View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Enlighten: Publications

Thompson, J., and Neil, D.M. (2012) Potential Nutraceuticals in Mussel Waste. Project Report. University of Glasgow, Glasgow, UK.

Copyright © 2012 University of Glasgow

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

Content must not be changed in any way or reproduced in any format or medium without the formal permission of the copyright holder(s)

When referring to this work, full bibliographic details must be given

http://eprints.gla.ac.uk/81400

Deposited on: 24 June 2013

Enlighten – Research publications by members of the University of Glasgow
http://eprints.gla.ac.uk

Potential nutraceuticals in mussel waste

A Scientific report by

Dr. John Thompson
Professor Douglas M. Neil

Institute of Biodiversity, Animal Health and Comparative Medicine,
College of Medical, Veterinary and Life Sciences, University of Glasgow

January 2012

Contents.

1. Introduction **1**

2. Materials & Methods **6**

3. Results **8**

4. Discussion **13**

5. Recommendations **15**

References **16**

Appendices **19**

1. Introduction

1.1 Distribution and life history of the Blue mussel, *Mytilus edulis*.

The Common or Blue mussel, *Mytilus edulis* (Linnaeus) is a marine, semi-sessile, boreo-temperate, epibenthic, bivalve mollusc (class Bivalvia; family Mytilidae) ubiquitous to the shores of North Western Europe, the Arctic and the Atlantic/Pacific coasts of North America (Newell, 1989; Hayward *et al*, 1996; Little & Kitching, 1996). Naturally found on rocky coasts from mean tide level into the shallow sublittoral zone (<99m), generally in dense beds (Hayward *et al*, 1996). The extent of these beds and the age distribution of the population depend on environmental factors, as well as spatial competition and predation (Hayward *et al*, 1996; Little & Kitching, 1996). The species can tolerate significant variations in salinity and is common in polyhaline to mesohaline estuarine habitats as well as on rocky coasts (Newell, 1989). *M. edulis* is also commonly found biofouling manmade structures below mean tide level. In addition, *M. edulis* is also widely cultured commercially in both North America and NW Europe (Hayward *et al*, 1996). High shore specimens, not subject to predation, can be long lived, (up to 20 years) (McKenzie, 1986). *M. edulis* exhibits an unusually flexible life strategy, by coupling high fecundity with an iteroparous life cycle it can rapidly colonise and thrive as a relatively short lived opportunist, or as a long lived community dominant (McKenzie, 1986). It is this flexibility that allows *M. edulis* such wide geographical and local distributions, over a range of habitats and substrates.

The Blue mussel, both as a planktotrophic larva and an adult, is an active suspension feeder, deriving its nutrition from particulate organic material (POM) suspended in the water column (Newell, 1989).

M. edulis are typically dioecious, but very rarely exhibit hermaphroditism (ca. 1%) (Uzmann, 1953; Sugiura, 1962; McKenzie, 1986). The reproductive cycle is annual, though multiple bouts of gametogenesis and spawning may occur in a given year (McKenzie, 1986). Reproduction in *M. edulis* is a succession of events dependent on numerous complex interactions between exogenous (e.g. food availability, temperature, salinity, etc.) and endogenous (nutrient reserves, hormonal cycle, genotype, etc.) variables (Newell *et al*, 1982). It is the interaction between these variables that determines the commencement and duration of the various phases of the cycle and thus ensures the synchronicity of gamete development within a given population (Newell *et al*, 1982). Each bout begins with the initiation of gametogenesis and culminates with spawning (release of eggs/sperm via the exhalent siphon) this is then followed by a vegetative rest period (Bayne *et al*, 1976, Newell, 1989). The onset of spawning is highly variable in wild populations, but typically occurs during the summer months, between April and October (Bayne *et al*, 1976). Fertilisation occurs within the water column, with sperm outnumbering eggs by a ratio of 10,000:1, despite this, however, large numbers of eggs remain unfertilised (Thompson, 1979). The spherical eggs (68-70µm diameter) on contact with the free swimming sperm are penetrated by an acrosome filament which allows the male gamete access to fertilise the ovum (Newell, 1989). In 5 to 24 hours the fertilised egg transforms into the ciliated, motile Trochophore, which in turn develops into the Veliger larval stage (Newell, 1989). The Veliger stage remains planktonic, feeding on nanoplankton using its ciliated velum for up to 35 days during which it goes through several developmental changes. First the shell gland secretes a thin transparent shell (exhibiting a straight hinge) and the larva enters the Prodissoconch I. substage of development (Newell, 1989). The Veliger continues to develop and eventually the cells of the mantle assume their adult role in shell formation, producing the characteristic umbo seen in the adult form (Prodissoconch II.). The larva then subsequently develops the pigmented ‘eye spots’ and foot of the adult form (Pediveliger). When the larva attains a size of approximately 0.25mm it temporarily attaches to a filamentous substrate (typically filamentous algae and hydroids). This primary settlement may last up to 6 months until the animal attains a size of

approximately 1.5mm (Bayne, 1976; Newell, 1989). When a suitable filamentous substrate is found the animal is observed to metamorphoses into the Plantigrade (juvenile) stage. Once a juvenile has attained a size, ca.1.5mm, it detaches from the filamentous substrate and re-enters the plankton. It has been suggested that primary settlement enables juvenile mussels to develop in an environment free from adult spatial competition (Thorson, 1957). The Plantigrade is passively carried by currents until adult mussels are encountered, upon contact the Plantigrade extrudes new byssus threads and attaches itself to either the substrate or the shells of other mussels, this is referred to as secondary recruitment (Bayne, 1976).

M. edulis typically remain sexually immature juveniles for the first two years, however, maturity may be attained within the first year (dependent on environmental conditions) with reproduction continuing until death (up to 20 years) (McKenzie, 1986; Newell, 1989). Adults display consistent growth up to an approximate maximum of 100mm (Newell, 1989).

1.2 Anatomy of *M. edulis*

1.2.1 External anatomy

Adult *M. edulis* exhibit bilaterally symmetrical (along the midline), elongated, roughly triangular shells (Figure 1) dimension and shape however, can vary considerably between individuals and populations (Newell, 1989; Hayward *et al*, 1996). The longest dimension (up to 100mm) is along the anterior-posterior axis from the narrow point of the umbo to the broad posterior shell margin (Figure 1). The outer surface of the shell is covered by a shiny proteinaceous membrane, the periostracum, which is dark blue to black in colour and exhibits fine concentric growth lines. When the periostracum becomes abraded (common on the umbo region of mature animals) the white, inner, aragonitic layer is exposed (Carter, 1980). A small percentage of individuals in a given population will possess light brown and/or striped valves. The valves are linked via a non-calcified, elastic, protein band called the hinge ligament, which together with the two valves forms the animals shell (Ruppert & Barnes, 1994).

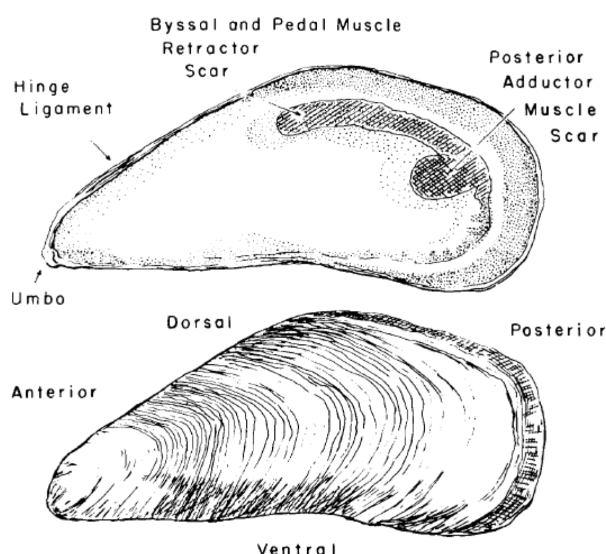

Figure 1. Interior and exterior characteristics of a Blue mussel, *Mytilus edulis*, valve (Newell, 1989).

1.2.2 Internal anatomy

Adult *M. edulis* are semi-sessile, epibenthic suspension feeders that anchor themselves to variable substrates (including other mussels) via collagenous, secreted protein strands known as byssus threads (Newell, 1989). The protein is secreted from glands in the animal's foot (Figure 2.) with the threads passing through a small notch, the pedal gape, in the middle of the ventral junction of the two valves when the shell is closed (Newell, 1989). The animal is allowed a limited degree of movement via the adjustment of existing threads and the secretion of new strands. This mobility is largely utilised to adjust the animals position in relation to water currents, in order to maximise feeding efficiency and also to avoid burial through sedimentation (Seed, 1976; Yonge, 1976).

The animal's interior anatomy is also highly distinctive; with the posterior adductor muscle significantly larger and better developed than the anterior. Located at the centre of the visceral mass (the animal's soft tissue) is the foot, which the animal may extend to secrete fresh byssus thread (Newell, 1989). The region of tissue extending around the margins of the visceral mass and along the entire periphery of both shell valves is known as the mantle (Figure 2) and is responsible for initiating new shell growth (Wilbur & Saleuddin, 1983). In two posterior regions the mantle is modified to form inhalant and exhalent siphonal apertures, these are utilised to direct feeding currents into and out of the mantle cavity and also to release gametes during spawning (Newell, 1989). The lining of the mantle is ciliated and lined with tracts responsible for conveying waste particles encased in mucus (pseudo-faeces) out of the mantle cavity via the exhalent siphon (Harris, 1990).

The gonads are closely associated with the mantle and can vary greatly in colour between individuals, from pale green/brown to bright orange/yellow. Lying within the mantle cavity on either side of the visceral mass are the filibranch gills or ctenidia and the labial palps which lie to either side of the animal's 'mouth' (Harris, 1990; Ruppert & Barnes, 1994). As well as gas exchange, the (ciliated) gills are utilised as feeding apparatus to filer and trap POM (Ruppert & Barnes, 1994). Such POM is trapped in mucus and conveyed toward the labial palps and mouth (Ruppert & Barnes, 1994). The central region of the visceral mass houses the digestive gland, intestine, heart, nephridium (crude kidney) and a portion of the gonads (Figure 2.) (Harris, 1990; Ruppert & Barnes, 1994). The nervous system in bivalves is bilateral, comprising three pairs of ganglia and two pairs of long nerve cords (Ruppert & Barnes, 1994).

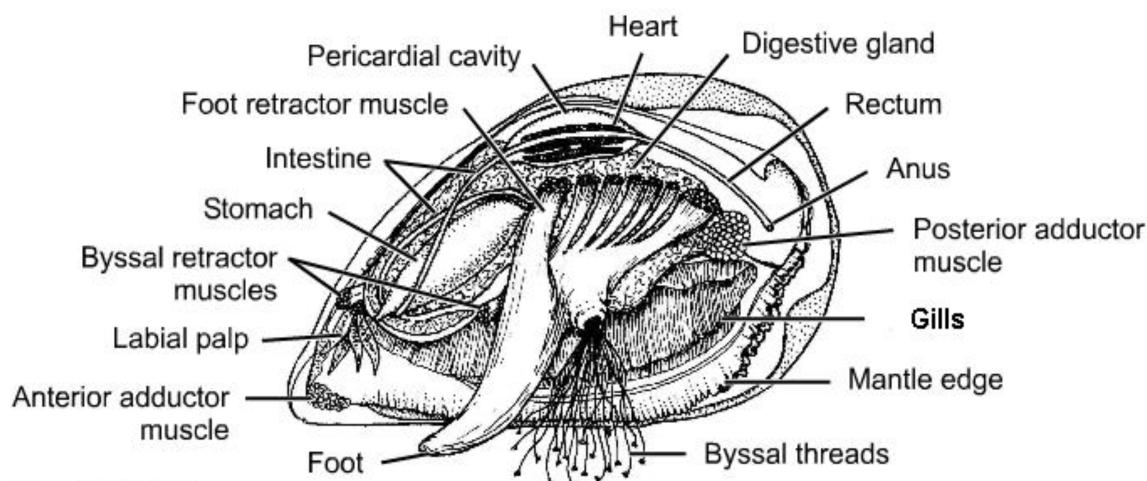

Figure 2. Major internal anatomical features of Blue mussel, *Mytilus edulis*. (source: BIODIDAC)

1.3 Commercial culture of *M. edulis*

Mussel species are commercially cultured worldwide with the majority of farms relying on off-bottom suspension systems, utilising either raft or long-line methods. Mussels grown in such suspension systems exhibit a more rapid rate of growth as they are provided with better access to current bourn POM (Jensen & Patursson, 2011). Long-line mariculture of *M. edulis* was first developed in North America in the mid 1970’s (Rice, 2010). However, this method did not become widely utilised by commercial growers until 1981 (Anon, 2003). The most critical factor in any commercial shellfish culture operation is the selection of a grow-out site that will produce good quality, market-sized animals within an acceptable time frame. The selection of such a site depends on understanding the specific local environmental factors that will influence production levels. Such factors will include how exposed a site is, water temperature, current velocity, sediment loading and food availability, as well as specific regional environmental factors, such as tidal amplitude, ice coverage, seasonal weather patterns and climate (Anon, 2003; Jenson & Patursson, 2011). Despite this even a culture operation possessing optimum grow-out sites and utilising the best harvesting methods available will not be 100% efficient. Consequently, the production of organic waste (both damaged and undersized animals) is an unavoidable by-product.

M. edulis production in Scotland has increased significantly over the last twenty five years; from 262 tonnes in 1986 to 4,219 tonnes in 2006 (Diaz *et al*, 2009). Given this and the fact that Europe is responsible for 50% of worldwide mussel production; over 600,000 tonnes from aquaculture sources in 2002 (Beaumont *et al*, 2007; Diaz, 2009) means that organic waste from such mariculture operations could prove a vast and economic source of useful substances. Such substances would likely take the form of lipids (triglycerides and fatty acids) along with the possible discovery of other compounds with commercial applications, i.e. so called ‘nutraceuticals’ (Badiu *et al*, 2008(a); Rice, 2010). The soft tissues of *M. edulis* have been show to be a good source of Iron, Thiamin, Riboflavin and vitamin B-12 as well as low in cholesterol and high in protein (Rice, 2010). The animals shell could also be utilised as a source of calcium, magnesium and phosphorus (Rice, 2010). Furthermore, lipid extracts from a closely related species, *Mytilus galloprovincialis* (Lmk), have been demonstrated as containing bioactive compounds (Badiu *et al*, 2008(b); Badiu *et al*, 2009). These extracts have been demonstrated as being suitable for use as emollients in products used in the management/treatment of dry skin disorders (e.g. eczema, psoriasis or skin dryness following chemotherapy or radiotherapy) (Badiu *et al*, 2009). Their effectiveness in the healing of induced skin burns in Wistar rats has also been established (Badiu *et al*, 2008(b)).

Therefore, extracts of *M. edulis* tissue may well contain compounds with potential applications in the production of nutritional supplements/food additive, skin-care products, cosmetics and/or pharmaceutical preparations.

1.4 Metabolomics

Metabolomics is a relatively young member of the “omics” family of biosciences. Others include; transcriptomics, involving the analysis of the composition of transcription factors. Also proteomics, in which the amino acid composition and structure of proteins is determined. As well as genomics in which the nucleotide composition and sequence of genomes (genetic material) is analysed.

Metabolomic analysis is the tool used to determine the composition of metabolites (the ‘metabolome’) in a given sample of tissue. The metabolome is regarded as a snapshot reflecting organ function and the physiological processes that were ongoing at the time of sampling, for example feeding, reproduction and cellular metabolism (Samuelsson & Larsson,

2008). Such analysis can be performed on a range of biological samples in order to detect low molecular weight compound compositions in any sort of biological material, including cells, tissues and biofluids (Nadler, 2011). The primary strength of this methodology is its ability to answer biological questions in a broad, untargeted manner via examination of the entire metabolome, allowing compounds of interest and biomarkers to be determined (Viant, 2008). Primarily, metabolomic analysis has been used to look for specific biomarkers in habitat indicator species. Such 'environmental' metabolomics has proven highly effective in highlighting how such organisms interact with their environment and is particularly relevant with regard to pollution exposure and toxicity testing (Viant *et al*, 2006; Viant, 2008). However, only recently has the vast potential of metabolomic analysis become fully apparent, with the potential to diagnose illness and disease through rapid, non-invasive testing of bio-samples (e.g. urine, blood, saliva, etc). As with the early stages of all the 'omics' sciences, there are hurdles. Currently interpretation of metabolome profiles (spectra) is difficult and will remain so until the creation of a metabolite database or library, as has occurred in the fields of genomics and proteinomics (Viant, 2007). Furthermore, metabolites unlike genes, transcripts and proteins are a highly physically and chemically diverse group of compounds of which, currently, no single bio-analytical technique is capable of blanket detection (Viant, 2007).

Currently, the two most widely utilised methods are ^{1}H nuclear magnetic resonance spectroscopy, or ^{1}H NMR and mass spectroscopy (Viant, 2007). Both techniques have considerable value in metabolomic analysis, however, neither has yet been fully developed for this application and consequently each has strengths and weaknesses (Viant, 2007). For example, of the estimated several thousand metabolites in a typical cellular metabolome; current NMR methods are believed to detect ca. 100 (less than 10%) and mass spectrometry up to 1000 (Viant, 2007).

1.5 Project Aims

- Determination of the proportional lipid content of *M. edulis* soft tissue waste as well as that exhibited by the primary tissue types (mantle, posterior adductor muscle, gonads and gill).

- Examination of the biochemical profile ('metabolome') of the soft tissue component of mussel waste produced by Blueshell Mussel Limited., with particular attention paid to any lipids, fatty acids and antioxidants present.

- To give an indication of likely key compounds within *M. edulis* soft tissue that may be used in other industrial processes/products, or represent potential 'nutraceuticals', the value of which may offset the costs for disposal of these wastes.

2. Materials and Methods

2.1 Sample selection

From the ca. 2 kg (wet weight) of frozen *M. edulis* waste material supplied by Blueshell Mussel Limited, Shetland Islands, UK; a subset of 45 animals were randomly selected for analysis. Of these 40 animals were analysed in their entirety (entire visceral mass, minus shell) with the remaining five dissected to provide samples of the four primary tissue types; mantle, gill, gonad and posterior adductor muscle. Whole wet weight (visceral mass plus shell) and length (point of umbo to broad posterior shell margin) were recorded.

2.2 Preparation of tissue samples

The soft tissue (visceral mass) of each animal was carefully removed from the shell using a scalpel and its mass recorded. Tissue samples were then excised from the five selected animals with care taken to ensure the tissue/organ was removed as completely as possible, before a determination of mass was made. All samples were assigned an identification code then transferred to individual (pre-weighed) 10 ml glass vials and frozen at -25°C.
Once frozen, the samples were freeze-dried over a period of 8 days using an Edwards Freeze Dryer Modulyo cooled to -40°C attached to an Edwards two stage #5 vacuum pump. Upon removal from the freeze dryer the samples were re-weighed to determine the water free mass of the tissue, then manually ground and stored at -25°C.

2.3 Lipid extraction

A chloroform: methanol extraction based on the Folch method (Folch, 1957) was utilised to isolate the lipid component of the freeze-dried samples. Twenty millilitres of HPLC grade chloroform: HPLC grade methanol (2:1, v/v) was added to 0.1 g to 0.5 g of freeze dried sample, this mixture was then homogenised on ice for 40-60 seconds using an Ultra-Turrax© T25 disperser (IKA™, UK). The resultant suspension was left on ice for 1 hr, before adding 5 mL of Potassium chloride solution (KCl; 0.88% w/v). The suspension was vortexed until homogenised (approximately 20 seconds) and left on ice for a further 5 min. The samples were then centrifuged in a Jouan™ C412 bench centrifuge (1500 rpm; 5 min; 4°C) in order to separate the aqueous and organic fractions. The upper water and methanol (aqueous) fraction was pipetted off (with care taken not to disturb the organic fraction) into 10 mL glass vials and stored, under nitrogen, at -25°C for possible later analysis. The lower organic/chloroform fraction was then passed through a pre-washed (in HPLC grade chloroform: HPLC grade methanol (2:1, v/v)) 11 cm Whatman™ no.1 filter paper. The solvent within the filtered organic fraction was then evaporated off under nitrogen flow, using a heating block (25°C). Once all the remaining solvent had evaporated the sample tubes were capped with aluminium foil (pierced with a single hole) and left to desiccate under vacuum for approximately 18 hr (Edwards two stage #2 vacuum pump linked to a silica gel lined dome desiccator).
The following day the mass of the organic fraction (i.e. lipid) was measured and its relative proportion within the original sample subsequently determined. The organic fraction was then resuspended in HPLC chloroform: HPLC methanol (2:1) with BHT (0.01%, w/v) at a concentration of 10 mg/mL^{-1} and stored in 3.5 mL glass vials, under nitrogen at -25°C for later analysis.

2.4 ^{1}H NMR Spectroscopy

Randomly selected whole animal, mantle, gonad, gill and posterior adductor muscle lipid extractions as well as a single (whole animal) water/methanol sample were analysed using ^{1}H NMR Spectroscopy. Individual lipid extracts from nine whole *M. edulis* and the collective mantle, gonad, gill and posterior adductor muscle samples were analysed.
5 mg ± 1 mg of each lipid sample was resuspended in deuterated chloroform ($CDCl_3$) and transferred to 5mm diameter NMR tubes, before their spectra at 400 MHz was determined using a Jeol™ ECX400 NMR spectrometer (1D sequence with pre-saturation of the water resonance). Further, subsequent analysis was performed on whole animal extract using a 2D NMR method, (^{1}H-^{1}H homonuclear correlation spectroscopy (COSY)) in order to confirm the spin systems of each of the components found in the spectra.
The 1D NMR spectra obtained were rendered using the MNova (v6.2.1) computer program prior to metabolomic analysis using Alice 2 software. Identification of the likely compounds present was then determined based on the chemical shift and integration of the spectra's peaks. A brief description of the theory behind interpretation of NMR spectra is outline in the appendices.

3. Results

3.1 Lipid quantification

The sampled animals supplied by Blueshell Mussel Limited were highly consistent with regards to length (S.D. less than 10% of mean), but displayed a greater variability regarding mass (S.D. ca. 26% of mean) (Table 1). This variability is likely a result of damage (many animals had portions of shell missing) and the varying degrees of desiccation suffered as a result.

Table 1. Biometrics of sampled *Mytilus edulis*; including mean length (long axis), mean whole animal mass and mean mass of soft tissue.

	Mean value ± St.Dev.
Length (mm)	59.93 ± 5.85
Whole animal (g)	10.863 ± 2.816
Soft tissue (g)	5.112 ± 1.544

Table 2. Average lipid content of whole *M. edulis* (including & excluding shell) and the four major tissue types.

	Mass (g) ± St.Dev.	**% of wet weight ± St.Dev.**	
Whole animal	$9.64 \times 10^{-2} \pm 4.71 \times 10^{-2}$	**(incl. shell)** **(excl. shell)**	0.88 ± 0.33 1.87 ± 0.67
Gonadal tissue	$1.37 \times 10^{-2} \pm 8.11 \times 10^{-3}$	2.00 ± 0.34	
Mantle	$5.58 \times 10^{-3} \pm 3.78 \times 10^{-3}$	1.03 ± 0.24	
Gill	$4.25 \times 10^{-3} \pm 1.62 \times 10^{-3}$	0.99 ± 0.14	
Posterior aductor Muscle	$3.62 \times 10^{-3} \pm 3.95 \times 10^{-3}$	0.69 ± 0.35	

Of the four primary tissue types analysed, *M. edulis* gonadal tissue exhibited the highest concentration of lipid (Table 2). The gonads contained, on average, approximately 14% of the animal's total lipid content, at least double that of the other tissues tested.

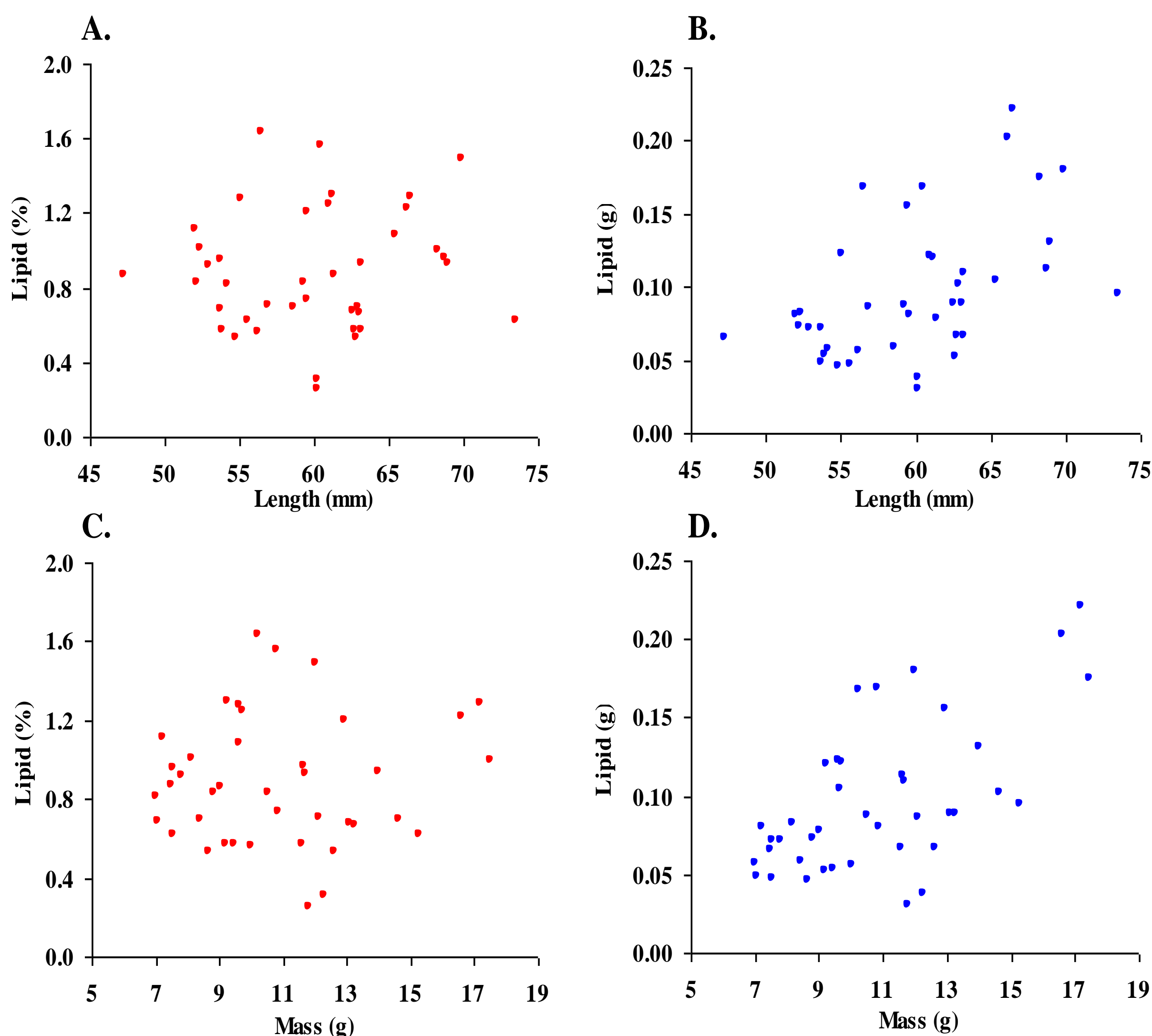

Figure 1. Relationship of soft tissue lipid content to animal size. **A.** % whole animal lipid content versus animal length. **B.** Mass of lipid in soft tissues versus animal length. **C.** % whole animal lipid content versus mass (including shell). **D.** Mass of lipid in soft tissues versus mass of animal (including shell).

As expected there was a clear positive correlation between the mass of lipid present in the soft tissues and animal size (length and mass) (Figure 1). This correlation appears more defined when plotted against whole animal mass as opposed to length. Consequently, it is recommended that measurements of animal mass rather than length should be utilised when determining estimates of lipid yield.

No pattern could be discerned in the relationship between animal size (either mass inclusive of shell or length) and percentage lipid content. Hence, there does not appear to exist an optimum size range for *M. edulis* with regard to percentage lipid content/yield.

3.2 ^{1}H NMR spectroscopy of *M. edulis* lipid extractions

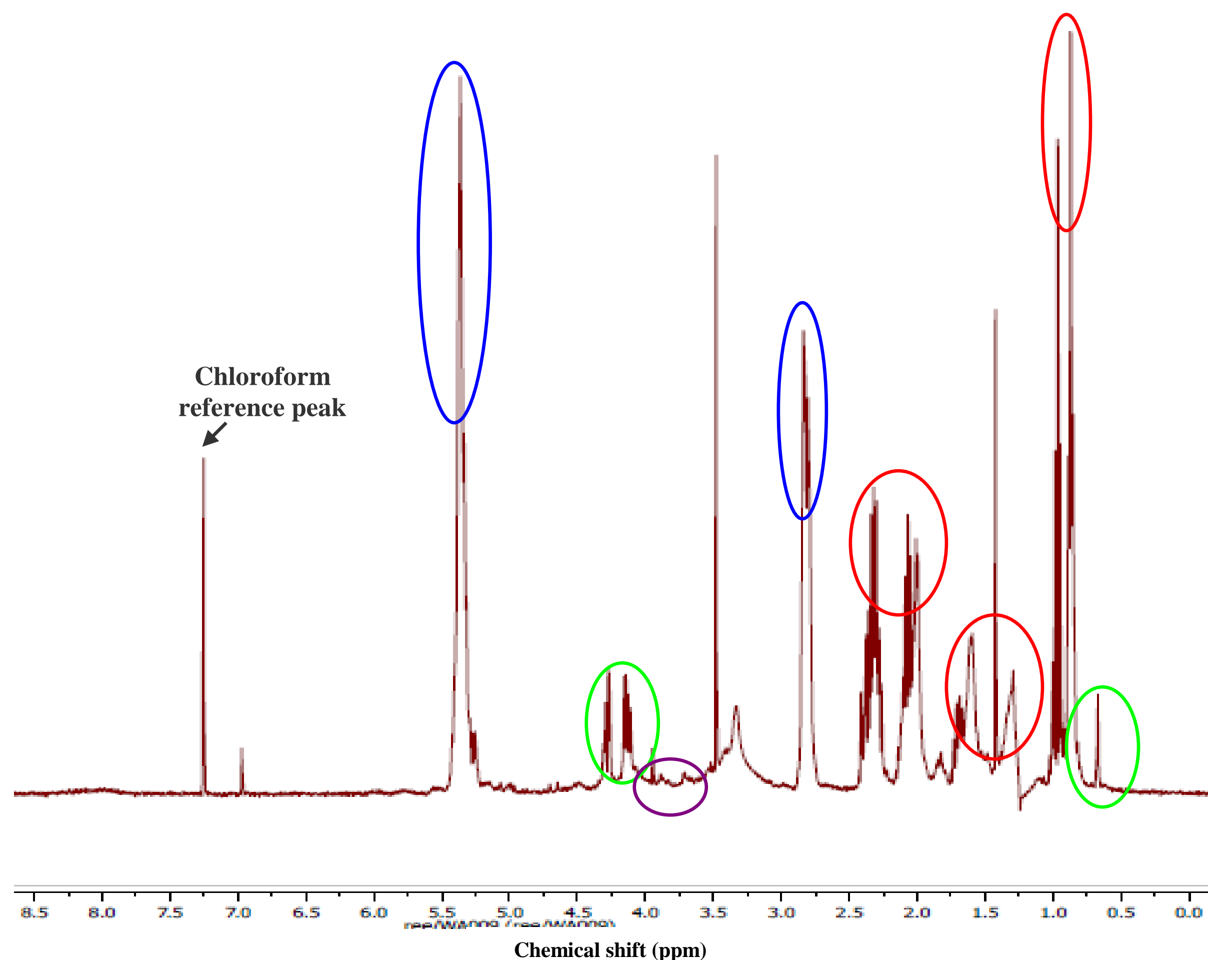

Figure 2. 1D (^{1}H) NMR spectrum obtained for whole *M. edulis* lipid extract including peaks indicating the likely presence of chitin (**O**), triglycerides (**O**) and fatty acids (**O**) as well as low molecular weight polysaccharides (**O**). All spectra for whole animal samples were highly consistent, exhibiting comparable compounds.

1D (^{1}H) NMR analysis indicated that the lipid extract obtained from *M. edulis* was dominated by triglycerides and fatty acids, but also contains chitin and small quantities of low molecular weight polysaccharides. These compounds are represented by multiple peaks with differing chemical shifts due to the multiple proton spin systems present within their molecular structure. Peaks labelled 'triglycerides' in Figure 2 represent portions of the compound's glycerol backbone. Those peaks labelled 'fatty acid' correspond to molecular components associated with the various fatty acids in the sample; these may be present as either components of the triglycerides or as 'free' fatty acids.

The 2D (^{1}H-^{1}H) COSY NMR analysis further confirmed the dominance of fatty acids and triglycerides within the lipid extracts obtained from Blueshell Mussel Ltd. *M. edulis*. Figure 3 clearly illustrates related peaks, i.e. those representing molecular components associated with one another within specific compounds.

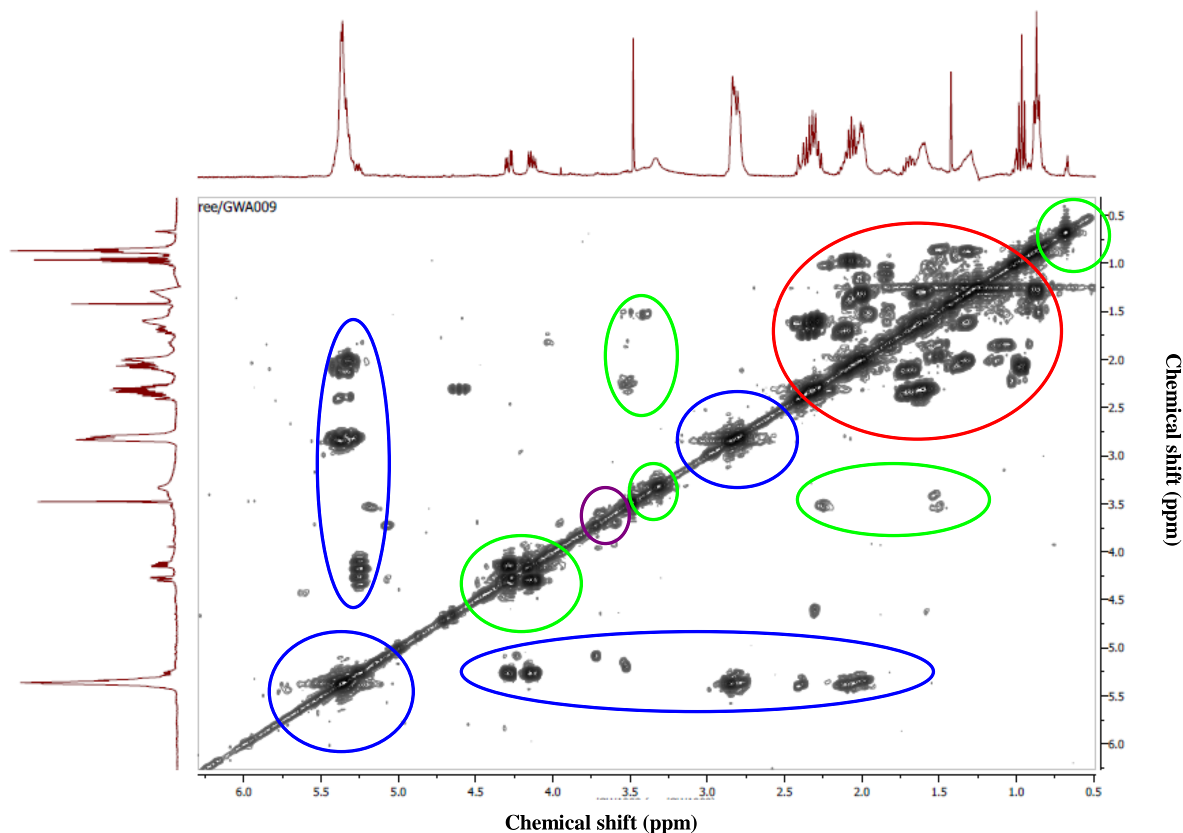

Figure 3. 2D (^{1}H-^{1}H) COSY NMR spectrum obtained using whole *M. edulis* lipid extract, peaks plotted in two dimensions for increased resolution of related regions. Regions of association are categorised as follows; chitin (**O**), triglycerides/glycerol (**O**), fatty acids (**O**) and low molecular weight polysaccharides (**O**).

A single sample of whole animal aqueous fraction was analysed to ensure no compounds of significance were overlooked within the methonal/H_2O soluble component, the 1D NMR spectra obtained for this sample is displayed in Figure 4.

The aqueous fraction contained small quantities of what may be a derivative of the amino acid tryptophan; indicated by evidence of an aromatic indole group combined with the presence of electronegative atoms of nitrogen (a component of both indole and amino groups). However, it is impossible to confirm the identity of this compound without further (mass spectroscopic) analysis. If it is indeed a tryptophan derivative the compound is likely the result of bacterial metabolism rather than a true component of the *M. edulis* metabolome.

The majority of the aqueous fraction appears to be composed of low molecular weight, monosaccharides and disaccharides. Mannose is an epimer of glucose and is important in glycoprotein biosynthesis. Rhamnose is a deoxy sugar largely produced by plants and microorganisms and is a common constituent of the type of micro algae that *M. edulis* would typically ingest (Fearman *et al*, 2009). While β-Glucose is a pyranose sugar and the monomer of chitin, a ubiquitous polysaccharide in the marine environment that would be a common constituent of suspended POM and of which *M. edulis* is capable of digesting (Birkbeck & McHenery, 1984).

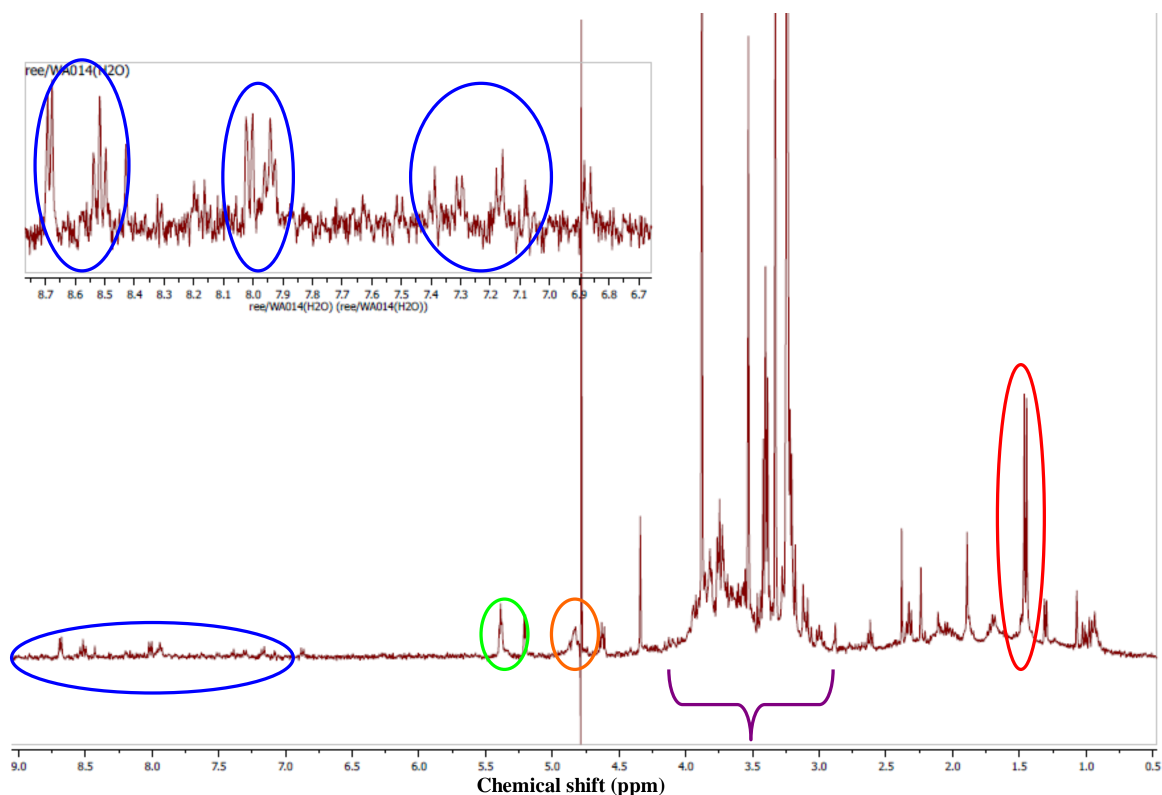

Figure 4. 1D (^{1}H) NMR spectrum obtained for whole *M. edulis* aqueous (methanol/H_2O) extract with peaks denoting the likely presence of a tryptophan derivative (O), as well as low molecular weight sugars ({) with indicators for; β-glucose (O), Rhamnose (O) and Mannose (O).

4. Discussion

The data gathered supports the conclusion that *M. edulis* waste material provided by Blueshell Mussel Limited contains a relatively low proportion of lipid; with lipid comprising an average of 1.87% of (soft tissue) wet weight. However, this value for lipid content appears consistent with previously published data from the U.S. Department of Agriculture, which lists the total fat content of raw mussel meat at 2.3% (Rice, 2010). Indeed, *M. edulis* is noted for possessing a very high protein to lipid ratio (Dare & Edwards, 1975; Rice, 2010). *M. edulis* collected from the Conwy Estuary, North Wales, have been shown to contain 8.4 times more protein and 3.2 times more carbohydrate in their dried flesh, than lipid (Dare & Edwards, 1975).

Despite the relatively low quantities *M. edulis* lipids are comprised of a high percentage of Omega-3 and Omega-6 polyunsaturated type, essential fatty acids, or EFAs. With omega-3 present in what is regarded as being a beneficially high proportion (Rice, 2010). *Mytilus galloprovincialis* (Lmk.), a closely related species, has been demonstrated as containing a high proportion of the Omega-3 fatty acids, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Badiu *et al*, 2009(b)). Furthermore, lipid extracts from *M. galloprovincialis* (Lmk.) have been shown to contain bioactive compounds demonstrated as viable for use as both emollients in skin treatments/beauty products and as an ingredient of salves used to aid the healing of burns (Badiu *et al*, 2008(b); Badiu *et al*, 2009(a)). However, the scale of this current investigation was not sufficient to identify the individual constituents comprising *M. edulis* lipid extract. Further laboratory analysis in the form of high performance liquid chromatography (HPLC) and mass spectrometry, would be necessary to identify specific components.

It is now generally considered that a healthy diet is one containing a ratio of between 1:1 and 1:4, Omega-3: Omega-6 EFAs (Daley *et al*, 2010). Given that such beneficial fatty acids may be present in *M. edulis* in such a favourable ratio, a cost effect means of rendering such waste could provide material of a suitable quality for use as both human and animal diet/feed supplements. However, the relatively low lipid yields observed during this project raises questions as to whether lipid extraction for dietary supplementation would prove a cost effective means of waste disposal for Blueshell Mussel Limited. Nevertheless, the possibility of combining *M. edulis* lipid extract with that obtained from other commercially exploited species, for example the Norway Lobster (*Nephrops norvegicus*) could render this a more viable option. *N. norvegicus* head waste has been shown to comprise 5% - 15% lipid, with significant levels of Omega-3 fatty acids and carotenoids (Albalat *et al*, 2009; Nadler, 2011). Also through combining multiple waste sources, processing costs would theoretically be lessened, i.e. the greater the volume processed the lower the unit cost and therefore, the wider the potential market. Consequently, further investigation is required to determine the economic viability of available commercial extraction methods and the marketability of/demand for the resultant product.

A more cost effective means of disposal (requiring minimal transportation and processing) might be the inclusion of *M. edulis* waste as a component in aquaculture feed. Salmon is currently Scotland's number one food export, with a current value of £285 million per annum (Anon, 2011). Numerous commercial salmonid aquaculture facilities are located in the Shetland Isles, all of which require a regular supply of feed (Anon, 2011). The majority utilise formulated pellet feeds with very high protein content (approx. 60%); an attribute shared by mussel flesh. Such feeds are becoming increasing expensive, due to mounting demand for their constituents, primarily fish meal and fish oil; both having been traditionally used in the production of agricultural feed (www.iffo.net). Currently, 50% of the world's fish oil production is used to produce salmon feed (Anon, 2008). Although efforts have been made to substitute these oils with material from plants sources (e.g. vegetable oils) this ultimately results in an end product lower in EFAs, particularly Omega-3 fatty acids. Consequently, *M. edulis* waste could provide a cost effective, protein rich, source of EFAs for salmonid culture, provided an effective means of delivery to cage farmed fish can be developed.

5. Recommendations

- Further investigation of the constituents of *M. edulis* lipid extract using more focused analytical methods such as high performance liquid chromatography (HPLC) and mass spectrometry.

- Assessment of the viability of combining *M. edulis* waste with the organic waste from other commercial marine species, both to reduce processing/logistical costs and to create a more marketable product.

- Evaluation of commercially available rendering/extraction processes to determine the cost-effectiveness and overall logistical viability of extracting marketable lipid products from Blueshell Mussel Limited waste material.

- Investigation of the viability of using the mussel waste generated by Blueshell Mussel Limited, locally, as feed material within the salmonid aquaculture industry of the Shetland Isles.

Acknowledgements

This project was funded in part by a Business Innovation Grant from Highlands and Islands Enterprise, with Blueshell Mussels Ltd. The company supplied the material for the analyses conducted.

References

Albalat, A., Watts, A., Neil, D.M., 2009. Potential use of the wasted heads of Norway lobsters (*Nephrops norvegicus*), 3rd TAFT Conference, Copenhagen, Denmark.

Anon, 2003. Profile of the Blue Mussel (*Mytilus edulis*). Canadian Government - Department of Fisheries and Oceans, Moncton, New Brunswick, Canada., pp. http://www.glf.dfo-mpo.gc.ca/Gulf-Region/Economic-Profiles/Past_Issues/2003-Mussel#2022.

Anon, 2008. SOFIA: The State Of World Fisheries and Aquaculture, 2008. Fisheries and Aquaculture Department; FAO [Food and Agriculture Organisation of the United Nations], Rome, pp. 176.

Anon, 2011. 'Top salmon farmers to develop innovative sea lice solution', Scottish Sea Farms Business News (http://www.scottishseafarms.com/en/news.html).

Badiu, D., Roncea, F., Rosoiu, N., 2009(a). Formulation and Pharmaceutical Evaluation of Three W/O Emulsions with *Mytilus galloprovincialis* Lmk. and *Rapana venosa* Lipid Extracts. Farmacia 57, 212-217.

Badiu, D., Roncea, F., Roşoiu, N., 2009(b). *Mytilus galloprovincialis* Lmk. and *Rapana venosa* lipid extracts in nutraceuticals system. Farmacia 57, 109-115.

Badiu, D., Coatu, V., Oros, A., Rosoiu, N., Barbes, L., 2008(a). Sanitary Comparative Characterization of Lipid Extracts From Mediterranean Mussel (*Mytilus galloprovincialis* Lmk.) and Hard-Shell Clam (*Rapana venosa*) of the Black Sea Coast. Cercetari marine 38, 269-277.

Badiu, D., Balu, A.M., Barbes, L., Luque, R., Nita, R., Radu, M., Tanase, E., Rosoiu, N., 2008(b). Physico-Chemical Characterisation of Lipids from *Mytilus galloprovincialis* (L.) and *Rapana venosa* and their Healing Properties on Skin Burns. Lipids 43, 829-841.

Bayne, B.L., Widdows, J., Thompson, R.J., 1976. Marine Mussels: Their Ecology and Physiology. Cambridge University Press, New York, USA., 495 pp.

Beaumont, A.R., Gjedrem, T., Moran, P., 2007. Blue Mussel *Mytilus edulis*, Mediterranean mussel *M. galloprovincialis*. In: Svasand, T., Crosetti, D., Garcia-Vasquez, E., Verspoor, E. (Eds.), Genetic impact of aquaculture activities in native populations. Genimpact Final Scientific Report, pp. 62-69.

Carter, G.A., 1980. Selected mineralogical data for the Bivalvia. In: Rhoads, D.A., Lutz, R.A. (Eds.), Skeletal growth of aquatic organisms. Plenum Press, New York, USA., pp. 627-643.

Daley, C.A., Abbott, A., Doyle, P.S., Nader, G.A., Larson, S., 2010. A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. Nutrition Journal 2010 9:10, 1-12.

Dare, P.J., Edwards, D.B., 1975. Seasonal changes in flesh weight and biochemical composition of mussels (*Mytilus edulis* L.) in the Conwy Estuary, North Wales. Journal of Experimental Marine Biology and Ecology 18, 89-97.

Dias, P.J., Bland, M., Shanks, A.M., Beaumont, A., Piertney, S.B., Davies, I.M., Snow, M., 2009. *Mytilus* species under rope culture in Scotland: implications for management. Aquaculture International 17, 437-448.

Harris, V.A., 1990. Sessile Animals of the Sea Shore. Chapman & Hall, London, UK., 381 pp.

Hayward, P., Nelson-Smith, T., Shields, C., 1996. Sea Shore of Britain & Europe, 352 pp.

Jenson, F., Patursson, E.J., 2011. Blue Mussel (*Mytilus edulis*) in Faroese Fjords: Biology and Farming Potential., Faculty of Science and Technology. University of the Faroe Islands, pp. 74.

Little, C., Kitching, J.A., 1996. The Biology of Rocky Shores. Oxford University Press, Oxford, 240 pp.

McKenzie, J.D., 1986. The Reproductive Cycle of *Mytilus edulis* L. From Lough Foyle. The Irish Naturalists' Journal 22, 13-16.

Nadler, L.E., 2011. Metabolomics of different tissues from the Norway lobster, *Nephrops norvegicus*: A first approach to determine uses for fishery waste and biomarkers of environmental health in a crustacean, Institute of Biodiversity, Animal Health and Comparative Medicine. University of Glasgow, Glasgow, UK., pp. 87.

Newell, R.I.E., 1989. Species Profiles: Life Histories and Environmental Requirements of Coastal Fishes and Invertebrates (North and Mid-Atlantic) - Blue Mussel, Fish and Wildlife Service Biological Report. U.S. Department of the Interior, pp. 25.

Newell, R.I.E., Hilbish, T.J., Koehn, R.K., Newell, C.J., 1982. Temporal variation in the reproductive cycle of *Mytilus edulis* L.(Bivalvia, Mytilidae) from localities on the east coast of the United States. Biological Bulletin 162, 299-310.

Rice, M.A., 2010. Cultured Mussels of the Northeast. NRAC Publication 210, 1-4.

Ruppert, E.E., Barnes, R.D., 1994. Invertebrate Zoology. Saunders College Publishing, 1056 pp.

Samulsson, L.M., Larsson, D.G.J., 2008. Contributions from metabolomics to fish research. Molecular Biosystems 4, 974-979.

Seed, R., 1976. Ecology. In: Bayne, B.L. (Ed.), Marine Mussels: Their Ecology and Physiology. Cambridge University Press, New York, USA., pp. 13-65.

Sugiura, Y., 1962. Electrical Induction of Spawning in Two Marine Invertebrates (*Urechis unicinctus*, Hermaphroditic *Mytilus edulis*). Biological Bulletin 123, 203-206.

Thompson, R.J., 1979. Fecundity and Reproductive Effort in the Blue Mussel (*Mytilus edulis*), the Sea Urchin (*Strongylocentrotus droebachiensis*), and the Snow Crab (*Chionoecetes opilio*) from Populations in Nova Scotia and Newfoundland. Journal of the Fisheries Research Board of Canada 36, 955-964.

Thorson, G., 1957. Bottom communities (sublittoral or shallow shelf). Geological Society of America. Memoirs 67, 461-534.

Uzmann, J.R., 1953. *Cercaria milfordensis nov. sp.*, a microcercous trematode larva from the marine bivalve, *Mytilus edulis* I. with special reference to its effect on the host. Journal of Parisitology 39, 445-451.

Viant, M.R., 2007. Metabolomics of aquatic organisms: the new 'omics' on the block. Marine Ecology Progress Series 332, 301-306.

Viant, M.R., 2008. Recent developments in environmental metabolomics. Molecular Biosystems 4, 980-986.

Viant, M.R., Pincetich, C.A., Tjeerdema, R.S., 2006. Metabolic effects of dinoseb, diazinon and esfenvalerate in eyed eggs and alevins of Chinook salmon (*Oncorhynchus tshawytscha*) determined by 1H NMR metabolomics. Aquatic Toxicology 77, 359-371.

Wilbur, K.M., Saleuddin, A.S.M., 1983. Physiology, Part 1 Shell formation. In: Saleuddin, A.S.M., Wilbur, K.M. (Eds.), The Mollusca. Academic Press, New York, USA., pp. 235-287.

Yonge, C.M., 1976. The 'Mussel' Form and Habit. In: Bayne, B.L. (Ed.), Marine Mussels: Their Ecology and Physiology. Cambridge University Press, New York, USA., pp. 1-12.

Appendices

Appendix 1. The theory and analysis of ^{1}H NMR and COSY Spectroscopy 20

Appendix 2. Final Report Form 21

Appendix 1.

The theory and analysis of ^{1}H NMR and COSY Spectroscopy

Analysis of ^{1}H NMR spectra involves the examination of two main determinants: chemical shift and spin-spin coupling.

Chemical shift is utilised to locate a given peak within the NMR spectrum, it is represented by δ, quantified in parts per million (ppm) and generally ranges from 0 to 12. Chemical shift is calculated by dividing the nuclear shielding of a proton by the magnetic field applied during NMR. Under such a magnetic field the electrons within a given molecule rearrange themselves in order to better shield the various atoms' nuclei. Consequently, the magnetic force felt by these nuclei is, by varying degrees, less than the field applied and therefore results in a varying chemical shift. Peaks denoting a high chemical shift are referred to as 'downfield' while those exhibiting a low chemical shift are said to be 'upfield'. It is through this behaviour, using the chemical shifts exhibited by the numerous peaks making up a spectrum, that the composition of parent molecules can be estimated. Any electronegative group or atom within a molecule will cause a reduction in the nuclear shielding of associated 'unprotected' protons (i.e. those lacking in valent electrons) thereby causing the peaks for those regions to be pushed downfield. Examples of such are Olefin and aromatic groups. Furthermore, the degree of separation of the chemical shifts of each peak is directly proportional to the frequency of the imposed magnetic field (Abraham *et al*., 1988).

Spin-spin coupling refers to an effect observed in ^{1}H NMR spectra where the small magnetic fields generated by adjacent nuclei within a molecule interact. Such interactions generate what are referred to as spin systems between adjacent protons. These additional spin systems result in a splitting of the resonance (i.e. the peak) so that multiple peaks may be present at a given chemical shift. These split peaks, or 'multiplets', are essential in determining the number of nuclei adjacent to a specific nucleus. The peaks seen within a muliplet are equal to the number of adjacent protons, plus one. For example, if a proton has two adjacent protons, it would exhibit a triplet, i.e. a peak split into three).

Via the utilisation of known reference spectra and of course experience, the chemical shift and spin-spin coupling systems detected through NMR can be used to identify compounds in a given sample (Abraham *et al*., 1988). In 2-dimensional (^{1}H-^{1}H) homonuclear correlation NMR spectroscopy, or COSY, samples are exposed to two frequency pulses, separated by a defined time period. These pulses produce two 1-dimensional (^{1}H) spectra, which can then be correlated as axes. The resultant 2-D spectrum plots the peaks along each axis as coordinates, creating so-called "diagonal peaks". Peaks whose coordinates are not found plotted on the diagonal are referred to as cross peaks. Cross peaks will be found on either side of the diagonal peaks, with each pairing representing the same compound. Consequently, peaks associated with the same compounds can be more readily identified utilising such 2D plots via the connection of cross and diagonal peaks (Abraham *et al*., 1988).

Abraham, R.J., Fisher, J., Loftus, P. (1988) Introduction to NMR Spectroscopy. Essex: John Wiley & Sons, Ltd.

Appendix 2.

Final Report Form

Business Innovation Grant Project – Interim Report – 19th December 2011

Academic partner: University of Glasgow (PI: Professor D.M. Neil; RA: Dr John Thompson)
Commercial partner: Blueshell Mussels Ltd.

Project Details

Project Title: The identification and quantification of potentially useful marine ingredients in Mussel waste

Project Brief: This project will determine the biochemical profile ('metabolome') of the soft tissue component of mussel waste produced by BlueShell Mussels Ltd., with particular attention to the amounts of Omega 3 lipids and antioxidants. It will thus identify key compounds that may be used in other industrial processes or products, or represent valuable 'nutraceuticals', and hence may offset the costs for disposal of these wastes.

Background: The organic waste generated by BlueShell Mussels production process is currently disposed of via standard means. The components of this waste, primarily the soft tissues, but also the shell and any associated seaweed may be rich sources of natural products that could potentially be extracted for use in other industries. This would be beneficial to the company as it would effectively dispose of the waste in an environmentally sound manner while providing an additional revenue stream, which would at the very least partially offset the company's processing/disposal costs.

Methodology: In order to gauge the types of compounds present and their relative quantities within the soft tissue component of the waste, a profile of the metabolome (biochemical profile) will be determined using 1D (^{1}H) and 2D (^{1}H-^{1}H) correlation (COSY) NMR (Nuclear Magnetic Resonance) spectroscopy. Although other techniques are available, at this stage of investigation NMR Spectroscopy has been identified as the most viable and cost effective method to determine the commercial viability of processing waste material.

Progress to date:

- Period 06/04/2011 to 02/05/2011 - Commencement of a review of literature encompassing metabolomics in marine invertebrates, metabolomic analysis methods (including 1D ^{1}H NMR Spectroscopy, 2D ^{1}H-^{1}H homonuclear correlation spectroscopy (COSY) and HPLC with full scan HRFTESIMS-orbitrap) and nutraceuticals extracted from marine sources.

- Period 27/07/2011 to 02/08/2011 - Preparation of laboratory space, consumables and equipment required for processing, extraction and analysis of *Mytilus edulis* tissue samples.

- 03/08/2011 - Receipt of 2kg of mussel waste from BlueShell Mussels Ltd. (single source sample, as opposed to 3 samples from separate farms).

- 04/08/2011 to 17/08/2011 - Processing of soft tissue samples, involving extraction of whole undersized and damaged *M. edulis* as well as dissecting out complete samples of

mantle, gill, gonad and Aductor Muscle for individual component analysis. Samples were prepared by freeze drying at -40°C for 10 days before being homogenised and stored at -25°C prior to lipid content analysis.

- Period 19/09/2011 to 24/11/2011 - Analysis of total lipid content of samples carried out using a solvent extraction technique modified from the Folch method as well as preparation of samples for 1D (^{1}H) & 2D (^{1}H-^{1}H) NMR spectroscopic analysis at the SULSA Scottish Metabolomics Facility (ScotMet) University of Strathclyde.

- Period 25/11/2011 to 16/12/2011 - 1D (^{1}H) & 2D (^{1}H-^{1}H) NMR spectroscopic analysis of samples at the SULSA Scottish Metabolomics Facility (ScotMet) University of Strathclyde.

Preliminary results & discussion:

1. Lipid quantification

Table 1. Biometrics of sampled *Mytilus edulis*; including mean length (long axis), mean whole animal mass and mean mass of soft tissue.

	Mean value ± St.Dev.
Length (mm)	59.93 ± 5.85
Whole animal (g)	10.863 ± 2.816
Soft tissue (g)	5.112 ± 1.544

Table 2. Average lipid content of whole *M. edulis* (including & excluding shell) and the four major tissue types.

	Mass (g) ± St.Dev.	% of wet weight ± St.Dev.
Whole animal	$9.64 \times 10^{-2} \pm 4.71 \times 10^{-2}$	**(incl. shell)** 0.88 ± 0.33 **(excl. shell)** 1.87 ± 0.67
Gonadal tissue	$1.37 \times 10^{-2} \pm 8.11 \times 10^{-3}$	2.00 ± 0.34
Mantle	$5.58 \times 10^{-3} \pm 3.78 \times 10^{-3}$	1.03 ± 0.24
Gill	$4.25 \times 10^{-3} \pm 1.62 \times 10^{-3}$	0.99 ± 0.14
Posterior aductor Muscle	$3.62 \times 10^{-3} \pm 3.95 \times 10^{-3}$	0.69 ± 0.35

- *M. edulis* gonadal tissue possessed the highest concentration of lipid, containing on average approximately 14% of the animal's total lipid content, at least double that observed in the other tissues tested.

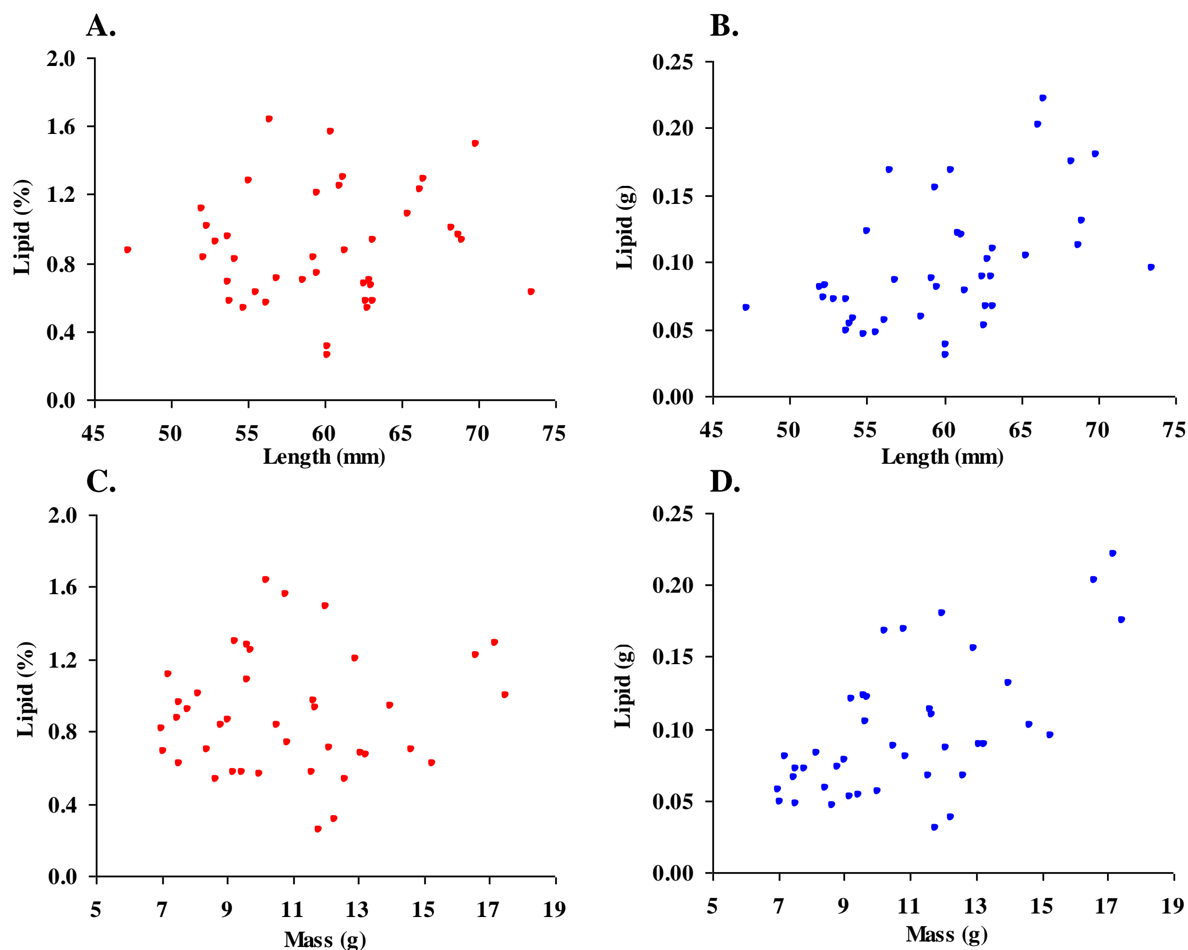

Figure 1. Relationship of soft tissue lipid content to animal size. **A.** % whole animal lipid content versus animal length. **B.** Mass of lipid in soft tissues versus animal length. **C.** % whole animal lipid content versus mass (including shell). **D.** Mass of lipid in soft tissues versus mass of animal (including shell).

- As expected there is a clear positive correlation between the mass of lipid present in the soft tissues and animal size. This correlation appears more defined when plotted against whole animal mass as opposed to length. Consequently, it is recommended that measurements of animal mass rather than length should be used when determining estimates of lipid yield.

- No pattern could be discerned in the relationship between animal size (either mass inclusive of shell or length) and percentage lipid content. Hence, there does not appear to exist an optimum size range for *M. edulis* with regard to percentage lipid content/yield.

2. NMR spectroscopy of *M. edulis* lipid extracts

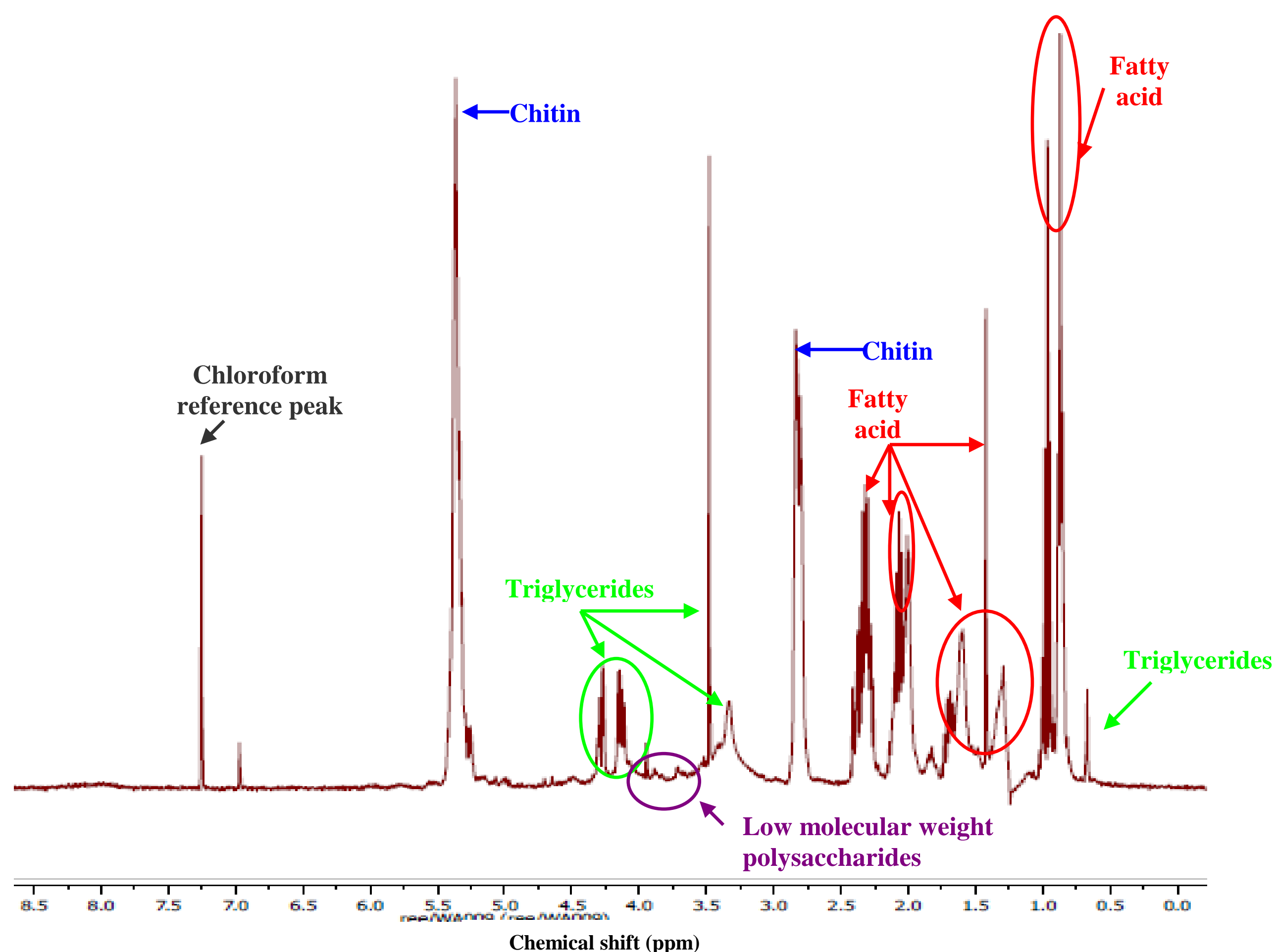

Figure 2. 1D (^{1}H) NMR spectrum obtained from whole *M. edulis* lipid extract including peaks indicating the likely presence of chitin, triglycerides and associated fatty acids as well as low molecular weight polysaccharides. All spectra for whole animal samples were very similar, exhibiting highly comparable compounds.

- 1D (^{1}H) NMR analysis indicates that the lipid extract obtained from whole *M. edulis* is dominated by triglycerides, but also contains chitin and small quantities of low molecular weight polysaccharides. These compounds are represented by multiple peaks with differing chemical shifts due to the multiple proton spin systems present within their molecular structure. Thus, the peaks labelled ‘triglycerides’ in Figure 2 represent regions of the compound’s glycerol backbone, whereas, peaks labelled ‘fatty acid’ correspond to molecular components associated with the various fatty acids present in the compounds.

- The 2D (^{1}H-^{1}H) COSY NMR analysis further confirms the dominance of triglycerides within the lipid extracts obtained from *M. edulis*. Figure 3 clearly illustrates related peaks, i.e. those representing molecular components that are associated with one another within specific compounds.

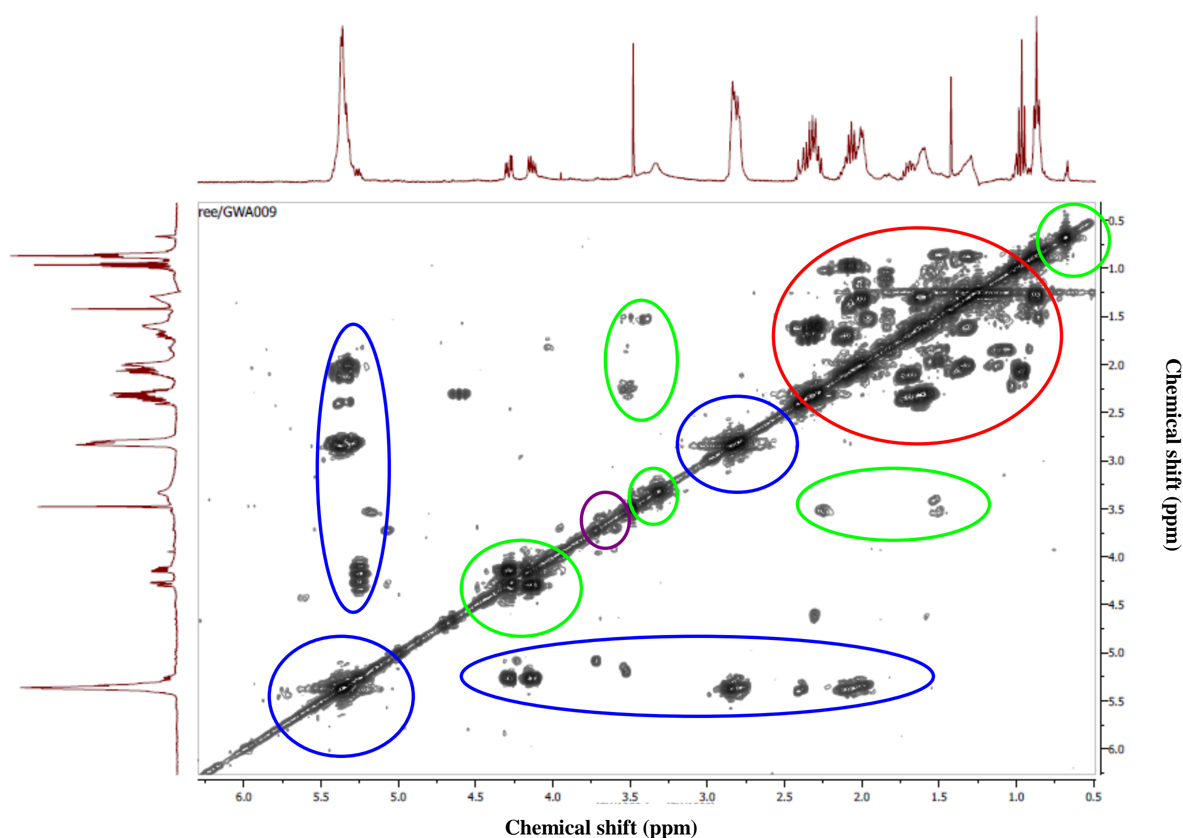

Figure 3. 2D (^{1}H-^{1}H) COSY NMR spectrum obtained using whole *M. edulis* lipid extract, peaks plotted in two dimensions for increased resolution of related regions. Regions of association are categorised as follows; O – Chitin, O – Triglycerides/glycerol, O – Fatty acids and O – Low molecular weight polysaccharides.

Preliminary conclusions & recommendations

The data gathered thus far support the conclusion that the lipid present within *M. edulis* waste (at 1.87% of soft tissue wet weight) comprises a relatively low proportion of the total material. This percentage lipid content appears consistent with data previously published by the U.S. Department of Agriculture, listing the total fat content of raw mussel meat at 2.3% (Rice, 2010). Indeed, *M. edulis* is noted as possessing a very high protein to lipid ratio (Dare & Edwards, 1975; Rice, 2010). *M. edulis* from the Conwy Estuary, North Wales, were shown to contain 8.4 times more protein and 3.2 times more carbohydrate in their dried flesh, than lipid (Dare & Edwards, 1975).

M. edulis lipids do, however, contain a large proportion of omega-3 and omega-6 polyunsaturated type essential fatty acids (EFAs) with omega-3 present in what is regarded as being a beneficially high proportion (Rice, 2010). *Mytilus galloprovincialis* Lmk., a closely related species, has been demonstrated as containing a high proportion of the omega-3 fatty acids, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Badiu *et al*, 2009).

It is currently not possible to identify specific compounds within the *M. edulis* lipid extract without further laboratory analysis in the form of high performance liquid chromatography (HPLC) and mass spectrometry. However, given the low proportion of lipid recovered from

the waste and the dominance of triglycerides within the extract, the likelihood of further analysis identifying any new compounds of economic significance is unlikely.

It is now generally considered that a healthy diet is one containing a ratio of 1:1 to 1:4, omega-3:omega-6 EFAs (Daley *et al*, 2010). Given that such beneficial fatty acids may be present in *M. edulis* in a favourable ratio, a cost-effective means of rendering waste animals could provide material of a suitable quality for use in diet/feed supplements. However, given the relatively low yields observed during this project, further investigation is required to determine the economic viability of such commercial extraction procedures.

References:

Badiu, D., Roncea, F., Roşoiu, N., 2009. *Mytilus galloprovincialis* Lmk. and *Rapana venosa* lipid extracts in nutraceuticals system. Farmacia 57, 1, 109-115.

Daley, C. A., Abbott, A., Doyle, P. S., Nader, G. A., Larson, S., 2010. A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. Nutrition Journal 2010 9:10, 1-12.

Dare, P.J., Edwards, D.B., 1975. Seasonal changes in flesh weight and biochemical composition of mussels (*Mytilus edulis* L.) in the Conwy Estuary, North Wales. Journal of Experimental Marine Biology and Ecology 18, Issue 2, 89-97.

Rice, M. A., 2010. Cultured Mussels of the Northeast. NRAC Publication 210, 1-4.