-alone means of flavour recovery, rather than through a reduction in fillet lipid.

Although this study confirms depuration as a reliable method of flavour recovery in farmed barramundi, the spatial distribution of GSM within the fillet provides a further means of mitigating off-flavour taint. For example, the ventral belly section contained approximately three times as much GSM as either the dorsal shoulder or posterior tail sections. In many cases, this region would possess a GSM concentration above the threshold of human sensory detection whilst the remainder of the fillet would be below this threshold. In this case, the off-flavour tainted ventral belly section should be removed thereby leaving the untainted dorsal shoulder and posterior tail sections for consumption.

The spatial distribution of GSM within barramundi fillets is a key consideration for researchers and producers alike. Producers often assess fish for off-flavour tainting prior to harvest to avoid the sale of off-flavour tainted cohorts (Johnsen & Kelly, 1990; Van der Ploeg, 1991; Tucker & Van der Ploeg, 1999). The collection of tissue for analytical and/or sensory assessment should be carefully considered, otherwise misleading and highly variable results may occur.

4.5 Summary and Conclusions

This study has revealed that the uptake of GSM by tropical barramundi is exceptionally rapid such that an exposure time of only minutes, to a GSM concentration of 15.1µg kg^{-1}, is sufficient for fish tissue to become off-flavour tainted above the threshold of human sensory detection. Barramundi tissue reached a maximum concentration of 8.8µg kg^{-1} after 3 hours of exposure. In contrast, depuration is a protracted process with GSM loss from tissue having a half-life of approximately four days. Upon return to water free of off-flavour taint, market sized barramundi (1.5-2.0 kg) with a GSM concentration of 8.25µg kg^{-1} required more than 2 weeks of depuration at 27°C for GSM concentration to fall below the threshold of human sensory detection. However for fish exposed to GSM within the range most frequently encountered on-farm (≤2.15µg kg^{-1}), off-flavour tainting can be eliminated after 8 days of depuration at 27°C. This study also provides the first insight into the spatial distribution of GSM within barramundi fillets. The spatial distribution of GSM provides a further avenue for flavour recovery via removal of the heavily tainted ventral belly region. This may circumvent the need for depuration or could be used in conjunction with depuration protocols to further reduce the likelihood of off-flavour taint being experienced by the end consumer.

The spatial distribution of GSM within fish fillets is also a critical consideration for researchers investigating off-flavour tainting and for producers using sensory assessment to detect off-flavour tainted cohorts prior to harvest. The precise location from which samples are collected for instrumental analysis and/or sensory assessment must be carefully considered in order to take into account the spatial distribution of off-flavour tainting compounds.

4.6 References

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CHAPTER 5

Flavour enhancement of freshwater farmed barramundi, (*Lates calcarifer*), through dietary enrichment with cultivated sea lettuce, *Ulva ohnoi*.

Preface

Chapters 2, 3 and 4 provided an understanding of the causative factors affecting off-flavour tainting in Australian farmed barramundi and identified potential management strategies to mitigate its impacts. However, farmed fish have also been observed to lack the flavour complexity often noted in wild-caught fish. The following chapter details the potential to enhance the flavour of farmed barramundi by including a significant fraction of marine algae into the diet for a short period of time prior to harvest, thereby optimising flavour quality.

This chapter has been published in *Aquaculture.* The full citation is:

Jones, B., Smullen, R. & Carton, A.G. (2016). Flavour enhancement of freshwater farmed barramundi *(Lates calcarifer*), through dietary enrichment with cultivated sea lettuce, *Ulva ohnoi. Aquaculture, 454,* 192-198.

The contribution of co-authors to the published manuscript are presented in Table 1.4.

5.1 Introduction

The organoleptic quality of wild caught seafood and aquaculture products is profoundly influenced by dietary factors occurring over the pre-harvest period (Ackman et al., 1972; Boyle et al., 1993; Levasseur et al., 1994; Whitfield et al., 2002; Ma et al., 2005). The diet of wild-caught seafood is often diverse and this is thought to contribute to the complex and distinctive flavour of seafood products (Boyle et al., 1992; Whitfield et al., 1997; Whitfield et al., 1998). Aquaculture systems however typically utilise manufactured feeds, accordingly fish reared in captive systems lack the diversity of wild ecological diets. Resultantly, the flavour of fish reared in aquaculture systems can be noticeably different from their wild-caught counterparts (Whitfield et al., 1997; Grigorakis et al., 2003; Ma et al., 2005; Grigorakis, 2007; Carton & Jones, 2013).

Whilst the flavour quality of aquaculture products is highly acceptable, consumers have reported a difference between the flavour of some aquaculture fish and wild-caught fish (Whitfield et al., 1997; Grigorakis et al., 2003; Ma et al., 2005; Grigorakis, 2007; Carton & Jones, 2013). The organoleptic quality of some aquaculture products has been described as less complex and lacking ocean or ‘sea-fresh’ characteristics (Whitfield et al., 1997; Ma et al., 2005). There have however been recent attempts to enhance the organoleptic attributes of aquaculture products by enriching manufactured artificial diets with critical flavour compounds (Ma et al., 2005; Kim, et al., 2007; Fuller et al., 2008).

A wide array of compounds has been implicated in the organoleptic quality of aquatic animals. Of particular interest are bromophenol compounds (BPs) and dimethylsulfide (DMS). These compounds appear to be unique in their ability to produce a characteristic sea-like or sea-fresh aroma, have been credited with giving

the sea its distinctive smell and are known to contribute desirable flavour characteristics to seafood (Ackman & Hingley 1968; Brooke et al., 1968; White, 1982 cited in Van Alstyne et al., 2009; Iida & Tokunaga, 1986 cited in Hill, et al., 2000; Boyle, et al., 1993; Whitfield et al., 1997; Ma et al., 2005; Kim et al., 2007; Wolfe, 2014).

Several studies have investigated the importance of bromophenols in seafood and aquaculture products. For example Whitfield et al. (1997) identified that farmed prawns lacked the distinctive ocean-flavour of wild-caught prawns, this was attributed to the absence of bromophenols in the diet. However Kim et al. (2007) enhanced bromophenol levels in cultured green grouper by enriching diets with marine algae while Ma et al. (2005) were successful in replacing ocean-flavour in cultured silver sea bream, again through dietary enrichment with marine algae high in BPs.

At present, dietary enrichment with DMS for the purposes of organoleptic enhancement remains unexplored. Many species of marine algae are known to synthesise a high concentration of dimethylsulphoniopropionate (DMSP), which cleaves to form DMS (Kwint & Kramer, 1996) and acrylic acid (Iida, 1988 cited in Kawai & Sakaguchi, 1996). It is also well understood that DMS and DMSP are passed through the food chain by herbivory and predation (Ackman et al., 1966; Ackman et al., 1972; Levasseur et al., 1994). Although DMS has been identified as a flavour volatile in several species of marine fish and prawns (Mansur, et al., 2003), the implications of feeding captive fish diets containing marine algae rich in DMSP or DMS remains unexplored.

Ulva is a genus of coastal marine green algae distributed throughout temperate and tropical regions (Kirkendale et al., 2013, Lawton et al., 2013). Species within this genus are known to synthesise a large number of volatile organic compounds and

olfactory compounds including BPs, DMSP and DMS (Sugisawa et al., 1990; Flodin et al., 1999; Whitfield et al., 1999 b). *Ulva ohnoi* is a species of the genus *Ulva* which is successfully cultivated in aquaculture systems (Mata et al., 2015) and is considered an ideal species to target bioremediation in land based aquaculture farms (Lawton et al., 2013). *U. ohnoi* has also been used as a bioremediation tool in abalone waste water tanks with the produced biomass being used as a feed for the abalone (Bolton et al., 2009). Diets incorporating a relatively high inclusion level of *Ulva* are readily consumed by finfish (Pereira et al., 2012; Marinho et al., 2013; Wassef et al., 2013) and may have the potential to alter organoleptic properties of cultured species. The ability to manipulate these properties has the potential to increase marketability and financial returns to producers. In addition, as aquaculture feed manufacturers seek to replace wild-sourced fish meal and oil with terrestrial plant based products, organoleptic quality issues may become significant as such diets could possibly be devoid of key flavour and aroma compounds.

This study explores the application and potential of the green seaweed *Ulva ohnoi* in a short-term finishing diet for the purpose of altering the organoleptic attributes of cultured freshwater fish prior to harvest. Short term finishing diets are used to actively modify traits that have high consumer appeal. For example finishing diets high in fish oil have been shown to restore highly desirable fatty acid profiles of farmed Atlantic salmon, red sea bream and Senegalese sole previously reared on plant oil based diets (Glencross et al., 2003; Bell et al., 2004; Reis et al., 2014).

Lates calcarifer, known as Asian sea bass or barramundi, was selected to assess the efficacy of an organoleptic enhancing diet enriched with *Ulva*. Barramundi is an important food fish in tropical regions with a total global harvest approaching ~164,000 tonnes per annum, 40% (~66,000t) of which originates from captive

aquaculture production (FAO, 2012). Whilst farmed barramundi is generally acknowledged to possess a favourable fresh fish flavour as observed in untainted fish in Chapters 2, 3 and 4 wild-caught barramundi are known to possess significantly stronger shellfish (prawn) characteristics (Frank et al., 2009) and typically achieve a higher sale price in Australian fish markets.

The objectives of this study were to:

1. Investigate the palatability of *Ulva* in aquaculture diets and determine the maximum inclusion rate by quantifying feed intake at varying dietary levels.
2. Determine if feeding fish diets enriched with *Ulva* affects aroma and flavour, characterise any organoleptic changes and investigate key flavour compounds in the muscle tissue of *Ulva* fed barramundi.
3. Determine the optimal inclusion level of *Ulva* in the diet, with respect to organoleptic changes, and to explore the temporal response of key flavour attributes to dietary manipulation.

5.2 Materials and Methods

5.2.1 Preparation and formulation of experimental diets

Dried, powdered *Ulva ohnoi* was supplied by MBD Energy and was used at four inclusion levels to formulate experimental diets. *U. ohnoi* was initially harvested from the Pacific Reef Fisheries production facility (MBD Energy, Ayr, Australia) and subsequently grown in continuous culture in 10,000 L parabolic tanks (Mata et al., 2015). A sufficient volume of the macroalgae was harvested and oven dried (24 hours at 60°C) before being milled and screened (<1mm) prior to use in the experimental diets. Cold-pressed diets were prepared by milling a commercially available

barramundi grower diet (Ridley Agriproducts Pty Ltd. Aquafeed, Narrangba, Australia) to a fine powder (< 1mm) and reconstituting with pre-gel maize starch (10%) and dried, powdered *Ulva*. Inclusion levels of *Ulva* were 0% (reference diet), 10%, 20%, 30% and 50%. Water was incorporated to form a pliable dough and pressure pelleted through a 10mm die (Hobart Corp, Troy, USA). Pellets were manually cut to ~10mm length prior to oven drying at 60°C until a constant weight was achieved. After 3 days of storage 100 randomly selected pellets from each diet were individually weighed to determine average pellet weight. Diets incorporating 10, 20 and 30% *Ulva* were used to characterise the flavour profile, and undertake sensory evaluations. The 30% and 50% diets were used to test the palatability of *Ulva* as a feed ingredient.

5.2.2 Fish supply

All fish in this study were supplied by a local freshwater barramundi farm (Good Fortune Bay Fisheries, Kelso, Australia), fish were netted from growout ponds and transported to the Marine Aquaculture Research Facilities Unit (MARFU) at James Cook University.

5.2.3 Preliminary assessment of *Ulva* as a feed additive

A total of 27 market sized barramundi (800-1000 g) were initially acclimated for a period of 7 days in 9 x 500 L fibreglass tanks (n = 3 fish per tank) supplied with continuous flow-through dechlorinated municipal water at ambient temperature. All fish were fed a cold pressed pelleted diet (0% *Ulva*) during the period of acclimation. Following this, diets comprising of 0% (reference diet), 30% and 50% *Ulva* were randomly allocated to each tank such that each diet was fed to a total of 3 tanks (n=9). Fish were hand fed to satiation once daily for a period of 30 days. Daily feed consumption was determined as the difference between the number of pellets supplied

to each tank and the number of uneaten pellets remaining after feeding activity had ceased. Average pellet weight was used to calculate the weight of feed consumed per tank, and average daily feed consumption over the 30 day feeding period was used to evaluate the relative palatability of each diet. Following 30 days of feeding, all fish were euthanized according to Australian industry standards (see Carton & Jones, 2013) and filleted on both sides within 30 minutes of harvest. Fillets were immediately frozen (-18°C) and stored for a period of two weeks prior to sensory assessment.

5.2.4 Sensory assessment

In order to determine if the organoleptic properties of fish fed a standard commercial diet and fish fed a diet containing *Ulva* differed, triangle tests were carried out, as detailed in Chapter 4, according to the Australian standard for sensory analysis (AS 2542.2.2-2005 Sensory analysis - Specific methods - Triangle test; ISO 4120:2004). Two separate triangle tests were carried out during which untrained panellists were presented with a set of three 20 g portions removed from the dorsal shoulder region of fillets. The first test assessed the aroma of raw barramundi flesh and the second assessed the flavour of cooked barramundi flesh. Cooked barramundi portions were prepared by wrapping individual portions in aluminium foil and cooking in a fan-forced oven for 10 minutes at 200°C. Panellists were asked to justify their selection of the “different” sample by recording descriptive terms that they felt best described why this sample was different from the other two. Samples were only identifiable by a randomly generated three digit blind code. Barramundi fed the 50% *Ulva* diet were not subjected to triangle testing as feed consumption was low and highly variable over the 30 day feeding period (see Results).

5.2.5 Determination of organoleptic enhancement and descriptive sensory assessment

In order to accurately describe the specific flavour, aroma and aftertaste imparted by dietary enrichment with *Ulva* a total of 48 market sized fish (1800-2000 g) were randomly allocated into 12 x 500 L fibre glass tanks (n = 4 fish per tank), and acclimated as previously described. Individual tanks were randomly allocated one of four experimental diets, 0% (reference diet), 10%, 20% or 30% *Ulva*, such that each diet was allocated to three individual tanks (n=12). Fish were hand fed to satiation once daily for a period of 21 days. Daily apparent feed consumption was determined as the weight of feed supplied to each tank until feeding activity had ceased. A single fish was removed from each tank at the commencement of the trial (day 0) and again after 7, 14 and 21 days of feeding and sampled as outlined above. Fillets were stored (-18°C) prior to sensory assessment, additional tissue samples (~100 g) were collected from the dorsal shoulder region of each fillet and stored (-18°C) prior to instrumental analysis for several key flavour compounds.

The organoleptic characteristics of fish fed each diet were assessed using human sensory evaluation following the methods detailed in Chapter 2. Nine panellists were selected from an initial group of 22 and explicitly trained in the sensory assessment of barramundi. Initial training sessions used both wild caught and farmed barramundi. Participants were trained to identify and describe the most significant organoleptic properties (aroma, flavour and aftertaste) present in each sample (organoleptic attributes defined by participants are presented in Fig. 1 with definitions presented in Appendix 3). Cooked samples, as described above, were randomly assigned to assessors and only identifiable using a blind randomly generated three digit code, at no time were participants aware of the nature of the trial. Each sensory descriptor was

evaluated along a 150 mm ungraded line ranging from 0 (absent) to 150 (intense). Distilled water and flat bread were used to clean the palate between samples.

5.2.6 Instrumental analysis of key flavour compounds

Tissue samples from barramundi fed 0% and 30% *Ulva* for 21 days were subject to instrumental analysis. The abundance of DMS and total BPs in barramundi muscle tissue was assessed using methods developed by Steve Fuller, Crop & Food Science, Department of Agriculture and Fisheries, Queensland.

The relative abundance of DMS was assessed using headspace gas chromatography – mass spectrometry (GC-MS). Barramundi fillets were minced using a blender, and then 5 g was accurately weighed into each of 2 ball mill cups. A 10 mL volume of a 0.021 ug mL^{-1} water solution of thiophene was added to each of the ball mill cups. The cups were sealed and attached to the mill, which was then run at 30 cycles s^{-1} for 60s. The contents of both cups were transferred to a 50 mL glass beaker and then mixed using a glass rod. A 10g quantity of the homogenate was weighed into a 20 mL headspace vial, 2 mL of water was also added followed by the addition of 2 g of sodium chloride. The vial was then securely capped and the contents mixed for 30 s using a vortex stirrer.

Static headspace GC-MS analysis of the extracts was performed using a Shimadzu GC-2010 gas chromatograph coupled with a Shimadzu GCMS-QP2010S mass selective detector (MSD). The system was controlled by Shimadzu GC-MS Solutions software (version 2.53). Headspace sampling was undertaken by Solid-phase microextraction (SPME) using a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by Cycle Composer software (CTC Analytics, version 1.5.2). The SPME fibre was a 50/30 μm divinylbenzene / carboxen/ polydimethylsiloxane (DVB/ PDMS/CAR), StableFlex, Supelco, Bellefonte, PA.

Prior to headspace sampling, the vials containing the homogenates were equilibrated at 60 °C for 2 min. During extraction, the SPME fibre was exposed to the sample headspace for 2 min at 60 °C, then inserted into the heated GC inlet, and desorbed at 250 °C in splitless mode. After 30 s, a 1:50 split ratio was programmed and maintained for the duration of the analysis. The GC column oven was fitted with a DB-1 capillary column (50 m× 0.22 mm i.d., 1 μm phase) SGE, Australia. The carrier gas was helium set to a flow rate of 1.3 mL min^{-1}, linear velocity 32 cm s^{-1}. The initial oven temperature was 40°C for 0.5 min, then ramped at 10°C min^{-1} to 200°C and held for 3.5 minutes. The interface temperature was set to 280 °C. Detection of DMS and thiophene was achieved with the MSD in Selective Ion Monitoring (SIM). The ion source was set at 70 eV and electron multiplier at 1350 V. The selected ions for DMS were m/z <u>62</u>, 47(98), 45(49), 46(41) and 35(34), and for thiophene, m/z <u>84</u>, 58(75), 45(45), 39(29) and 57(13). Positive identification was confirmed by the presence of both target and qualifier ions at the correct retention time and with the correct ion ratios. Results are expressed as the ratio of the DMS peak area to the thiophene peak area.

Measurement of BP compounds was performed according to the methods of Fuller et al. (2008) for fish tissue. The method combines simultaneous distillation–extraction followed by alkaline back extraction of a hexane extract and subsequent acetylation of the BPs. Analysis of the bromophenol acetates was accomplished by headspace solid phase microextraction and gas chromatography–mass spectrometry using selected ion monitoring.

Analysis for DMS and BPs was performed at Agri-Science Queensland, Department of Agriculture and Fisheries.

5.2.7 Statistical analysis

Differences in the consumption of experimental diets, the intensity of organoleptic attributes and the relative abundance of flavour compounds was assessed by ANOVA. Significant differences between treatments during triangle testing were determined by calculating p-values for each triangle test according to standard methods (AS 2542.2.2-2005 Sensory analysis - Specific methods - Triangle test; ISO 4120:2004). The level of significance was defined at $p<0.05$. Results are reported as the mean ± the standard error of the mean (SEM).

5.3 Results

5.3.1 Preliminary assessment of *Ulva* as a feed additive, feed consumption

Average daily feed consumption per fish during 30 days of feeding at each inclusion level is shown in Table 5.1. The use of *Ulva* at an inclusion level of 30% did not significantly affect feed consumption (ANOVA, $p>0.05$), when compared to the reference diet. Feed consumption was however significantly reduced at the 50% inclusion level (ANOVA, $p<0.05$).

Table 5.1 Effect of *Ulva* inclusion level on daily feed consumption by barramundi. Different superscripts between rows indicate significant differences in feed consumption ($p<0.05$ ANOVA).

Ulva inclusion level	Average daily feed consumption (g feed fish^{-1})
0%	6.56 ± 1.17^{a}
30%	4.78 ± 0.39^{ab}
50%	2.12 ± 0.98^{b}

5.3.2 Preliminary assessment of *Ulva* as a feed additive, sensory assessment

The flavour of cooked barramundi portions ($p=0.033$) and the aroma of raw barramundi portions ($p=0.008$) were significantly different between fish fed the 30%

Ulva diet and the reference diet (Table 5.2). Strong fish, ocean/seafood and shellfish flavour and aroma were identified as the primary reasons for the difference.

Table 5.2 Results from triangle testing performed on barramundi portions from fish fed either the reference diet (0% *U. ohnoi*) or the 30% *U. ohnoi* diet.

	Raw fillet Aroma	Cooked Fillet Flavour
Number of assessors	35	34
Correct responses	19	17
***p* value**	0.0085	0.033

5.3.3 Determination of organoleptic enhancement and descriptive sensory assessment

Average daily apparent feed consumption per fish during 21 days of feeding at each inclusion level is shown in Table 5.3. Apparent feed consumption was not significantly affected by the *Ulva* inclusion level (ANOVA, $p>0.05$).

Table 5.3 Daily apparent feed consumption by barramundi at differing *Ulva* inclusion levels. No significant differences in feed consumption were observed ($p<0.05$ ANOVA).

Ulva inclusion level	Average apparent daily feed consumption (g feed fish^{-1})
0%	26.47±1.13
10%	20.36±2.77
20%	19.07±1.20
30%	21.61±2.19

Barramundi fed the 10% *Ulva* diet had a similar organoleptic profile to those fed the reference diet (Fig. 5.1). Clear differences in the organoleptic profile were apparent in fish fed the 20% *Ulva* diet. Significant increases (ANOVA, $p<0.05$) in the intensity of crab like/seafood flavour, crab like aroma, crab like aftertaste, sweet aroma and flavour complexity were all observed (see Appendix 3 for definitions of these descriptive terms). Overall desirability was also seen to increase. A comparable flavour profile was observed in fish fed the 30% *Ulva* diet, although sweet aroma did

not show parallel elevations and the increase in cooked crab aroma was also less pronounced (Fig. 5.1).

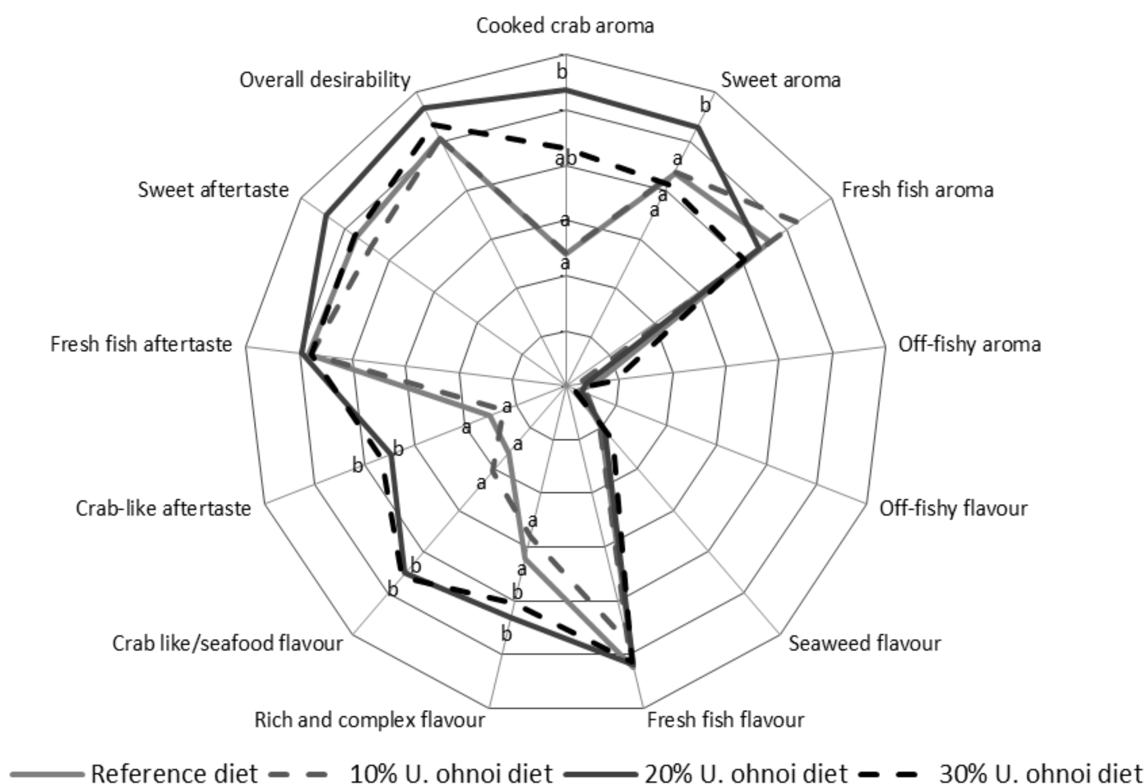

Fig. 5.1 Sensory attributes of barramundi fed four inclusion levels of *U. ohnoi*. Solid light grey line denotes barramundi fed the reference diet (0% *U. ohnoi*), dashed light grey line represents barramundi fed a 10% inclusion level, solid dark grey line denotes barramundi fed a 20% inclusion level and dashed black line represents barramundi fed the 30% *U. ohnoi* diet. Different superscripts indicate significant differences in the intensity of sensory attributes.

Crab-like/seafood flavour was observed to increase significantly (ANOVA, $p<0.05$) in intensity after the first seven days of feeding with a diet containing 30% *Ulva*, compared to at the commencement of the feeding period. Similarly, the intensity of this attribute increased during the first seven days of feeding with the 20% inclusion level, although this increase was approximately half of that observed for the 30% diet and the intensity of crab-like/seafood flavour at this time was not significantly greater than at the commencement of feeding (ANOVA, $p>0.05$).The intensity of this attribute continued to increase with feeding time and was significantly more intense after 21 days feeding on both diets than at the commencement of the feeding period

(Fig. 5.2). By day 21 the intensity of crab-like/seafood flavour was almost identical in fish reared on both of these diets. No significant change in crab-like/seafood flavour was observed in fish fed the 10% *Ulva* diet during 21 days of feeding (Fig. 5.2).

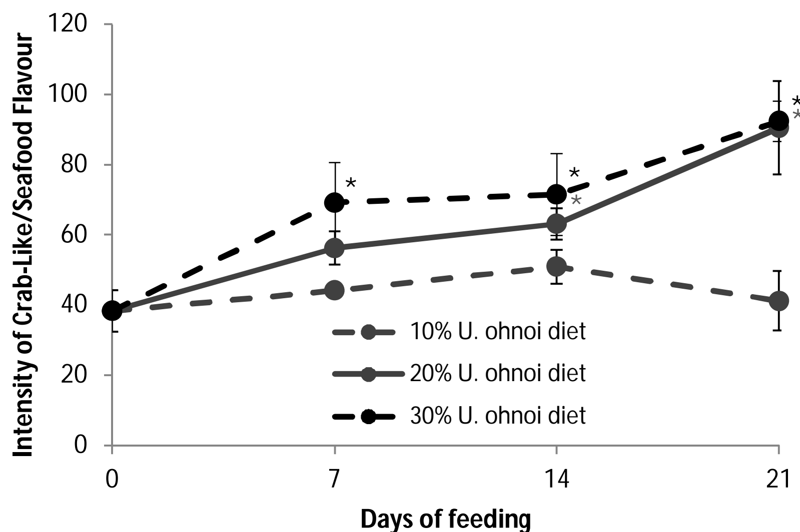

Fig. 5.2 Changes to the intensity of Crab-like/Seafood flavour of barramundi flesh (n = 3) during 21 days of feeding *U. ohnoi*-enriched diets. Dashed grey line denotes fish fed the 10% *U. ohnoi* diet, solid grey line represents fish fed the 20% *U. ohnoi* diet and dashed black line denotes fish fed the 30% *U. ohnoi* diet. Error bars represent the standard error of the mean. *Indicates the intensity of Crab-like/Seafood flavour was significantly different from that observed at the commencement of the feeding period.

5.3.4 Instrumental analysis

Barramundi fillets from fish fed the reference diet and the 30% *Ulva* diet were analysed for DMS and BPs. The concentration of DMS in the flesh of barramundi fed 30% *Ulva* for 21 days was more than 8 times higher than in fish fed the reference diet. This elevation in DMS was commensurate with an approximate 3 times greater intensity of crab-like/seafood flavour (Fig. 5.3).

Bromophenols were not detected in the flesh of barramundi reared on either diet.

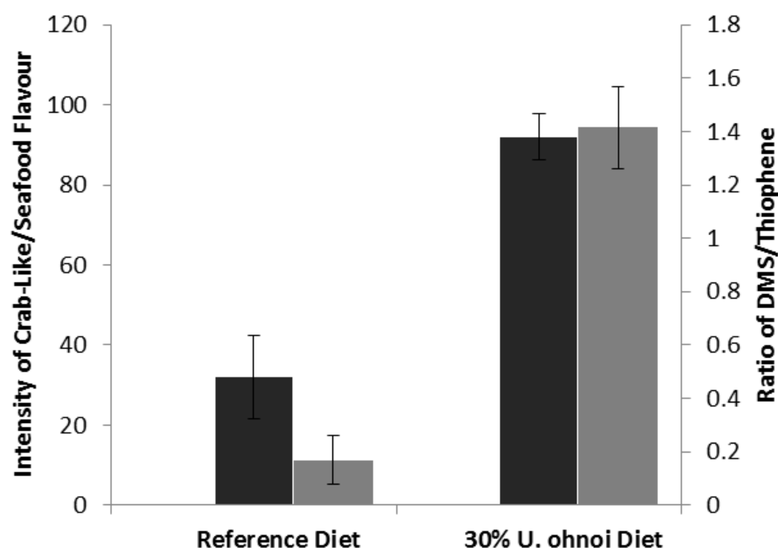

Fig. 5.3 Intensity of crablike-seafood flavour and relative abundance of DMS in the flesh of barramundi fed the reference diet (0% *U. ohnoi*) and the 30% *U. ohnoi* diet. Dark grey bars denote the intensity of crab-like/seafood flavour and light grey bars represent the relative abundance of DMS measured in barramundi flesh. Error bars represent the standard error of the mean.

5.4 Discussion

This study confirms that diets enriched with macroalgae have great potential to enhance the flavour of freshwater farmed fish. Feeding cultured barramundi a diet with ≥ 20% inclusion of *Ulva* induced a change in organoleptic attributes with increases in seafood-like attributes and desirability.

Feed consumption was evaluated to investigate the palatability of *Ulva ohnoi* in a finishing diet. Feed consumption for fish fed the reference diet was similar to commercially expected rates (Glencross & Bermudes, 2012), indicating that fish were acclimated and unstressed within the experimental system. Feed consumption reduced when *Ulva* was included into the diet at 30%, although this was not significantly different (ANOVA, $p>0.05$) from consumption of the reference diet. Feed consumption was however drastically reduced (ANOVA, $p<0.05$) when *Ulva* was increased to 50% inclusion, with consumption reducing by 67% when compared to the reference diet. Given this data it appears that the upper limit for dietary inclusion

of *Ulva* is ~30%. *Ulva spp.* has been found to be palatable for other species at relatively high inclusion levels, for example Nile tilapia (≤ 30%) (Pereira et al., 2012; Marinho et al., 2013), and other carnivorous species such as European sea bass (≤15%) (Wassef et al., 2013) and rainbow trout (≤ 30%) (Pereira et al., 2012). Over the 30 day feeding period barramundi fed all diets remained in good health with no mortalities recorded. The inclusion of *Ulva* in the diet did not cause any overt signs of stress, as would be indicated by differences in mortality or behaviour between the diet treatments.

The organoleptic properties of barramundi fed the 30% *Ulva* diet were initially compared with fish fed the reference diet. Triangle testing identified a significant difference ($p<0.05$) in the organoleptic properties of both raw and cooked barramundi samples between these diet treatments. The sensory panel reported strong fish, seafood and shellfish flavours as being responsible for this difference. Ma et al. (2005) observed a similar affect when cultured silver sea bream were fed diets containing a 30% inclusion of the marine brown macroalgae *Sargassum siliquastrum* over an 8 week period. Triangle testing identified a significant difference between the flavour of fish fed a diet containing *S. siliquastrum* and those fed a standard commercial diet, seafood flavours were again highlighted as the underlying cause of this difference.

To better characterise the differences in flavour and resolve optimal inclusion levels of *Ulva*, fish were fed diets containing 0% (reference), 10%, 20%, and 30% *Ulva* over a 3 week period. The organoleptic attributes of fish from each of these treatments were then assessed by a trained sensory panel. Differences in organoleptic attributes were not evident until the inclusion level of *Ulva* reached 20%. Flavour, aroma and aftertaste attributes of fish fed the 20% and 30% diets were similar (Fig. 5.1). The

inclusion of 20-30% *Ulva* in the diet resulted in the enhancement of five desirable organoleptic attributes (Fig. 5.1), cooked crab aroma, sweet aroma, crab-like aftertaste, crab-like/seafood flavour and complex flavour. Notably, the intensity of cooked crab aroma and sweet aroma were less pronounced in fish fed 30% *Ulva* than in fish fed 20% *Ulva* diets, the cause of this is difficult to resolve. It is possible, although speculative, that this is related to changes in the sensorial nature of critical flavour compounds at increasing concentration. For example, the aroma of DMS is described as sea-like at low concentration and sulphur/chemical/rotting shellfish at high concentration (Brooke et al., 1968; Ackman et al., 1972; White, 1982 cited in Van Alstyne et al., 2009; Levasseur et al., 1994;). Changes in the organoleptic attributes of critical flavour compounds at increasing concentration have the potential to alter the aroma profile of fish tissue.

The ability to enhance the flavour of cultured finfish has clear benefits for aquaculture producers. Consumers have commented on differences in flavour between aquaculture species and their wild-caught counterparts with some aquaculture products lacking flavour complexity (Whitfield et al., 1997; Grigorakis et al., 2003; Ma et al., 2005; Grigorakis, 2007; Carton & Jones, 2013). This study reinforces the findings of Ma et al. (2005), that enriching the diets of finfish with marine macroalgae has the potential to enhance the organoleptic properties and therefore the overall desirability of the final post-harvest product. These results clearly show that dietary manipulation can be used to enhance or modify organoleptic attributes prior to harvest. However to be used effectively the temporal response to dietary manipulation must be clearly understood. Application of the diet must be of a sufficient time period for organoleptic modification or enhancement to occur, however feeding for prolonged periods may reduce somatic growth or increase feed conversion ratio

(FCR) as the algal fraction of the diet is likely to have low digestibility compared to traditional dietary ingredients (Pereira et al., 2012; Marinho et al., 2013; Wassef et al., 2013).

A clear increase in crab-like/seafood flavour was observed in barramundi fed the 30% *Ulva* diet after only 7 days of feeding. Similarly, when the 20% *Ulva* diet was applied crab-like/seafood flavour increased after 7 days, although the magnitude of this increase was approximately half of that observed for the 30% diet. The intensity of crab like/seafood flavour continued to increase, and remained higher in fish fed the 30% *Ulva* diet than in fish fed the 20% *Ulva* diet after 14 days of feeding. The highest intensity of crab-like/seafood flavour was observed after 21 days of feeding and, at this time, was comparable in fish fed the 20% or 30% *Ulva* diets. This demonstrates that increasing the dietary inclusion level of *Ulva* to 30% accelerates organoleptic enhancement, although this effect is limited to the first 14 days of feeding.

Questions remain as to the exact mechanisms driving the change in organoleptic attributes. In natural ecosystems, variation in diet is considered to be a key driver of flavour complexity (Boyle et al., 1992; Whitfield et al., 1998). For example, Ackman et al. (1972), Whitfield et al. (1998; 1999a), and Levasseur et al. (1994) observed the effects of alga-derived compounds on fish flavour. In these cases, BPs (Whitfield et al., 1998, Whitfield et al., 1999a) and DMS (Ackman et al., 1972; Levasseur et al., 1994) were observed to affect the flavour of carnivorous fish.

In the current study, BPs were not detected in barramundi flesh at any time. The total BP content of the cultured *Ulva* used in experimental diets was however low, being previously measured at 41.0 $\mu g\ kg^{-1}$ (unpublished data). The lack of deposition of BPs in muscle tissue in this experiment is most likely the result of low levels in the

ingredient, for example Whitfield et al. (2002) has shown that only a fraction of the total available dietary BPs are deposited in muscle tissue.

DMS was found to be present in the muscle tissue of fish fed both the reference and the 30% *Ulva* diet (Fig 5.3.). However there was wide disparity in the level of accumulation with *Ulva* fed fish having an eight fold higher DMS tissue level than fish fed the reference diet (Fig 5.3.). A wide-ranging change in organoleptic attributes was observed concurrently with this increase in DMS, most notably a threefold increase in the intensity of crab-like/seafood flavour (Fig 5.3.). Whilst DMS has previously been associated with off-flavour in fishes (Ackman et al., 1972; Levasseur et al., 1994), this is the first study to report a link between dietary DMS enrichment and organoleptic enhancement of finfish.

The concentration of DMS in fish tissues, as with other environmental compounds, will reflect the balance between uptake and natural depuration. The uptake and depuration of DMS by fish remains relatively unexplored but warrants further investigation as this compound appears to be an important organoleptic component. Uptake rates are clearly important in determining the time required for organoleptic changes to occur following dietary manipulation, however the rate of depuration is also critical as DMS in muscle tissue is likely to reduce if dietary enrichment is discontinued. This remains unexplored but is clearly of significance as losses of DMS prior to harvest may negate the organoleptic effects of dietary enrichment.

The flavour of seafood can also be influenced by the uptake of DMSP (Hill et al., 2004; Smit et al., 2007). The organoleptic attributes of DMSP are unresolved, but considered to be minimal (Hill et al., 2000), however its breakdown to DMS during the post-mortem period has previously been associated with organoleptic changes in seafoods (Hill et al., 2004; Smit et al., 2007). Significant increases in DMS

concentration, attributed to the breakdown of DMSP, were observed in giant clam muscle (Hill et al., 2004) and abalone meat (Smit et al., 2007) during the post-mortem period and were associated with the development of potent odours. In both of these cases, increases in DMS were associated with elevated storage and processing temperatures (>25°C). DMSP is converted to DMS enzymatically, for example by bacterial DMSP lyase, (Ledyar et al., 2003) or nonenzymatically (Dancey & Blough., 1987). In either case, temperature is likely to influence the rate of breakdown.

The post-mortem breakdown of DMSP to DMS may be a critical concern for the organoleptic enhancement of farmed barramundi using diets enriched with *Ulva*. *Ulva* is rich in DMSP (439.2mg kg^{-1}) and following consumption, approximately 84% of dietary DMSP in *Ulva*-enriched diets is digested by barramundi (Jago et al. 2014). DMSP was not investigated in the current study however an elevated level of DMS was observed in flesh samples that were frozen (-18°C) immediately post-mortem. Further research is required to investigate if DMS concentration has the potential to fluctuate during the post-mortem storage period and if this can influence organoleptic properties.

This study has demonstrated that the inclusion of macroalgae in the diet can enhance the organoleptic characteristics of fish post-harvest. However caution should be exercised in the use of this ingredient. In this study, elevated levels of DMS were observed in the tissue of flavour enhanced fish, however the nature of the effect appears concentration dependent (Hill et al., 2000) with levels ≥100 nmol g^{-1} considered commercially problematic (Motohiro, 1962; Iida & Tokunaga., 1986; Hill et al., 2000). For example levels of DMS in clams and oysters have previously been described as a positive flavour component (Ronald & Thomson 1964; Ackman & Hingley, 1968; Brooke et al., 1968; Iida & Tokunaga, 1986), whereas DMS is often

identified as a flavour problem in fish and shellfish (Motohiro, 1962; Sipos & Ackman, 1964; Ackman et al., 1966; Levasseur et al., 1994; Hill et al., 2004; Smit et al., 2007).

Before this ingredient can be used in a commercial context, during the final stage of the production cycle, more information is required, especially a finer scale understanding of the relationship between changes in organoleptic attributes and the concentration of DMS deposited in the muscle tissue.

It is also important to understand if post-harvest handling and storage techniques influence the breakdown of DMSP to form DMS in tissue. For example storage temperature may influence the conversion of DMSP to DMS in the muscle tissue and therefore cause variations in key organoleptic attributes at the point of consumption (Hill et al., 2004; Smit et al., 2007). Furthermore, Ackman et al. (1966) has suggested that DMS is readily released during the cooking process, this warrants further investigation to determine if cooking has the potential to affect DMS levels.

The short term application of diets with moderate inclusion levels of *Ulva*, during the final stages (weeks) of the production cycle, appears to be a promising mechanism to enhance or actively modify key flavour attributes pre-harvest and increase consumer acceptance.

5.5 Conclusions and outcomes

The findings of this study confirm that the flavour of a freshwater farmed fish can be enhanced through the short-term application of a diet enriched with marine algae. The overall desirability of barramundi was increased by imparting rich and complex seafood-like flavours during three weeks of feeding with diets containing *Ulva ohnoi*. This flavour change occurred concurrently with an increase in the concentration of

DMS in fish flesh. The use of a short-term finishing diet may provide a mechanism to produce low cost freshwater fish with flavours that are more characteristic of wild caught marine species.

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CHAPTER 6

Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi (*Lates calcarifer*).

Preface

Chapters 2-5 in this thesis are primarily concerned with optimising the flavour quality of farmed barramundi at the time of harvest. However, product quality is known to gradually deteriorate during the post-harvest transportation and storage period due to oxidative, enzymatic and/or bacterial processes. This is especially important in the Australian barramundi aquaculture industry as production typically occurs in sparsely populated regions with protracted transporation times to major markets. In response, potential management strategies were investigated that have the potential to fortify farmed barramundi against spoilage during the post-harvest period, prior to human consumption. The outcomes of this research are presented in the following chapter.

This chapter was published in *Aquaculture*. The full citation is:

Jones, B. C. & Carton, A. G. (2015). Effects of dietary enrichment with alpha-tocopherol acetate and post-harvest filleting on lipid oxidation and flesh quality of tropical farmed barramundi (*Lates calcarifer*). *Aquaculture, 448*, 280-287.

The contributions of co-authors to the published manuscript are presented in Table 1.5.

6.1 Introduction

The quality and storage life of fish products is profoundly influenced by pre-harvest factors and post-harvest processing and storage methods (Fletcher & Hogdson, 1988; Frigg et al., 1990; Undeland et al., 1998; Huidobro et al., 2001; Ruff et al., 2003; Wilkinson et al., 2008; Erikson et al., 2011; Carton & Jones, 2013). Changes in quality often occur gradually over the period of post-harvest storage as a result of oxidative, enzymatic and/or bacterial processes. These processes degrade flavour, taste and aroma qualities and serve to promote changes to texture, colour, pH and nutritional status, which can ultimately render fish products unfit for consumption and unmarketable (Gram, 1992; Gram & Huss, 1996; Olafsdóttir et al., 1997; Bonilla et al., 2007).

Fish are acutely prone to lipid oxidation due to high levels of polyunsaturated fatty acids (PUFAs) in the flesh (Hultin, 1994; Undeland, 2001), which limits the storage life of fish products (Jittinandana et al., 2006). The oxidation of lipid can result in a loss of product quality through the development of off-flavours, potent odorants, and a loss of colour and texture (Ladikos & Lougovois, 1990; Liu et al., 1995; Gray et al., 1996). This is especially important in aquacultured fish where lipid content of the flesh can often be considerably higher than in wild counterparts. Enriching tissues with anti-oxidants such as α-tocopherol acetate added to the diet prior to harvest has been shown to slow the rate of lipid oxidation in various meat products (Liu et al., 1995), including fish (Frigg et al., 1990; Baker & Davies, 1996; Harare et al., 1998; Ruff et al., 2003; Chen et al., 2008). Anti-oxidants such as α-tocopherol acetate are effective in scavenging free radicals in both the initiation and propagation steps of autoxidation (St. Angelo, 1996) thereby restraining lipid oxidation and post-harvest

deterioration in quality. This has been shown to improve the quality and storage life of the product (Frigg et al., 1990).

Slowing the rate of lipid oxidation can also be achieved by limiting the exposure to or complete exclusion of molecular oxygen, and is often accomplished through vacuum or modified atmosphere packaging (Khayat & Schwall, 1983; Siah & Ariff, 2002). However any post-harvest processing that results in the disruption of tissues, such as filleting and skinning has the potential to accelerate lipid oxidation, to this extent filleted fish have been shown to have low lipid oxidation stability (Hutlin, 1994). Maintaining the fish whole and/or the skin intact following harvest has been suggested to limit the availability of molecular oxygen, and has been demonstrated to reduce the rate of lipid oxidation in herring fillets (Undeland et al., 1998).

Barramundi, known throughout the Asia Pacific region as Asian Sea Bass (*Lates calcarifer*) is an important food fish in tropical regions with a total global harvest reaching ~164,000t per annum, 40% of which originates from captive aquaculture production (FAO, 2012b). In Australia, the total aquaculture production of barramundi is approximately ~4000t per annum (Savage & Hobsbawn, 2015). Production typically occurs in sparsely populated regions in the tropical north of Australia, a considerable distance from major metropolitan markets (as previously discussed). This geographical isolation results in protracted transportation and storage periods, with the product often reaching consumers as many as nine days after harvest, with consumption recommended within 14 days of harvest (pers comm, E. Poole). During the transportation period as many as 4-5 transfers through the supply chain can occur during which time fish can also be exposed to sub-optimal storage conditions.

Barramundi grown in Australia is sold chilled and unfrozen, either as whole ungutted fish or as skinned fillets (Harrison et al., 2013). Due to considerable geographic isolation and storage concerns during transit, the Australian barramundi aquaculture industry has developed harvest methods assumed to limit lipid oxidation and quality degradation. Farmed barramundi are chilled rapidly at the point of harvest by ice immersion and thermal conditions during handling and packing are controlled to limit product spoilage (Carton & Jones, 2013). There is however a paucity of information relating to quality changes following packing, during transport and the extended storage period. Furthermore, techniques to prevent quality degradation during this period have not been investigated. There is a need to understand and thereby improve the susceptibility of barramundi products to lipid oxidation and changes in quality over the post-harvest storage and transportation period.

The objectives of this study were to:1) understand changes in quality and lipid oxidation over the post-harvest storage period, (2) determine the efficacy of dietary α-tocopherol acetate enrichment as a means of fortifying tissues against lipid oxidation, (3) evaluate the effect of post-harvest processing (skinned fillets and whole ungutted fish) on changes in quality and lipid oxidation over the post-harvest storage period, and (4) identify the combined effects of dietary α-tocopherol acetate enrichment and post-harvest processing during chilled storage.

Lipid oxidation was measured, as a critical indicator of product quality, at day 9 and day 14 this representing critical time points in the supply chain. Other post-harvest quality parameters such as flesh surface colour, pH and bacterial activity were also selected as indicators of product quality and assessed after 5, 9 and 14 days of chilled storage.

6.2 Materials and Methods

6.2.1 Diets and diet formulation

Diets were commercial barramundi rearing diets formulated and manufactured by Ridley Agriproducts Pty Ltd. (Narangba, Australia) (Wenger 7 head X185 extruder, Wenger, Sabetha, USA) and were identical in proximate composition (50% protein, 16% fat). The two diets were enriched (pre-extrusion) with 192 mg (standard diet) and 628 mg (enriched diet) α-tocopherol acetate kg^{-1} and extruded as 10 mm floating pellets.

2.2. Fish and rearing conditions

The experiment was carried out at a commercial barramundi aquaculture facility, North Queensland, Australia (Pejo Enterprises, Innisfail, Australia). A total of 252 market sized barramundi (800-1000 g) were obtained from a single rearing pond. Individual fish were then weighed and randomly allocated across six rectangular plastic floating cages (4 m^3) suspended within an earthen culturing pond (70m x 42 x 1.5m). Each cage housed a total of 42 fish and experienced water quality conditions identical to those occurring during normal commercial aquaculture production (temperature 24- 28°C, dissolved oxygen 3-6 mg/L). Mean fish weight and length was not significantly different (ANOVA, $P>0.05$), between treatment cages. Individual cages were assigned either the standard (192 mg α-tocopherol acetate kg^{-1}) or enriched diet (628 mg α-tocopherol acetate kg^{-1}), in a balanced order to block for possible cage location effects. Fish were hand fed to satiation once daily for the duration of the 169 day feeding period, with apparent feed consumption (AFC) calculated as the weight of feed supplied to each cage, and recorded weekly.

6.2.3 Sampling of fish and collection of flesh samples

Immediately following stocking, three individual fish were randomly selected from each cage to determine the initial concentration of α-tocopherol acetate present in the tissue. Fish were hand netted and euthanized using standard commercial methods (immersion in ice slurry) (Carton & Jones, 2013). Following this, weights and lengths of all fish were recorded and a tissue sample (~5.0 g) removed from the shoulder region (Fig. 6.1), using a 2cm (dia) stainless steel coring tool. Samples were taken from both the left and right sides of the fish. Tissue samples of individuals from the same cage were then pooled, homogenised and a 10 g subsample of the homogenate was immediately frozen in liquid nitrogen. On return to the laboratory homogenised samples were removed from liquid nitrogen and stored at -80°C.

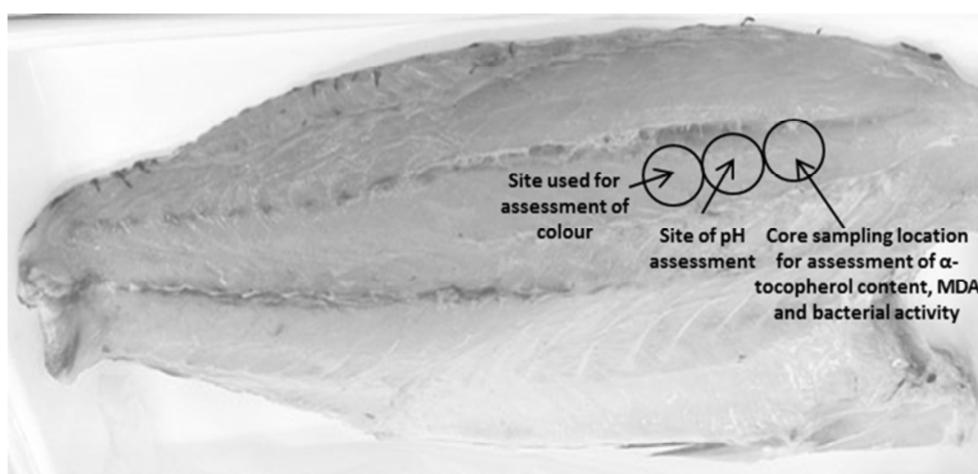

Fig. 6.1 Location of assessment of α-tocopherol, lipid oxidation (MDA), bacterial activity, pH and colour.

6.2.4 Assessment of post-harvest changes in quality

The effect of dietary α-tocopherol acetate enrichment on lipid oxidation and flesh quality over the post-harvest storage period was evaluated for two processing methods; 1) whole ungutted fish, and 2) skinned fillets. At 169 days all fish were harvested from cages and euthanized as described above. Following this fish were removed from the ice slurry and either filleted immediately or maintained as whole

ungutted fish and stored at 2°C in a temperature controlled cold room (6m x 2.5m, Coldroom Supplies Pty, Australia). Storage conditions were identical for fillets and whole ungutted fish. Lipid oxidation was measured after 0, 9 and 14 days of chilled storage, with bacterial activity, tissue pH and tissue colour measured after 0, 5, 9 and 14 days of chilled storage.

6.2.5 Physical and chemical analyses

6.2.5.1 Measurement of α-tocopherol acetate uptake

To quantify the uptake of α-tocopherol acetate from the diet into the flesh three fish were randomly selected from each cage and tissue samples taken (as described above). Fish were sampled after 14, 28, 42, 56, 140 and 169 days of feeding. Weights and lengths of individual fish were also recorded to assess fish condition at the time of sampling. The α-tocopherol content of flesh samples was determined by HPLC methods performed at the Lincoln Marine Science Centre (Port Lincoln, South Australia), Flinders University based on Huo et al. (1999) (D'Antignana et al., 2012). α-tocopherol was double extracted from homogenised fish flesh in 60 mL of methanol (meth) - butylhydroxytoluene (BHT) solution (1mL of meth to 1 mg of BHT). A 0.95 – 1.05g subsample of frozen minced flesh was added to 40mL meth/BHT then homogenised by Omni-Prep (Omni International, USA). This first extraction continued for three hours on ice. The solution was vortex stirred hourly during this time for a period of 15 seconds. After this period, the homogenate was centrifuged for four minutes at 4500 rpm. A 3.9 mL volume of the resulting supernatant was pipetted into a 10 mL tube and placed on ice. The remaining supernatant was discarded. The tissue pellet was re-suspended by vortex stirring in 20 mL of meth-BHT solution for the second extraction. The homogenate was placed on ice for 30 minutes, after which it was centrifuged as previously described. A 2.1 mL volume of the supernatant was

combined with the 3.9 mL from the first extract. This was vortex stirred for 30 seconds prior to being filtered (0.45 µm). Samples were then eluted through a Prevail C18 5µm column (Waters, 150 mm x 4.6 mm) with methanol:distilled water (98:2) as the mobile phase. α-tocopherol was detected using a Waters 2475 fluorescent detector (filters: 296 nm excitation and 340 nm emission) connected to a Waters 2695 separations module refrigerated at 4°C. The concentration of α-tocopherol in the flesh (mg kg^{-1} wet weight) was calculated using an α-tocopherol standard of known concentration using the following equation:

$$Vitamin\,E = \frac{\left(\left(\frac{[Area\ of\ vit\ E\ std]}{Area\ of\ sample}\right) \times Dilution\ factor\right)}{(Extracted\ Muscle\ ww\ g)}$$

6.2.5.2 Lipid Oxidation

Core tissue samples (5.0 g) were collected from the shoulder region from both the left and right sides of two randomly selected whole ungutted stored fish from each cage (n = 6 for both diet treatments). Tissue samples originating from each individual cage were then combined, homogenised, and stored at -80°C prior to lipid oxidation analysis. Tissue samples (5.0 g) were also collected from four randomly selected fillets from each cage (n = 12 for both diet treatments), and treated as outlined above.

Lipid oxidation was measured by a distillation-colorimetric technique, the 2-thiobarbituric acid method (TBARS) modified from Wong et al. (1991) (D’Antignana et al., 2012), as used in previous studies to measure lipid oxidation in teleosts (Frigg et al., 1990; Ruff et al., 2002; Jittinandana et al., 2006; Musgrove et al., 2011). Analyses were performed at the Lincoln Marine Science Centre (Port Lincoln, School of Biological Sciences, Flinders University). Malonaldehyde concentration in fish flesh was determined using a spectrophotometric method modified after Wong et al.

(1991) on a Multiskan Ascent (Thermo Labsystems) multi-plate reader (D'Antignana, 2007). For each sample, minced fish flesh (~1g) was placed into a 10 ml centrifuge tube along with 5 mL of cold 0.6 M perchloric acid and vortex stirred for 15 seconds. The sample was then allowed to extract on ice for a period of 20 minutes. Following this time, samples were centrifuged for 5 minutes at 4000 rpm. The resulting supernatant was filtered (0.45 µm) into 2ml cryogenic vials prior to being stored at 80°C until analysis.

For analysis, sample extracts were removed from -80°C storage and thawed at room temperature. A serial dilution of tetraethoxypropane (malonaldehyde inclusive) standards (Sigma) appropriate to the expected range of sample results was prepared. A 500 µL volume of 0.02M 2-thiobarbituric acid solution was added to 500 µL of sample extract or the standards in 10 mL vials. These vials were vortex stirred before being incubated in a 100°C water bath. After 35 minutes of incubation, the vials were removed from the water bath and allowed to stand for 15 minutes at room temperature. 300 µL aliquots were taken in duplicate and transferred from each vial to a 96 well micro plate. Their absorbance was measured at 540 nm. Malonaldehyde concentration (mg.kg-1 wet weight flesh) was calculated from the standard curve using the following formula:

$$X = \left(\frac{1000}{A}\right) \times \left(\frac{B}{C}\right) \times 0.00072$$

where A = weight of flesh extracted (g), B = volume of perchloric acid the flesh was extracted in, C = volume used in assay, and X = concentration of malonaldehyde in the tissue (mg kg^{-1} wet weight flesh).

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6.2.5.3 Crude fat

At the beginning of the storage period, a subsample of homogenised tissue originating from each cage was collected and stored at -80°C. Samples were analysed for crude fat using ethyl acetate extraction – gravimetric determination methods, based on the Norwegian Standard Method (NS 9402 E) (NSA 1994), by the Lincoln Marine Science Centre (Port Lincoln, School of Biological Sciences, Flinders University) (see section 4.2.3.4).

6.2.5.4 Bacterial counts

After 0, 5, 9 and 14 days of chilled storage, tissue samples were collected from whole ungutted fish and fillets as previously described. Homogenised tissue samples were placed into sealed plastic bags, wrapped in absorbent paper and stored on ice. Bacterial activity was assessed via bacterial counts performed by the Innovative Food Solutions and Technologies Group (Queensland Department of Agriculture, Fisheries and Forestry, Cairns, Australia), using the Australian Standard Method of Measurement (Food microbiology - Examination for specific organisms - Standard plate count AS 5013.12.3-2004).

6.2.5.5 pH

Tissue pH of randomly selected whole ungutted fish (n=6 for both diet treatments) and fillets (n=12 for both diet treatments) was assessed using a pH Spear probe (Eutech Instruments PTE LTD, Singapore) after 0, 5, 9 and 14 days of chilled storage for each diet. Tissue pH was measured in the shoulder region on both the left and right sides of whole fish or fillets immediately adjacent to the tissue sampling location for lipid oxidation (Fig. 6.1).

6.2.5.6 Colour

Tissue surface colour was measured with a Minolta Chromameter CR-300 (Minolta Camera Co., Osaka, Japan), following 0, 5, 9 and 14 days of chilled storage. Whole ungutted fish (n=6 for both diet treatments) were filleted on both sides and the skin removed. The resulting fillets were then assessed for surface colour along with stored fillets (n=12 for both diet treatments). Colour measurement was performed at the shoulder region immediately adjacent to the tissue sampling location for lipid oxidation and pH (Fig. 6.1). The colour variables calculated were Hunter L*, a* and b* (Hunt, 1977), where L* describes lightness (100 = white, 0 = black), a* describes red-green chromaticity (+a* = red, -a* = green) and b* describes yellow-blue chromaticity (+b* = yellow, -b* = blue), (Jittinandana et al., 2006).

6.2.6 Statistical analyses

Results for α-tocopherol uptake, lipid oxidation, bacterial growth, pH development and colour degradation were analysed using ANOVA. To determine significant differences in means, Tukey's post-hoc analysis was applied. In order to determine the effects of diet and processing method and to look for interactive effects on fillet quality after 14 days of storage, two-way ANOVA was performed for lipid oxidation (MDA), bacterial growth, pH and colour (L*, a*and b*) with diet and processing method as fixed factors. One way ANOVA was subsequently performed for each processing method and diet to determine if values observed after 14 days of storage were significantly different from the value obtained at the commencement of storage. One way ANOVA was used to analyse the results obtained for fish growth, feed consumption, and crude fat content of fish. The level of significance was defined at $P<0.05$. All results are reported as the mean ± the standard error of the mean (SEM).

6.3 Results

6.3.1 Fish growth and feed consumption

During the feeding period (169) days, mean fish weight increased from 940.6±6.3 g to 1221.1±54.4 g for the enriched diet and 925.7±9.6 g to 1149.3±22.8 g for the standard diet (Table 6.1). Final fish weight (ANOVA, *P>0.05*), AFC and feed conversion ratio (FCR) (ANOVA, *P>0.05*) were not significantly different between diet treatments (Table 6.1).

Table 6.1 Effects of different dietary α-tocopherol acetate inclusion levels on apparent feed consumption, growth and FCR. Apparent feed consumption, growth and FCR did not vary significantly between diets (*P*>0.05).

Diet	Mean initial fish weight (g)	Mean final fish weight (g)	Mean apparent feed consumption per cage (g)	Feed conversion ratio (FCR)
Standard	925.7±9.6	1149.3±22.8	18357±285	3.66±0.39
Enriched	940.6±6.3	1221.1±54.4	18153±994	2.69±0.31

6.3.2 Deposition of α-tocopherol into barramundi flesh

The deposition of α-tocopherol acetate increased markedly over time in fish fed the enriched diet (Table 6.2), increasing from an initial value of 13.67±0.50 to 17.27±0.83 mg kg^{-1} after only 14 days of feeding. Alpha -tocopherol acetate continued to increase reaching a maximum of 24.18±1.06 mg kg^{-1} after 56 days of feeding, after which no further increase was observed. No significant changes in α-tocopherol were observed in fish fed the standard diet (Table 6.2).

Table 6.2 Effects of different dietary α-tocopherol acetate inclusion levels on the α-tocopherol concentration in barramundi flesh. Different superscript letters within rows indicates significant differences in α-tocopherol concentration, different superscript numbers within columns indicates that values were significantly different (*P*<0.05).

	Alpha-tocopherol concentration in fish flesh (mg kg^{-1})						
Diet	**Day 0**	**Day 14**	**Day 28**	**Day 42**	**Day 56**	**Day 140**	**Day 169**
Standard	14.03±1.93^{1}			13.89±0.57^{1}			12.52±0.34^{1}
Enriched	13.67±0.50^{a,1}	17.27±0.83^{b}	20.06±1.31^{b,c}	16.50±1.14^{a,b,1}	24.18±1.07^{c}	23.69±2.65^{c}	17.89±1.48^{a,b,2}

6.3.3 Lipid oxidation

After 9 days of chilled storage (2°C) lipid oxidation was found to be comparable in both whole ungutted fish and fillets, and was independent of the level of α-tocopherol in the rearing diet (Fig. 6.2). Values across all treatments were not significantly different (ANOVA, *P>0.05*) from malonaldehyde (MDA) values measured immediately following harvest. Malonaldehyde values averaged 2.06x10^{-2} ±0.0027 mg kg^{-1} with a minimum value of 1.67x10^{-2} ± 0.0018 mg kg^{-1} observed in whole ungutted fish that were fed the enriched diet and a maximum value of 2.60x10^{-2}±0.0095 mg kg^{-1} observed in filleted fish fed the standard diet (Fig. 6.2).

Significant increases (ANOVA, *P*<0.05) in MDA were seen in filleted fish after 14 days of chilled storage. Fish fed the standard diet and filleted immediately post-harvest, recorded the maximum MDA value of 4.6x10^{-2}± 0.0091 mg kg^{-1}, representing an approximate doubling in MDA over the 14 days of storage. A similar trend of increasing MDA was observed in fish that were fed the enriched diet and filleted prior to storage reaching 3.33x10^{-2} ± 0.0048 mg kg^{-1} after 14 days of chilled storage (Fig. 2). The increase in MDA for filleted fish fed the enriched diet was approximately half that recorded for filleted fish that were fed the standard diet, although this difference was not significant (ANOVA, *P>0.05*). Filleting fish prior to storage resulted in

significant increases in lipid oxidation over the 14 days of storage and appears to be somewhat constrained by enrichment with α-tocopherol in the rearing diet.

MDA values of fish that were stored whole and ungutted (Fig. 6.2) were unaffected by either the duration of storage or the level of α-tocopherol in the rearing diet (ANOVA, $P>0.05$). In general MDA values on day 9 and 14 were comparable to values obtained immediately after harvesting.

Differences in MDA were observed between post-harvest processing methods (skinned fillets and whole ungutted fish) after 14 days of storage, and this was independent of the level of α-tocopherol in the rearing diet. For example after 14 days of storage MDA levels in filleted fish fed the enriched diet were ~85% higher than in whole ungutted fish fed the enriched diet, although the difference was not significant (ANOVA, $P>0.05$), this trend was also observed for whole ungutted fish and fillets fed the standard diet (Fig. 6.2).

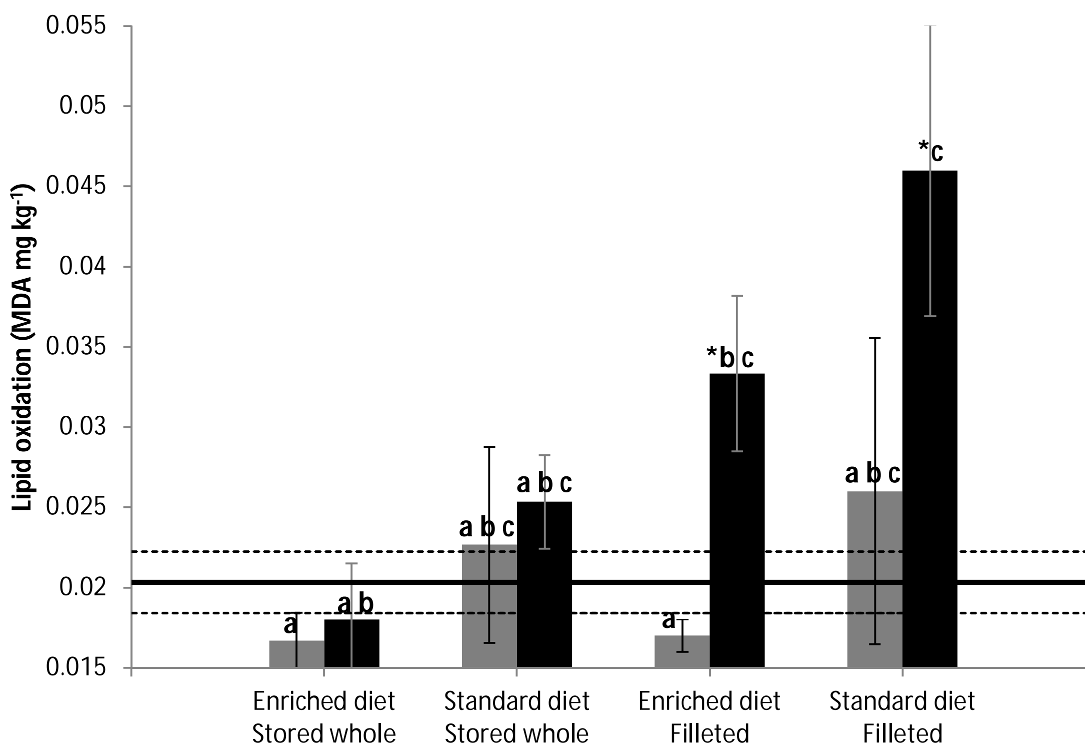

Fig. 6.2 Effect of α-tocopherol acetate enrichment and processing method on lipid oxidation (MDA) in barramundi flesh (n = 3) during chilled storage (2°C). Mean MDA concentration in barramundi flesh, collected from both diet treatments, immediately after harvest is represented by solid line with dashed lines representing the standard error of the mean, MDA concentration following 9 days of chilled storage is represented by grey bars, MDA concentration following 14 days of chilled storage is represent by black bars. Error bars represent standard error of the mean. *MDA values were significantly different from values observed immediately after harvest. Different letters above bars denotes statistically significant differences (ANOVA, $P<0.05$) between treatments.

The largest difference in MDA was observed between whole ungutted fish fed the enriched diet and filleted fish fed the standard diet. After 14 days of storage, MDA in filleted fish fed the standard diet was significantly higher (ANOVA, $P<0.05$), reaching $4.6 \times 10^{-2} \pm 0.0091$ mg kg^{-1}, than in whole ungutted fish fed the enriched diet that reached $1.8 \times 10^{-2} \pm 0.0091$ mg kg^{-1}. The combined effect of pre-harvest enrichment with α-tocopherol acetate with whole ungutted storage had the most significant impact in reducing lipid oxidation after 14 days of chilled storage.

6.3.4 Crude fat

At the commencement of the storage period, considerable variation in crude fat content was observed between individual cages although there was no significant difference between fish fed standard or α-tocopherol enriched diets (ANOVA, $P>0.05$). For cages fed the standard diet, the average crude fat content of adipose tissue was 3.87% with crude fat content of tissue samples obtained from individual cages observed to be 1.53%, 5.17% and 4.9%. For cages fed the α-tocopherol enriched diet, average crude fat content was 4.06% with samples obtained from individual cages containing 0.56%, 4.32% and 7.31% crude fat.

6.3.5 Bacterial counts

Bacterial counts remained low throughout the 14 days of storage, never exceeding 8.6×10^4 CFU g^{-1} in any individual treatment which is substantially below food safety guidelines ($<10^6$-10^7 CFUg^{-1})(Huss, 1995). In general bacterial counts increased across all treatments (Table 3) over the period of storage. The highest bacterial counts were observed in fillets after 14 days of storage. Despite this no significant differences (ANOVA, $P>0.05$) resulting from changes in diet or in processing method were observed (Table 6.3).

Table 6.3 Effect of dietary α-tocopherol acetate, storage method and storage time on bacterial growth (CFUg^{-1}). No statistically significant effects were observed ($P>0.05$).

		Bacterial growth ± SEM (CFU g^{-1})							
Diet	Storage method	Day 0		Day 5		Day 9		Day 14	
Standard	Whole	200	±100	200	±100	1500	±1253	300	±100
	Fillet	200	±100	667	±33	633	±186	30633	±27686
Enriched	Whole	300	±100	700	±306	4733	±3641	400	±0
	Fillet	300	±100	1033	±120	1700	±513	25333	±9871

6.3.6 pH

No significant changes in pH were observed in either whole ungutted fish or fillets after 5 days of storage. After 9 days the pH of whole ungutted fish increased significantly from values observed on day 0 (Fig. 6.3). This increase was for both the standard and the enriched diet. No further changes in pH were recorded. No significant (ANOVA, *P>0.05*) changes in pH were observed in filleted fish over the 14 day storage period.

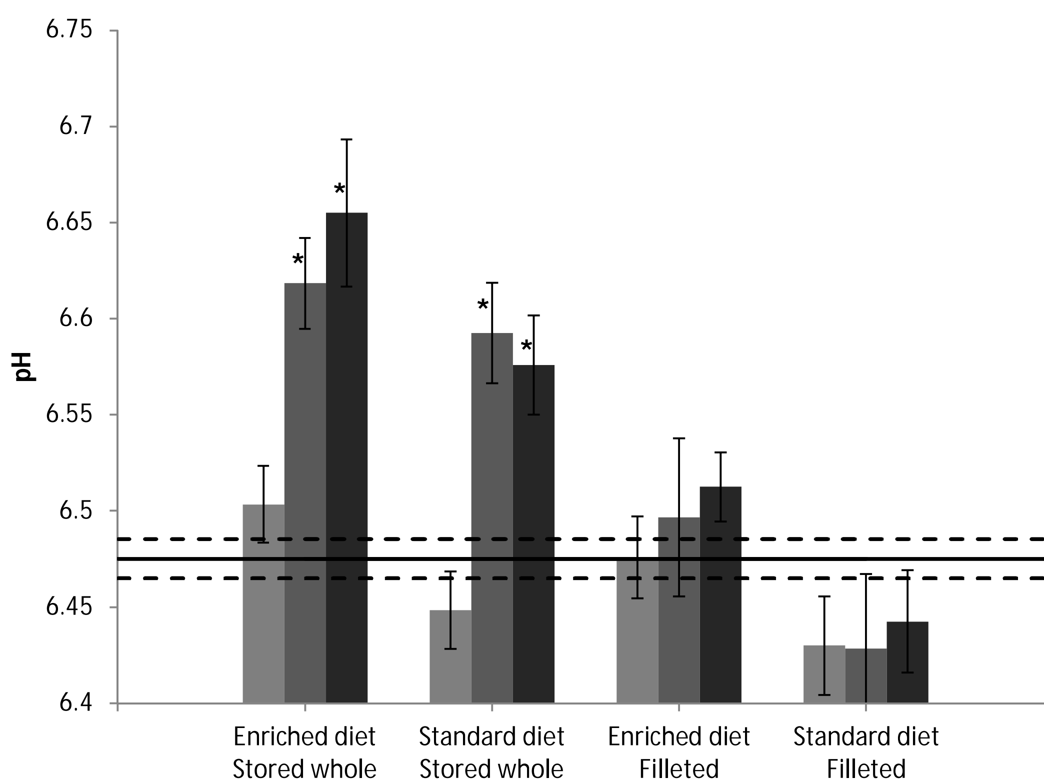

Fig. 6.3 Effect of α-tocopherol acetate enrichment and processing method on pH of barramundi flesh during chilled storage (2°C). Mean pH in barramundi flesh, collected from both diet treatments, immediately after harvest is represented by solid line with dashed lines representing the standard error of the mean, pH following 5 days of storage is represented by light grey bars, pH following 9 days of chilled storage is represented by dark grey bars, pH following 14 days of chilled storage is represent by black bars. Error bars represent standard error of the mean. *pH values were significantly different from values observed immediately after harvest.

6.3.7 Colour

6.3.7.1 Lightness (L*)

Lightness score (L*) immediately post-harvest was not affected (ANOVA, $P>0.05$) by diet with lightness scores of 40.97±1.31 L* and 41.02± 0.98 L* for fish fed the standard and enriched diet, respectively. Whole ungutted fish became paler during storage although this effect was restricted to the first 9 days of storage (Fig. 6.4a). Following this no further changes in lightness scores were observed (ANOVA, $P>0.05$). After 14 days of storage lightness scores were comparable in both groups of whole ungutted fish fed the standard diet and enriched diet, with L* scores of 43.73±0.8 and 44.18±0.39, respectively. In contrast fish filleted prior to storage became progressively paler over the full duration of the 14 day period, reaching L* scores of 46.99±1.02 and 45.54±1.37 for fish fed the standard and enriched diets, respectively (Fig. 6.4a). The observed difference in L* scores between storage methods was not significant (ANOVA, $P>0.05$).

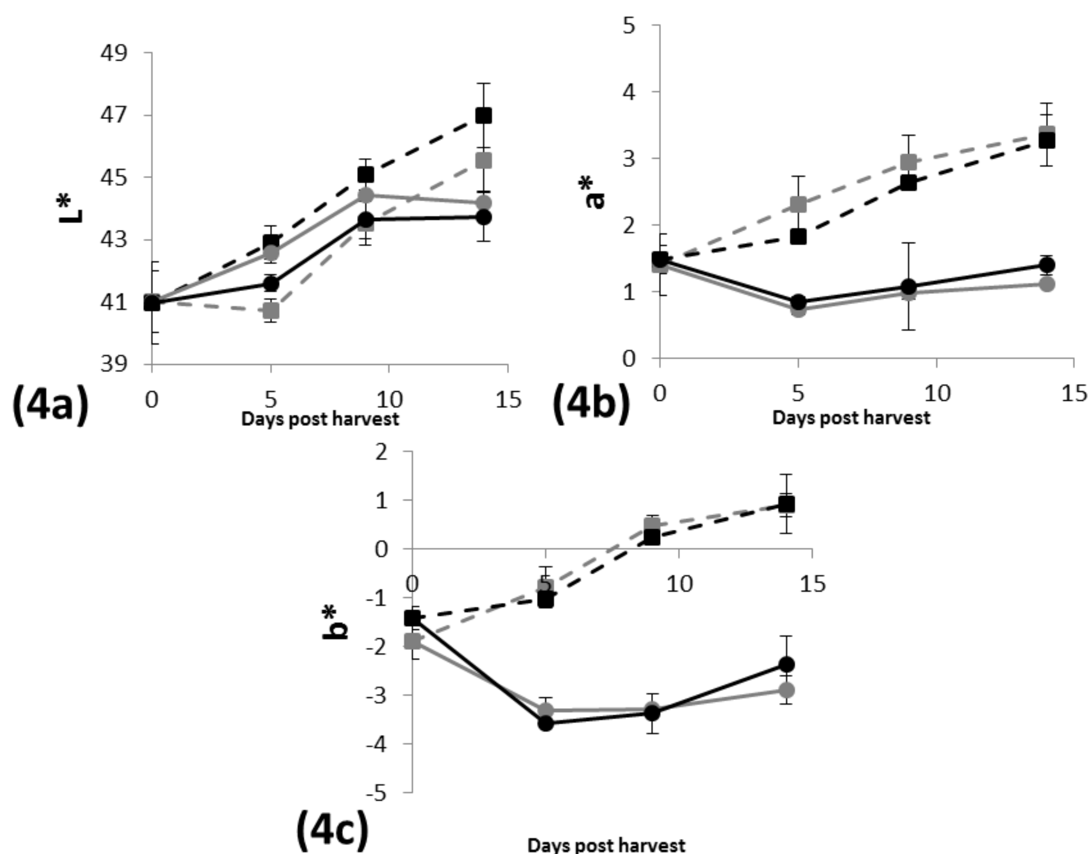

Fig. 6.4 Changes to lightness (6.4a), a* (6.4b) and b* (6.4c) colour scores of barramundi flesh (n = 3) during 14 days of chilled storage (2°C). Colour scores for the flesh of barramundi stored whole are represented by circles; solid black circles indicate that fish were fed the standard commercial diet while solid grey circles indicate that fish were fed the α-tocopherol acetate enriched diet. Solid black squares represent colour scores for barramundi fed the standard commercial diet and filleted prior to chilled storage whilst solid grey squares represent colour scores for barramundi fed the α-tocopherol acetate enriched diet and filleted prior to chilled storage. Error bars represent the standard error of the mean.

6.3.7.2 Colour (a* and b*)

Immediately post-harvest a* and b* colour scores were not significantly different between diets (Fig. 6.4b and c). However, clear changes in flesh colour were observed as early as 5 days after harvesting. Filleted fish trended towards higher a* and b* values, indicating that fillets were becoming more red (+a*) and more yellow (+b*) throughout the period of storage. Filleted fish reared on both diets were significantly redder and yellower after 14 days of chilled storage when compared to initial colour

scores. This was unaffected by diet with fish fed the standard and enriched diets displaying near identical trends.

In contrast a* and b* scores of whole ungutted fish were considerably more stable over the period of storage. The a* scores of whole ungutted fish showed a marginal decrease during the first five days of storage. Following this, a* scores stabilised over the remaining 9 days reflecting values (ANOVA, $P>0.05$) similar to those measured immediately post-harvest. Diet did not affect a* scores with fish fed the standard and enriched diets possessing comparable values (ANOVA, $P>0.05$) throughout the storage period. A similar overall trend was observed for b* scores for whole ungutted fish although values were significantly reduced (ANOVA, $P>0.05$) during the first 5 days of storage. This indicates that the flesh of whole ungutted fish became more blue (-b*) over the initial period of storage, after which time b* values largely stabilised. After the 14 day storage period, b* values for fish stored whole and ungutted and fed the enriched diet were not significantly different (ANOVA, $P>0.05$) from values recorded immediately after harvest. There was, however, a significant reduction in b* values (ANOVA, $P<0.05$) in whole ungutted fish fed the standard diet, although the magnitude of change was less than that observed for filleted fish.

6.4 Discussion

Barramundi fed both standard and enriched α-tocopherol acetate diets remained in good health over the duration of the study, with no mortalities recorded. Growth was approximately equivalent to commercially expected rates of growth (Glencross & Bermudes, 2012). The enhanced α-tocopherol acetate diet did not cause any overt signs of stress, as would be indicated by differences in mortality and growth between the two diets. This is similar to that observed for rainbow trout (*Oncorhynchus mykiss*,

Jittinandana et al., 2006) and Atlantic halibut (*Hippoglossus hippoglossus*, Ruff et al., 2002).

Uptake of α-tocopherol into the tissues of fish fed the enriched diet (628 mg kg^{-1} α-tocopherol) was rapid with a significant increase in tissue α-tocopherol concentration in the fillet after only 14 days of feeding. Tissue α-tocopherol reached a plateau after 8 weeks of feeding, indicating that the tissues had reached equilibrium with the diet. Following this, α-tocopherol fluctuated over time. These fluctuations may be attributed to a sub-clinical outbreak of *Chilidonella sp.* which was observed in cultured fish in the surrounding pond during this period. Immune function in fish is thought to be impacted by dietary α-tocopherol (Blazer & Wolke, 1984; Wise et al., 1993) and the presence of protozoan parasites may have led to α-tocopherol being sequestered from the flesh. However fluctuations in tissue α-tocopherol have also been observed by Watanabe et al. (1981) and Ruff et al. (2002). As highlighted by Ruff et al. (2002), the uptake and inclusion of α-tocopherol is determined by a diverse combination of species-specific factors and rearing conditions. Despite these fluctuations, tissue α-tocopherol concentration in the fillet broadly reflected the level of enrichment in the diet with tissue uptake being ~2-3% of the dietary level.

This study is the first account of lipid oxidation in whole, ungutted barramundi or barramundi fillets during chilled storage. Overall MDA values were remarkably low (<0.05 mg kg^{-1}) after 14 days of storage and this was irrespective of either diet or post-harvest processing technique. For example Ruff et al. (2002) recorded ~0.6mg kg^{-1} MDA in halibut fillets following 9 days of cold storage when fed 613 mg kg^{-1} α-tocopherol. Jittinandana et al. (2006) recorded 0.69 mg kg^{-1} MDA in trout fillets after 4 weeks cold storage. Onibi et al. (1996) observed 2-4 mg kg^{-1} MDA in Atlantic salmon fillets after 9 days of cold storage when fed 70-170 mg kg^{-1} α-tocopherol.

Low MDA values for barramundi appears to be indicative of the inherent oxidative stability of farmed barramundi when harvested under standard industry protocols. This could be attributed to the lower lipid content of barramundi flesh and/or the rapid chilling of fish at harvest (ice immersion), a technique known to extend the shelf life of fish flesh during storage (Olafsdóttir et al., 2006, Bao et al., 2007).

The optimal storage conditions employed during the experiment (continual 2°C) are also likely to have contributed to low lipid oxidation. However, storage conditions are often not optimal during normal commercial transport and handling, with barramundi being subjected to as many as 4-5 transfers through the supply chain (Carton & Jones, 2013). During these transfers it is common for boxed fish to be removed from chilled storage for redistribution. These unavoidable break points along with handling of fish by wholesalers, retailers and end users would result in higher, sub-optimal storage temperatures. This is likely to accelerate lipid oxidation in barramundi flesh. As such, it would be expected that the results obtained by this study would be further magnified, and the protective effects of α-tocopherol enrichment amplified above that observed in the current study. This is an area that requires further investigation.

In general, considerable variability was observed in MDA concentration within treatments. The source of this variability is difficult to resolve, although variations amongst individual fish with regard to tissue concentrations of α-tocopherol and/or muscle lipid are likely sources. For example, after 140 days of feeding on the enriched diet, α-tocopherol values ranged between 21.6 and 25.7 mg kg^{-1}, while crude fat values at the commencement of storage ranged from 0.56% to 7.31% in enriched fish and 1.53% and 5.17% for non-enriched fish. Such variability could be attributed to variability in feed intake, activity levels and natural variation between individual fish within treatments.

Increased tissue concentrations of α-tocopherol appeared to constrain the production of MDA in stored fillets, thereby providing a degree of protection against post-harvest lipid oxidation. This demonstrates that the protective effects of α-tocopherol in constraining lipid oxidation as shown in coldwater and subtropical fish (Frigg et al., 1990; Stéphan et al., 1995; Undeland et al., 1998; Gatta et al.,2000; Scaife et al., 2000; Ruff et al., 2002; Jittinandana et al., 2006) also extend to tropical fish cultured and harvested at much higher temperatures.

Lipid oxidation was most strongly influenced by post-harvest processing technique. MDA concentrations in stored fillets increased significantly after 14 days of storage whereas MDA concentrations in whole ungutted fish did not increase from initial levels. This is not surprising considering that lipid oxidation is dependent upon the availability of molecular oxygen. For example Undeland et al. (1998) demonstrated that storing herring with the skin intact reduces lipid oxidation in tissue underlying the skin layer, presumably by inhibiting the passage of oxygen into subcutaneous flesh.

In this study, the combined effect of α-tocopherol enrichment and storing fish whole and ungutted provided the greatest benefit in terms of reducing lipid oxidation. Lipid oxidation did not increase in enriched, whole ungutted fish during 14 days of chilled storage, in contrast, a pronounced increase in lipid oxidation occurred in fish fed the standard diet and filleted immediately post-harvest . This demonstrates the benefits of an integrated approach for the prevention of lipid oxidation that considers the application of strategies/techniques both pre and post-harvest. A combined strategy of pre-harvest enrichment of α-tocopherol and post-harvest storage of whole ungutted fish has the potential to deliver the highest quality product to end users, by inhibiting lipid oxidation and the resulting deterioration of flavour, aroma, texture and nutritional value of barramundi.

Flesh colour is also known to be affected by lipid oxidation in fish (Li et al., 1998; Hamre et al., 2003; Wetterskog and Undeland, 2004; Sohn et al., 2005; Guillerm-Regost et al., 2006; Buchanan and Thomas, 2008). A lightening of colour is frequently reported for fish flesh over prolonged storage periods (Robb et al., 2000; Ruff et al., 2002; Chouberta & Baccaunaud, 2006; Guillerm-Regost et al., 2006). Barramundi flesh in this study generally became lighter (increased L*) over the 14 days of storage, although lightness of whole ungutted fish peaked at day 9 before stabilising. Lightening in fillets over the storage period was somewhat constrained by α-tocopherol enrichment, although the most pronounced reduction was achieved via post-harvest processing technique with whole ungutted fish showing the least changes during storage. Progressive lightening is likely related to muscle structure alterations during rigor (Guillerm-Regost et al., 2006), causing alterations in light scattering properties (Erikson & Misimi, 2008), although Stien et al. (2005) highlight reductions in the translucency of fish muscle, leading to alterations in light-absorbing and reflecting properties (Ozbay et al., 2006).

Colour changes of fillets and whole ungutted fish during storage were clearly divergent. Fillets experienced progressive colour deterioration while the colour of whole ungutted fish remained relatively stable over the period of storage. Colour changes during storage were independent of dietary α-tocopherol. Similar results have been observed for Atlantic halibut (Ruff et al., 2002) and rainbow trout (Jittinandana et al., 2006).

Changes in muscle colour during storage are thought to be primarily driven by the oxidation of myoglobin to metmyoglobin (Chen & Chow, 2001). This process is accelerated at low pH (6.3-6.5) (Chow et al., 2009) and ultimately depends on myoglobin content, which differs between red (slow oxidative) and white (fast

glycolytic) muscle. Chaijan et al. (2005) demonstrated a decrease in redness index (a^*/b^* ratio) in sardine and mackerel as storage time increased, as a result of the destruction of the heme protein through autoxidation. However these findings are for red / slow oxidative muscle which has higher levels of myoglobin than white / fast glycolytic muscle. Similarly surface browning in cod fillets is caused by the formation of a denatured form of methemoglobin, resulting from blood contaminating the surface of the fillet (Kelly & Little, 1966). The timing and role of post-harvest processing is also important as contraction of the fillets during rigor is known to affect the light scattering properties of muscle as well as chemical light absorption (Skjervold et al., 2001; Veiseth-Kent et al., 2010).

Deterioration in lightness and colouration are used by consumers as a visual and rapid means of assessing freshness and desirability (Olafsdóttir et al., 2004). Any shift away from the perceived colour of freshly harvested fish is often perceived as a reduction in freshness. In this study, the greatest shift in colour and lightness during storage occurred in fish that were filleted prior to chilled storage. These fillets exhibited the greatest degree of paling during storage and displayed the greatest shift in a* and b* chromaticity.

Decreased muscle pH in fish flesh has been demonstrated to contribute to colour degradation (Chow et al., 2009) and can also increase muscle gaping, blood spotting, flesh texture alterations and drip loss (Robb & Kestin, 2002; Wilkinson et al., 2008). The pH of fish flesh is frequently observed to increase during chilled storage (Gildgerg, 1978; Ruff et al., 2002; Sarika et al., 2012). This can be attributed to the production of volatile bases by bacteria within the tissues as glycogen is utilised as an energy source, as well as to the actions of heterofermentative lactic acid bacteria which grow and degrade amino acids with the formation of carbon dioxide and other

decarboxylation products (Sarika et al., 2012). In this study, increases in pH in whole ungutted fish was observed whilst the pH of filleted fish remained stable and appeared to decrease in unenriched fillets during storage. The pH of the flesh appeared to be inversely proportional to the degree of lipid oxidation occurring. It is conceivable that when lipid oxidation proceeds, bacteria are able to take advantage of the resulting oxidative products as an energy source rather than glycogen and their other normal substrates. As a result, the production of volatile bases may be reduced thus preventing associated pH increases. The lower pH observed in filleted fish after 9 and 14 days of chilled storage has the potential to advance quality degradation and may have contributed to the colour degradation observed.

Bacterial growth was observed to be highest in filleted fish after 14 days of chilled storage although a high degree of variability within treatments was observed. In general, the flesh of live healthy fish is considered to be sterile as the fish's immune system prevents bacteria from proliferating (Huss, 1995). However microorganisms are present on all external surfaces (gills and skin) and in the intestines. Upon death, surface bacteria colonise scale pockets and can invade the flesh by entering between muscle fibres. However Murray & Shewan (1979) found that a very limited number of bacteria invaded the flesh during iced storage. The rate of microbial invasion during iced/chilled storage is thought to be influenced by skin thickness as well as the environmental temperature from which the fish has been harvested with bacterial proliferation proceeding more slowly in tropical, warm water species (Gram et al., 1990). Since barramundi are a dense-skinned, tropical species, bacterial invasion during chilled storage of whole, ungutted fish is expected to proceed slowly during 14 days of chilled storage. This was reflected in the current study where bacterial growth in whole ungutted barramundi remained very low throughout storage. However,

filleting and skinning have the effect of removing the protective capacity of the skin. As such, bacteria are introduced into the underlying flesh and can proliferate. The proliferation of bacteria in iced/chilled tropical fish often passes through a lag phase of 1-2 weeks as the bacteria present are not adapted to the cold temperature (Gram et al., 1990). This was reflected in the current study where the greatest increase in bacterial growth occurred in stored fillets between 9 and 14 days although these increases were not statistically significantly.

6.5 Conclusions

Dietary enrichment with α-tocopherol acetate, combined with whole ungutted storage, is effective in preventing lipid oxidation and associated deteriorations in product quality in farmed barramundi flesh during chilled storage. Storing fish whole and ungutted also prevented reddening and yellowing of fillets during storage and appeared to reduce lightening of flesh between 9 and 14 days of storage. Bacterial proliferation after 14 days of chilled storage was also reduced by storing fish whole and ungutted. Further research investigating higher dietary inclusion of α-tocopherol acetate would be beneficial in determining if the time required for enrichment could be compressed or if higher levels of enrichment could be achieved. Investigating the impacts of sub-optimal storage conditions, simulating industry realities, would also be beneficial and could be investigated by forcing oxidation through elevated temperatures during storage.

6.6 References

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CHAPTER 7

General Discussion

Preface

This thesis is directly concerned with applying scientific research to an industry issue. The primary objective of the research program was to address quality deficits in farmed barramundi. The research has been successful in this respect, generating data that can underpin the development of a quality optimisation strategy for the Australian barramundi aquaculture industry. This success relied on a high level of collaboration with the Australian barramundi farming industry.

7.1 Research – Producer – Industry collaboration

The research program involved close collaboration with members of the Australian barramundi aquaculture industry, their peak body, the Australian Barramundi Farmers Association (ABFA) and Ridley Agriproducts Pty Ltd., a large commercial manufacturer of aquaculture diets. The research was strengthened by this collaboration and farmers and industry leaders have shown a strong interest in the research process and findings. The Australian Barramundi Farmers Association and Ridley Agriproducts Pty Ltd., along with industry members, have supported the research project by contributing cash funding, fish, pond space, cages, tanks, feed and knowledge of fish husbandry. Research plans and findings have been disseminated to industry through presentations at successive annual ABFA conferences between 2010 and 2015 and at research and development meetings and workshops. This has also facilitated feedback from industry that has strengthened research outcomes while at the same time giving legitimacy to the project. At all times the independence and integrity of the scientific research conducted for this thesis was respected by all

stakeholders. This collaboration has continued beyond the completion of the current research program with additional studies currently underway and future research planned. This future research will be facilitated by the strong relationships developed during the course of this research program.

Although the primary focus of this project was to address quality deficits in farmed barramundi, the findings presented have wide-ranging implications that extend beyond the Australian barramundi aquaculture industry. The findings presented in this thesis have important implications across three related fields: advances in the field of aquaculture research; aquaculture production and product quality globally, across a diverse range of species and geographic locations; and practical applications in the Australian barramundi aquaculture industry. A brief summary of the key findings of this research program will be presented followed by a detailed discussion of these findings in the context of these three related fields.

7.2 Summary of key findings

This thesis has identified causative agents for flavour quality concerns in farmed barramundi and considered practical management responses to these issues. The results of detailed investigations of off-flavour tainting, flavour complexity and deteriorations in product quality during post-harvest storage have been presented.

The underlying factors leading to off-flavour tainting in pond-reared barramundi were investigated, with findings presented in Chapter 2. Geosmin (GSM) was identified as the primary compound associated with off-flavour tainting of pond-reared barramundi. Concentrations of this compound, in pond waters, generally persisted at levels up to ~2.0 $\mu g\ L^{-1}$ and were observed to exceed 14 $\mu g\ L^{-1}$ (Fig. 2.1). This is consistent with the findings of Exley (2014) who investigated off-flavour compounds throughout the geographic range of Australian freshwater barramundi

aquaculture over a three-year period. Barramundi housed in pond water accumulated GSM in edible tissue with the level of accumulation being highly dependent on the concentration in holding water (Fig. 2.2, $R^2 = 0.97$). Human sensory evaluation of pond-reared barramundi demonstrated that the intensity of off-flavour tainting was highly correlated with the concentration of GSM present in flesh (Fig. 2.4, $R^2 = 0.94$). The threshold level of sensory detection for GSM in farmed barramundi was estimated to be below 0.74 ug kg^{-1}. This is comparable to the sensory threshold of GSM in trout (0.9 µg kg^{-1}, Robertson et al., 2005), channel catfish (0.25 - 0.5 µg kg^{-1}, Grimm et al., 2004), bream (0.90 µg kg^{-1}, Persson, 1980) and sea trout (0.59 µg kg^{-1}, Persson, 1980). As the level of GSM in barramundi tissue increased, the intensity of off-flavour tainting also increased, throughout the range of concentrations assessed (0.74 - 4.47 µg kg^{-1}) (Fig. 2.4).

In order to further investigate specific aspects of off-flavour tainting in farmed barramundi, a novel method of imparting off-flavour into captive fish was developed and is presented in Chapter 3. This simple, yet precise, reproducible and low cost method for producing natural GSM utilised laboratory cultures of the common cyanobacterium *A. circinalis*. When fish were exposed to these cultures in a controlled environment, they developed off-flavour tainting that was identical to that occurring in fish exposed to GSM in aquaculture pond water across ten key organoleptic attributes (Fig. 3.1). This method affords precise control over the level of GSM to which fish are exposed, can be used to manipulate the GSM content of fish tissues and can generate fish with varying intensities of off-flavour tainting on-demand.

The ability to manipulate water GSM and generate off-flavour tainted fish was used to investigate the uptake, depuration and spatial distribution of GSM in barramundi

fillets. The findings of this research are presented in Chapter 4. Uptake of GSM was observed to progress rapidly when fish were exposed to GSM at the upper limit of concentrations observed in pond water (15.1 $\mu g\ L^{-1}$), exceeding the threshold of sensory detection within 3 minutes of exposure and plateauing after 3 hours (Fig. 4.3).

On return to clean water, depuration was slower than uptake with a half-life of GSM in fish flesh of approximately 4 days being observed and residual GSM, above the predicted threshold of detection remaining after 14 days (Fig. 4.4). This pattern of rapid uptake and prolonged depuration is consistent with several other species of fish (Martin et al., 1988; Lloyd & Johnsen, 1992; Yamprayoon & Noomhorn, 2000; Robertson et al., 2005). Although depuration is more protracted than uptake, sensory assessment revealed that fish exposed to GSM within the range most frequently encountered in pond water (2.15 $\mu g\ L^{-1}$) could be depurated of off-flavours within 8 days (Table 4.1).

A distinct pattern in the spatial distribution of GSM in barramundi fillets was also observed. While the dorsal shoulder and posterior tail regions were similar in this regard, the ventral belly region was profoundly richer in GSM (Fig. 4.5). This spatial distribution of GSM was associated with crude fat content (Fig. 4.6) which showed a very similar spatial pattern (Fig. 4.5). This supports the observation of Percival et al. (2008), that off-flavour tainting was strongest in the belly region of cage reared barramundi from Lake Argyle, northern Australia.

The spatial distribution of off-flavour tainting has also been observed in cultured channel catfish. Grimm et al. (2015) observed that MIB was elevated in the skin section of fillets, presumably due to the greater abundance of crude fat in this region. Zimba & Grimm (2015) also observed a pattern in the spatial distribution of GSM and MIB in channel catfish. The fillet region closest to the head was observed to possess

the highest level of these compounds with successively lower concentrations occuring in the mid section and tail end of the fillets (Zimba & Grimm, 2015). It is possible that this pattern of spatial distribution is also associated with elevations in crude fat in the belly region of the fillet. Further research is required to determine this relationship.

Having established the potential to mitigate off-flavour tainting in farmed barramundi, the ability to further enhance flavour quality through dietary manipulation was investigated and is presented in Chapter 5. It was confirmed that the flavour of freshwater fish could be enhanced by the short-term application of a diet enriched with marine algae. The overall desirability of barramundi was increased by imparting rich and complex seafood-like flavours (Fig. 5.1) that increased in intensity during three weeks of feeding with diets containing ≥20% *Ulva ohnoi* (Fig. 5.2). This flavour change was associated with an increase in the concentration of DMS in fish flesh (Fig. 5.3).

Understanding the underlying factors affecting off-flavour tainting, developing strategies to mitigate off-flavour tainting and enhancing flavour through dietary manipulation have been shown to optimise flavour quality at the point of harvest. However it is critical that flavour and quality are not degraded during the post-harvest storage period, prior to human consumption. Although the majority of Australian farmed barramundi is grown in geographically remote locations with protracted transportation periods to major markets, this aspect of the supply chain was previously unexplored.

The storage stability of farmed barramundi as well as pre- and post-harvest management strategies to optimise storage life was investigated for farmed barramundi with findings presented in Chapter 6. Dietary enrichment with α-tocopherol acetate, combined with whole ungutted storage prevented lipid oxidation,

and the associated deteriorations in product quality, in farmed barramundi during 14 days of chilled storage (Fig. 6.2). Storing fish whole and ungutted also prevented reddening and yellowing of fillets during storage and appeared to reduce lightening of flesh between 9 and 14 days of storage (Fig. 6.4). Bacterial proliferation after 14 days of chilled storage was also reduced by storing fish whole and ungutted (Table 6.3).

7.2 Advances in the field of aquaculture research

The data presented in this thesis have important implications for aquaculture research globally. The key findings of this thesis will be discussed with respect to flavour quality research addressing: off-flavour tainting, flavour enhancement and product deteriorations during the post-harvest storage period.

7.2.1 Off-flavour tainting

Off-flavour tainting of pond-reared barramundi was linked to the presence of GSM in culture water. Although GSM is well known to cause off-flavour tainting in fish (e.g. Tucker, 2000; Howgate, 2004; Percival et al., 2008; Carton & Jones, 2013), this is the first time that GSM has been implicated in the off-flavour tainting of tropical farmed barramundi. Fish tainted with GSM were described as having a muddy and earthy aroma and a muddy/earthy flavour. These descriptors are consistent with most off-flavour events associated with GSM exposure (e.g. Tucker 2000, Howgate 2004, Percival et al. 2008, Carton & Jones, 2013). However, sensory assessment also revealed rotting vegetation aroma, rotting weed flavour and weedy aftertaste in off-flavour tainted fish. These descriptors are not generally associated with GSM accumulation, although Percival et al. (2008) identified weedy odour in off-flavour tainted barramundi exposed to MIB.

Weedy-type aroma and flavour descriptors are now reported in the only published studies that describe off-flavour tainting of farmed barramundi (Chapter 2: Jones et

al., 2013; Percival et al., 2008). This highlights the potential for variability in the description of off-flavour tainting between different species of fish. However, the use of different descriptive terms may also arise due to cultural or demographic factors. These published studies, investigating off-flavour tainting in farmed barramundi, were conducted in Australia using trained sensory panels. Trained sensory assessment of Australian silver perch has also reported weedy flavours in pond cultured fish (Allan et al., 2000; Allan & Rowland, 2005). Anecdotal reports of off-flavour tainting in a diverse range of wild-caught Australian freshwater fish also include comments on weedy-type flavours (Pers. com. E. Poole; *About Fishing NQ Magazine*, vol. 1, 2010). Researchers should be aware of the potential for off-flavour tainting to be described by varying descriptors in different species of fish and in different demographic groups.

The thesis also presents a new method for intentionally imparting off-flavour tainting into captive fish (Chapter 3). This method overcomes difficulties arising from the erratic nature of on-farm off-flavour events. It also eliminates impediments associated with the use of synthetic GSM such as possible divergence in the kinetics of uptake and depuration and human ethical constraints, while ensuring authenticity of organoleptic attributes associated with off-flavour tainting. The ability to impart natural off-flavour taint into cultured fish “on demand”, at predetermined levels will enable researchers to perform targeted experiments in a wide range of culture species. This will greatly extend our knowledge of off-flavour tainting and facilitate the development of practices to alleviate the problem of off-flavour tainting in cultured fish.

Using this method, several important aspects of off-flavour tainting in farmed barramundi were investigated and are reported in Chapter 4. Rates of uptake and

depuration were investigated for fish exposed to GSM at the upper limit of concentrations known to occur in culture water. The spatial distribution of GSM within the white muscle tissue of off-flavour tainted barramundi was also examined and its relationship with crude fat explored.The time required to recover flavour quality in moderately tainted fish was also investigated to determine the potential to eliminate off-flavour tainting.

Following exposure to an extreme level, GSM accumulated rapidly in barramundi tissue with a significant increase in flesh GSM observed within only 3 minutes of exposure. After 3 hours, GSM accumulation plateaued with no further increase in flesh GSM observed beyond this time. This is markedly similar to the rapid uptake and prolonged depuration observed in temperate and cold-water species including rainbow trout and channel catfish (Lloyd & Johnsen, 1992; Martin et al., 1988; Robertson et al., 2005). This indicates that uptake and depuration rates can be similar in various species of fish cultured at varying latitudes. This supports the findings of Yamprayoon & Noomhorn (2000) who observed a similar pattern in the uptake and depuration of GSM in tropical tilapia. Researchers should be aware that the uptake of GSM is likely to proceed extremely rapidly in tropical, temperate and cold-water species.

The spatial distribution of GSM in fish fillets observed in this thesis has important implications for aquaculture research. It is critical that the region from which samples are collected for analytical and sensory assessment is tightly controlled and documented. Failure to do so may cause misleading or variable results. This is also a key consideration when reviewing previous publications. Findings that do not specify the region of the fillet sampled and/or do not provide results for a range of regions should be treated with caution.

7.2.2 Flavour complexity

It has also been confirmed that the flavour of farmed fish can be actively enhanced by dietary manipulation (Chapter 5). This supports the findings of Ma et al. (2005) who reported sensorial effects in silver sea bream after 8 weeks of feeding on diets containing marine algae. In the current research program a significant change in flavour was observed after only 7 days of feeding with flavour intensity increasing throughout the three week feeding period. This finding has significant implications for aquaculture research. It indicates the potential for flavour manipulation within a relatively short time frame. Hence, it is recommended that future studies investigating flavour enhancement through dietary manipulation should assess sensory attributes at frequent intervals, including during the first three weeks of feeding.

The potential causative relationship observed between the potent flavour compound DMS and flavour enhancement also has implications for aquaculture research. Most previous studies have implicated BPs as the predominant flavour compound associated with flavour complexity in fish (e.g. Boyle et al., 1992; 1993; Whitfield et al., 1997; 2002). Additionally, a number of studies have investigated the ability to enhance BP levels in cultured fish to enhance flavour (Ma et al., 2005; Kim et al., 2007; Fuller et al., 2008). While BPs are clearly an important flavour compound in wild and captive fish species, DMS may also have the potential to impart desirable flavours. Future research should include an investigation of the abundance of DMS in tissues.

7.2.3 Product deterioration during the post-harvest storage period

Feeding barramundi a diet enriched with α-tocopherol acetate increased the α-tocopherol content of farmed fish and minimised lipid oxidation during the post-harvest period (Chapter 6). This confirms that that the fortifying effects of α-

tocopherol frequently observed in cold and temperate water species (Frigg et al., 1990; Harare et al., 1998; Ruff et al., 2003; Chen et al., 2008) also apply to tropical fish.

The most effective strategy in preventing deteriorations in product quality was achieved by implementing management strategies during both the pre-harvest period, by dietary α-tocopherol acetate enrichment, and the post-harvest period, by maintaining fish whole and ungutted. It is important that researchers take into account the effects of both pre- and post-harvest practices, as well as considering possible interactive effects, on quality indices during the post-harvest storage period.

7.2.4 Summary

In summary, the thesis presents a number of advances in the field of aquaculture research that have implications for researchers investigating product quality. Implications for aquaculture research have been presented across the three major quality issues typically investigated: off-flavour tainting, flavour complexity, and product deterioration during the post-harvest transport and storage period.

7.3 Implications for aquaculture production and product quality

As well as having wide-ranging implications for aquaculture research, this thesis presents new information that can be used to enhance product quality in a diverse range of cultured species and aquaculture production systems, across a broad geographic range. Critical information has been presented with respect to off-flavour tainting, flavour enhancement and post-harvest quality preservation.

7.3.1 Off-flavour tainting

In tropical barramundi ponds, GSM was observed to persist continuously throughout the sampling period, being detected in 88% of samples collected (Chapter 2). The abundance of GSM in these ponds was not correlated with solar radiation or

temperature fluctuations (Chapter 2). Exley (2014) identified a similar trend with GSM repeatedly observed in tropical freshwater barramundi ponds over a three-year period and concentrations not appearing to be related to seasonal factors (Pers com., P. Exley). In contrast, off-flavour episodes in temperate locations are known to be somewhat seasonal with GSM production supressed during periods of low temperature and solar radiation (Lovell et al. 1986, Robertson et al. 2006, Robin et al. 2006).

In the tropics, temperature and solar radiation are relatively stable throughout the year and are unlikely to limit the growth of taint producing microbes. Under such conditions, off-flavour tainting is unlikely to exhibit seasonal trends. This is a critical consideration for aquaculture systems located in tropical regions. Growers must be aware of the potential for off-flavour tainting to occur year-round with little impact from seasonal factors.

Extreme GSM levels, above 4.0µg L^{-1} were observed in tropical barramundi ponds (Chapter 2). Such high levels of off-flavour taint are generally regarded as being exceptionally high, although similar levels have been observed in channel catfish (*Ictalurus punctatus*) culture ponds (Martin et al. 1988, Van der Ploeg & Boyd, 1991; Van der Ploeg et al., 1992). The highest GSM concentration observed in tropical barramundi pond water was 14.37 µg kg^{-1}. As the intensity of off-flavour tainting is known to be tightly correlated with exposure level (Chapter 2), very intense off-flavour tainting is likely to persist at this extreme concentration. The accumulation of GSM in fish exposed to an extreme level (15.1 µg L^{-1}) was extremely rapid, with the tissue concentration of GSM exceeding the predicted threshold of human sensory detection within three minutes of exposure (Chapter 4). This indicates that even very short-term exposure to the extreme GSM levels occurring in tropical aquaculture

ponds can impart clearly discernible off-flavour tainting. Aquaculture producers must be aware of the potential for off-flavour tainting to occur within minutes of exposure to GSM. This may be especially important in tropical locations where GSM concentrations are likely to persist for extended periods of time and have the potential to reach exceptionally high concentrations.

The prolonged depuration of GSM observed in this research program (Chapter 4) also has impliations for producers. The findings of this thesis show that under extreme conditions, GSM can remain in edible tissues beyond 14 days after fish are returned to clean, untainted water. This is a critical concern for producers and demonstrates the potential for off-flavour tainting to persist in pond reared fish for extended periods of time, even when GSM in pond water abates.

However, it has also been demonstrated that for fish exposed to a moderate level of GSM (2.15µg L^{-1}), flavour quality can be recovered by depurating fish in clean water. Off-flavour tainting was eliminated from moderately off-flavour tainted fish after 8 days of depuration. This shows the potential for depuration to recover flavour quality in cultivated fish. As the rate of depuration is likely to be influenced by a range of species-specific factors (Howgate, 2004), further research on individual species will be required to optimise depuration procedures.

The spatial distribution of GSM in fish fillets, presented in Chapter 4, impacts aquaculture farmers in two ways. The spatial distribution of off-flavour compounds in the fillet must be carefully considered where fish are assessed for flavour and quality prior to harvest. It is critical that the most heavily tainted region of the fish that is usually sold and consumed is used for sensory assessment procedures. In farmed barramundi, the ventral belly region of the fillet was observed to be more heavily tainted with GSM compared to other fillet regions. This was a consequence of the

high abundance of crude fat in this region. The spatial distribution of off-flavour tainting may vary between species of fish. Grimm et al. (2015) found that off-flavour tainting compounds were most abundant in the skin section of channel catfish fillets while Zimba & Grimm (2015) found that off-flavour compounds were most elevated at the head-end of channel catfish fillets and declined towards the tail-end. Such variations in the spatial distribution of GSM should be investigated and considered so that producers can make informed decisions during sensory assessment procedures.

The spatial distribution of off-flavour tainting may also be exploited during processing to minimise the sale of off-flavour tainted flesh. It may also be possible to differentiate the various regions of the fillet for marketing purposes. For example, in barramundi, the shoulder and tail portions may be considered to be of the highest quality and therefore marketed differently from the potentially less desirable belly region.

7.3.2 Flavour complexity

The potential to enhance the flavour of farmed fish has clear benefits to fish farmers. The application of a diet enriched with marine algae increased flavour complexity and seafood flavour in freshwater fish (Chapter 5). This could potentially give producers the ability to produce low-cost freshwater fish with the organoleptic attributes of marine-caught seafood.

Further research is required to facilitate commercial application of such a diet. However, the potential to enhance flavour in farmed fish through dietary manipulation has been clearly demonstrated. Flavour effects were observed after only 7 days of feeding. This is advantageous as it allows producers to alter the flavour of their product rapidly, in response to customer demands. The potential to enhance flavour rapidly also alleviates the potential loss of growth during the flavour-enhancing

period. This is a consequence of marine algae often being poorly digestible by fish (Pereira et al., 2012; Marinho et al., 2013; Wassef et al., 2013). As a result, diets composed of a significant fraction of marine algae are likely to retard fish growth. However, by restricting the feeding period, the loss of growth during the flavour-enhancing period will be minimised.

A significant benefit of using *Ulva* in a flavour enhancing diet is that many species within this genus are commercially harvested and cultivated. Many *Ulva* products are commercially available and can be incorporated into aquaculture diets without the need to establish new industries to produce dietary ingredients. The potential for *Ulva* to be used in waste water remediation is a further advantage. There is great potential for *Ulva* to be produced in bioremediation systems at marine aquaculture facilities, thereby improving environmental outcomes (Lawton et al., 2013). The algae produced in these systems may then be incorporated into aquaculture diets to optimise product quality and economic benefits for producers.

7.3.3 Product deterioration during the post-harvest storage period

Aquaculture farmers frequently undertake management practices to ensure that their product is fortified against deteriorations in quality during post-harvest transport and storage. This is often achieved by rapid chilling at the point of harvest and maintaining optimal storage conditions during the post-harvest period (Carton & Jones, 2013). Quality deterioration during post-harvest storage was investigated in farmed fish and results are presented in Chapter 6. This research examined the effects of management strategies applied during both the pre-harvest and post-harvest periods.

Pre-harvest dietary enrichment with α-tocopherol acetate restricted lipid oxidation and the associated deteriorations in product quality during 14 days of chilled storage.

Diets enriched with supra-nutritional α-tocopherol acetate are advantageous to producers due to their ease of application. Such diets are also readily produced by feed manufacturers: α-tocopherol acetate is routinely included in aquaculture diets to provide basal nutritional requirements of culture animals. It is also a relatively low-cost ingredient with elevated inclusion levels presenting no difficulties to the manufacture of extruded diets (pers com. R. Smullen). This method is effective in reducing lipid oxidation during storage, is low cost and is easily applied.

Storing fish whole and ungutted was also effective in constraining lipid oxidation, preventing pH decline, limiting colour deterioration and minimising bacterial proliferation in fish flesh during storage. Aquaculture producers who process their fish prior to sale can exploit this information by ensuring that fish are maintained whole and ungutted for as long as possible. In doing so, lipid oxidation, and the associated deteriorations in product quality, may be minimised, thereby yielding higher quality at the point of consumption. Those producers who do not have direct control over processing procedures after sale now have the data required to provide recommendations to third party processors. By providing fish processors with the information presented in this thesis, at it pertains to maintaining quality during chilled storage, it may be possible to ensure that higher quality is maintained through to the end consumer.

7.3.4 Summary

In summary, the thesis provides critical knowledge to aquaculture producers globally, across a wide range of cultivated species and geographic locations, that can be used to develop methods to optimise product quality. It has addressed the three most significant quality issues facing aquaculture producers; off-flavour tainting,

flavour complexity, and product deteriorations during the post-harvest transport and storage period.

7.4 Applications for the Australian barramundi aquaculture industry

Drawing on the discussion of this thesis with respect to advances in aquaculture research and implications for production and product quality, a number of key recommendations for the Australian barramundi farming industry will now be identified.

The Australian barramundi farming industry faces a unique set of challenges with respect to optimising product quality. The majority of Australian barramundi farms are located in the tropics where high water temperature and solar radiation persist for the majority of the year. As microbial populations that cause off-flavour tainting are known to thrive under such conditions (Paerl & Tucker, 1995), this is likely to increase the frequency and severity of off-flavour tainting events. This was confirmed by the observation of GSM persisting in pond waters at moderate to extreme concentrations throughout the sampling period (Chapter 2) and is supported by the findings of Exley (2014).

In Australia barramundi is a highly valued food fish. As well as being extensively cultivated across tropical regions, fish are commercially harvested by wild-capture fisheries across northern Australia. Cultivated fish compete directly with wild caught marine and estuarine fish in the market place. In some instances, these wild-caught specimens have been noted as having a higher level of flavour complexity as well as possessing stronger shellfish (prawn) flavour characteristics (Frank et al., 2009) and frequently achieve higher prices in the market place (pers com, E. Poole). The barramundi farming industry would benefit from the development of a reliable

technique to increase flavour complexity and add seafood-like flavours to harvested fish.

Most barramundi farms in Australia are also located in geographically remote locations with protracted transport times to major metropolitan markets. This increases the potential for quality deterioration to occur prior to retailing and human consumption. The industry would benefit from the development of an industry-wide strategy to prevent quality deterioration during chilled storage.

The research findings set out in this thesis suggest that the Australian barramundi farming industry should develop a management strategy to optimise the quality of cultured barramundi. The details of any such strategy are beyond the scope of this thesis. However the research findings suggest that the industry should develop best practice guidelines, quality standards, educational programs for producers and related initiatives. To address quality deficits in farmed barramundi, a quality optimisation strategy must address off-flavour tainting, flavour complexity and deterioration in product quality occurring during the post-harvest period.

7.4.1 Off-flavour tainting

Having observed GSM to be the predominant off-flavour compound in Australian pond reared barramundi a management strategy is required to prevent the harvest and sale of off-flavour tainted fish. Three potential management strategies for preventing the sale of off-flavour tainted fish are presented:

1) Detection of off-flavour tainted cohorts prior to harvest

The first approach to preventing the sale of off-flavour tainted fish is to identify off-flavour tainted cohorts prior to harvest, by sensory assessment, and exclude them from harvest. Although this approach may appear to be simple, there are several confounding factors that must be recognised. Three factors that must be addressed for

sensory assessment to be effective are: assessors must be familiar with the organoleptic characteristics of off-flavour tainted fish; the region of the fillet from which samples are collected for sensory assessment must be carefully considered to ensure that the sample accurately detects off-flavour tainting in all normally consumed tissues; and the number of fish collected must also adequately represent the entire cohort.

The organoleptic nature of GSM induced off-flavour tainting in Australian farmed barramundi is presented in Fig. 2.3 with definitions of sensory descriptors appearing in Appendix 1. This list of sensory attributes could be used by Australian barramundi farmers as a reference to which off-flavour tainting can be compared. The off-flavour attributes identified in the thesis by the trained sensory panel were: muddy aroma, rotting vegetation aroma, earthy aroma, muddy-earthy flavour, rotting weed flavour, weedy aftertaste and earthy aftertaste. These descriptors should be used by barramundi farmers to familiarise panellists with the organoleptic attributes that they may experience during sensory assessment.

Tissue samples for sensory assessment should be collected from the ventral belly region of fish. This region was observed to possess the highest concentration of GSM of the three fillet regions assessed (Chapter 4). If off-flavour tainting is not detected in this region then it can be assumed with a high level of confidence that off-flavour tainting will not be detected in the remainder of the fillet.

It is also necessary that a sufficient number of fish be tasted to ensure that the sample collected is representative of the entire population. Considerable variability in the concentration of GSM in fish tissue and the intensity of off-flavour tainting was observed between individual fish (refer to chapters 2, 3 & 4). This is most likely related to the high variability of crude fat observed between individual fish (as

discussed in Chapter 6) which is known to affect the uptake and deposition of GSM in tissue (as discussed in Chapter 4). Although variability in the intensity off-flavour tainting was not investigated with respect to optimising sensory assessment procedures in this research program, Zimba & Grimm (2015) present a statistical approach to optimising the detection of MIB off-flavour in cultured channel catfish. Zimba & Grimm (2015) reported that 40 individual fish were required to detect the presence of off-flavour when <3% of fish in the population were off-flavour tainted while 11 fish was sufficient when >20% of the population was off-flavour tainted. In general, it was observed to be more accurate to assess more fish at fewer sampling times than to assess a smaller number of fish at regular intervals. The information reported by Zimba & Grimm (2015) can be used as a guide for Australian barramundi farmers, until a species-specific approach can be developed for GSM induced off-flavour in cultured barramundi.

2) Depurating off-flavour from fish prior to harvest

Although identifying and excluding off-flavour tainted cohorts may be an effective method of preventing the harvest of off-flavour tainted fish, this method may be unsuitable under certain conditions. For example, in order to maintain the supply of fish to customers, an alternative, untainted cohort of fish must be available for harvest. In this thesis, off-flavour tainting was observed to persist almost continuously in barramundi ponds with multiple ponds affected simultaneously (Fig. 2.1). As a result, off-flavour tainting may persist across all market-ready cohorts of fish simultaneously.

When the situation arises that all market-ready cohorts of fish are off-flavour tainted simultaneously, it may be necessary to eliminate off-flavour tainting from fish prior to harvest. This is can be achieved by depurating fish in clean, untainted water prior to

harvest. GSM levels in barramundi ponds were observed most frequently below ~2 μg L^{-1} (Fig. 2.1). For market sized fish (1.5-2kg) exposed to these concentrations, depuration eliminated off-flavour tainting after 8 days at 27°C (Table 4.1).

The rate of depuration is affected by a range of factors, principally, fish size, lipid content and water temperature. Large, fatty fish will take longer to depurate than small, lean fish and the rate of depuration will be reduced for all fish at lower water temperatures (Howgate, 2004). The depuration rate and time to recover flavour observed in this research program should be used as a guide for producers. However the time required to recover flavour quality may vary depending on the afore-mentioned factors.

Although the time required to completely remove GSM from tissues may vary according to a range of uncontrolled factors, depuration in clean water for any period of time will reduce the concentration of GSM present and hence the intensity of off-flavour tainting. This is advantageous to barramundi producers. However, as a precaution, it may be prudent to undertake sensory assessment, as previously discussed, in conjunction with depuration to ensure that off-flavour tainting has been eliminated from the fish.

3) Removal of the heavily tainted ventral belly section

Most barramundi farms in Australia do not process their fish prior to sale. The majority of fish are sold whole for later processing by wholesalers and retailers. It may therefore be beneficial for producers to disseminate to processors the information contained in the thesis relating to the spatial distribution of off-flavour tainting. In some cases the dorsal shoulder and posterior tail regions may be untainted even though off-flavour persists in the ventral belly region. In these cases, removing the

ventral belly region may be sufficient to prevent off-flavour tainting in the remaining fillet.

The most suitable strategy for preventing off-flavour tainting is likely to vary from farm to farm. This thesis is intended to provide information that individual producers can use to develop strategies to mitigate the detrimental effects of off-flavour tainting.

The three potential strategies outlined above may be used independently or in conjunction with each other. For example, depuration may be used to reduce the intensity of off-flavour tainting; however, if subsequent sensory assessment detects a low-level taint in the belly region, this portion may be removed during processing to yield an untainted fillet for sale.

7.4.2 Flavour complexity

Producers should also be aware of the potential for flavour enhancement by dietary manipulation. Dietary manipulation could be used in conjunction with the above methods to add flavour complexity and marine flavours to farmed barramundi prior to sale. Further research is required to develop a finishing diet, for the purpose of flavour enhancement, that could be applied in a commercial context. In Australia, barramundi are reared on extruded, floating diets. These diets are most suitable for commercial production as they are highly stable during storage and allow feeding activity to be visually monitored. Although diets used in the current research program were cold-pressed, sinking pellets, there are no obvious obstacles to incorporating *Ulva* into extruded, floating pellets. Further research is required to determine if the flavour enhancing effects observed in this study can be achieved using diets more suited to commercial farming systems.

7.4.3 Product deterioration during the post-harvest storage period

The α-tocopherol acetate enriched diets used in this study were extruded, floating diets. These were fed to farm reared fish in conditions identical to those experienced in normal commercial production. This thesis highlights the benefits of pre-harvest dietary α-tocopherol enrichment in combination with storing fish whole and ungutted (Chapter 6).

The Australian barramundi aquaculture industry currently relies upon the rapid chilling of fish at the point of harvest and maintaining low temperature during the storage period to optimise freshness (Carton & Jones, 2013). The majority of fish are not processed on-site but are sold whole and ungutted.

Storing fish whole and ungutted for the duration of the storage period was observed to be an effective method of limiting lipid oxidation, mitigating colour changes and minimising bacterial activity (Chapter 6). This is a critical factor in preventing quality deteriorations and the Australian barramundi aquaculture industry should provide recommendations to wholesalers and retailers regarding the importance of this factor.

The research program also found that lipid oxidation in barramundi flesh can be reduced during chilled storage by dietary enrichment with α-tocopherol acetate during the pre-harvest period (Chapter 6). Including this powerful antioxidant into the diet at 628 mg kg^{-1} increased the concentration of α-tocopherol in edible tissue from 13.67 mg kg^{-1} to 17.27 mg kg^{-1} after 2 weeks of feeding. A comparable α-tocopherol concentration of 17.89 mg kg^{-1} was observed after 169 days of feeding. This reduced lipid oxidation during 14 days of chilled storage in both stored fillets and whole ungutted fish. Fish that were enriched with α-tocopherol acetate and stored whole and

ungutted had the highest oxidative stability with lipid oxidation not progressing during 14 days of chilled storage.

It is therefore recommended that Australian barramundi farmers use diets enriched with supranutritional α-tocopherol acetate to fortify their products against lipid oxidation. It is also recommended that fish be stored whole and ungutted throughout the supply chain to optimise freshness, mitigate colour deterioration and prevent bacterial proliferation.

7.4.4 Summary

In summary, it is recommended that Australian barramundi producers implement a strategy that addresses off-flavour tainting, either through the use of depuration procedures or by assessing the flavour of fish prior to harvest to ensure that off-flavour tainted fish are not harvested. In addition, it may be possible for producers to liaise with fish processors to remove the ventral belly region from fillets prior to sale to further reduce the likelihood of off-flavour tainting being experienced by the end user. Diets enriched with supra-nutritional α-tocopherol acetate could also be used by producers to limit lipid oxidation during the post-harvest storage period. Producers can use the information presented in this thesis to inform wholesalers and retailers of the fortifying effects of storing fish whole and ungutted to prevent quality deterioration during the post-harvest storage period.

It is also recommended that further research be performed to commercialise the production of extruded, floating diets for the purpose of enhancing flavour in farmed barramundi. Such research can draw on the outcomes presented in this thesis. Future research should address the potential to manipulate flavour ‘on farm’ using diets that are suitable to commercial production. Additionally, the storage stability of manufactured diets should be addressed to ensure consistent results are achieved

throughout the storage-life of the diet. The storage stability of flavour enhanced fish should also be assessed to determine the potential for fluctuations in flavour during post-harvest storage.

7.5 Future research objectives

This thesis presents a set of original research findings related to optimising the quality of farmed barramundi. This information presents advances in the field of aquaculture research, has implications for aquaculture production and product quality globally and can underpin the development of a quality optimisation strategy for the Australian barramundi aquaculture industry. However, the thesis also identifies several key areas requiring additional research.

This thesis revealed GSM to be the most common off-flavour compound present in freshwater barramundi aquaculture ponds. At the time that this research was completed, a broader survey of pond water across the geographic range of barramundi culture in Australia was required, to determine whether GSM is the primary off-flavour compound in farmed barramundi across a wider range of environmental settings. This has been completed by research staff at the Queensland Department of Agriculture and Fisheries. Exley (2014) confirmed the findings presented in this thesis. GSM was identified to be the most abundant off-flavour compound in pond waters with moderate (~2 $\mu g\ L^{-1}$) concentrations observed most frequently and the potential for GSM to reach extreme concentrations (~15 $\mu g\ L^{-1}$) (Exley, 2014).

It would also be useful to undertake additional research investigating a range of pond management strategies to limit the proliferation of off-flavour compounds in barramundi culture water. Previous attempts to prevent off-flavour tainting microbes have had low and variable levels of success (Exley, 2014). However, having shown that depuration can recover flavour quality within 8 days, after exposure to a moderate

GSM concentration (2.15 μg L^{-1}), it may be advantageous to explore pond management strategies that constrain GSM levels within this range. This would facilitate an integrated response to off-flavour tainting that would minimise exposure to GSM, by pond management procedures, followed by depuration to recover flavour quality. This concept has great potential with Exley (2014) identifying a number of methods, including the addition of lysine to pond water, that could constrain GSM levels in pond water within this moderate concentration range.

It may also be beneficial to investigate methods of accelerating depuration. The rate of depuration is known to be affected by gill ventilation rate. Gill ventilation rate is affected by environmental factors such as metabolic rate, water temperature and oxygen availability (Howgate, 2004). Manipulating these factors during depuration may accelerate depuration thereby compressing the overall time required to recover flavour quality. The influence of factors such as fish size and tissue lipid on depuration rate should also be investigated to enable more accurate predictions of the time required to recover flavour quality.

The research presented in this thesis demonstrates that diets enriched with marine algae have the potential to further enhance the organoleptic properties of fish. Further research could be undertaken to investigate if the results observed in this study can be replicated under commercial conditions. Initially, it will be necessary to determine if marine algae such as *Ulva* can be successfully included into the extruded, floating diets most frequently used in commercial aquaculture systems. If extruded diets can be manufactured, the palatability and flavour enhancing effects of such diets must be investigated. These diets must also be assessed with respect to storage stability to determine if storage time and conditions (e.g. temperature, humidity, light) affect their flavour enhancing potential. The storage stability of flavour-enhanced fish is also

unknown while the effects of cooking on flavour enhancement are unexplored. These factors should also be addressed by future research programs.

In this research program, the potent aroma compound, DMS, was associated with an increase in the intensity of crab-like/seafood flavour. Further research is required to establish if the intensity of seafood flavour in barramundi tissue is correlated with DMS throughout a range of tissue concentrations.

Numerous other aroma compounds also have the potential to affect flavour in cultivated fish. It would be beneficial to assess the suite of aroma compounds present in flavour enhanced fish tissues to determine their relative influence on flavour characteristics. This can be achieved using gas chromatography-olfactometry. This technique couples chemical analysis with human sensory assessment to characterise aroma-active as well as character-impact compounds, responsible for the aroma characteristics of a food sample (Zellner et al., 2008).

By understanding the suite of compounds responsible for flavour enhancement, it may be possible to establish a standardised, analytical approach to assessing the level of flavour enhancement in fish without the need to undertake time consuming sensory assessment procedures. Further research is required to determine the capacity to develop such an approach.

Understanding the full suite of compounds associated with flavour enhancement would also facilitate a broader exploration of the raw materials used in the production of flavour enhancing feeds. It may be possible to establish the suitability of raw materials by undertaking chemical analysis of the aroma compounds present. This would enable a broad range of raw materials to be rapidly assessed. Such an approach could also be used to investigate the potential variability of dietary ingredients with respect to the presence of critical compunds. This would facilitate the development of

a rapid method of assessing the suitability of raw materials prior to the manufacture of flavour enhancing diets.

Having observed that diets enriched with marine algae can add flavour complexity to farmed fish, it may be possible to use these diets to mask off-flavour tainting in taint-affected fish. The threshold of sensory detection of off-flavour compounds in fish flesh is thought to be affected by the relative intensity of natural background flavour in fish tissues (Tucker, 2000). It is likely that adding flavour complexity, through the application of a finishing diet enriched with marine algae, will increase the threshold of sensory detection of off-flavour compounds. At low concentrations of off-flavour compounds, this may serve to completely mask off-flavour tainting, thus avoiding the need to depurate off-flavour. Alternatively, flavour enhancement may be used in conjunction with depuration to further optimise product quality. The potential of flavour enhancing diets to mask off-flavour tainting should be investigated to determine its efficacy in recovering flavour quality in taint-affected fish.

Dietary enrichment with α-tocopherol acetate appeared to reduce lipid oxidation during the post-harvest storage of whole ungutted fish and stored fillets. Tissue levels of α-tocopherol increased significantly from baseline levels after 14 days of feeding and reached a maximum concentration after 56 days. When fish were fed a diet containing 628 mg kg^{-1} α-tocopherol acetate, a tissue concentration of 17.89 mg kg^{-1} was observed at harvest, this reduced lipid oxidation during the post-harvest period. It may, however, be possible to optimise α-tocopherol enrichment by investigating higher dietary inclusion levels. This may have the potential to increase the level of uptake or may compress the time required to adequately enrich tissues.

It may also be advantageous to investigate the fortifying effects of α-tocopherol enrichment during sub-optimal storage conditions, simulating industry realities. This

could be investigated by forcing oxidation through elevated temperatures during the post-harvest storage period.

7.6 Conclusion

The primary objective of this research program was to address quality deficits in farmed barramundi. This was achieved by undertaking integrated research that addressed the three major factors impacting on product quality for farmed barramundi: off-flavour tainting, lack of flavour complexity and quality deterioration during the post-harvest period.

The studies reported in this thesis identify the wide range of factors that cause off-flavour and poor quality in cultivated barramundi in Australia. Off-flavour tainting was found to be primarily caused by exposure to GSM, which was observed to persist in pond-waters for extended periods of time and had the potential to reach extreme levels. A lack of flavour complexity in farmed barramundi appears to be related to a deficiency in critical flavour compounds. Flavour-affecting BPs were not detected in barramundi tisue while an elevation in the concentration of DMS was observed to be associated with increased seafood flavour. Deterioration in product quality during the post-harvest period was observed to be affected by the dietary availability of α-tocopherol acetate and processing method. This is a significant consideration given the geographic isolation of many barramundi farms and the protracted transportation time to major markets.

This thesis also provides a body of scientific evidence to underpin improvements to industry practices that would holistically address the flavour and quality of barramundi cultivated in Australia (Fig 7.1). Off-flavour tainting of farmed barramundi can be mitigated by depurating fish in clean water prior to slaughter, by developing sensory assessment procedures that identify and exclude off-flavour

tainted cohorts from harvest; and by removing the most heavily tainted ventral belly region prior to consumption. Flavour complexity can be enhanced by feeding farmed barramundi a diet composed of 20-30% *Ulva ohnoi*. This has the potential to enhance flavour quality by adding seafood like flavours and increasing desirability during 1-3 weeks of feeding. The post-harvest product can be fortified against quality degradation by enriching fish with dietary α-tocopherol acetate and preventing tissue disruption prior to chilled storage, ensuring higher quality is maintained to the point of human consumption.

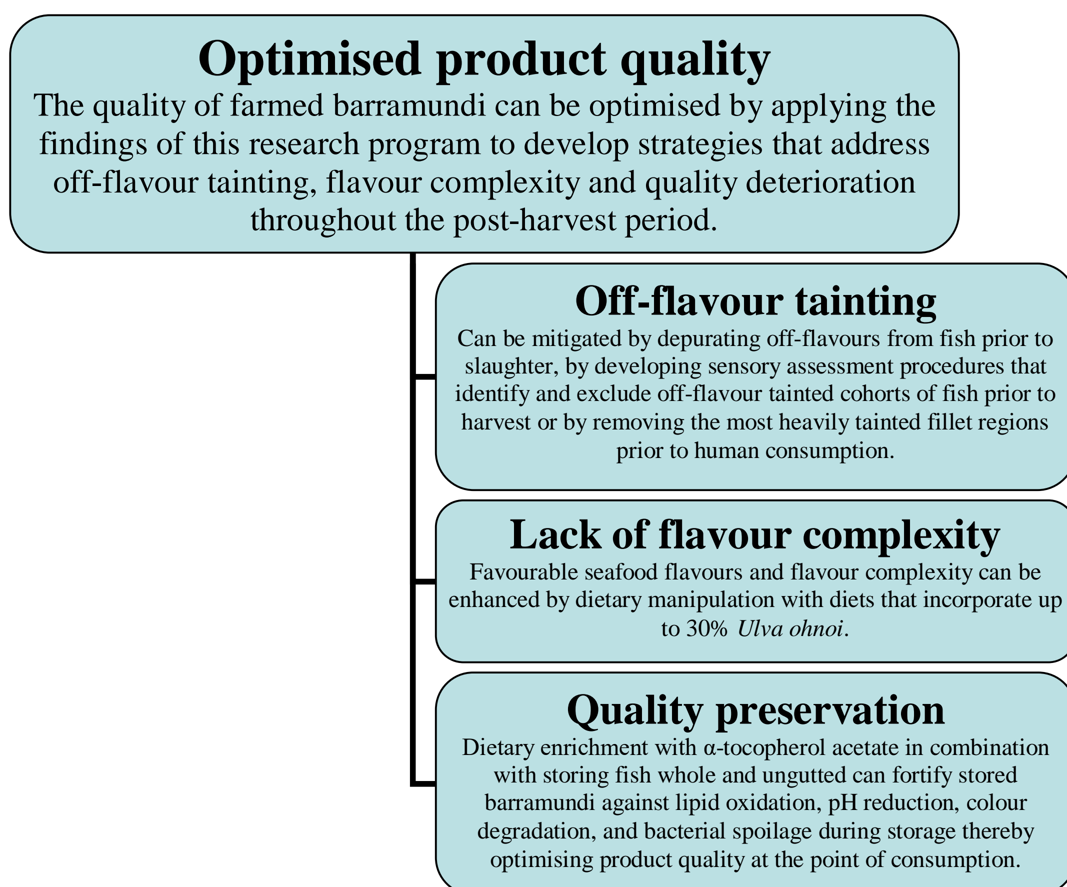

Fig. 7.1 An integrated approach designed to optimise flavour and quality of Australian farmed barramundi.

While further research is required to validate and refine the findings of this thesis, a research foundation has been developed that has the potential to improve the flavour and quality of farmed barramundi in Australia. This will enable the industry to more efficiently and effectively compete against similar domestic products and overseas producers of cultivated fish. A high quality premium product also provides a point of differentiation for Australian producers as they compete for domestic and international markets.

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APPENDICES

Appendix 1 Sensory attributes defined by the trained panel used for barramundi exposed to geosmin.

Attribute	Definition
Aroma	
Muddy aroma	The aroma of freshwater pond mud.
Rotting vegetation aroma	The aroma of rotting freshwater vegetation.
Fresh fish aroma	The aroma of freshly cooked white fleshed fish.
Earthy aroma	The smell of soil or earth when rainfall first starts falling.
Steamed aroma	The aroma of steamed fish or chicken.
Flavour	
Sweet flavour	A sweet flavour experienced when the sample is in the mouth.
Metallic flavour	A metallic/iron or blood flavour experienced when the sample is in the mouth.
Fresh fish flavour	The flavour of freshly cooked white fleshed fish experienced when the sample is in the mouth.
Muddy/earthy flavour	Tastes like freshwater pond mud or earth experienced when the sample is in the mouth.
Rotting weed flavour	Tastes like rotting freshwater weeds experienced when the sample is in the mouth.
Off fishy flavour	Reminiscent of old/stale fish experienced when the sample is in the mouth.
Aftertaste	
Metallic after taste	The lingering taste of metal/iron or blood after the sample has been swallowed.
Weedy after taste	Leaves the taste of freshwater weeds in your mouth after the sample has been swallowed.
Earthy after taste	A flavour reminiscent of soil when rainfall first starts remaining in the mouth after the sample has been swallowed.
Overall Desirability of Sample	The sample is to be rated for its overall appeal in terms of aroma, taste and aftertaste.

Appendix 2 Definitions of selected sensory attributes used for barramundi exposed to geosmin.

Attribute	Definition
Aroma	
Muddy aroma	The aroma of freshwater pond mud.
Rotting vegetation aroma	The aroma of rotting freshwater vegetation.
Earthy aroma	The smell of soil or earth when rainfall first starts falling.
Steamed aroma	The aroma of steamed fish or chicken.
Flavour	
Sweet flavour	A sweet flavour experienced when the sample is in the mouth.
Fresh fish flavour	The flavour of freshly cooked white fleshed fish experienced when the sample is in the mouth.
Muddy/earthy flavour	Tastes like freshwater pond mud or earth experienced when the sample is in the mouth.
Rotting weed flavour	Tastes like rotting freshwater weeds experienced when the sample is in the mouth.
Aftertaste	
Weedy after taste	Leaves the taste of freshwater weeds in your mouth after the sample has been swallowed.

Appendix 3 Sensory attributes defined by the trained panel used for barramundi fed diets incorporating a significant fraction of *Ulva ohnoi*.

Attribute	Definition
Aroma	
Crab-like aroma	The aroma of fresh boiling crabs.
Sweet aroma	A sweet aroma reminiscent of sugar being heated.
Fresh fish aroma	The aroma of freshly cooked white fleshed fish.
Off-fishy aroma	Aroma that is reminiscent of old/stale fish.
Steamed aroma	The aroma of steamed fish or chicken.
Flavour	
Sweet flavour	A sweet flavour experienced when the sample is in the mouth.
Crab-like/seafood flavour	A flavour very similar to that of freshly cooked crab meat, also described as a favourable seafood flavour.
Fresh fish flavour	The flavour of freshly cooked white fleshed fish experienced when the sample is in the mouth.
Seaweed flavour	A flavour reminiscent of salt water algae or seaweed experienced when the sample is in the mouth.
Rich and complex flavour	Refers to the complexity of flavour present as opposed to "plainer" samples that lack complexity.
Off-fishy flavour	Reminiscent of old/stale fish experienced when the sample is in the mouth.
Aftertaste	
Crab-like aftertaste	The lingering taste of freshly cooked crab after the sample has been swallowed.
Fresh fish aftertaste	Leaves the taste of freshly cooked white fleshed fish in your mouth after the sample has been swallowed.
Sweet aftertaste	A sweet flavour remaining in the mouth after the sample has been swallowed.
Overall Desirability of Sample	The sample is to be rated for its overall appeal in terms of aroma, flavour and aftertaste.