The microbiological safety of seaweed and insect larvae as novel and sustainable fish feed ingredients

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STATEMENT OF ORIGINALITY

I hereby confirm that this PhD thesis is an original piece of work conducted independently by the undersigned and all work contained herein has not been submitted for any other degree.

All research material has been duly acknowledged and cited.

Signature of candidate……

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Date…….....

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ABSTRACT

The capacity of global aquaculture to feed nine billion people by 2050 requires replacement of unsustainable fishmeal and plant ingredients in aquafeed with innovative ingredients such as seaweed-fed insect larvae. Dipteran (fly) larvae offer a protein composition similar to fishmeal, whilst seaweed provides omega-3, essential for both fish and human health. However, seaweed is readily colonised in the coastal environment by potentially pathogenic bacteria, and there are no bacteriological standards for seaweed manufactured for animal feed. Bacteriological standards for insect products are not yet adequate given the unknown risks associated with different insect species. To demonstrate the public health safety of seaweed-fed dipteran larvae entering the feed and food chain, this thesis sought to produce a bacteriological risk assessment of the entire production chain. Seaweed flies (Coelopidae) were shown to be capable of enhancing the spatio-temporal distribution and persistence of *E. coli* O157:H7 in decaying wrack and beach sand, thus increasing opportunities for contamination of living seaweed. Screening of seaweed-fed black soldier fly larvae (*Hermetia illucens*; BSFL) for bacteriological hazards during trial production demonstrated that incoming raw feed materials and the production environment are sources of bacteriological contamination, which processing of BSFL into finished products can eradicate. Simulated manufacture of meal from seaweed supporting biofilms of pathogenic bacteria revealed that drying seaweeds at a temperature that maintains their nutritive content (50 °C) can encourage pathogen persistence in stored powder due to the interacting effects of temperature, water activity, bacterial species and strain during processing. BSF prepupae reared on pathogen contaminated seaweed powder supplement selectively reduce *E. coli* levels in their guts. A survey of the seaweed industry suggested that feed producers and the public currently rely on remoteness of harvesting sites from anthropogenic disturbance as a measure of bacteriological water quality and thus product safety. Based on the identification of critical control points (CCPs) throughout the feed-food production chain, it is recommended that existing microbiological criteria for Shellfish Harvesting areas should be applied to freshly harvested seaweed. Microbiological criteria for ready-to-eat (RTE) food products are recommended as standards for freeze-thawed seaweed for

Coelopidae, and powdered seaweed for BSFL. By demonstrating that seaweed-fed insect products pose no bacteriological threat to consumers, this thesis will contribute to transformation of global aquaculture into a sustainable food production system.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF APPENDICES

LIST OF ABBREVIATIONS

The abbreviations listed below are detailed in full only on their first appearance in the text.

AMR	Anti-microbial resistance
ANFs	Anti-nutritional factors
ANOVA	Analysis of variance
APW	Alkaline Peptone Water
ARG	Antimicrobial resistance genes
a_w	Water activity
BSE	Bovine Spongiform Encephalitis
BSF	Black soldier fly
BSFL	Black soldier fly larvae
C	Carbon
CCP	Critical control point
CEF	Controlled environment facility
CFU	Colony forming units
CT	Cefixime and potassium tellurite
DHA	Docosahexaenoic acid
DM	Dry matter
DW	Dry weight
EAA	Essential amino acid
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
EPS	Extracellular polysaccharide
ESBL	Extended-spectrum β-lactamase
EU	European Union
FA	Fatty acid
FC	Free chlorine

FIOs	Faecal indicator organisms
FM	Fishmeal
FMFO	Fishmeal and fish oil
FO	Fish oil
FPE	Feed or food production environment
FNS	Food and nutrition security
FSS	Food Standards Scotland
GAP	Good agricultural practices
GHP	Good hygiene practices
GIT	Gastrointestinal tract
GMP	Good manufacturing practices
HACCP	Hazard Analysis and Critical Control Point system
HUS	Haemolytic uremic syndrome
IMTA	Integrated multi-trophic aquaculture
LB	Luria-Bertani broth
LC-PUFA	Long Chain Polyunsaturated fatty acid
MLGA	Membrane Lactose Glucuronide Agar
N	Nitrogen
n-3	Omega-3
n-6	Omega-6
NGS	Next generation sequencing
OA	Ocean acidification
PAPs	Processed animal proteins
PBS	Phosphate buffered saline
PCBs	Polychlorinated biphenyls
POPs	Persistent organic pollutants
PUFAs	Polyunsaturated fatty acids
RLU	Relative light units/ luminescence
rm	Repeated measures

RTE	Ready-to-eat
SDG	Sustainable Development Goal
SHAs	Shellfish harvesting areas
SMAC	Sorbitol MacConkey Agar
sp.	Single species
spp.	Several species within the genus
STEC	Shiga toxin-producing *E. coli*
THB	Total heterotrophic bacteria
UN	United Nations
VBNC	Viable but non-culturable
WGS	Whole genome sequencing
XLD	Xylose lysine deoxycholate agar

Chapter 1 | General Introduction

1.1 GLOBAL FOOD PRODUCTION: CHALLENGES TO SUSTAINABILITY, SECURITY AND HEALTH

The current global food system presents a threat to both human health and the environment (Willett *et. al.,* 2019). Sustainable diets exert minimal environmental impacts, allow for intergenerational food security (the reliable and adequate supply of food) and nutrition, and are safe and healthy (Meybeck and Gitz, 2017). In 2015, the United Nations (UN) Sustainable Development Goal (SDG) 2 outlined an international ambition to achieve sustainable agriculture and food security, improve nutrition and end hunger, and SDG 12 called for sustainable production and consumption practices (Stephens *et. al.,* 2018). Yet these targets for long-term sustainability of food production systems are challenged by the triple threat of global environmental deterioration and change, population growth (projected to reach 9.7 billion by 2050) and food price fluctuations (Stephens *et. al.,* 2018; Bene *et. al.,* 2015).

The current system of food production is the primary driver of global environmental degradation. For example, it is responsible for: (i) land use conversion to cropland and pasture with associated losses of biodiversity and ecosystem services; (ii) depleted quality and quantity of exploited freshwater resources; and (iii) greenhouse gas emissions associated with livestock, fertilisers, tilling and land clearance (Willett *et. al.,* 2019). The shift towards diets high in energy but micronutritionally poor, with increasing dependence on animal based foods high in saturated fats, has resulted in malnourishment in the form of both undernourishment and obesity amongst one-third of the global population (Lindgren *et. al.,* 2018). The current emphasis on the nutritional value of foods however may shift the focus to increasing production of foods more beneficial to health (Ramankutty *et. al.,* 2018).

One area of food production that offers great potential for addressing future food and nutrition security (FNS) globally is aquaculture, which has experienced unprecedented growth over the last 40 years: in 2010, every second fish consumed was farmed, and protein sourced from fish exceeded that sourced from poultry and cattle (Bene *et. al.,* 2015). Fish provide essential amino acids, and more micronutrients than mammalian meat or plants, including vitamins and minerals (Mohanty *et. al.,* 2017). Increasing

consumption of oily finfish such as Atlantic salmon is driven largely by the human health benefits of omega-3 Long Chain Polyunsaturated fatty acids (*n*-3 LC-PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the only source of which is the marine food chain (Calder, 2018; Sprague *et. al.,* 2016). Juvenile marine fish also require EPA and DHA for growth and survival, and farmed fish traditionally attain these via fish oil (FO) contained in fishmeal (FM) (Tocher, 2010; Tacon and Metian, 2008a).

However, escalating prices for FM and FO (FMFO) resources have resulted from overdependence on a declining supply of wild marine fish catch (Pauly and Zeller, 2016). Cheaper replacement plant proteins and oils in commercial feeds can lack essential amino acids, and introduce anti-nutritional factors (ANFs), which either directly or via the metabolic products they produce, impede an animal's use of food and therefore their growth and health (Shepherd *et. al.,* 2017; Francis *et. al.,* 2001; Makkar, 1993). Furthermore, plant-based feed ingredients compete with human food resources; approximately 60 % of crop biomass edible by humans is diverted for animal feed (Makkar, 2018). Reduction of FMFO in aquafeeds and thus EPA and DHA in farmed fish may necessitate future increases in human consumption of oily fish to maintain *n*-3 LC-PUFA intake, yet European Union (EU) citizens are opposed to genetic modification of plants to contain *n*-3 LC-PUFA (Sprague *et. al.,* 2016). Animal feed accounts for 60 – 70 % of the total costs of production of food-producing animals, and the price and availability of feed ingredients represents a bottleneck in future global expansion of aquaculture (Barragan-Fonseca *et. al.,* 2017; Pelletier *et. al.,* 2018). It is clearly desirous that the aquaculture industry breaks its dependence on environmentally and commercially unsustainable feed sources. However, the choice of alternative feed sources for producers is dictated not only by price, but also the nutritional requirements of the farmed fish species and the EU regulatory framework prohibiting certain feed ingredients for food-producing animals (Naylor *et. al.,* 2009; EFSA, 2015).

1.2 FUTURE-PROOFING THE INDUSTRY: INSECTS AND SEAWEED IN AQUAFEED

One potential substitution for FMFO and plant proteins and oils are insects, which constitute natural dietary components of most economically important freshwater fish, including several key farmed anadromous species such as Atlantic and chum salmon

(Magalhaes *et. al.,* 2017; Henry *et. al.,* 2015). The requirements of carnivorous fish diets, e.g. high in protein and lipids, and low in carbohydrate, are better met by animal-derived feed ingredients (Gatlin *et. al.,* 2007; Lock *et. al.,* 2018). The advantages of farming insects as a feed resource are multiple: insects can reduce and valorise organic waste streams, such as manure, food waste and plant matter; as poikilotherms, insects expend less energy and are thus more efficient at converting food into protein than mammals; and finally insects are highly productive yet their production potentially exerts minimal environmental impact in the form of feed and water inputs (Makkar *et. al.,* 2014; Premalatha *et. al.,* 2011; Sanchez-Muros *et. al.,* 2014; Rumpold and Schlüter, 2013). However, the main input into the production of insect meal is the feedstock, and current EU Regulations prohibit the rearing of farmed insects on cost effective organic waste streams (Pelletier *et. al.,* 2018; EC, 2001; EC, 2009a and c). The environmental sustainability credentials of farming insects therefore risk being undermined by the current requirement to rear insects on environmentally unsustainable commercial feeds which are typically plant-based, such as soybean meal (Smetana *et. al.,* 2016).

Insects provide protein, lipids, vitamins and minerals, in quantities dependent on the insect species, diet, rearing conditions, life stage and the processing and extraction methods used during production (Nogales-Merida *et. al.,* 2018). Currently in Europe, commercially feasible, large scale production of insects as aquafeed ingredients has focussed on the black soldier fly (BSF; *Hermetia illucens*), the common housefly (*Musca domestica*) and the yellow mealworm (*Tenebrio molitor*) (Lock *et. al.,* 2018). BSF are, however, currently the primary insect whose larvae are exploited for animal feed by ~ 80 % of EU insect production companies (Derrien and Boccuni, 2018). The protein, essential amino acid (EAA), lipid, and essential fatty acid (FA) requirements of fish vary between species and differ according to whether a species is marine or freshwater, carnivorous or omnivorous, cold- or warm-water, and stage of development (Henry *et. al.,* 2015). However, most insect species provide a higher proportion of protein than soybean meal, though less than fishmeal, and Diptera (particularly BSF and the common housefly) offer an EAA composition closer to that of fishmeal when compared with Coleoptera (beetles) and Orthoptera (grasshoppers, locusts and crickets) which are more similar to soybean meal (Barroso *et. al.,* 2014). Fish farmers perceive insects as

'natural' constituents of fish diets, and of benefit to fish welfare and environmental sustainability (Verbeke *et. al.,* 2015). Feed trials have successfully replaced the fish or soybean diets of farmed fish in whole or in part with Dipteran proteins, including BSF larvae (BSFL) for seabass and Atlantic salmon, and housefly larvae for tilapia (Magalhaes *et. al.,* 2017; Belghit *et. al.,* 2018; Wang *et. al.,* 2017). Complete substitution of fishmeal with BSFL meal in the diets of Atlantic salmon for example had no detrimental effect on fish growth or health, or on fillet texture, smell or taste (Lock *et. al.,* 2015).

However, insects generally contain a much lower proportion of *n*-3 fatty acids and higher proportion of *n*-6 fatty acids than fishmeal, and terrestrial Diptera do not naturally contain EPA or DHA (Barroso *et. al.,* 2014; Fontaneto *et. al.,* 2011). Yet, insect larvae can be enriched in EPA and DHA by dietary inclusion of fish offal, fishmeal and seaweed (St-Hilaire *et. al.,* 2007a and 2007b; Sealey *et. al.,* 2011; Barroso *et. al.,* 2017; Liland *et. al.,* 2017). Seaweed as aquafeed would be cheaper than enriching insects as an intermediate step, however, complex carbohydrates in seaweeds can reduce protein digestibility, impairing fish growth and health, particularly of farmed carnivorous fish (Henry *et. al.,* 2015; MacArtain *et. al.,* 2007; Kamalam *et. al.,* 2017). Insects, conversely, can efficiently convert carbohydrate-rich organic matter such as seaweed into high value protein and lipids (Pastor *et. al.,* 2015), which is also more economically and environmentally rational than enriching insects with LC-PUFA using FMFO.

1.3 INSECTS AS ANIMAL FEED: THE EU RESPONSE

In response to growing interest in the potential of new, alternative proteins potentially entering the feed and food chain, the European Food Safety Authority (EFSA) and the food safety bodies of several EU countries were incentivised to undertake a comprehensive review of the potential risk to human consumers of undesirable substances being introduced to feed and food production chains by novel feed ingredients (EFSA, 2015). This risk profile represents a call for data collection to generate further evidence of the potential hazards associated with utilising insects as feed and food (Belluco *et. al.,* 2018). There are many unknowns about the safety of insects as feed material or complete feed or food, and the use of novel feed substrate for insects potentially introduces multiple feed and food safety hazards (Van der Spiegel

et. al., 2013). The risk to public health will arise from either increased exposure to expected hazards or exposure to previously undocumented hazards (Belluco *et. al.,* 2018).

1.4 THE SEAWEED RESOURCE

Seaweeds are diverse multicellular photosynthetic macroalgae typically inhabiting the intertidal and subtidal zones of marine and estuarine ecosystems, and as primary producers form the basis of marine food webs (Baweja *et. al.,* 2016). Seaweeds are categorised as brown (Phaeophyta), which includes wracks (Fucales) and kelps (Laminariales); green (Chlorophyta), or red (Rhodophyta) algae. Seaweeds have been exploited as feed for livestock for thousands of years (Makkar *et. al.,* 2016). In Europe, there are three potential sources of seaweed that the livestock and food industries can exploit. One possible source is detached seaweed deposited on beaches, although this is intermittently available, and inconsistent in quality. Seaweed cultivation is dominated by southeast Asian countries, but is still a relatively nascent industry in Europe, constrained by inadequate infrastructure and investment, as well as international competition, though with great future potential (White and Wilson, 2015; Taelman *et. al.,* 2015; Rebours *et. al.,* 2014). Therefore, the European seaweed feed and food sectors currently depend on wild harvesting of natural seaweed stocks, primarily *Ascophyllum nodosum* and *Laminaria* spp. (Rebours *et. al.,* 2014; Kadam *et. al.,* 2015).

As feedstock, seaweeds are underutilised despite representing a renewable and abundant form of organic biomass, which do not require freshwater or fertiliser to grow, utilise minimal growing space compared with terrestrial crops, and do not compete for land with crops grown for food or feed (Rajauria, 2015; Liland *et. al.,* 2017). Seaweed is protein-rich, with a relatively complete amino acid profile, contains significant concentrations of PUFAs (EPA can comprise up to 34 % of seaweed FAs), vitamins and minerals, and is a natural source of nutrition for many aquatic animals (Maehre *et. al.,* 2014). Several seaweed species have therefore been tested as fish feed supplements, driven in part by the PUFA content and associated benefits for fish growth, health, survival and fillet colour (Rajauria, 2015).

1.5 THE USE OF DIPTERAN SPECIES IN AQUAFEED

1.5.1 Black soldier fly (BSF) Hermetia illucens, Stratiomyidae

Hermetia illucens (L.) (Stratiomyidae) or the black soldier fly (BSF) (Fig. 1.1) is endemic to southern USA but is found throughout the tropics, subtropics and warm temperate zones (Makkar *et. al.,* 2014). BSF do not vector human diseases, and are classed as a non-nuisance fly (Cickova *et. al.,* 2015; Diener *et. al.,* 2009). The short-lived adult flies are weak fliers, and require only water, no food, relying on stored body fat for nutrition (Sheppard *et. al.,* 2002). Within two days of mating, females oviposit in dry crevices near feed substrate, typically laying between 320 – 620 eggs each (Sheppard *et. al.,* 1994; Tomberlin *et. al.,* 2002). The detritivorous BSFL can feed and develop on a multitude of organic materials including animal and human manure, decaying plant matter, abattoir, kitchen and brewery waste (Zhou *et. al.,* 2013; Banks *et. al.,* 2014; Sheppard *et. al.,* 1994; Nguyen *et. al.,* 2015; Webster *et. al.,* 2016). Larvae pass through six larval instars during development and take two to four weeks to reach the prepupal stage depending on environmental conditions (e.g. feed quality, temperature and humidity), at which time the prepupae egress in search of dry pupation sites (Hall and Gerhardt, 2002; Myers *et. al.,* 2008; Kenis *et. al.,* 2018).

Figure 1.1. Lateral view of *Hermetia illucens* (L.). Image taken from www.befbiosystems.eu.

The protein and fat content of BSFL are influenced by their diet, although variation exists depending on strain, species, life stage processed and method of processing (Liu *et. al.,* 2017; Zhou *et. al.,* 2013). BSFL can convert organic matter to protein at an exceptional rate, and mass reared BSFL can comprise on average approximately 42 % protein (dry weight (DW)) and approximately 29 % fat (DW), although the latter is deficient in LC-PUFAs (Diener *et. al.,* 2009; St-Hilaire *et. al.,* 2007a; Wang and Shelomi, 2017). Fish offal in BSFL diets produced a lipid content in the larvae of 30 % (of which 3 % was *n*-3 FAs) within 24 hours (St-Hilaire *et. al.,* 2007b). This lipid content was 43 % more than that produced in manure-fed BSFL (St-Hilaire *et. al.,* 2007b). This highlights the ability of BSFL to receive nutrients from feed materials which are not suitable for humans or other animals (Liland *et. al.,* 2017). Seaweed (*A. nodosum*) added as a feed supplement at ≤ 50 % produced BSFL with EPA content positively correlated with EPA content in the feed (Liland *et. al.,* 2017). *A. nodosum*-enriched BSFL meal and lipids fed to Atlantic salmon did not impair digestibility, feed conversion, whole body protein, lipid, amino acid and mineral content or growth performance of the fish (Belghit *et. al.,* 2018).

An alternative to BSF is the common housefly (*M. domestica*), which feeds and reproduces in human and animal faeces, food waste including fish offal, and other organic matter, and has been trialled as aquafeed due to its great reproductive potential and rapid growth rate (Ganda *et. al.,* 2019; Cickova *et. al.,* 2015). However, BSFL exhibit greater adaptability than houseflies to new diets (Nogales-Merida *et. al.,* 2018), which might explain the lack of attempts to enrich housefly larvae with LC-PUFA from novel sources. Moreover, the synanthropic common housefly is an important vector of animal and human diseases (Cickova *et. al.,* 2015; Forster *et. al.,* 2007). As such, despite the lack of evidence that housefly larvae meal can transfer undesirable microbes to fish, the potential for inclusion of housefly larvae in the feed and food chain is tainted by the burden of perceived and potentially actual risk to animal and human health (Makkar *et. al.,* 2014).

1.5.2 Seaweed fly (Coelopidae)

Coelopidae (Diptera) or seaweed flies are distributed worldwide (Smith, 1989), and two main European species, with sympatric ranges, are the larger and northerly *C. frigida*

(Fabricius) and the smaller and southerly *C. pilipes* (Halliday) (Fig. 1.2), although *C. frigida* is the dominant UK species (Dobson, 1974a; Edward *et. al.,* 2007; Edward *et. al.,* 2008). The sole habitat of Coelopidae is detached and decaying seaweed wrack, which in the UK is primarily comprised of the brown seaweed genera *Fucus* spp. and *Laminaria* spp. and accumulates along the strandline of beaches due to storm and tidal action (Edward and Gilburn, 2007). Wrack beds are the site of breeding, egg laying, larval feeding and growth, pupation and adult emergence, with both species co-habiting in high abundance (Dobson, 1974a; Smith, 1989). *C. frigida* for example form dense populations of approximately 1000 larvae kg^{-1} of seaweed (Butlin *et. al.,* 1984). Coelopids are attracted to wrack beds within hours of deposition, particularly by the heat generated by algal decay (Dobson, 1974a). Exposure to decaying seaweed induces male mating activity and female ovipositioning, with females of both species utilising brown seaweeds to lay their eggs, although *C. frigida* favour *Laminaria* spp. and *C. pilipes* prefer *Fucus* spp. (Dunn *et. al.,* 2002). Furthermore, both species can breed on a mixture of *Laminaria* spp. and *Fucus* spp., but not on *Fucus* spp. alone, although *C. frigida* can also breed on *Laminaria* spp. alone (Dobson, 1974b).

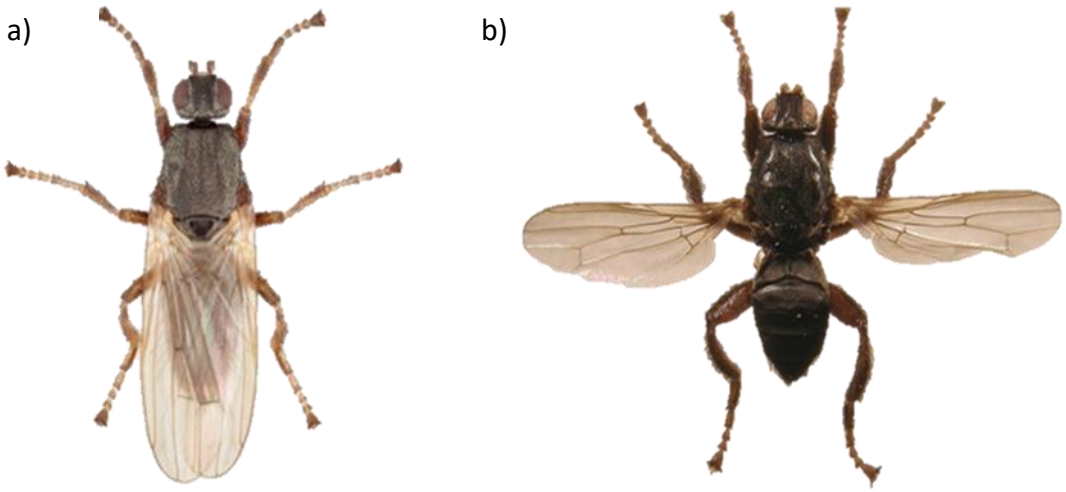

Figure 1.2. Dorsal view of *Coelopa frigida* (a) and *Coelopa pilipes* (b).
Images by Malcolm Storey (a) and Steven Falk (b).

A female *C. frigida* can lay an average of three clutches comprised of up to 80 eggs each, whereas *C. pilipes* females lay single eggs (Burnet and Thompson, 1960; Butlin *et. al.,* 1984). Coelopidae larvae of both species have similar life histories, and although *C. frigida* egg to adult development is more rapid, the duration for both species can be two to three weeks depending on the sustained wrack bed temperature (Dobson, 1974a and 1974b). *C. frigida* in particular is easily cultured, due in part to its high fecundity (Burnet and Thompson, 1960). Larvae hatch within approximately 24 hours and pass through three instar stages, after which pupation occurs in drier zones within the wrack bed (Dobson, 1974a). The internal temperature of decaying wrack beds can reach 40 °C, and *C. frigida* and *C. pilipes* larvae inhabit the cooler and warmer zones of the same wrack bed respectively, reflecting different spatial niche distributions (Phillips *et. al.,* 1995).

The larvae of both species are frequently washed out of their transient wrack bed habitats into nearshore waters where they serve as natural prey for fish (Dobson, 1974a). Furthermore, although mass migration of adult *C. frigida* over considerable distances has been documented (Egglishaw, 1961), Coelopidae are not pests of humans. Finally, as specialist feeders of seaweed, it is likely that Coelopidae naturally bioaccumulate LC-PUFAs from their marine diet (Fontaneto *et. al.,* 2011). Coelopidae are therefore exceptional candidates for introducing larval protein and LC-PUFAs to farmed carnivorous fish. However, mass production of Coelopidae has not yet been commercially attempted, and only one study investigating the nutritional potential of this insect family as feed for animals, including fish, has been undertaken (Biancarosa *et. al.,* 2018a). *C. frigida* and *C. pilipes* larvae reared on *F. serratus* contained higher protein and FA levels than those grown on *L. digitata*, reflecting the different nutritional composition of the seaweed species (Biancarosa *et. al.,* 2018a).

The protein (8 – 9 % dry matter (DM)) content of *C. frigida* and *C. pilipes* grown on *F. serratus* was far less than that of BSFL reared on a 10 % seaweed diet (28.6 % DM), although the amino acid composition of both species of Coelopidae larvae was suitable for animal nutrition (Biancarosa *et. al.,* 2018a; Liland *et. al.,* 2017). Lipid content of *C. frigida, C. pilipes* and BSFL was, however, similar at 18 % (DM) in Coelopidae, and 22.2 % (DM) in BSFL fed seaweed at a 50 % inclusion level (Biancarosa *et. al.,* 2018a; Liland

et. al., 2017). The composition of PUFAs in *C. frigida* and *C. pilipes* larvae did not reflect that of the seaweed to the same degree as in BSFL, probably due to physiological differences between the fly species (Biancarosa *et. al.,* 2018a). However, ~ 3.5 % of *C. frigida* and *C. pilipes* larvae FAs were EPA bioaccumulated from the seaweed, exceeding ≤ 1 % achieved in seaweed-enriched BSFL (Biancarosa *et. al.,* 2018a). Importantly, both species of Coelopidae larvae require a diet comprised exclusively of seaweed, representing a potential advantage over rearing BSFL in terms of feed costs.

1.6 SAFETY OF SEAWEED IN THE FEED AND FOOD CHAIN

Insect rearing, harvesting and processing are vulnerable to contamination by, and potential bioaccumulation, growth and transmission of, microbiological (e.g. pathogens and mycotoxins), viral, prion, chemical (e.g. heavy metals and pesticides) and parasitical hazards (Belluco *et. al.,* 2013; EFSA, 2015; Van der Spiegel, 2013). The feed substrate used to mass-rear insects represents a key determinant of the microbial hygiene of insect products for feed and food (Belluco *et. al.,* 2018; Fraqueza and Patarata, 2017). The vulnerability of manufactured insect products to contamination via raw feed materials and finished feed emerged as a common theme in the European biological risk assessments (EFSA, 2015; NVWA, 2014; FASFC, 2014; ANSES, 2015). Several reviews have assessed the safety of some commercially important insect species primarily for food but also for feed (Belluco *et. al.,* 2013; Van der Spiegel *et. al.,* 2013; Van Raamsdonk *et. al.,* 2017; Dobermann *et. al.,* 2017). The consensus is that major knowledge gaps regarding the potential transfer of undesirable substances from feed substrate to insects exist. Although data concerning risks to livestock (including insects) and human health from incorporating seaweed in diets do exist (Van der Spiegel *et. al.,* 2013), research primarily focuses on heavy metals and this is reflected in EU legislation (Table 1.1).

The Codex Alimentarius Commission develops international recommendations for food safety standards to inform national systems for protecting consumer health (Tacon and Metian, 2008b). The Codex Code of practice on good animal feeding states that a risk assessment of animal feed ingredients must be undertaken if none exists in order to establish that the levels of environmental contaminants, if present, do not pose a risk

to human consumers at the end of the feed and food chain (FAO/WHO, 2004). EU regulations pertinent to ensuring the safety of seaweed as a feed and food material entering the human food chain are outlined in Table 1.1. There are currently no regulations stipulating maximum allowed levels of bacterial pathogens in seaweed feed or food, yet there is growing evidence that seaweeds represent potential reservoirs of environmental bacteria potentially hazardous to human health (Ishii *et. al.,* 2006; Mahmud *et. al.,* 2007; Quilliam *et. al.,* 2014).

Table 1.1. The regulatory framework governing the use of seaweed as animal feed and human food in the EU.

Sector	EU Regulation	Relevance to utilisation of seaweed resource	Reference
Feed	Commission Regulation (EU) 68/2013 of 16 January 2013 on the Catalogue of feed materials	Seaweed dried and milled into powder can be marketed as 'seaweed meal' for animal feed; feed listed in the catalogue must comply with all EU legislated feed safety requirements	EC, 2013a; Van Raamsdonk *et. al.,* 2017
	Council Regulation (EC) 767/2009/EC of 13 July 2009 on the placing on the market and use of feed	Seaweed processed any other way than dried and milled is a 'novel feed ingredient' and subject to Reg (EC) 767/2009; manufacturers must make public any such product not listed in the Catalogue of Feed Materials Register; Regulation lists prohibited animal feed materials (e.g. faeces and domestic waste) but states that it cannot be assumed that materials not included on this list (such as seaweed) are safe; crude ash content of seaweed meal must be stated; no restrictions on the species of seaweed which can be used as animal feed; animal feed placed on the market must be safe	EC, 2009a; Wan *et. al.,* 2018
	Regulation (EC) 1831/2003 of 22 September 2003 on additives for use in animal nutrition	If seaweed meal is categorised as an additive rather than feed, the product requires authorisation by the EFSA before it can be marketed in Europe	EC, 2003
	Council Regulation (EC) 178/2002 of 28 January 2002 laying down the general principles and requirements of food law, establishing the European	Food safety legislation emphasises the necessity of considering the entire food production chain from	EC, 2002a

	Food Safety Authority and laying down procedures in matters of food safety	primary production (which includes harvesting of wild products) to sale to consumers	
	Council Regulation (EC) 183/2005/EC of 12 January 2005 laying down requirements for feed hygiene	Feed manufacturers are required to ensure feed hygiene and safety from 'farm-to-fork', from primary production of raw feed ingredients to production of food producing animals, which includes determining microbiological criteria based on scientific risk assessment	EC, 2005a
	Council Directive (EC) 2002/32/EC of 7 May 2002 on undesirable substances in animal feed	Guidance on the maximum allowed limits in feed material generally of toxic metals (e.g. arsenic, lead and mercury), toxic elements (e.g. cadmium), mycotoxins and persistent organic pollutants (e.g. dioxins and polychlorinated biphenyls (PCBs)), but not bacterial pathogens; the maximum levels of mercury, cadmium, arsenic and lead in seaweed as a feed material are 0.1, 1, 2 and 10 mg kg^{-1}	EC, 2002b
	Council Regulation (EC) 396/2005/EC of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin	The maximum levels of pesticides permissible in plant or animal based materials entering the feed and food chain	EC, 2005b
	Commission Regulation (EU) 1275/2013 of 6 December 2013 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for arsenic,	Maximum limit for arsenic in seaweed meal and feed materials derived from seaweed is 40 mg kg^{-1}; maximum limit for arsenic in complementary feed and complete feed containing seaweed meal and feed materials based on seaweed is 10 mg kg^{-1}	EC, 2013b

	cadmium, lead, nitrates, volatile mustard oil and harmful botanical impurities		
	Directive (EC) 60/2000 of 23 October 2000 establishing a framework for Community action in the field of water policy; Regulation (EC) 834/2007 of 28 June 2007 on organic production and labelling of organic products; Commission Regulation (EC) 710/2009 of 5 August 2009 amending Regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007, as regards laying down detailed rules on organic aquaculture animal and seaweed production	Seaweed can be classified as organically produced if grown in locations free of pollutants or substances that would undermine organic status, and if the growing site is of high ecological quality	EC, 2000; EC, 2007; EC, 2009b
	FAO/WHO (2004) The Codex code of practice on good animal feeding	Assures the safety of feed for food-producing animals from the level of primary production, throughout manufacturing, to distribution to animals	FAO/WHO, 2004
	FAO/WHO (2003) Recommended international code of practice- General principles of food hygiene; Regulation (EC) 853/2004 of 29 April 2004 laying down specific hygiene rules for food of animal origin	Established the Hazard Analysis and Critical Control Points (HACCP) methodology, a mandatory tool in the EU for identifying biological, chemical and physical hazards in all materials and processes during processing of food, establishing critical limits, and controlling, monitoring and preventing, eliminating or reducing the hazards to acceptable levels. The result is an HACCP tool unique to a specific product, process and feed or food	FAO/WHO, 2003; FAO/WHO, 2004; FAO/WHO, 2009; FAO/WHO, 2015; EC, 2004b; Fraqueza and Patarata, 2017

		production environment (FPE), but importantly, applies to post-harvest feed and food processing *after* farm-level primary production; Regulation recommends that primary producers apply HACCP as far as is practicable, and follow general hygiene requirements, including good hygiene practices (GHP) and good agricultural practices (GAP)	
Food	Regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs; Commission recommendation (EU) 464/2018 of 19 March 2018 on the monitoring of metals and iodine in seaweed, halophytes and products based on seaweed	No maximum limits for arsenic, cadmium, iodine, lead or mercury in edible seaweed or food products based on seaweed (with the exception of seaweed based food supplements); manufacturers of edible seaweed products are advised to monitor concentrations of metals and iodine in their foodstuffs	EC, 2006a; EU, 2018a
	EFSA (2006) Tolerable upper intake levels for vitamins and minerals	Upper limits for dietary intake of iodine ranges between 200 and 500 µg day^{-1} depending on age group	EFSA, 2006
	Regulation (EU) 460/2018 of 20 March 2018 authorising the placing on the market of *Ecklonia cava* phlorotannins as a novel food under Regulation (EU) 2015/2283	Edible *Ecklonia cava* is subject to regulations which establish maximum intake levels, and heavy metal and microbiological criteria, due to its iodine content	EU, 2018b
	Directive (EC) 60/2000 of 23 October 2000 establishing a framework for Community action in the field of water policy; Regulation (EC) 834/2007	Seaweed can be classified as organically produced if grown in locations free of pollutants or substances	EC, 2000; EC, 2007; EC, 2009b

	of 28 June 2007 on organic production and labelling of organic products; Commission Regulation (EC) 710/2009 of 5 August 2009 amending Regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007, as regards laying down detailed rules on organic aquaculture animal and seaweed production	that would undermine organic status, and if the growing site is of high ecological quality	
	FAO/WHO (2003) Recommended international code of practice- General principles of food hygiene; FAO/WHO (1997) Principles and guidelines for the establishment and application of microbiological criteria related to foods; Regulation (EC) 853/2004 of 29 April 2004 laying down specific hygiene rules for food of animal origin	The key guidelines for assuring food hygiene; established the HACCP methodology, a mandatory tool in the EU for identifying biological, chemical and physical hazards in all materials and processes during processing of food, establishing critical limits, and controlling, monitoring and preventing, eliminating or reducing the hazards to acceptable levels. The result is an HACCP tool unique to a specific product, process and FPE, but importantly, applies to post-harvest feed and food processing *after* farm-level primary production; recommended that primary producers apply HACCP as far as is practicable, and follow general hygiene requirements, including GHP and GAP	FAO/WHO, 1997; FAO/WHO, 2003; FAO/WHO, 2004a; FAO/WHO, 2009; FAO/WHO, 2015; EC, 2004b; Fraqueza and Patarata, 2017

1.6.1 Bacteriological risks

Coastal waters from which seaweed is harvested for animal feed is typically inhabited by resident autochthonous bacteria, and by transient allochthonous microbes often originating from the terrestrial environment, e.g. via faecal sources. Faecally-derived microorganisms include human pathogens and non-pathogenic faecal indicator organisms (FIOs) (commensal *E. coli* and intestinal enterococci), which indicate the presence of human or non-human faecal contamination (Cho *et. al.,* 2016). Faecal microbes can enter coastal waters from point sources such as the overflow of untreated or partially treated wastewater, or via diffuse sources associated with agricultural practices or wildlife faecal contributions (Cho *et. al.,* 2016; Tondera *et. al.,* 2015). FIOs have been used to estimate the risk of enteric pathogenic microbes also being present in the environment, due to the logistical challenges of detecting typically low concentrations of pathogenic bacteria of public health importance in environmental matrices (Zhang *et. al.,* 2016; Cho *et. al.,* 2016; Ahmed *et. al.,* 2018). The primary route of transmission to humans of opportunistic waterborne pathogens is through environmental exposure, including faecal-oral transmission via contaminated food or water (Brouwer *et. al.,* 2018). FIOs and bacterial pathogens do not necessarily correlate predictably in the environment due to the potential for their differential survival in various environmental matrices (Bradshaw *et. al.,* 2016; O' Mullan *et. al.,* 2017). FIOs and bacterial pathogens may also both emanate from sources other than faecal (Zhang *et. al.,* 2016). Thus, in order to assess the disease risk associated with bacterial contamination of the aquaculture feed-human food chain, the persistence and transfer of key pathogens in terrestrial and marine feed and food producing landscapes should be investigated separately from FIOs (Bradford *et. al.,* 2013).

The fate of bacteria in coastal and intertidal waters is mediated by physical and biological transfer between seawater and sand, both well-established FIO reservoirs, and opportunities for environmental persistence and growth are facilitated by favourable abiotic and biotic conditions (O'Mullan *et. al.,* 2017; Whitman *et. al.,* 2014; Solo-Gabriele *et. al.,* 2016). Genes of pathogenic *E. coli*, *Listeria monocytogenes*, *Clostridium perfringens* and *Campylobacter jejuni* have all been isolated from swash zone sand of freshwater beaches, and wave action can mobilise *E. coli* cells attached to

sand particles, leading to bacterial resuspension in overlying seawater (Zhang *et. al.,* 2016; Vogel *et. al.,* 2016). Faecally contaminated seawater can also transfer bacteria to the surface of living and decaying seaweed, where the bacteria readily form persistent biofilms and can become concentrated relative to levels in surrounding seawater (Ishii *et. al.,* 2006; Byappanahalli *et. al.,* 2015; Shapiro *et. al.,* 2014; Van den Heuvel *et. al.,* 2010).

The nutritionally rich surfaces of seaweeds provide sheltered harbourage highly susceptible to epiphytic and endophytic attachment and potential growth by opportunistic planktonic microbiota, including bacteria which are typically early epiphytic colonisers of submerged surfaces (Hollants *et. al.,* 2013; Lachnit *et. al.,* 2011). Depending on seaweed species and season, dense bacterial biofilms can develop on seaweeds (Bengtsson *et. al.,* 2010). Bacterial communities typical of marine waters and associated with seaweeds can differ between seaweed species sharing the same habitat, and do not necessarily mirror the surrounding planktonic bacterial communities (Singh and Reddy, 2014; Lachnit *et. al.,* 2009). This can be due to seasonal variation in planktonic microbial assemblages, the relative attachment efficiency of different bacteria, bacterial interactions with pre-existing microbial colonisers which can outcompete or inhibit attachment by successive colonisers, or production of species specific chemicals by seaweeds to inhibit or attract certain bacterial colonisers (Singh and Reddy, 2014; Lachnit *et. al.,* 2009; Steinberg *et. al.,* 2002).

In coastal environments, detached and decaying algae can function as reservoirs of FIOs and pathogens, such as *Clostridium botulinum*, and provide sites for growth of pathogenic *E. coli* (Olapade *et. al.,* 2006; Byappanahalli *et. al.,* 2003; Chun *et. al.,* 2013; Chun *et. al.,* 2017). Fresh, attached *Cladophora* (a freshwater species of macroalgae) can harbour *E. coli*, *Campylobacter*, *Shigella*, *Salmonella* and *C. botulinum*, although *a priori* pathogen growth was not demonstrated (Byappanahalli *et. al.,* 2009; Ishii *et. al.,* 2006). Greater bacterial abundance within biofilms attached to macrophytes inhabiting brackish water compared with the same macrophyte species inhabiting freshwaters, was associated with plant nutrient and chemical content, and thus resources available to bacteria, differing between the two environments (Hempel *et. al.,* 2008). Salinity is the main driver of aquatic bacterial community diversity, and surface colonisation by

bacteria including *E. coli* is driven by species-specific signals adapted to the environmental conditions favoured by the bacterial species (Lozupone and Knight, 2007; Stanley and Lazazzera, 2004). Thus, the efficacy of attachment by non-halophilic bacteria to freshwater *Cladophora* may differ in the context of seaweed in a saline environment.

Senescing brown, red and green seaweeds facilitated *E. coli* survival in seawater, and *E. coli* replication can occur in the presence of *Ulva* sp*., Sargassum* sp*. and Undaria* sp*.* leachates (Quilliam *et. al.,* 2014; Quero *et. al.,* 2015). Certain seaweed species however, also contain antimicrobial properties active against select bacteria: for example, extracts of *L. digitata* inhibited growth of *L. monocytogenes* significantly more effectively than exudates from red or green seaweed, and extracts of *U. reticulata* inhibited growth of *E. coli* and *V. parahaemolyticus* (Cox *et. al.,* 2010; Vairappan and Suzuki, 2000). High concentrations of *L. digitata* extracts killed both Gram-positive and –negative bacteria (Gupta *et. al.,* 2010). However, this efficacy was negatively correlated with the temperature at which the seaweed was dried beforehand (Gupta *et. al.,* 2010).

FIOs and pathogenic bacteria can also be introduced to seaweed feed material from the feed production environment (FPE) (Muhterem-Uyar *et. al.,* 2015). The factors that make opportunistic bacteria effective colonisers of surfaces in the natural environment facilitates their exploitation of habitat niches and formation of biofilms in FPEs, which provides opportunities for contamination and recontamination of feed materials during manufacture (Bridier *et. al.,* 2015). Following harvesting, the factors that determine the potential for growth and inactivation of bacteria present in seaweed meal can be categorised as, (a) the intrinsic physicochemical nature of the feed, e.g. water activity (a_w), pH, structural and nutritional composition, and seaweed antimicrobial exudates; (b) extrinsic factors, e.g. temperature, presence of other microbes and larval antimicrobial activity, which can affect the ecology of the target microbe; (c) implicit factors, i.e. the range of intrinsic conditions in which a microbe can grow and resist stress, physiological cell state, and historical cell stress which determine bacterial species-, strain- and even cell-specific growth rates in feed, and (d) processing factors, such as the duration and nature of treatment, e.g. washing, slicing, heating, storage,

which can alter the properties of the feed and therefore the microbial habitat (Ross, 2008; Besten *et. al.,* 2017).

For terrestrial animals such as insects, seaweed must be processed into a digestible form from which nutrients can be absorbed (Packer *et. al.,* 2016). In the EU, this typically involves fresh seaweed being washed by hand to remove epiphytes and dirt; tunnel or convective oven drying to reduce bulk and prevent deterioration, but preferably at low temperatures to retain beneficial nutritional properties; finally milling of dried seaweed into powder for storage of up to one year (Radulovich *et. al.,* 2015; Kadam *et. al.,* 2015; Makkar *et. al.,* 2016; McHugh, 2003). This typical sequence of fluctuating environmental conditions to which any pathogenic bacterial contaminant of seaweed would be exposed during processing and storage will determine the level of consumer (both animal and human) risk posed by the final product (Ross, 2008).

1.6.2 Heavy metal risks

Due to the biosorption properties of their cell walls, seaweeds are vulnerable to heavy metal sequestration from surrounding seawater (Davis *et. al.,* 2003). Heavy metals can enter the environment through industrial and agricultural activities, and from geological sources (Lopez-Alonso, 2012). Lead, mercury and cadmium, and the metalloid arsenic, are of animal and public health concern due to their capacity to transfer through food chains and cause adverse physiological and biochemical effects via sublethal dietary exposure (Lopez-Alonso, 2012). The capacity of seaweed to accumulate metals is due primarily to the abundance of metals in the water and the sequestration capacity of the seaweed for a specific metal (a product of seaweed metabolic processes combined with local environmental conditions) (Sanchez-Rodriguez *et. al.,* 2001). Heavy metal concentrations in seaweed are mainly specific to seaweed species, and the capacity for metal accumulation capacity follows the order brown > red > green seaweeds (Ryan *et. al.,* 2012). Also, the concentration of arsenic is higher in smaller sizes of powdered *A. nodosum* particles (250 – 850 µg) compared with larger particle sizes (850 – 1940 µg) (Mac Monagail *et. al.,* 2018). This may have implications for the safety of insects if fed *A. nodosum*, depending on the required grain size.

1.6.3 Risks from other contaminants

Human health hazards from consuming seaweeds also include exposure to toxins and ANFs, high levels of iodine and ammonium, persistent organic pollutants (POPs), pesticides, radioactive isotopes and microplastics (Van der Spiegel *et. al.,* 2013). ANFs present in seaweeds for example include lectins which can interact with cells of the human digestive tract and cause adverse systemic reactions, and kainic acid, which naturally occurs in seaweed and can cause neurotoxic effects at high doses (de Oliveira *et. al.,* 2009; Holdt and Kraan, 2011). Geological sources of iodine in seawater can be bioaccumulated by seaweeds, and though essential for human metabolism, can, when ingested in excess of recommended daily intake levels, result in thyroid dysfunction and affect reproduction (Nitschke and Stengel, 2015; Delange, 2002).

1.7 SAFETY OF INSECTS IN THE FEED AND FOOD CHAIN

Insects as feed for farmed animals currently play only a minor role in the EU feed sector due primarily to restrictive legislation stemming from concern about the safety of processed animal proteins (PAPs) fed to food-producing animals following the 1990s Bovine Spongiform Encephalitis (BSE) outbreak (EFSA, 2015) (Table 1.2). However, the recent decision by the European Commission to permit the inclusion of insects in aquaculture feed (Reg (EC) 893/2017; EC, 2017a), came into effect on 1st July 2017, and marks a progressive step change amongst policy makers (Table 1.2). Categorisation of insects as 'farmed animals' (Reg (EC) 1069/2009; EC, 2009c) which cannot be reared on organic waste streams reframes the potential for commercial exploitation of seaweed as a rearing substrate for farmed insects, since there are no such restrictions on utilising seaweed meal as feed to produce insect PAPs for aquafeed (Table 1.2). A significant lack of knowledge regarding the biological and chemical safety of insect products, and outmoded regulations, has hampered commercial exploitation of insect proteins as sustainable, next generation feed and food ingredients and products. Research into the potential of insects as feed and food has thus far focussed largely on their nutritive value, with concern about safety of insects in the food chain lagging behind (Rumpold and Schlüter, 2013).

Published data on industrial-scale insect rearing and processing is not widely available, and there are currently no standardised insect husbandry methods (Riddick, 2014; Van Huis *et. al.,* 2015). Therefore, guidelines for good manufacturing practices (GMP) for insect production are still being developed. Yet it is widely accepted that undesirable substances can be present in mass reared insects and that their occurrence and relative concentrations are dependent on the insect species, feed substrate, life stage at harvest, the nature of the hazard, production practices and the hygienic condition of the FPE (Lock *et. al.,* 2018; Van der Spiegel *et. al.,* 2013). Feed for insects may be contaminated with microbes and other hazards which the insects may accumulate or convert when feeding, which can increase or decrease the concentration of and thus risk posed by the contaminant (Van der Spiegel *et. al.,* 2013). Ultimately, microbiological criteria specific to each insect species must be established following a full risk assessment of identified hazards (Belluco *et. al.,* 2017). Hazard Analysis and Critical Control Point (HACCP) guidelines unique to each particular production system of a specific company will therefore need to be developed to control hazards during insect rearing, processing, storage and distribution to ensure safe products enter the feed and food chain.

Table 1.2. Evolution of the regulatory framework governing the use of insects as animal feed and human food in the EU.

Sector	EU Regulation	Relevance to utilisation of insect resource	Reference
Feed	Council Regulation (EC) 999/2001 of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies	Forbade the use of processed animal proteins (PAPs) as feed for ruminants and non-ruminants other than for fur animals; since insect PAPs were not explicitly excluded from this Regulation, their inclusion as prohibited feed for food-producing animals was assumed	EC, 2001; Van Raamsdonk *et. al.,* 2017
	Commission Regulation (EU) 56/2013 of 16 January 2013 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies	Allows non-ruminant animals, including terrestrial insects (albeit those which are non-pathogenic to humans and other animals), to be utilised as feed for non-ruminant livestock and aquaculture species	EC, 2013c
	Commission Regulation (EU) 893/2017 of 24 May 2017 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as regards the provisions on processed animal protein	Permits the inclusion of insects in aquaculture feed; lists seven insect species in the Catalogue of Feed Materials permitted to be used as aquaculture feed (BSF, common housefly, yellow mealworm, lesser mealworm, house cricket, banded cricket and field cricket); list can be expanded with the proviso that candidate insect species are not recognised vectors of human, animal or plant pathogens, or are protected or invasive species	EC, 2017a; EC, 2001; EC, 2011

	Commission Regulation (EC) 1069/2009 of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption; Council Regulation (EC) 999/2001 of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies; Council Regulation (EC) 767/2009/EC of 13 July 2009 on the placing on the market and use of feed	Categorises farmed insects as 'farmed animals'; PAPs intended as animal feed cannot be reared on the following Category 1 and 2 feed substrates: ruminant PAPs, meat or bone meal, catering waste, meat or fish discard from food processing plants or supermarkets, and human and animal manures and digestive tract contents; insect PAPs utilised as feed must be safe; limits permissible feedstock for rearing insects to vegetable substrate, although does include commercial animal feed including fishmeal, or former food still safe for human consumption, together with some limited products of animal origin (e.g. eggs and fishmeal)	EC, 2009a ; EC, 2009c; EC, 2001
	Council Regulation (EC) 183/2005/EC of 12 January 2005 laying down requirements for feed hygiene	Insect PAPs must meet feed hygiene standards	EC, 2005a
	Council Regulation (EC) 396/2005/EC of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin; Council Directive (EC) 2002/32/EC of 7 May 2002 on undesirable substances in animal feed	Insect PAPs must not exceed maximum allowed levels of pesticides, toxic metals (e.g. arsenic, lead and mercury), toxic elements (e.g. cadmium), mycotoxins or persistent organic pollutants (e.g. dioxins and PCBs); the maximum allowed levels of mercury, arsenic, cadmium and lead are 0.1, 2, 2 and 10 mg kg^{-1} in feed materials for fish, and 0.1, 4, 0.5 and 5 mg kg^{-1} in complete fish feed	EC, 2005b; EC, 2002b

	Commission Regulation (EU) 68/2013 of 16 January 2013 on the Catalogue of feed materials	Terrestrial insects can be used in all their life stages, with or without processing, provided that they meet EU legislative standards on contaminants and undesirable substances in animal feed	EC, 2013a
	Commission Regulation (EC) 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive	Insect PAPs are required to be tested after application of any processing method only for *C. perfringens* (absent in 1 g of the product); insect PAPs that have been stored prior to dispatch must be tested for *Salmonella* spp. and Enterobacteriaceae; *Salmonella* spp. must be absent in five samples (25 g each), and it is unsatisfactory if Enterobacteriaceae exceed 300 colony forming units (CFU) in 1 g. However, out of the five samples, it is acceptable if in two samples the Enterobacteriaceae count is between 10 - 300 CFU g^{-1}, and is < 10 CFU g^{-1} in the remaining three samples.	EC, 2011
	FAO/WHO (2004) The Codex code of practice on good animal feeding	Assures the safety of feed for food-producing animals from the level of primary production, throughout manufacturing, to distribution to animals	FAO/WHO, 2004
	FAO/WHO (2003) Recommended international code of practice- General principles of food hygiene; Regulation (EC) 853/2004 of 29 April	Established the HACCP methodology, a mandatory tool in the EU for identifying biological, chemical and physical hazards in all materials and processes during processing of food, establishing critical limits,	EC, 2004a; FAO/WHO, 2003; FAO/WHO, 2004; FAO/WHO, 2009; FAO/WHO, 2015;

	2004 laying down specific hygiene rules for food of animal origin	and controlling, monitoring and preventing, eliminating or reducing the hazards to acceptable levels. The result is an HACCP tool unique to a specific product, process and FPE, but importantly, applies to post-harvest feed and food processing *after* farm-level primary production; recommended that primary producers apply HACCP as far as is practicable, and follow general hygiene requirements, including GHP and GAP; the rearing of insects falls within the category of primary production and is therefore not subject to HACCP requirements, however slaughter, processing, storage, transport etc. must comply with HACCP guidelines	Fraqueza and Patarata, 2017
Food	Council Regulation (EC) 258/1997 of 27 January 1997 concerning novel foods and novel food ingredients	Based on the criteria established in this Regulation and despite not being specifically mentioned in the Regulation, insects are categorised as 'novel foods' since they were not widely consumed within the Community before 15th May 1997	EC, 1997
	Commission Regulation (EU) 2283/2015 of 25 November 2015 on novel foods	Streamlines approval for traditional foods from countries outside the EU, such as insects and insect ingredients, provided proof of previous safe consumption can be demonstrated	EC, 2015

	Commission Regulation (EU) 2470/2017 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2283/2015 of the European Parliament and of the Council on novel foods	Once a food is included on the Union List of novel foods it can enter the EU market whilst adhering to the rigors of food safety legislation	EU, 2017b; Belluco *et. al.,* 2017
	Regulation (EC) 853/2004 of 29 April 2004 laying down specific hygiene rules for food of animal origin	Establishes rules for hygiene of food of animal origin; insects fall within this remit if provided live to the consumer or used to produce food, but not if processed in any way	EC, 2004b; Belluco *et. al.,* 2017
	FAO/WHO (2003) Recommended international code of practice- General principles of food hygiene; FAO/WHO (1997) Principles and guidelines for the establishment and application of microbiological criteria related to foods; Regulation (EC) 853/2004 of 29 April 2004 laying down specific hygiene rules for food of animal origin	The key guidelines for assuring food hygiene; established the HACCP methodology, a mandatory tool in the EU for identifying biological, chemical and physical hazards in all materials and processes during processing of food, establishing critical limits, and controlling, monitoring and preventing, eliminating or reducing the hazards to acceptable levels. The result is an HACCP tool unique to a specific product, process and FPE, but importantly, applies to post-harvest feed and food processing *after* farm-level primary production; recommended that primary producers apply HACCP as far as is practicable, and follow general hygiene requirements, including GHP and GAP as applied in the traditional livestock sector; the rearing of insects falls within the category of primary	EC, 2004a; FAO/WHO, 1997; FAO/WHO, 2003; FAO/WHO, 2004; FAO/WHO, 2009; FAO/WHO, 2015; Fraqueza and Patarata, 2017; Schlüter *et. al.,* 2017

		production and is therefore not subject to HACCP requirements, however slaughter, processing, storage, transport etc. must comply with HACCP guidelines	
	Commission Regulation (EC) 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs; FASFC (2014) Food safety aspects of insects intended for human consumption	Currently, in the absence of insect-specific microbiological criteria, producers of edible insects must abide by existing food safety Regulations. The maximum allowed levels of *Salmonella* in meat (absent in 25 g during shelf-life) and *L. monocytogenes* (absent in 25 g before leaving the production environment) in ready-to-eat foods could be applied to edible insects, although EU Regulations separate 'meat' from 'offal', whereas insects are often eaten whole.	EC, 2005c; FASFC, 2014; Belluco *et. al.,* 2017

1.7.1 Bacteriological risks

Concerns about the transfer of food-associated pathogens between multiple species in feed and food chains pose both an animal and human health concern (Wang and Shelomi, 2017). Bacteria colonise insects either vertically (parentally) or horizontally from their environment, e.g. from the rearing and processing environment, including from human handling, and the feedstock substrate (Schlüter *et. al.,* 2017). As a result, autochthonous bacteria and allochthonous opportunistic bacteria (including human pathogens) are harboured in the insect gastrointestinal tract (GIT), which together with the mouthparts and body surface is the main niche for insect-associated bacteria (Schlüter *et. al.,* 2017). An increasing number of publications have documented the diversity of the microbiota associated with the main groups of insects produced as human food, whilst a limited number of studies have assessed the effects of commercial processing on microbial hazards in edible insects.

The autochthonous bacterial communities of farmed insects typically include Enterobacteriaceae along with *Bacillaceae*, *Pseudomonaceae* and pathogenic members of *Enterococcaceae* (Osimani *et. al.,* 2018a; Vandeweyer *et. al.,* 2017a; Grabowski and Klein, 2017). Commensal, food spoilage and potentially pathogenic bacteria, including *B. cereus* and *Clostridium* sp., have been isolated from various edible insects including BSFL (Jeon *et. al.,* 2011; Osimani *et. al.,* 2016; Giaccone, 2005; Stoops *et. al.,* 2016). BSFL harbour a unique GIT microbiota of 48 bacterial species, including *Pseudomonas* spp. and *Bacillaceae*, regardless of the influence of microbiota in their feed (Jeon *et. al.,* 2011; Wynants *et. al.,* 2018a). *Salmonella* spp. and *L. monocytogenes* are rarely detected in farmed insects by culture-dependent methods (Vandeweyer *et. al.,* 2017b; Wynants *et. al.,* 2018b; Osimani *et. al.,* 2018b), although molecular analysis has identified *Listeria* spp. in powdered cricket (Garofalo *et. al.,* 2017). *Vibrio* sp. are rare in most edible insects but have been detected in edible giant water bugs, and mealworm frass (Osimani *et. al.,* 2018a and 2018b). However, insects cannot express and therefore biologically vector mammalian prions (EFSA, 2015).

The gut microbiome of insects, though strongly related to the bacterial diversity of their feed and environment, does not precisely mirror these external microbial communities (Engel and Moran, 2013). Insect GITs naturally host commensal and symbiotic

microflora, as well as opportunistic colonisers which may aid digestion, serve as direct nutrition, and in some cases survive, replicate and be excreted in faeces as viable cells in high concentrations into feed (Engel and Moran, 2013; Wynants *et. al.,* 2018a and 2018b). Feeding and developing BSFL can reduce, though not eradicate, levels of *E. coli*, *E. coli* O157:H7 and *Salmonella* spp. in livestock and human faeces, depending on the manure pH (Liu *et. al.,* 2008; Erickson *et. al.,* 2004; Lalander *et. al.,* 2013; Lalander *et. al.,* 2015). Importantly, these bacteria did not accumulate in the BSFL, possibly due to digestion or initiation of an antibacterial immune response to the presence of non-native microbes which can induce resistance to bacterial colonisation in insect GITs generally, particularly during stages of incomplete exoskeleton development (Cirimotich *et. al.,* 2011; Jeon *et. al.,* 2011; De Smet *et. al.,* 2018; Dillon and Dillon, 2004). Antibacterial extracts from BSFL exhibit inhibitory effects on various Gram-negative and –positive bacteria (Choi *et. al.,* 2012; Park *et. al.,* 2014).

Different rearing environments provided by insect companies, including larval density, play a role in structuring the microbial communities of edible insects, particularly of insects reared in contact with their faeces (Vandeweyer *et. al.,* 2017a; Stoops *et. al.,* 2016). Microbial differences between batches of the same insect species produced under similar rearing conditions by a single company may be indicative of bacterial contamination from the environment, such as feed and handling (Vandeweyer *et. al.,* 2017b). The concentration of a bacterial contaminant in the feed may overwhelm GIT antibacterial action or outcompete native GIT microbiota, thus enabling pathogen survival in the feed and colonisation of (and subsequent growth in) larval GITs (Wynants *et. al.,* 2019). Dissimilarities between microbial diversity and abundance in larvae and their feed reflects abiotic and biotic selective pressures on feed microbiota, and species- and strain-specific effects on ingested bacteria of the BSFL GIT environment, including selection for e.g. enterococci, which is highly adapted to insect GITs (Wynants *et. al.,* 2018b; Garofalo *et. al.,* 2017). Increasing expression of GIT antimicrobials during development, and expulsion of gut contents by prepupae prior to pupation, can also reduce abundance of BSFL-associated bacteria, including *Salmonella* spp. and enterococci obtained from feed, during transition from larvae to pupae (Zheng *et. al.,* 2013; Lalander *et. al.,* 2013). Differences in microbial communities and concentrations

between larvae reared on different feeds in different locations was attributed to the influence of microbial communities unique to each substrate and FPE on BSFL GIT microflora, and microbial responses in feed to BSFL-mediated biotic and abiotic modifications of the feed (Wynants *et. al.,* 2018a).

Studies on interactions between Coelopidae larvae and bacteria are scarce. The specialist larvae develop by feeding on the diverse bacterial assemblages populating decaying seaweed, and can dramatically suppress the growth of natural seaweed microflora (Dobson, 1974a; Cullen *et. al.,* 1987; Egan *et. al.,* 2013). Coelopidae larval digestive tracts can harbour bacterial assemblages of > 20 species, including *Bacillus*, *Staphylococcus*, *Enterobacter*, and two *Vibrio* spp. (Cullen *et. al.,* 1987). Larvae fail to develop on sterile seaweed or non-algal marine plants, suggesting that environmentally sourced seaweed-specific microbes and possibly seaweed metabolites are important for survival (Cullen *et. al.,* 1987). However, Coelopa larvae can survive on a monospecific diet of isolated larval gut bacteria (*B. subtilis*) as well as *E. coli* (Cullen *et. al.,* 1987).

Bacterial concentrations in Dipteran intestinal tracts are typically reduced during metamorphosis between life stages due to the immune response, native microfloral competition in the GIT and physiological modifications. This can result in initially low populations of gut bacteria in newly emerged adults (Greenberg *et. al.,* 1970), although newly emerged adults can rapidly be contaminated with pathogens from their food and wider environment (Shane *et. al.,* 1985). Commensal *E. coli* can persist trans-stadially during the process of metamorphosis in houseflies and stable flies (*Stomoxys calcitrans*), enabling them to function as vectors immediately on emergence (Rochon *et. al.,* 2005). Ultimately, however, evidence suggests that the majority of human pathogenic bacteria cannot replicate in insect alimentary canals, with the greatest contamination risk coming from environmental sources, and the direct transmission of zoonotic diseases from insects to humans has not been recorded (Vallet-Gely *et. al.,* 2008; EFSA, 2015; Belluco *et. al.,* 2015; Rumpold and Schlüter, 2013). Synanthropic flies, such as houseflies, and non-synanthropic fruit flies can spread and transmit human pathogens including *Salmonella*, pathogenic *E. coli* and possibly *Vibrio* either directly or indirectly (via the environment) to humans (Pace *et. al.,* 2017; Janisiewicz *et. al.,* 1999). The vector potential of a fly depends on the microbial ecology of the bacterial species,

whether the bacterial contaminant is sited externally or internally, the fly's capacity for antibacterial action, and the ingested dose of bacteria (Nayduch *et. al.,* 2013; Nayduch and Burrus, 2017). Bacterial contamination of flies and subsequent transmission can be either mechanical (and temporary) via carriage on mouthparts (Kobayashi *et. al.,* 1999) or the body surface (Sukontason *et. al.,* 2006), or biological in saliva, or faeces via survival of ingestion, possible internal replication and subsequent transmission (Pava-Ripoll *et. al.,* 2012).

Post-harvest processing must adequately decontaminate insect products since it is impossible to eradicate human pathogens from insects merely through controlled breeding or gut voidance by pre-harvest starvation, and removal of insect GITs is unfeasible (Schlüter *et. al.,* 2017; Wynants *et. al.,* 2017). During processing, crushed larvae can release GIT bacteria into the product, and heating at ≥ 90 °C can achieve log reductions of Enterobacteriaceae in larvae including in GITs but not eradicate spore-forming bacteria (Klunder *et. al.,* 2012; Rumpold *et. al.,* 2014). It is however assumed that processing techniques for larval protein and lipid extraction will eradicate microbes from BSFL products (Schlüter *et. al.,* 2017). The shelf-life of dried and powdered BSFL meal depends on the storage temperature which determines water activity in the product, a key control of bacterial growth in feed and food materials (Kamau *et. al.,* 2018).

1.7.2 Risks from other contaminants

BSFL can accumulate cadmium, lead, mercury and arsenic, and *C. frigida* and *C. pilipes* can accumulate cadmium, lead and arsenic, from seaweed feed substrate, and concentrations of certain heavy metals in Coelopidae can exceed maximum EU levels for feed, surpassing original concentrations in the seaweed (Biancarosa *et. al.,* 2018a and b). Different feed substrates produced distinct fungal communities in BSFL reared on them, including the human pathogenic genus *Trichosporon* (Boccazzi *et. al.,* 2017). Various Dipteran species mass reared on a range of waste organic materials produced larvae containing undesirable substances, including veterinary medicine and the pesticide Chlorpyrifos in housefly larvae, although levels were generally within safe limits and could be removed by processing (Charlton *et. al.,* 2015). Mild to fatal allergic reactions in humans due to an immune response to various Arthropod species are

associated with proteins which occur in all vertebrates and invertebrates (Belluco *et. al.,* 2013).

1.8 Conclusion

The potential bacteriological risks associated with various Diptera used, and proposed for use, in the feed and food chain are unique to each combination of insect species, feed material and FPE. The hygienic quality of feed substrate used to rear insects plays a key role in determining the safety of insect products, and a comprehensive risk assessment will be required to identify any hazards not only in seaweed feed, but also in any insect reared on that feed. A 'farm-to-fork' approach should identify the critical control points (CCPs) where potentially pathogenic microbes can enter the production chain during primary and secondary production, and where manufacturing processes act to inhibit or encourage microbial survival in the novel feed materials and products made from them. Complex dynamics between autochthonous insect GIT microbiota and environmentally-sourced allochthonous bacteria may influence the eventual pathogen load of BSFL and Coelopidae larvae at the point of harvest.

1.9 Research rationale, aim and objectives

Aquaculture plays a pivotal role in the global food system, supplying protein to 4.5 billion people (Bene *et. al.,* 2015). Yet future demand by a growing global population for farmed carnivorous marine fish in particular will encounter a bottleneck in aquafeed due to a diminishing supply of FMFO and the nutritional and environmental issues associated with utilising alternative plant ingredients. The EU has recognised the potential of terrestrial insects, particularly Diptera, as an alternative, sustainable protein source in aquafeed, but not the capacity of insects to convert low quality organic matter into high quality protein. Additionally, insects will need to be enriched in marine *n*-3 LC-PUFAs essential to the health of both marine fish and human consumers. Seaweed, a recognised animal feed in the EU, is abundant, nutritious, contributes to minimising the environmental impacts of food production, and has a proven ability to enrich the Diptera *H. illucens* and Coelopidae with *n*-3 LC-PUFAs. However, the safety of sustainable diets for insects is paramount, and there is growing evidence that potentially pathogenic bacteria readily colonise seaweed in the natural environment.

Yet there are no manufacturing guidelines or associated microbiological standards for seaweed meal produced in the EU, and microbiological standards for insect products do not necessarily reflect the range of bacteria that seaweed feed could introduce to the production chain. The interplay between a bacterial hazard in insect feed, the insect species, life stage at harvest, substrate type, the FPE and processing methods involved produces a level of bacteriological risk associated with insect products at the point of manufacture that is unique to that specific combination of conditions.

Therefore, the overarching aim of this thesis is to quantify the risk of human pathogenic bacteriological contaminants being transferred throughout the seaweed-fed insect meal production chain, from seaweed harvesting sites in the coastal zone to the manufacture of feed pellets incorporating seaweed-fed insect products. This aim is addressed through the following objectives:

- Determine how the specialist seaweed fly Coelopidae and its larvae interact with a human pathogenic bacteria present in its natural habitat and food resource, wrack beds (Chapter 2).
- Undertake a farm-to-fork risk assessment of the presence and persistence of FIOs and pathogenic bacteria during trial production of seaweed-fed BSFL to determine the bacteriological safety of seaweed-fed insects in the feed and food chain (Chapter 3).
- Determine essential production parameters for Coelopidae to be reared on an industrial scale, by evaluating how larval mass, yield, survival and development rate can be maximised by identifying optimal post-harvest processing of the seaweed substrate, as well as optimal larval rearing density (Chapter 3).
- Assess the effects of typical post-harvest industrial processing practices on the survival of an FIO and key pathogenic bacterial contaminants attached to recently harvested seaweed (Chapter 4).
- Examine the risk of rearing BSFL on a seaweed powder supplement contaminated with an FIO and pathogenic bacteria (Chapter 4).
- Gauge the relative importance of bacteriological water quality to the UK and Irish seaweed industry and advisory organisations in selection of sites for harvesting and cultivating seaweed for feed and food (Chapter 5).

- Develop GAP or GMP guidelines for each stage of the seaweed-fed insect production chain, and make recommendations for establishing microbiological criteria for seaweed feed, and for seaweed-fed insects (Chapter 6).

Chapter 2 | The seaweed fly (Coelopidae) can facilitate survival and transmission of *E. coli* O157 at sandy beaches

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R. Quilliam, D. Oliver and A. Gilburn supervised the project. F. Barreiro designed and carried out the experiment entitled *Persistence and activity of E. coli O157:H7 in the presence of Coelopidae colonies*, and I. swinscoe analysed and interpreted the data produced. I. Swinscoe designed and implemented all other experiments and undertook all subsequent data analysis and interpretation. All authors commented on draft versions of this manuscript. The published version is presented here.

2.1 Abstract

The appropriate management of recreational beaches is essential for minimising risk of human exposure to microbial pathogens whilst simultaneously maintaining valuable ecosystem services. Decaying seaweed on public beaches is gaining recognition as a substrate for microbial contamination, and is a potentially significant reservoir for human pathogens in close proximity to beach users. Closely associated with beds of decaying seaweed are dense populations of the seaweed fly (Coelopidae), which could influence the spatio-temporal fate of seaweed-associated human pathogens within beach environments. Replicated mesocosms containing seaweed inoculated with a bioluminescent strain of the zoonotic pathogen *E. coli* O157:H7, were used to determine the effects of two seaweed flies, *Coelopa frigida* and *C. pilipes*, on *E. coli* O157:H7 survival dynamics. Multiple generations of seaweed flies and their larvae significantly enhanced persistence of *E. coli* O157:H7 in simulated wrack habitats, demonstrating that both female and male *C. frigida* flies are capable of transferring *E. coli* O157:H7 between individual wrack beds and into the sand. Adult fly faeces can contain significant concentrations of *E. coli* O157:H7, which suggests they are capable of acting as biological vectors and bridge hosts between wrack habitats and other seaweed fly populations, and facilitate the persistence and dispersal of *E. coli* O157:H7 in sandy beach environments. This study provides the first evidence that seaweed fly populations inhabiting natural wrack beds contaminated with the human pathogen *E. coli* O157:H7 have the capacity to amplify the hazard source, and therefore potential transmission risk, to beach users exposed to seaweed and sand in the intertidal zone. The risk to public health from seaweed flies and decaying wrack beds is usually limited by human avoidance behaviour; however, seaweed fly migration and nuisance inland plagues in urban areas could increase human exposure routes beyond the beach environment.

2.2 Introduction

Shiga-toxin (*stx*) producing *Escherichia coli* (STEC) serotype O157:H7 is often carried in the digestive tracts of various animal reservoirs including cattle and other ruminants (Ferens and Hovde, 2011). Human infection by *E. coli* O157:H7 can cause acute

gastrointestinal illness, presenting primarily in the form of diarrhoea, but can also cause haemolytic uremic syndrome (HUS) and lead to permanent liver damage (Griffin and Karmali, 2017). Importantly, infection can be caused by extremely low infectious dose rates (< 10 - 50 viable cells), and can be fatal for young children or those with compromised immune systems (Teunis *et. al.,* 2004; Lim *et. al.,* 2010). There is also growing concern about the multiple antimicrobial resistance (AMR) of shiga toxin-producing *Escherichia coli*, due in part to indiscriminate application of antibiotics to livestock and the various direct and indirect pathways by which humans can become infected (Hoelzer *et. al.,* 2017). Cattle, human, environmental and food sources of 129 *E. coli* O157:H7 isolates have exhibited resistance to at least five antimicrobials (Srinivasan *et. al.,* 2007). Coupled with the increased risk of antibiotic dosing provoking HUS in clinical patients (Freedman *et. al.,* 2016), there is an important public health risk posed by under-reported reservoirs and undocumented vectors of *E. coli* O157:H7 in the environment. Human *E. coli* O157:H7 infection most commonly occurs through consumption of contaminated food and water, person-to-person contact, or exposure to animal carriers (Kintz *et. al.,* 2017). The epidemiology of *E. coli* O157:H7 is shaped by multiple routes of exposure throughout the wider environment in which human-animal ecological niches overlap, which coupled with the specific survival characteristics of *E. coli* O157:H7 in non-host habitats prevents accurate prediction of the spatio-temporal fate of this pathogen in the environment (Chapman *et. al.,* 2017; van Elsas *et. al.,* 2011). Hence, our incomplete understanding of the survival capacity of *E. coli* O157:H7 in hostile secondary environments, together with a lack of accurate quantification tools, hampers efforts to manage its public health risk (Quilliam *et. al.,* 2011a; Young, 2016).

The level of risk of human infection by a zoonotic pathogen such as *E. coli* O157:H7 is partly determined by the prevalence of infection amongst disease reservoirs and secondary (bridge) hosts (Lloyd-Smith *et. al.,* 2009). Important bridge hosts known to spread and transmit *E. coli* O157:H7 directly and indirectly to humans are synanthropic (e.g. houseflies) and non-synanthropic (e.g. fruit flies) species of fly (Diptera) (Pace *et. al.,* 2017; Janisiewicz *et. al.,* 1999). Fly larvae are typically nutritionally dependent on bacteria in their diet, although destructive gut enzymes and antimicrobial substances enable the larvae of some species to produce near-sterile faecal excretions (Mumcuoglu

et. al., 2001; Nayduch and Burrus, 2017). The environment is the principal source of bacterial contamination of adult flies, and often occurs via direct ingestion from a feeding surface or indirectly during grooming (Nayduch and Burrus, 2017). Thereafter, bacteria attached to the fly exoskeleton may be passively transferred to other surfaces, including from hairs, legs and adhesive feet, or deposited via regurgitation or faecal excretions if the bacteria are capable of surviving passage through the digestive tract (Sasaki *et. al.,* 2000; Graczyk *et. al.,* 2001; Sukontason *et. al.,* 2006). *E. coli* O157:H7 has been found to replicate on housefly mouthparts thus extending the duration of its expression in housefly faeces, and to grow on housefly exoskeletons and in vomit spots (Kobayashi *et. al.,* 1999; Wasala *et. al.,* 2013). The cumulative effect of these mechanical and biological interactions of flies with pathogens is to enhance their capacity for disease transmission.

Recreational beach environments are vulnerable to downstream transport of human pathogens, and virulence stx_2 genes of pathogenic *E. coli* have been isolated from swash zone sand of freshwater beaches (Cho *et. al.,* 2016; Bauer and Alm, 2012). The source of an outbreak of *E. coli* O157:H7 infection amongst seven children playing on a UK marine beach, for example, was identified as a contaminated stream draining an area of upstream cattle grazing, recently subjected to heavy rainfall (Ihekweazu *et. al.,* 2006). Although seawater and sand are known reservoirs of faecal bacteria (Solo-Gabriele *et. al.,* 2016), additional reservoirs for microbial pathogens within beach environments include decaying piles of seaweed (wrack), which can also enhance the persistence of *E. coli* in adjacent seawater and sand (Imamura *et. al.,* 2011; Quilliam *et. al.,* 2014). Stranded, decaying wrack is thus a potentially important reservoir for *E. coli* O157:H7 and can concentrate human exposure risks within recreational spaces such as bathing water beaches. In beach environments, the public often share their recreational space with seaweed flies (Coelopidae), which are attracted to decaying wrack beds within a few hours of deposition along the strandline (Dobson, 1974a). Seaweed flies undergo their entire life-cycle within wrack beds, and often form dense populations. In northern Europe, the dominant species are *C. frigida* (Fabricius) and *C. pilipes,* and detached seaweed induces both male mating behaviour and female ovipositioning, with *C. frigida* preferentially laying eggs on *Laminaria* spp. and *C. pilipes* favouring *Fucus* spp. (Dobson,

1974a; Edward *et. al.,* 2007; Dunn *et. al.,* 2002). Although the potential for decaying wrack beds to function as reservoirs of human pathogenic bacteria is gaining recognition (Quilliam *et. al.,* 2014; Russell *et. al.,* 2014), there are no published studies addressing the risk of seaweed flies disseminating human pathogens between wrack habitats.

Identification of all possible modes of direct and indirect transmission of human microbial pathogens in the coastal zone will enable more effective management of the potential public health risk in that environment (Young, 2016; Caron *et. al.,* 2015). Therefore, the aim of this study was to establish whether *C. frigida* and *C. pilipes* can influence the survival and transmission dynamics of *E. coli* O157:H7. Furthermore, the use of a chromosomally *lux*-marked (Tn5 *lux*CDABE) *E. coli* O157:H7 serotype (Ritchie *et. al.,* 2003) provided the opportunity to measure bioluminescence of the pathogen as a proxy for changes in its metabolic activity in decaying seaweed and in sand in the presence of flies and larvae, and in response to ingestion by both life stages. Specifically, the objectives were to determine whether the presence and feeding activity of multiple generations of flies and larvae respectively and of both species had consequences for the persistence and metabolic activity of *E. coli* O157:H7 on decaying seaweed and in beach sand; to determine the effect of *C. frigida* larval feeding, developmental stage and larval-associated native microbiota, and the competitive effect of natural wrack bed bacterial communities, on the survival and metabolic activity of *E. coli* O157:H7 in the larval gut, on decaying seaweed and in beach sand; to establish the capacity for *C. frigida* flies to transmit, and function as bridge hosts of, *E. coli* O157:H7, investigate whether vector competence differed between females and males, and determine the metabolic activity of the vectored pathogen, and finally to quantify the contribution of faecal excretion of metabolically active *E. coli* O157:H7 to transmission by *C. frigida* adults following pathogen ingestion, and identify whether capacity for biological transmission differed between females of different reproductive stage and compared with males. It was hypothesised that (i) the presence of seaweed flies and larvae facilitates the persistence and activity of *E. coli* O157:H7 in wrack beds and underlying sand; (ii) larval feeding suppresses *E. coli* O157:H7 populations and activity in their seaweed substrate by inactivating the pathogen during larval digestion, that this mode

of action is mediated both by larval developmental stage and the presence of native gut and exoskeleton bacteria, and that natural bacterial assemblages in wrack beds limit *E. coli* O157:H7 growth through competition; (iii) *C. frigida* flies, particularly females, are a bridge host and transmission pathway for metabolically active *E. coli* O157:H7, and (iv) metabolically active *E. coli* O157:H7 can be dispersed and survive in the environment via biological transmission in faecal excretions, females exhibit a greater capacity for this mode of transmission than do males, and females with developing eggs imbibe more *E. coli* O157:H7 than females with mature eggs.

2.3 METHODS

2.3.1 Preparation of Coelopidae colonies

Colonies of *C. frigida* and *C. pilipes* were cultured from wild larvae collected from stranded wrack beds on an exposed and natural sandy beach in Fife, Scotland (56°11.191'N, 2°48.679'W). Larvae were grown in a controlled environment cabinet (Reftech B.V., Netherlands) at 25 °C ± 2 °C, a relative humidity of 60 % and a photoperiod of 12 h, and fed with fresh, finely minced (0.5 cm^2) seaweed species characteristic of a stranded wrack bed: (*Laminaria digitata* (Hudson) (40 %), *Laminaria hyperborea* (Gunnerus) (20 %), *Fucus serratus* (L.) (20 %), *Ascophyllum nodosum* (L.) (10 %), *Saccharina latissima* (L.) (5 %), *Palmaria palmata* (L.) (3 %) and *Rhodomela confervoides* (Hudson) (2 %). Newly emerged adults were collected as virgins twice daily through attraction to a light box. Following 10 s anaesthesia with CO_2, flies were classified by species and sex, and stored at 4 °C in ventilated 150 ml plastic Erlenmeyer flasks containing cotton wool soaked in a 50 % glucose solution; all flies were used in experimental mesocosms within 96 h.

2.3.2 Experimental design

A total of four experiments were conducted. Three utilised mesocosms containing multiple individuals designed to investigate Coelopidae population level interactions with *E. coli* O157:H7 in simulated wrack bed habitat comprising decaying seaweed and underlying sand. In the first study, (i) *C. frigida* and *C. pilipes* flies were introduced to mesocosms to determine the effect of mixed species colonies (and multiple generations of flies and larvae) on *E. coli* O157:H7 persistence and activity in wrack bed habitat over

several months. The second mesocosm experiment (ii) sought to examine the effect of *C. frigida* larval feeding and development on *E. coli* O157:H7 persistence in simulated wrack bed habitat, the facilitatory role of the larvae's native exoskeleton and gut microflora on their capacity to digest the pathogen, and the competitive effect of natural wrack bed bacterial communities on *E. coli* O157:H7. The third mesocosm study (iii) was designed to investigate whether *C. frigida* flies were capable of transmitting *E. coli* O157:H7 between wrack bed habitats. A fourth experiment (iv) employing microcosms containing single adult individuals fed known concentrations of *E. coli* O157:H7 was intended to quantify at fine scale the role of biological transmission of the pathogen by the flies in their vectoring capability.

2.3.3 Materials for experimental mesocosms

Seaweed, sand and seawater were collected at low tide the day before starting each experiment. Recently deposited seaweed (*Laminaria* spp. (70 %) and *Fucus* spp. (30 %)), was gathered from the strandline; sand was collected from above the drift line and seawater from the surf zone. All environmental materials were stored at 4 °C prior to transfer to mesocosms. Background *E. coli* and total heterotrophic bacteria (THB) were enumerated in all seaweed, sand and seawater used in experimental mesocosms, and pH and water content measured in the seaweed and sand (Table 2.1). To quantify background *E. coli* and THB concentrations, four replicate samples of 10 g of seaweed or 5 g of sand were added to 10 ml or 5 ml of sterile seawater, respectively, and then vortexed for 1 minute. The supernatant was subsequently serially diluted with sterile seawater and 50 µl streaked onto Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid) to enumerate presumptive *E. coli*, or R2A agar (CM0906, Oxoid) to enumerate THB. Seawater samples (n = 4) were shaken and 100 ml vacuum-filtrated through a 0.45 µm cellulose nitrate membrane (Microsart CN-filter, Sartorius Stedim Biotech GmbH, Goettingen, Germany) and transferred onto MLGA. Plates for *E. coli* were incubated at 37 °C for 24 h and plates for THB incubated at 18 °C for 48 h. For sand and seaweed samples, bacterial concentrations were expressed as CFU (colony forming units) g^{-1} dry matter content (where representative seaweed and sand samples were dried at 80 °C for 24 h), or expressed as CFU 100 ml^{-1} for sea water samples.

Table 2.1. Characteristics of seaweed and sand used in mesocosm experiments. Values represent the means ± SE.

Experiment	Environmental parameter	Seaweed	Sand
1*	Water content (%)	74 ± 1	14 ± 0.2
	pH	-	9.6 ± 0.1
	E. coli (CFU g^{-1})	0	0
	Total heterotrophic bacteria (CFU g^{-1})	33 x 10^3 (± 0.23)	52 ± 0.44
2 and 3+	Water content (%)	81 ± 0.2	12 ± 0.4
	pH	-	8.0 ± 0.2
	E. coli (CFU g^{-1})	< 10	0
	Total heterotrophic bacteria (CFU g^{-1})	110 x 10^4 (± 0.36)	20 ± 0.13

* *E. coli* O157:H7 persistence

+ *E. coli* O157:H7 survival during larval development and *E. coli* O157:H7 transmission by flies

A non-toxigenic, chromosomally *lux*-marked (Tn5 *lux*CDABE) *E. coli* O157:H7 serotype (Ritchie *et. al.* 2003) was grown on Sorbitol MacConkey Agar (SMAC) (CM0813, Oxoid) supplemented with cefixime and potassium tellurite (CT) (SR0172, Oxoid) at 37 °C for 24 h for the selective isolation of *E. coli* O157:H7. Presumptive colonies of *E. coli* O157: H7 were confirmed by a latex agglutination test (DR0260, Oxoid), and then grown in Luria-Bertani (LB) broth (CM1018, Oxoid) at 37 °C, at 100 rev min^{-1}, for 18 h. Cells were washed three times in phosphate buffered saline (PBS) and re- suspended in PBS in preparation for use in experimental mesocosms. The bioluminescence phenotype of the *lux* biomarker of this strain of *E. coli* O157:H7 is dependent on the cellular energy status. As cellular metabolism requires energy, bioluminescence output can be used as a proxy for the metabolic activity of the population of cells, and thus, bioluminescence allows a

quick *in situ* estimation of how metabolically active the *E. coli* O157:H7 population is (Quilliam *et. al.,* 2012).

2.3.4 Persistence and activity of E. coli O157:H7 in the presence of Coelopidae colonies

This mesocosm experiment was designed to quantify the effect of the presence of multiple generations of *C. frigida* and *C. pilipes* flies and larvae on *E. coli* O157:H7 persistence and metabolic activity in seaweed and sand. A total of eight mesocosms were established, each consisting of a 5 L plastic container (Addis Ltd., UK) with paper towelling secured over a hole (10 cm x 10 cm) in the lid to allow gas exchange. Treatment mesocosms contained both *E. coli* O157:H7 and flies, whereas the control contained just *E. coli* O157:H7 with no flies. Both treatment and control consisted of four replicate mesocosms, and each mesocosm contained 1 kg of finely minced (0.5 cm^2) seaweed (approximately 5- 6 cm depth) laid over 2 kg of sand (approximately 3 cm depth). For each mesocosm, the *E. coli* O157:H7 inoculant was introduced by mixing the seaweed for 5 min in a stomacher bag with 200 ml of seawater contaminated with *E. coli* O157:H7 (1.84×10^9 CFU ml^{-1}). To each mesocosm containing flies, 10 male and 10 female individuals of both *C. frigida* and *C. pilipes* (*n* = 40) were added. All mesocosms were transferred to a controlled environment cabinet and maintained at 20 °C ± 2 °C, with a relative humidity of 60 % and a photoperiod of 12 h.

To enumerate bacterial concentrations from each replicate mesocosm, 10 g of seaweed or 5 g of sand were added to 10 ml or 5 ml of sterile seawater, respectively, and then vortexed for 1 minute. Luminescence (relative light units (RLU)) of the seaweed or sand supernatant was immediately measured using a SystemSURE 18172 luminometer (Hygiena Int., Watford, UK) to quantify relative *E. coli* O157:H7 metabolic activity. The remaining supernatant was serially diluted using sterile seawater, plated onto CT-SMAC plates, and incubated as described above. *E. coli* O157:H7 in both seaweed and sand was measured in each replicate mesocosm on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 15, 23, 31, 43 and 56; mesocosms containing the flies were not sampled on days 1 and 2 in order to allow mating to occur.

2.3.5 Survival and activity of E. coli O157: H7 in C. frigida larvae and wrack habitat during larval development

This mesocosm experiment aimed to quantify the influence of larval feeding and development on the persistence and activity of *E. coli* O157:H7 associated with larvae and their wrack habitat, and to examine the effect of the removal of the naturally occurring larval-associated microbiota on the ability of larvae to digest or inactivate *E. coli* O157:H7. Mesocosms (*n* = 240) comprising 4 treatments and 2 controls consisted each of 40 100 ml sterile plastic pots (Gosselin^{TM}, Fisher Scientific UK Ltd) containing 25 g of seaweed (approximately 4 cm depth) placed on 20 g of sand (approximately 1.5 cm), with lids comprised of paper towelling to allow gas exchange. Prior to placement within each treatment and control mesocosm, the seaweed was divided into 1 kg batches, and inoculated by homogenising it for 5 min in a stomacher bag with 200 ml of *E. coli* O157:H7 contaminated seawater (4.42 x 10^7 CFU ml^{-1}). In treatment mesocosms, a pair of unmated *C. frigida* female and male flies was introduced to each mesocosm and removed 4 days later. On day 5, the resulting larvae were removed from these mesocosms and treated in one of four ways, (i) untreated, (ii) surface sterilised, (iii) starved, or (iv) surface sterilised then starved. Untreated larvae and larvae subjected to surface sterilisation only were removed for 3 h before returning 10 to each mesocosm. Starvation involved moving larvae to an empty sterile container for 24 h, before returning 10 to each mesocosm. Thus sampling of mesocosms containing starved larvae lagged behind other treatments and the controls by 24 h. The aim of surface sterilisation was to reduce the microbial communities on the larval surface (although not completely eliminate them) and involved immersion in a 19:1 PBS:Ethanol solution for 1 min followed by two rinses in sterile PBS. Weak disinfectant was used in place of a potentially more effective stronger concentration in order to avoid incidental gut sterilisation of larvae due to larval ingestion of the disinfectant during immersion.

There were two control treatments from which larvae were absent: 40 mesocosms contained seaweed inoculated with *E. coli* O157:H7 laid on top of sand, and 40 mesocosms contained seaweed and sand both pre-sterilised by autoclaving (121 °C for 15 mins), after which the seaweed was inoculated with *E. coli* O157:H7. Destructive sampling of 10 larvae, 10 g seaweed and 5 g sand from replicate mesocosms (*n* = 4) from

treatments and controls began six days after initial inoculation of seaweed and continued for eight successive days. Larvae (and pupae) were handled with sterile forceps and ground in 2 ml PBS in a 1.5 ml Eppendorf tube for 30 s with a micro pestle (Anachem Ltd., Bedfordshire, UK). Luminescence of the supernatant was immediately measured, and the homogenate serially diluted and plated onto CT-SMAC agar as described above. *E. coli* O157:H7 concentration and relative activity were also enumerated in both seaweed and sand samples as described above. Mesocosms were maintained at 25°C ± 2°C, a relative humidity of 60% and a photoperiod of 12 h.

2.3.6 Transmission of E. coli O157:H7 by female and male C. frigida flies

Using mesocosms, vector competence of *C. frigida* flies for metabolically active *E. coli* O157:H7 was assessed by investigating the capacity of females and males to separately contaminate previously uncontaminated seaweed and sand. Mesocosms (*n* = 80) consisted of 100 ml sterile plastic pots (Gosselin™, Fisher Scientific UK Ltd) containing 25 g of seaweed (approximately 4 cm) placed on 20 g of sand (approximately 1.5 cm), with lids comprised of paper towelling. Prior to placement within each mesocosm, the seaweed was divided into 1 kg batches, and inoculated with 200 ml of *E. coli* O157:H7 contaminated seawater (6.9×10^7 CFU ml^{-1}). Ten *C. frigida* flies were added to each mesocosm; 40 mesocosms contained female flies, and 40 mesocosms contained male flies. After 24 h, all female and male flies were moved to 80 new mesocosms that contained 25 g of uncontaminated seaweed (approximately 4 cm), placed on 20 g of sand (approximately 1.5 cm), with paper towelling lids. After a further 24 h, eight replicate mesocosms (four female, four male) containing transplanted flies were destructively sampled, with 10 flies, 10 g seaweed and 5 g sand sampled from each mesocosm on nine successive days. Each fly was anaesthetised by 10 s exposure to CO_2 gas, and ground in 2 ml PBS in a 1.5 ml Eppendorf tube for 30 s with a micro- pestle. Luminescence of each fly supernatant was immediately measured, and the concentration of *E. coli* O157:H7 in the remaining supernatant determined as described above. *E. coli* O157:H7 was enumerated, and relative activity measured, in the seaweed and sand as described above. All mesocosms were maintained at 25 °C ± 2 °C, at a relative humidity of 60 % and a photoperiod of 12 h.

2.3.7 Contribution of faecal excretion to transmission of E. coli O157:H7 by female and male C. frigida flies

The potential for adult *C. frigida* faeces to facilitate the transmission and survival of *E. coli* O157:H7 was determined in mated females (*n* = 240), virgin females (*n* = 240) and virgin males (*n* = 240) in microcosms taking the form of individually enclosed Petri dishes (diameter 55 mm). Females were mated two days prior to the beginning of the experiment, and flies for all treatments were starved for 24 h before sampling began. Each Petri dish contained a sterile Eppendorf tube lid containing a feeding solution made from the liquid from decaying *L. digitata*. Half of the Petri dishes for each treatment (*n* = 120) contained feeding solution that had been contaminated by 250 µl of *E. coli* O157:H7 (1.09×10^2 CFU μl^{-1}), whilst the control groups (*n* = 120) received 250 µl of feeding solution not contaminated with *E. coli* O157:H7. Previous observations determined that most seaweed flies typically began producing faecal droplets 6 h after introduction to the feeding solution, and that fly mortality began after 24 h. Thus, flies from each experimental and control microcosm (*n* = 40 for each treatment) were sampled at 6 h, 12 h and 24 h, and *E. coli* O157:H7 concentration and relative activity measured in each individual fly, and in their faeces. Faecal droplets belonging to each individual fly were counted, and a sterile toothpick used to transfer their faeces to 5 ml of LB Broth. Faeces were enriched overnight for 18 h at 100 rpm at 37 °C; cells were centrifuged, washed three times and re-suspended in PBS. Luminescence was quantified, and the solution serially diluted and plated onto CT-SMAC media to enumerate *E. coli* O157:H7 concentrations. Petri dish microcosms were maintained at 25 °C ± 2 °C, at a relative humidity of 60 % and a photoperiod of 12 h.

2.3.8 Statistical analysis

Data were normally distributed following $\log_{10}$ transformation, and analysis of variance (ANOVA) was applied to the data (SPSS 24.0 software, SPSS Inc. Chicago, IL, USA). A repeated measures (rm) ANOVA was used to test the effect of Coelopidae presence on *E. coli* O157:H7 concentration and relative activity in seaweed and sand, and a factorial ANOVA followed by Tukey post-hoc tests used to analyse the effect of larval feeding on

E. coli O157 concentration, vector competency and the capacity of seaweed flies for biological transmission. Differences were considered significant at the $P \leq 0.05$ level.

2.4 Results

2.4.1 Persistence and activity of E. coli O157:H7 in the presence of Coelopidae colonies

The presence of *C. frigida* and *C. pilipes* flies significantly enhanced survival of *E. coli* O157:H7 attached to seaweed ($P < 0.001$) (Fig. 2.1a), and in the underlying sand ($P < 0.05$) (Fig. 2.1c), compared to mesocosms where flies were absent. Regardless of the presence or absence of flies, the concentration of *E. coli* O157:H7 peaked significantly in seaweed between days 9 and 15, and in sand on day 11 ($P < 0.05$). Subsequent *E. coli* O157:H7 die-off to day 23 in seaweed was rapid in both treatments, reaching a concentration ~1 log CFU g^{-1} lower in the absence of flies than in seaweed associated with flies ($P < 0.05$). *E. coli* O157:H7 levels in sand were ~1 log CFU g^{-1} higher in the presence of flies than in the absence of flies between days 11 and 23 ($P < 0.05$), but the rate *E. coli* O157:H7 die-off in sand over two months was not significantly different between treatments. *E. coli* O157:H7 remained detectable in both seaweed and sand up to day 56. The presence of flies exerted no influence on the luminescence of *E. coli* O157:H7 in seaweed or sand (Fig. 2.1b and d).

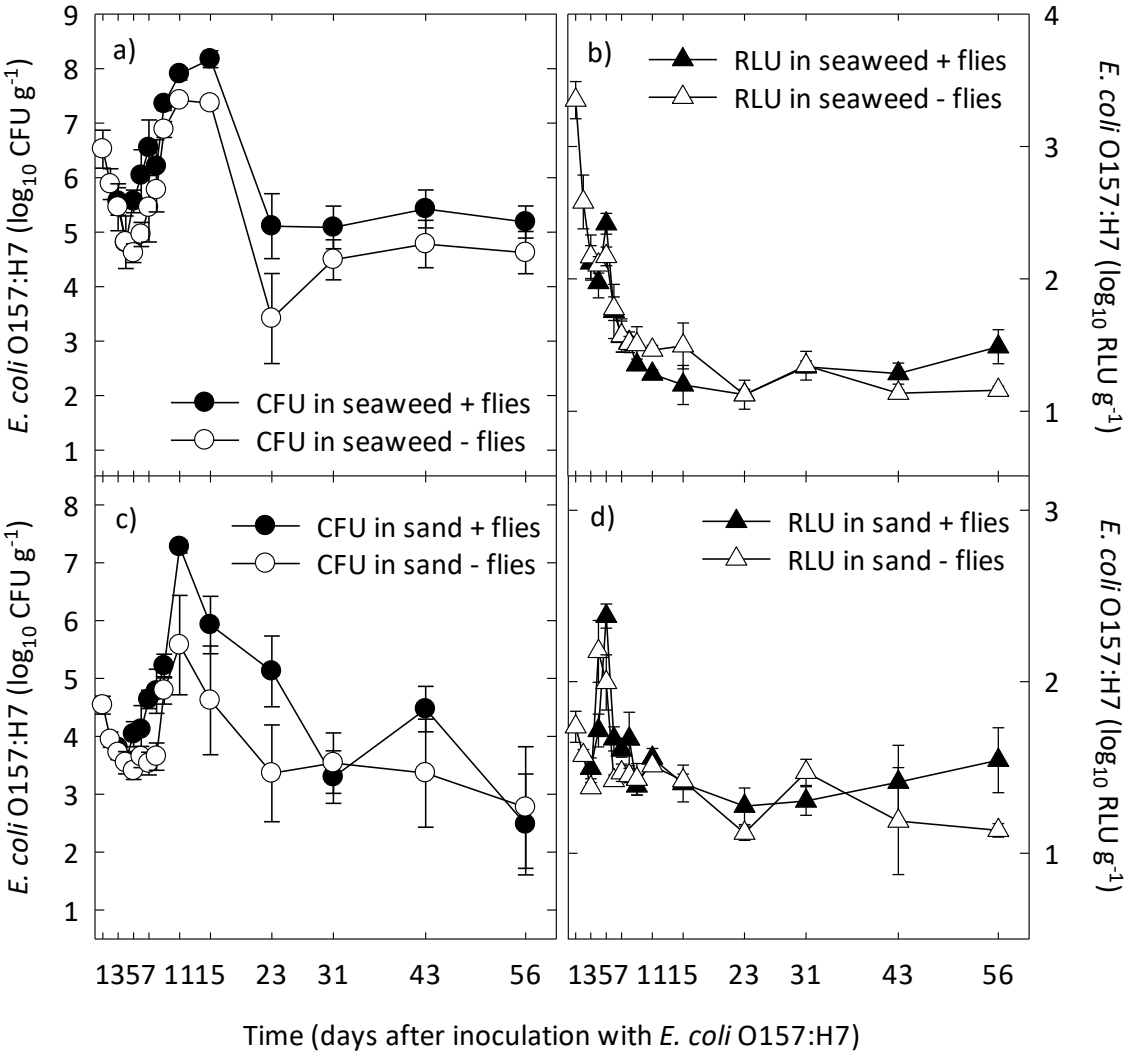


Fig. 2.1. Concentration in CFU (circles) in seaweed (a) and luminescence in RLU (triangles) in seaweed (b) of *E. coli* O157:H7 in mesocosms containing either flies (filled symbols) or no flies (open symbols). Concentration in CFU (circles) in sand (c) and luminescence in RLU (triangles) in sand (d) of *E. coli* O157:H7 in mesocosms containing either flies (filled symbols) or no flies (open symbols). Each mesocosm contained equal numbers of both *C. frigida* and *C. pilipes* flies. Data points represent the means ± SE.

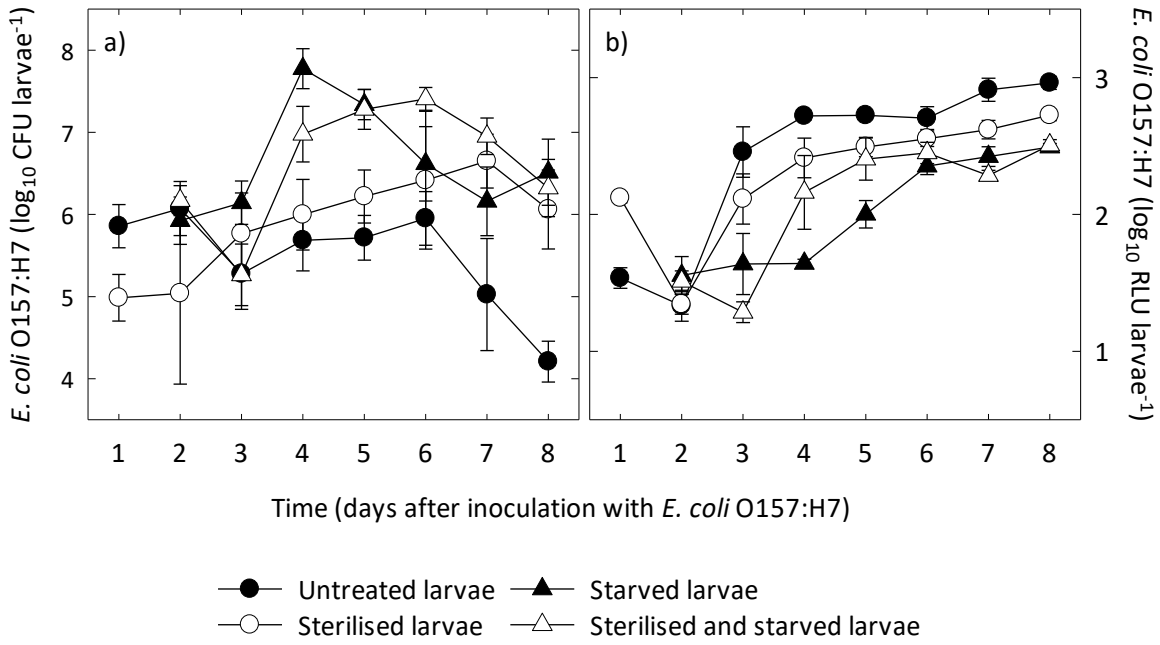
2.4.2 Survival and activity of E. coli O157: H7 in C. frigida larvae and wrack habitat during larval development

By day 7, *E. coli* O157:H7 concentrations associated with untreated larvae fell to ~1 log CFU below that associated with sterilised, and starved and sterilised, larvae ($P < 0.05$), and by day 8 was ~1.5 log CFU lower than levels detected in all treated larvae ($P < 0.05$) (Fig. 2.2a). Reduction of the gut microbiota of larvae due to 24 h starvation led to significantly higher levels (~2 log CFU) of *E. coli* O157:H7 associated with starved larvae compared with untreated and surface sterilised larvae on day 4 ($P < 0.05$). Luminescence of *E. coli* O157:H7 increased significantly in untreated and sterilised larvae between days 2 and 3, and in starved, and starved and sterilised larvae, between days 3 and 4 ($P < 0.001$) (Fig. 2.2b). Luminescence of *E. coli* O157:H7 associated with untreated larvae exceeded that of all larvae that had reduced gut microbiota on days 3, 4, 7 and 8 ($P < 0.05$), and of starved larvae on day 5 ($P < 0.05$).

The presence of larvae suppressed *E. coli* O157:H7 concentrations in seaweed, compared with the non-sterile larvae-free mesocosms in which the concentration of *E. coli* O157:H7 associated with the seaweed increased over 8 days to 2 – 3 log CFU g^{-1} higher than all mesocosms containing larvae ($P < 0.001$) (Fig. 2.2c). Concentrations of *E. coli* O157:H7 in seaweed associated with untreated larvae increased rapidly by ~2 log CFU g^{-1} between days 5 and 6 ($P < 0.05$), whereas no significant change over time was observed in *E. coli* O157:H7 levels in seaweed associated with treated larvae. The luminescence of *E. coli* O157:H7 associated with seaweed in the mesocosms containing larvae and in the larvae-free mesocosms increased significantly between days 2 and 4 ($P < 0.05$), before levelling off (Fig. 2.2d). Luminescence in seaweed in the mesocosms containing untreated larvae was significantly higher from day 3 onwards ($P < 0.05$), than in seaweed associated with larvae where the gut microbiota had been reduced. However, the absence of natural seaweed microflora, and the absence of larvae did not affect *E. coli* O157:H7 luminescence in seaweed. *E. coli* O157:H7 levels in sand were not influenced by the presence or absence of larvae (Fig. 2.2e); however, from day 7 the concentrations of *E. coli* O157:H7 in the sand of mesocosms that contained either larvae that had been both sterilised and starved, or the non-sterile mesocosms that contained no larvae, were significantly higher than in the sand of mesocosms that contained either

the surface-sterilised larvae or the starved larvae ($P < 0.05$). The luminescence of *E. coli* O157:H7 in sand associated with larvae and in larvae-free controls increased significantly between day 2 and 3 ($P < 0.05$), although there was no significant difference between mesocosms that contained larvae and those that contained no larvae (Fig. 2.2f).

In the absence of larvae and natural seaweed microflora, *E. coli* O157:H7 in seaweed and sand of the sterile control mesocosms significantly exceeded levels in seaweed and sand in all treatments containing larvae and of the non-sterile control on day 1 ($P < 0.001$). By day 8, subsequent die-off of *E. coli* O157:H7 in seaweed in the sterile control mesocosms resulted in the concentration being significantly lower than that of seaweed in the non-sterile control mesocosms ($P < 0.001$), whilst *E. coli* O157:H7 concentration in sand by day 8 was no different to that in any of the treatment or non-sterile control mesocosms. Luminescence of *E. coli* O157:H7 in sand was significantly enhanced by the absence of natural microflora in the sterile control compared with the non-sterile control on days 1, 2 and 4 ($P < 0.05$).



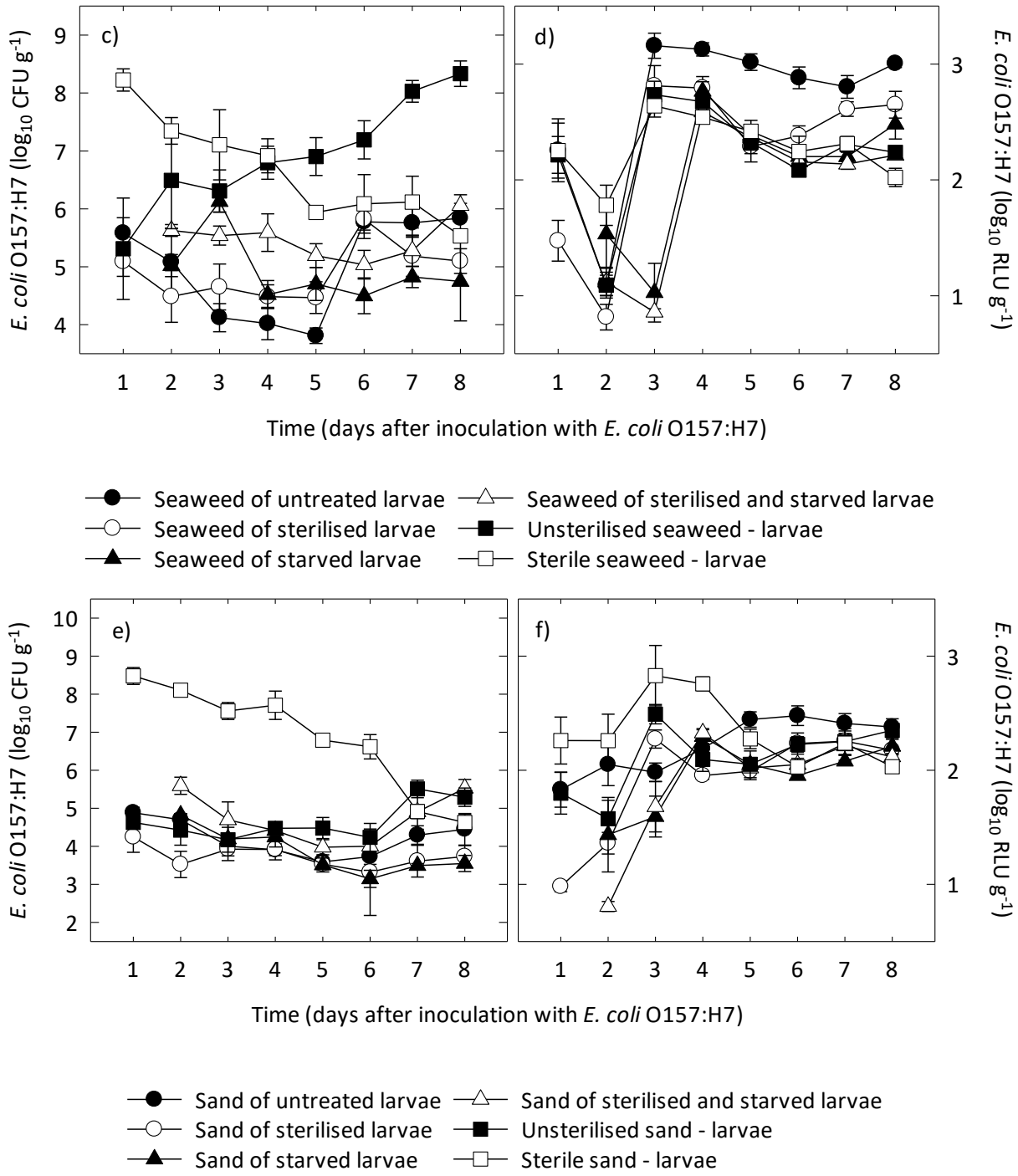


Fig. 2.2. Concentration in CFU (a, c and e) and luminescence in RLU (b, d and f) of *E. coli* O157:H7 in *C. frigida* larvae (a – b), seaweed (c - d) and sand (e - f) in mesocosms containing either untreated larvae (filled circles), surface sterilised larvae (open circles), starved larvae (filled triangles) or sterilised and starved larvae (open triangles). Each mesocosm contained equal numbers of larvae. Control mesocosms without larvae contained either unsterilized substrate (filled squares) or sterilised substrate (open squares). Data points represent the means ± SE.

2.4.3 Transmission of E. coli O157:H7 by female and male C. frigida flies

Female and male *C. frigida* flies transmitted *E. coli* O157:H7 from contaminated seaweed to mesocosms previously free of the pathogen at concentrations > ~3 log CFU g^{-1} to seaweed and > ~2 log CFU g^{-1} to sand (Fig. 2.3a). The sex of the fly made no significant difference to the concentration of *E. coli* O157:H7 associated with the flies following 24 h exposure to contaminated seaweed, or on the subsequent persistence of the pathogen in flies until day 8. However, the sex of the flies in the mesocosms significantly influenced *E. coli* O157:H7 concentrations in seaweed on day 3, at which time pathogen levels on seaweed in female mesocosms were ~3 log CFU g^{-1} higher than on seaweed in male mesocosms ($P < 0.05$). *E. coli* O157:H7 concentrations in sand in female mesocosms significantly exceeded that of sand in male mesocosms by ~2.5 log CFU g^{-1} on day 2 ($P < 0.05$). Luminescence of *E. coli* O157:H7 in female flies, and the seaweed and sand in their mesocosms, was significantly higher than in male flies, seaweed and sand ($P < 0.001$) and increased significantly over time in both female and male flies ($P < 0.001$) (Fig. 2.3b). Maximum levels of *E. coli* O157:H7 luminescence occurred in flies, seaweed and sand on days 8, 8 and 4 respectively in female mesocosms and on days 8, 6 and 6 respectively in male mesocosms, significantly exceeding luminescence levels recorded in flies, seaweed and sand at all preceding and subsequent sampling points ($P < 0.05$).

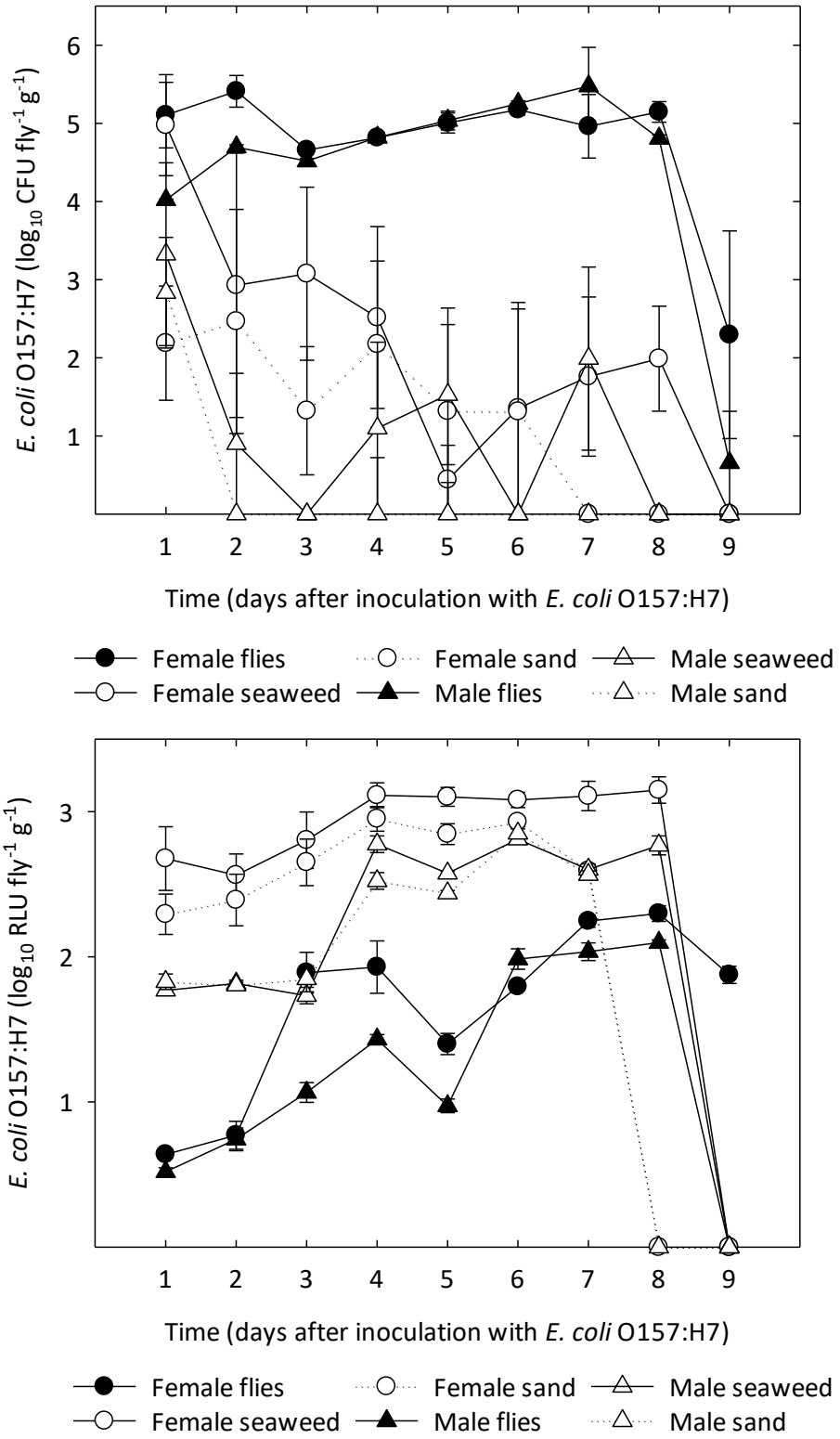


Fig. 2.3. Concentration in CFU (a) and luminescence in RLU (b) of *E. coli* O157:H7 in female flies (filled circles), male flies (filled triangles), seaweed (open circles) and sand (open circles with dotted line) in female *C. frigida* mesocosms, and seaweed (open triangles) and sand (open triangles with dotted line) in male *C. frigida* fly mesocosms. Data points represent the means ± SE.

2.4.4 Contribution of faecal excretion to transmission of E. coli O157:H7 by female and male C. frigida flies

After 6 h exposure to contaminated feeding solution, mated female flies were contaminated with significantly (~1 log CFU fly^{-1}) more *E. coli* O157:H7 than either virgin females or males ($P < 0.05$) (Fig. 2.4). By 12 h *E. coli* O157:H7 contamination had decreased in both females and males, with a significant reduction in mated females between 6 h and 12 h ($P < 0.001$). After 24 h exposure to *E. coli* O157:H7, 40 % of males, 7.5 % of mated females and 5 % of virgin females had died. The further decrease in contamination by *E. coli* O157:H7 between 12 h and 24 h was not significantly different between mated females and virgin males. However, between 12 h and 24 h *E. coli* O157:H7 concentrations in virgin females increased by ~1 log CFU fly^{-1}, and the final concentration at 24 h was significantly higher than levels associated with mated females and virgin males ($P < 0.05$). *E. coli* O157:H7 luminescence did not change significantly in female or male flies over 24 h, and there was no significant difference between mated and virgin females at 6 h, 12 h or 24 h; however, the luminescence of *E. coli* O157:H7 associated with males was consistently lower than in either female fly group ($P < 0.05$).

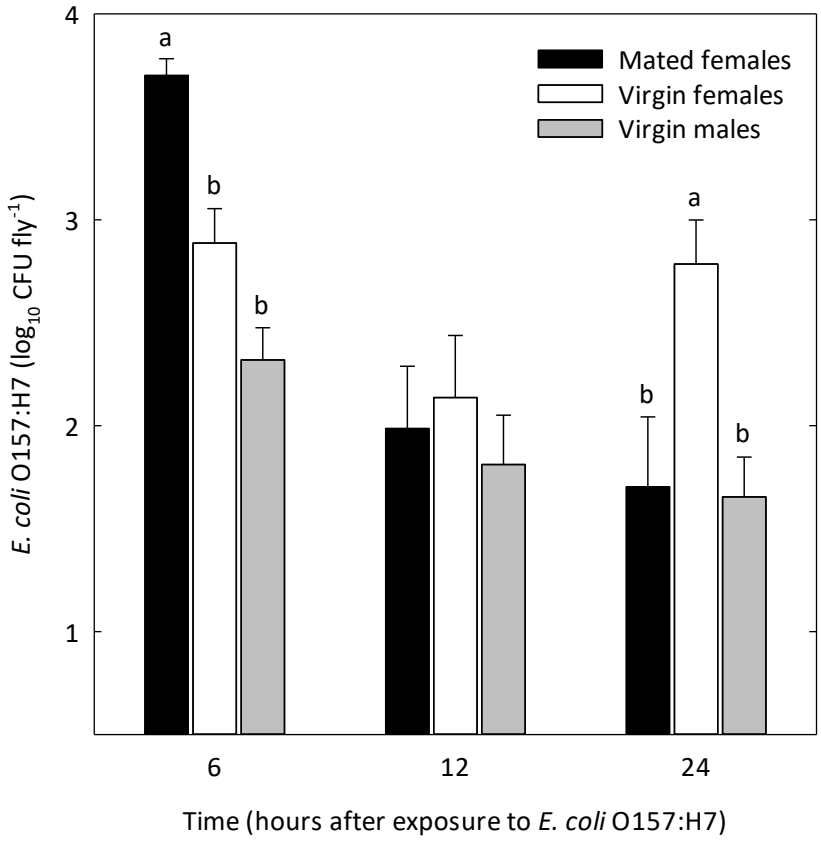


Fig. 2.4. *E. coli* O157:H7 concentrations in mated female flies (black bars), virgin female flies (white bars) and virgin male flies (grey bars) following exposure to inoculated feeding liquid. At each time point, bars that do not share a letter are significantly different from each other (two-way ANOVA, $P< 0.05$; Tukey's test, $P< 0.05$). Values represent the means +SE.

Ingestion of *E. coli* O157:H7 had no effect on excretion levels in mated females, virgin females or virgin males relative to controls, and the number of faecal excretions produced by all flies in all mesocosms significantly increased between 6 h and 12 h, and again between 12 h and 24 h ($P < 0.05$). Faecal biomass and excretion rate were unaffected by the extent of egg maturation in females, but significantly exceeded that of males over the entire 24 h period ($P < 0.05$). The *E. coli* O157:H7 load in fly faeces increased significantly between 6 h and 12 h in females and males by ~2- log CFU fly^{-1} ($P < 0.001$) followed by a significant reduction of 3 - 4 log CFU fly^{-1} in females and 2- log CFU fly^{-1} in males ($P > 0.001$) by 24 h (Fig. 2.5). The sex of the fly affected the concentration of *E. coli* O157:H7 in faecal excretions, with females producing ~4 log CFU

fly^{-1} more of the pathogen in their faeces than males at 6 h and 12 h, and ~2 - 3 log CFU fly^{-1} more than males at 24 h ($P < 0.001$). The extent of egg maturation in females also affected levels of the pathogen in female faeces after 24 h exposure, with concentrations in mated females being ~1 log CFU fly^{-1} greater than in virgin females ($P < 0.05$). Luminescence of *E. coli* O157:H7 in the faeces of both female and male flies peaked at 12 h, increasing significantly between 6h and 12 h in male faeces and falling significantly between 12 h and 24 h in virgin female faeces ($P < 0.05$). However, luminescence of *E. coli* O157:H7 in faeces did not differ significantly between mated and virgin females during the 24 h; luminescence in faeces from male flies remained significantly lower by comparison at all sampling times ($P < 0.001$).

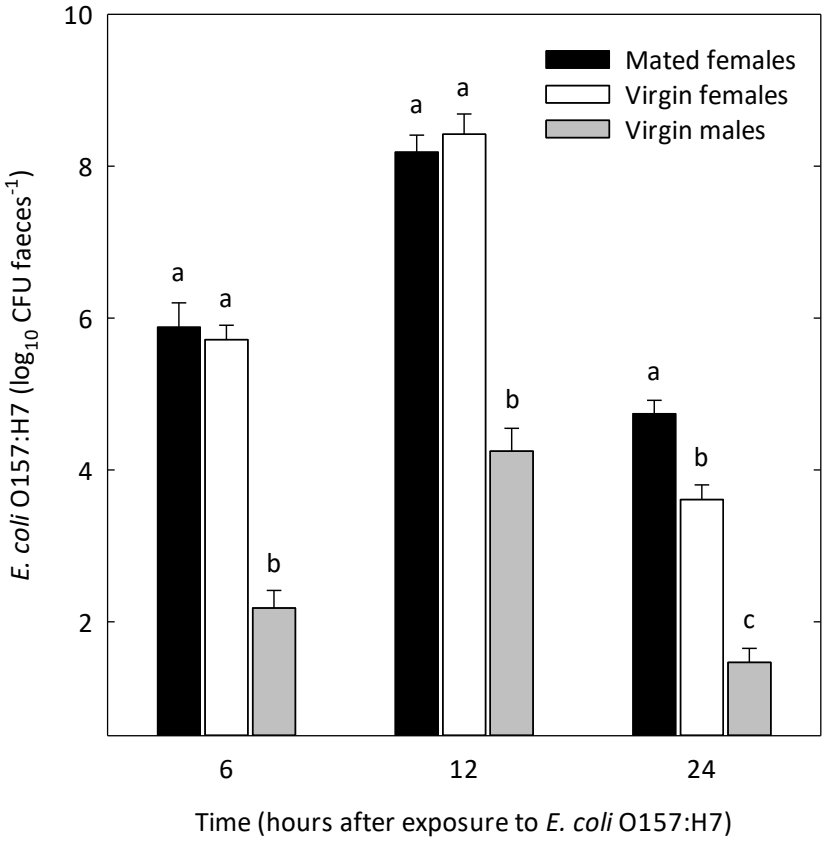


Fig. 2.5 *E. coli* O157:H7 concentration in the faeces of mated female flies (black bars), virgin female flies (white bars) and virgin male flies (grey bars) following exposure to inoculated feeding liquid. At each time point, bars that do not share a letter are significantly different to each other (two-way ANOVA, $P< 0.05$; Tukey's test, $P< 0.05$). Values represent the means +SE.

2.5 Discussion

The role of non-synanthropic Diptera in the environmental dissemination of human pathogenic bacteria has not been previously examined within a public health context. This study has demonstrated that an endemic species of seaweed fly (*C. frigida*) commonly found in dense populations on public beaches throughout Europe is capable of facilitating the dispersal of *E. coli* O157:H7 between individual seaweed habitats, and further transmission to beach sand. An important mechanism for this transmission is in faecal excretions by adult flies. Furthermore, the presence of both *C. frigida* and *C. pilipes* enhanced growth of *E. coli* O157:H7 in simulated wrack bed environments, and in the underlying sand. Activity by multiple generations of flies and larvae in decaying wrack beds modifies their habitat by altering the physio-chemical composition of the substrate, and can facilitate microbial growth and persistence (Cullen *et. al.,* 1987). This study provides the first evidence that seaweed fly populations inhabiting natural wrack beds contaminated with the human pathogen *E. coli* O157:H7 have the capacity to amplify the hazard source, and therefore potential transmission risk, to beach users exposed to seaweed and sand in the intertidal zone.

Following ingestion of high concentrations of *E. coli* O157:H7, seaweed flies were rapidly internally contaminated with the pathogen and within 6 h produced faeces containing viable (metabolically active) *E. coli* O157:H7 bacteria at concentrations exceeding the infectious dose for humans (Teunis *et. al.,* 2004). Homogenisation of whole flies in order to measure individual infection with *E. coli* O157:H7 prevented evaluation of the separate contributions of bacterial attachment to exoskeletons compared with ingestion to overall individual contamination. However, a degree of external carriage of the pathogen was highly probable as flies of both sexes were observed in and on feeding solution dishes. Therefore, greater carriage of *E. coli* O157:H7 after 6 h by mated females than virgin females, and *vice versa* at 24 h, cannot be solely attributed to differences in ingestion volume or rate, possibly due to stage of egg development (Sasaki *et. al.,* 2000). However, greater contamination of mated female flies than males at 6 h, and of virgin females than males at 24 h may indicate that reproductive biology influences ingestion volume and rate, most likely due to physiological requirements associated with egg production.

A distinction should be drawn between studies in which fly exposure to a pathogen is via a single food droplet, and those that allow constant feeding as in the present study, which more realistically reflects the availability of the source in natural wrack beds. The former might be expected to produce a steady decline over time in pathogen concentration in flies due to clearance from their digestive tracts, whereas the latter may generate a more variable result due to multiple feeding opportunities (Fleming *et. al.,* 2014). It is possible that the higher levels of activity of mated and virgin females compared with males in the Petri dish microcosms may have resulted in additional contamination of the female exoskeleton leading to higher overall *E. coli* O157:H7 concentrations on mated females at 6 h and virgin females at 24 h. Importantly, the concentration of *E. coli* O157:H7 associated with the flies did not consistently increase during exposure to the contaminated feeding solution; seaweed flies are therefore unlikely to be reservoirs of *E. coli* O157:H7, meaning that the pathogen load associated with individuals is dependent upon levels of contamination in seaweed, and is not influenced by disease maintenance amongst seaweed fly populations (Caron *et. al.,* 2015).

The lower luminescence, and hence, relative metabolic activity, of *E. coli* O157:H7 associated with male flies compared with females over 24 h may indicate that efficacy of inactivation of the pathogen in the seaweed fly gut is partly related to the sex of the fly. This is unlikely however, given that in Dipteran digestive tracts the efficacy of antibacterial effectors active against non-native gut bacteria (the innate response) depends primarily on the species of fly and the vulnerability of the bacterial species to that response (Nayduch and Burrus, 2017). The fate of ingested *E. coli* O157:H7 may also be dose-dependent, meaning that below or above a certain dose threshold, bactericidal substances in seaweed fly digestive tracts may be effective against ingested cells of the pathogen (Kumar and Nayduch, 2016). It is likely that external *E. coli* O157:H7 contamination of both mated and virgin female seaweed flies will be greater than males due to their higher physical activity. By contrast, if the majority of male contamination was internal and thus vulnerable to gut inactivation, this might account for the consistently lower metabolic activity of *E. coli* O157:H7 associated with male flies compared with females.

The mechanisms of bacterial transmission by flies to various surfaces via regurgitation and faecal excretion are well established (Pava-Ripoll *et. al.,* 2012), and passage of *E. coli* O157:H7 through the digestive tract of seaweed flies did not entirely inactivate this pathogen. Female *C. frigida* produced more faecal excretions on average than male *C. frigida*, suggesting a more rapid ingestion rate by females than males, which was also matched by a faster excretion rate. Clearance of *E. coli* O157:H7 from the digestive tracts of female *C. frigida* was more rapid than their ingestion rate, whereas males excreted *E. coli* O157:H7 at approximately the same rate as they ingested the pathogen. The excretion rate by both female and male flies approximately doubled between 6 - 12 h, and 12 - 24 h, although this was not mirrored by the concentration of faecal *E. coli* O157:H7, most likely due to the rate of pathogen die-off in the feeding solution. The concentration of *E. coli* O157:H7 in the faeces of both sexes may be underestimated due to desiccation of most excreta by 12 h which would have affected recovery, and the decreasing availability of the feeding solution due to evaporation over 24 h. However, these results do demonstrate that at 12 h after initial ingestion of *E. coli* O157:H7, both female and male seaweed flies present the greatest risk of pathogen transmission via faecal excretion.

Female and male seaweed flies were capable of vectoring *E. coli* O157:H7 to seaweed and sand 24 hours after exposure to the pathogen. The faster rate of faecal production by female *C. frigida*, and thus greater quantity of excretion of *E. coli* O157:H7 compared with males, represents the underlying mechanism for the greater pathogen load transmitted by females than males to simulated wrack habitats. Excretion droplets have been shown to be 'hotspots' of *E. coli* O157:H7 when the pathogen was fed to houseflies (Sasaki *et. al.,* 2000), and viable populations of this pathogen remained in seaweed fly faeces for at least 24 h after initiation of feeding on *E. coli* O157:H7. The persistence of *E. coli* O157:H7 on seaweed and sand demonstrates that seaweed fly excretions onto the surface of wrack and sand provided favourable conditions for *E. coli* O157:H7 persistence in these substrates. Survival of the pathogen in and on the flies is thus maintained by continual ingestion and recontamination of the exoskeleton from the wrack habitat.

Temperature is a key determinant of the distribution of the cold-favouring *C. frigida* and a northward shift in their northern European range in recent decades is a likely response, in part, to a simultaneous warming trend in this region (Phillips *et. al.,* 1995; Edward *et. al.,* 2007; IPCC, 2013). Mass migration of *C. frigida* adults over considerable distances has been reported, included nuisance inland plagues in urban areas, possibly driven by sub-optimal habitat conditions, or alternatively optimal conditions supporting high population densities (Egglishaw, 1961; Oldroyd, 1954). The phenomenon of inland emigration of seaweed flies indicates that the presence of decaying seaweed is not a pre-requisite attractant for their dispersal, although the absence of wrack habitat inland would prevent establishment of a population in that location. Female *C. frigida* can lay three clutches of up to 80 eggs each and in mainland Europe this species is normally more abundant, and experiences a faster egg to adult development time, than *C. pilipes* which lay single eggs (Dobson 1974a and b; Edward *et. al.,* 2007). *C. frigida* larvae typically occur at densities of approximately 1000 larvae kg^{-1} of seaweed, and in optimal conditions, *C. frigida* populations have the potential to increase by approximately 200 times with each generation (Butlin *et. al.,* 1984; Dobson, 1974a). Thus, the potential for *E. coli* O157:H7 transmission by migrating female and male *C. frigida* within and between beaches, and even inland, should not be underestimated. The ability of seaweed flies to vector *E. coli* O157:H7 from contaminated wrack beds on beaches to recently deposited seaweed, together with intraspecific transmission to other seaweed fly populations, therefore increases the spatial reach of the risk of public exposure to this pathogen.

Persistence and growth of *E. coli* O157:H7 in seaweed and sand both in the presence and absence of seaweed flies confirms that the simulated wrack environment facilitates long term survival of *E. coli* O157:H7. Both seaweed and sand provide a source of environmental exposure to the pathogen, which ensure that several generations of *C. frigida* and *C. pilipes* flies are continually externally and internally contaminated and re-contaminated (Graczyk *et. al.,* 2001). Thus, a single wrack bed could ensure the persistence of *E. coli* O157:H7 and subsequent vectoring by several generations of seaweed flies; however, wrack beds in the natural environment are transient habitats, often present for no more than a few days (Edward *et. al.,* 2007). Furthermore,

laboratory conditions protected *E. coli* O157:H7 from predation, ultraviolet radiation, and provided plentiful nutrients, water and a favourable temperature (O'Mullan *et. al.,* 2017). Therefore, depending on vulnerability to high tides and internal wrack bed temperatures attained, the observed growth of *E. coli* O157:H7 in seaweed and sand from day 4 may occur only sporadically in beach environments, meaning that production of a single cohort of *E. coli* O157:H7 contaminated seaweed flies from a single wrack bed is more likely than production of multiple cohorts. Additionally, the predominance of a single bacterial species in the larval diets, and presence of two seaweed species only, contrasts with the diverse microbial assemblage associated with the multiple seaweed species present in natural wrack beds (Edward *et. al.,* 2008). Restriction to a sub-optimal diet, however, affected all treatments equally, and seaweed fly larvae have been shown to survive on a monospecific diet of commensal *E. coli*, suggesting that feeding and development were not greatly impaired by these experimental conditions (Cullen *et. al.,* 1987).

Interestingly, *C. frigida* adult flies facilitated the survival of *E. coli* O157:H7 in wrack bed habitats over 56 days due to excretion of viable cells of the pathogen following ingestion, despite the presence of multiple generations of larvae, whilst *C. frigida* larvae alone initially suppressed populations of *E. coli* O157:H7 in the seaweed they inhabited. The onset of pupation on approximately day 6 coincided with reductions in the concentration of *E. coli* O157:H7 in larvae and pupae. This phenomenon, recorded for other species of fly larvae and human pathogens, could be caused by cessation of feeding and subsequent voiding of digestive tracts prior to pupation, and the destruction or inactivation of *E. coli* O157:H7 by gut microbes during metamorphosis (Lalander *et. al.,* 2013; Engel and Moran, 2013). The efficiency by which these mechanisms reduced pathogen loads in seaweed fly larvae, and resulted in increased *E. coli* O157:H7 on seaweed was dependent on larvae possessing a full complement of native gut microbiota. Examination of the possible contribution of loss of surface microbiota from seaweed fly larvae to reduction of *E. coli* O157:H7 concentrations within the larvae may have been confounded by ineffective surface disinfection of larvae. This may have contributed to the lack of distinction between detected pathogen loads in untreated and surface sterilised larvae throughout most of the sampling period.

In the absence of seaweed fly larvae and a diverse community of competing microbiota in seaweed and sand, the initial rapid growth of *E. coli* O157:H7 was not sustained. In a contamination scenario of decaying wrack beds contaminated with lower concentrations of *E. coli* O157:H7, seaweed fly larvae may be capable of greater levels of pathogen reduction in seaweed and in the underlying sand. However, concentrations of *E. coli* O157:H7 in or on larvae and pupae may still exceed that of their substrate during their development, and at any level of contamination may be capable of passive transmission of the pathogen between wrack habitats given that larvae washed by the sea from wrack beds can survive 48 h of immersion in seawater (Dobson, 1974a). Further research is required to determine if *E. coli* O157:H7 can be transmitted trans-stadially between seaweed fly pupae and newly eclosed adult flies, and at what concentration might *E. coli* O157:H7 ingested by larvae produce adults immediately capable of vectoring the pathogen between wrack habitats (Schuster *et. al.,* 2013).

The typical management response at popular recreational sandy beaches is to remove decaying seaweed, which also appeals to the public's aesthetic preferences (Quilliam *et. al.,* 2015), yet this has been shown to elicit either no change or an increase in faecal indicator organisms, such as *E. coli*, in nearshore water (Russell *et. al.,* 2014). In addition, wrack removal reduces richness of invertebrate species inhabiting wrack beds, including *C. frigida* and *C. pilipes* (Gilburn, 2012). Management of diffuse and point sources of *E. coli* O157:H7 in the environment can help to reduce *E. coli* O157:H7 inputs into beach environments, and farm-level strategies to reduce direct defecation by livestock and diffuse agricultural runoff to the coastal zone are important to mitigate the transfer of pathogens and nutrients to coastal environments (Young, 2016). Excessive nitrogen loading of coastal waters is a major cause of accelerated seaweed production, resulting in unnaturally high levels of wrack biomass accumulating along coastlines (Anderson *et. al.,* 2002). In such a scenario, and in combination with warmer temperatures as a result of climate change, the availability of seaweed biomass for attachment by human pathogens including *E. coli* O157:H7, combined with the subsequent growth of seaweed fly populations due to increased habitat availability, could potentially increase the opportunity for seaweed flies to function as bridge hosts and disseminate human pathogens at recreational beaches.

2.6 Conclusion

Seaweed flies and their larvae form large natural populations in recreational beach environments and can act as bridge hosts of the human pathogen *E. coli* O157:H7. However, they are restricted to decaying wrack beds and their dispersal is limited to beach environments where that habitat occurs. Therefore, despite seaweed flies facilitating long-term survival of *E. coli* O157:H7 in seaweed and sand, and flies and larvae potentially disseminating the pathogen amongst individual wrack beds and seaweed fly populations, both vectors and reservoirs are spatially constrained within the environment. The risk to public health from seaweed flies and decaying wrack beds is usually limited by human avoidance behaviour. However, beach sand can act as a significant reservoir with which the public make far more deliberate contact, particularly following beach grooming and the removal of seaweed.

Chapter 3 | Assessing the microbiological safety of seaweed-fed black soldier fly (*Hermetia illucens*) larvae, and optimising production of seaweed fly (*Coelopa frigida*) larvae, as feed for salmon aquaculture.

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3.1 Abstract

Sustainable ingredients for animal feed are becoming scarcer. Insects have emerged as a promising protein and lipid ingredient for fish feed, with two Dipteran (fly) species offering great potential as novel aquafeed ingredients. Black soldier fly (BSF; *Hermetia illucens*) can efficiently convert low quality organic matter into high value protein and fat and could therefore be used as a sustainable feed supplement in commercial aquaculture. However, BSF lack the essential long chain polyunsaturated fatty acids (LC-PUFAs) that farmed carnivorous marine fish attain from natural marine diets. Seaweeds are a sustainable source of both organic matter and complex carbohydrates, and feeding BSF larvae with varying concentrations of seaweed can provide a source of marine LC-PUFAs in their diet. Alternatively, there is the potential to utilise the larvae of the seaweed fly (Coelopidae), which naturally contain LC-PUFAs because their diet is comprised solely of seaweed. However, unlike BSF, the seaweed fly has never before been mass produced on a commercial scale, and essential parameters of their production need to be established. A critical challenge for incorporating seaweed into the diets of insect larvae produced as feed for the aquaculture industry is that pathogenic bacteria and faecal indicator organisms (FIOs) can readily attach to, and colonise seaweeds. Therefore, before this novel insect feed ingredient is advocated, microbiological risk assessments are warranted to ensure animal and public health protection from farm-to-fork. In this chapter, raw materials and finished products were screened for the presence of FIOs and pathogenic bacteria during the production of seaweed-fed BSF larvae (BSFL) and their formulation into fish feed pellets. Neither seaweed nor BSFL were found to present bacteriological hazards, although FIOs and *Listeria* spp. were introduced to various raw materials and finished products during handling, distribution and storage. However, microbial levels in finished products never exceeded microbiological quality standards for insect processed animal proteins. The effects of physical pre-treatment of seaweed (fresh, frozen, minced, un-minced and powdered) and rearing density on total *C. frigida* larval mass, yield, survival and development time were also tested. Optimal larval biomass output was produced by rearing 0.8 larvae g^{-1} of frozen-thawed and finely minced seaweed. Freezing for long-term storage does not impair seaweed nutritional quality for Coelopidae larvae,

whereas drying and powdering seaweed does. Critical control points for controlling bacteriological hazards during seaweed-fed BSF production were identified, and production parameters established to facilitate initial commercial testing of Coelopidae as aquafeed.

3.2 Introduction

The commercial production of insects to supply a protein source for the aquaculture feed industry has been permitted in the European Union (EU) since July 2017 (Reg (EC) 893/2017; EC, 2017a). Insects offer important advantages over increasingly expensive and scarce fishmeal and fish oil in aquafeed, and over alternative plant ingredients which can lack essential amino acids (EAA) and compete with human food demands (Jobling, 2016; Shepherd *et. al.,* 2017). Insects, by comparison, meet the high protein and fat, and low carbohydrate dietary requirements of carnivorous farmed fish, e.g. salmon (Henry *et. al.,* 2015; Lock *et. al.,* 2018). The EAA profile of Diptera (flies) such as black soldier fly (BSF; *Hermetia illucens*) is more similar to fishmeal than other tested insects, and can partially replace fishmeal in fish diets with no nutritional impairment (Barroso *et. al.,* 2014; Lock *et. al.,* 2016). Carnivorous marine fish require and obtain omega-3 Long Chain Polyunsaturated fatty acids (*n*-3 LC-PUFA) from their marine diets, which is essential for the health of fish, and human consumers of the fish (Tocher, 2010; Calder, 2018).

Terrestrial Diptera, such as BSF, typically contain a lower proportion of *n*-3 LC-PUFA than fishmeal, but can be enriched in these key nutrients via dietary inclusion of fishmeal, fish offal or seaweed (Barroso *et. al.,* 2014; St-Hilaire *et. al.,* 2007a and b; Sealey *et. al.,* 2011; Barroso *et. al.,* 2017; Liland *et. al.,* 2017). Alternatively, the larvae of the terrestrial seaweed fly (Coelopidae) feed solely on seaweed and thus naturally contain marine PUFAs at levels exceeding that achieved in BSFL (Biancarosa *et. al.,* 2018a). However, seaweed flies have been under researched as a potential aquafeed ingredient. Seaweed is a permitted feed material in the EU (Reg (EC) 68/2013; EC, 2013a), and represents a sustainable functional supplement in insect diets, as it is an abundant organic material which does not compete with human or livestock food resources (Rajauria, 2015; Liland *et. al.,* 2017). Importantly, seaweed harvested from

nearshore marine waters is being increasingly recognised as a potential reservoir of pathogenic microorganisms (Byappanahalli *et. al.,* 2009; Ishii *et. al.,* 2006). EU food safety legislation demands hygienic quality of all materials entering the food production chain from 'farm-to-fork' (EC, 2002; EC, 2005). However, microbiological standards have not yet been established for seaweed utilised as animal feed, and the feed substrate for insects is widely cited as a major source of undesirable substances in insects (Van der Spiegel *et. al.,* 2013). Demonstrating the microbiological safety of seaweed as insect feed, and the commercial potential of the specialist seaweed fly larvae as aquafeed, are two challenges to achieving industry and regulatory acceptance of these novel ingredients in sustainable aquafeed.

Seaweeds are vulnerable to surface colonisation by human pathogenic bacteria naturally present in seawater, e.g. *Vibrio* spp., or allochthonous bacteria such as *Listeria* spp. or toxigenic strains of *Escherichia coli* such as *E. coli* O157, originating from sewage, livestock or wild animals (Linke *et. al.,* 2014; Orruno *et. al.,* 2017). Non-marine bacteria may survive for significant periods in various extra-enteric environmental matrices, including seawater, and thus pose a risk of attachment to the surface of seaweeds (Lothigius *et. al.,* 2010; Mahmud *et. al.,* 2007). Importantly, the EU seaweed industry currently lacks standardisation of processing techniques, particularly with regard to drying processes. This has implications for potential survival of pathogens on seaweed throughout subsequent utilisation, particularly if not subjected to further microbial inactivation treatment after drying (Garcia-Vaquero and Hayes, 2016). Microbiological hazards associated with dried seaweed feed may therefore present animal and human health risks further along the feed and food chain.

All EU insect producers operate within the EU regulatory framework, which requires adherence to hygiene practices. Such practices are designed to control microbiological hazards during primary and secondary production, and are implemented according to a hazard analysis and critical control point (HACCP) system (Reg (EC) 183/2005; EC, 2005). Currently, microbiological quality standards for insect processed animal proteins (PAPs) require sampling of products for *Clostridium perfringens* (absent in 1 g of the product), *Salmonella* spp. (absent in 25 g) and Enterobacteriaceae (unsatisfactory if in excess of 300 colony forming units (CFU) in 1 g) (Reg (EC) 893/2017; EC, 2017a). There is a paucity

of data on microbiological risks associated with farmed insects, and the majority of safety assessments of BSFL as a feed material focus on mycotoxins and heavy metals (Derrien and Boccuni, 2018). The insect species, the production and processing methods, general environment, feed substrate and stage of insect harvest can all contribute to the microbiological risk profile of BSFL meal (Van Raamsdonk *et. al.,* 2017; Van der Spiegel *et. al.,* 2013). Feed substrate influences BSFL gut bacteria and *Bacillus cereus* have been detected in samples of dried and powdered larvae produced for the feed market (Grabowski and Klein, 2017; Jeon *et. al.,* 2011). BSFL are capable of reducing *E. coli* and *Salmonella* in their feed substrate when fed on manures (Erickson *et. al.,* 2004; Lalander *et. al.,* 2013, 2015; Liu *et. al.,* 2008; Zheng *et. al.,* 2013), and can produce antimicrobial substances active against Gram-positive and Gram-negative bacteria (Park *et. al.,* 2014). High hydrostatic pressure treatment of BSFL intended as animal feed was successful for controlling yeasts and moulds, but aerobic mesophilic bacteria proved more resistant (Kashiri *et. al.,* 2018). Although industrial protein and lipid extraction methods are likely to decontaminate raw insect materials, CCPs still need to be determined for all production and processing stages (Schlüter *et. al.,* 2017).

Under current EU Regulations, farmed insects are 'farmed animals' (Reg (EC) 1069/2009; EC, 2009c), which restricts their substrate to commercial animal feed or former food still safe for human consumption. This constrains the capacity of the insect industry to fully exploit the exceptional ability of BSFL to efficiently convert low quality and low cost waste organic matter such as animal manures into protein (Zhou *et. al.,* 2013). Attempts to enrich BSFL with EPA and DHA using seaweed still required wheat based feed to form the bulk of the diet, since the inclusion of seaweed powder at levels > 50 % detrimentally affected larval growth and nutritional targets (Liland *et. al.,* 2017). This highlights the fundamental issue with enriching a terrestrial insect's diet with seaweed which is not a natural dietary constituent of that insect. In contrast, the larvae of seaweed flies (Diptera: Coelopidae) are specialist consumers of seaweed and offer a lipid profile reflecting their marine diet (Biancarosa *et. al.,* 2018a). There is, however, a paucity of information regarding the mass rearing of Coelopidae for commercial exploitation, and fundamental knowledge gaps remain with regard to essential

parameters of their production, in contrast to BSFL where commercial production parameters are well established (Biancarosa *et. al.,* 2018a).

Laboratory cultures of *Coelopidae frigida* have been reared successfully from egg to adulthood on fresh, as well as frozen-thawed, *Laminaria* spp. (including *L. digitata*) and *Fucus* spp. (including *F. serratus* and *F. vesiculosus*), as mono-specific and mixed species diets, as well as rehydrated powdered kelp (Dunn *et. al.,* 2005; Biancarosa *et. al.,* 2018a; Cullen *et. al.,* 1987). Mincing seaweed increases mannitol release, which increases microbial proliferation and the rate of seaweed decomposition and thus food availability to larvae (Leggett *et. al.,* 1996; Dobson 1974a). The detrimental effect of feed sterilisation on larval development is indicative of the crucial role that microorganisms play in Coelopidae nutrition (Cullen *et. al.,* 1987). Although the fatty acid content of larvae was unaffected by the seaweed species utilised, a diet of *F. serratus* produced ~ 70 % greater *C. frigida* growth compared with a diet of *L. digitata* (Biancarosa *et. al.,* 2018a). However, *C. frigida* require *Laminaria* spp. to breed and develop successfully, thus Coelopidae diets must contain both genera (Phillips *et. al.,* 1995; Edward and Gilburn, 2012). Previous studies identified the optimal rearing density of *C. frigida* as 0.8 larvae g^{-1} of feed, although this may vary depending on the nature of the substrate (Burnet and Thompson, 1960; Leggett *et. al.,* 1996; Kenis *et. al.,* 2018).

The aims of this study were to (1) identify CCPs within commercial seaweed and BSF production chains and (2) provide preliminary data on essential production parameters necessary for mass production and commercial exploitation of Coelopidae for aquafeed. To address (1), a microbiological safety assessment was conducted throughout the manufacturing chain during trial production of fish feed pellets from seaweed-fed BSFL meal. Raw ingredients and finished products (from the harvesting of seaweed and rearing of BSFL, to the manufacture of feed pellets), were screened for pathogenic bacteria and FIOs that may originate from utilising seaweed as a feed substrate for BSFL. To address (2), the effect of physical pre-treatment of seaweed to extend feed shelf life and increase larval food availability, and the ideal rearing density of larvae from first instar to prepupae, on total mass, yield, survival and development time of *C. frigida* larvae was determined.

3.3 Materials and methods

3.3.1 Microbiological safety of seaweed-fed BSFL throughout the production chain

The production companies involved in this project were visited between October 2016 and August 2017. Samples were cultured on the following selective media: membrane lactose glucuronide agar (MLGA, CM1031; Oxoid, Basingstoke, UK) to quantify presumptive *E. coli;* Slanetz and Bartley medium (CM0377; Oxoid,) to quantify presumptive intestinal enterococci; sorbitol MacConkey agar (SMAC, CM0813; Oxoid) supplemented with cefixime and potassium tellurite (CT, SR0172; Oxoid) for isolation of *E. coli* O157; xylose lysine deoxycholate agar (XLD, CM0469; Oxoid) for determining *Salmonella* spp.; *Listeria* selective agar (Oxford formulation, CM0856; Oxoid) supplemented with modified *Listeria* selective supplement (SR0206; Oxoid) for quantifying *Listeria* spp.; and cholera TCBS medium (CM0333; Oxoid) to quantify *Vibrio* spp. All plates were incubated at 37 °C for 24 h (except Slanetz and Bartley plates for enterococci which were incubated at 44 °C). Bacterial CFU g^{-1} were normalised by obtaining the dry weights (80 °C for 24 h) of each substrate.

3.3.1.1 Seaweed harvesting and processing

The first phase in this production chain took place at a commercial seaweed harvesting facility in the Republic of Ireland (Fig. 3.1). Fresh, attached seaweed was hand harvested from the rocky intertidal shoreline of Finavarra beach, County Clare, Ireland at low tide in October 2016. The seaweed species collected were *Laminaria digitata*, *Fucus serratus*, *Ascophyllum nodosum*, *Palmaria palmata* and *Ulva lactuca.* Fresh seaweed was hand washed in cold, potable water to remove sand and visible epiphytic flora and fauna, laid in plastic trays and dehumidified overnight for 12 h at 40 ± 4 °C to achieve a moisture content of ≤ 12 %. Each seaweed species was separately milled to produce a powder of particle size 0.5 – 1 mm, and the powders from each individual species were subsequently combined in equal proportions. This dried seaweed powder mixture was packaged in plastic bags and transported to the BSFL rearing facility within two days.

Long term records indicated extremely low FIO levels at two neighbouring bathing water quality monitoring locations (Bishops Quarter Beach and Traught) of comparable

adjacent land use conditions to Finavarra (EPA, 2017). Coupled with the likelihood of highly dilute pathogen concentrations, if present in the seawater; the lack of necessary equipment for enrichment of samples before culturing in the improvised laboratory within the seaweed factory; and the potential for environmental stress, such as high salinity, to induce a viable but non-culturable state (VBNC) in the pathogenic bacteria and thus produce false negative results (Ramirez-Castillo *et. al.,* 2015), seawater and seaweed were screened for FIOs only (*E. coli* and enterococci). To assess the level of background FIOs in the harvesting water, four replicate 100 ml samples of seawater were collected and vacuum-filtrated through a 0.45 µm cellulose nitrate membrane (Sartorius, Göttingen, Germany), and transferred to selective media. To assess the level of background FIOs colonising seaweed, screening for *E. coli* and enterococci took place after: (1) harvesting; (2) washing; (3) drying; and (4) storage of the seaweed used for the feeding trial. At least 2 kg of four of the seaweed species *L. digitata*, *F. serratus*, *A. nodosum*, *P. palmata*, and approximately 100 g of *U. lactuca* were harvested from a wide stretch of the intertidal zone. A 500 g sample of each species (50 g of *U. lactuca*) was cut into 2 cm pieces, and individually homogenised (with no added liquid) for 2 minutes using a hand blender (MSM6700GB; Bosch, Stuttgart, Germany). Samples of 10 g (n = 4) were taken from each homogenised batch of each seaweed species, and vortexed in 10 ml of sterile seawater for 60 s, and 20 µl of the supernatant plated onto selective media, which were inverted and incubated. The remaining 1.5 kg of each seaweed species (50 g of *U. lactuca*) was washed and the seaweeds processed and prepared for microbiological testing as above. The remaining intact 1 kg of each species (25 g of *U. lactuca*) underwent overnight dehumidification followed by processing and microbiological testing as above. Finally, the milled and stored seaweed powders were microbiologically tested as described above.

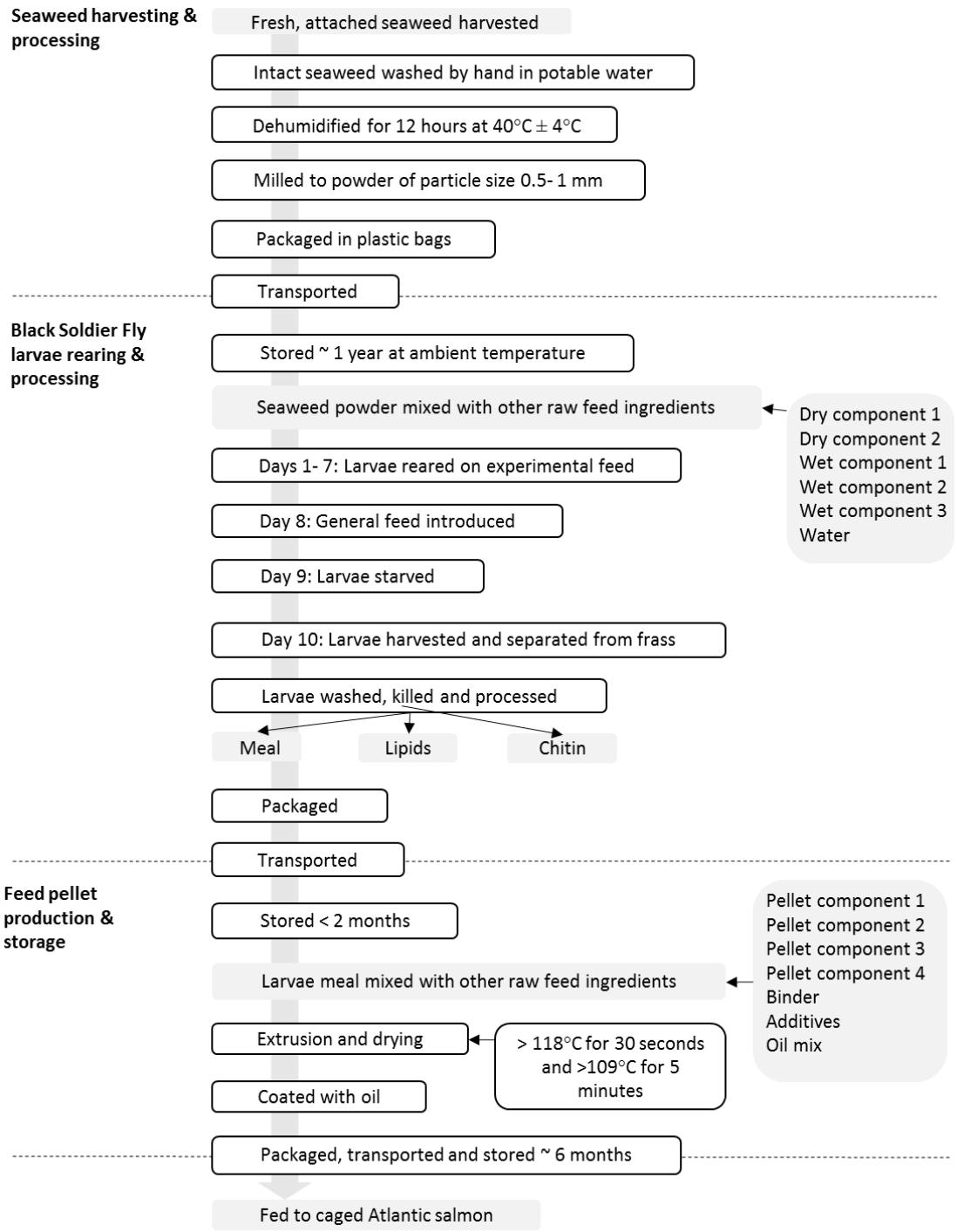


Figure 3.1. Schematic of production process of fish feed pellets manufactured using seaweed-fed black soldier fly larvae. Grey boxes = raw materials and finished products. White boxes = processing, packaging, distribution and storage.

3.3.1.2 BSFL rearing and processing

The microbiological safety assessment of larvae meal production was conducted at a BSF rearing and processing facility in the Netherlands during 2016. Environmental parameters during larval rearing and processing, physico-chemical conditions of raw materials and finished products, and quality control records of the facility were considered commercially sensitive and were therefore not disclosed. However, environmental swabs and sampling of final products were regularly undertaken according to Regulation (EC) 893/2017 (EC, 2017). The seaweed powder mixture had been stored for approximately one year in plastic bags at ambient temperature at the BSF production facility before being used in the feeding trial. The sequential stages of BSF fly breeding, egg laying, larvae rearing, larvae harvesting, washing, and killing, and the final processing of larvae into fish meal products are shown in Figure 3.1. Environmental conditions within the adult breeding rooms, larvae rearing facility and during washing and processing were not disclosed, and the composition of the BSFL feed is categorised as either dry or wet components to protect the commercial interests of the company.

The seaweed powder was divided between two separate feeding trials, although the feed recipe differed between Trials 1 and 2 due to limited availability of seaweed powder after Trial 1 (Table 3.1); however, the feeding regime was the same for both trials (Table 3.2). On day eight, larvae received 'general' feed containing approximately the same ingredients provided on the preceding seven days, with the exclusion of the seaweed powder, since pilot trials showed that any seaweed powder remaining in larval digestive tracts at the time of harvest congested the larvae processing machinery. No new feed was provided on day nine in order to starve larvae and thus encourage them to void their digestive tracts, and larvae were harvested on day ten. Adult BSF used to produce larvae for Trial 1 died before they could be screened for pathogens, and the larvae produced in Trial 2 were not processed due to the success of Trial 1. Therefore, a microbiological safety evaluation of one entire production chain from adult breed stock to processed larvae products was not possible. The materials screened for bacteria were as follows: the six ingredients (with the exception of potable water) of which the larvae feed was comprised, adult flies which produced the larvae utilised in

the feeding trial (Trial 2 only), whole larvae and their frass (mixed with waste feed), and finally the meal, lipids and chitin produced by processing of the larvae (Trial 1 only). Stored samples were unavailable, as the finished product from Trial 1 was transported to the feed pellet production facility immediately after processing of larvae.

Each adult fly or larvae sample consisted of three individuals, and 10 samples of each were taken. Three flies or larvae were added to a 1.5 ml Eppendorf tube containing 100 µl phosphate buffered saline (PBS), the contents were homogenised using a micropestle, and a further 100 µl of PBS added. Each tube was vortexed for 60 s, and 10 µl plated onto selective media and incubated. Chitin samples (500 g) were homogenised for 2 min, with no added liquid, using a hand blender. All other materials were pre-processed into a fine powder or a thick liquid, and would therefore not have benefitted from further homogenisation. From each 500 g sample of all materials, including chitin, 10 g (n = 10) were vortexed in 10 ml PBS for 60 s, and 50 µl plated onto selective media.

Table 3.1. The varying proportions of seaweed powder supplement added to other raw feed materials, and the seaweed-free general feed recipe, constituting the substrate provided for black soldier fly larvae during feeding trials 1 and 2.

	Ingredients	Quantity (kg)
Trial 1 Recipe	Seaweed powder	47
	Dry component 1	7.5
	Dry component 2	7.5
	Wet component 1	157
	Wet component 2	78
	Wet component 3	78
	Water	50
	Total	425
		Proportion (%)
General feed	Dry component 1	10
	Wet component 1	45
	Wet component 2	22.5
	Wet component 3	22.5
		Quantity (kg)
Trial 2 recipe	Seaweed powder	15
	Dry component 1	7.5
	Dry component 2	7.5
	Wet component 1	157
	Wet component 2	78
	Wet component 3	78
	Water	15
	Total	358
		Proportion (%)
General feed	Dry component 1	10
	Wet component 1	45
	Wet component 2	22.5
	Wet component 3	22.5

Table 3.2. Feeding regime for rearing black soldier fly larvae on seaweed-supplemented feed during feeding trials 1 and 2. One scoop was equivalent to ~0.9 kg of feed.

Day	Number of scoops of feed
1	4
2	4
3	9
4	9
5	9
6	6
7	6
8	3 + 3 kg of General Feed
9	0 + 1 L water
10	0

3.3.1.3 Feed pellet production and storage

A pathogen safety assessment of fish feed pellets manufactured using the larvae meal was undertaken at a commercial fish feed company in Norway in 2017. All raw ingredients (including larvae meal) were utilised within six months of receipt. The principle processing stages were the grinding and mixing of raw ingredients, the production of feed pellets from this mixture through high temperature and pressure extrusion followed by drying, and the coating of pellets in oil (Fig. 3.1). Eight raw ingredients were mixed to produce four batches of pellets, and two batches (BP90015101 and BP90015102) were used in this study, which contained the same raw ingredients sourced from the same containers. The primary drying stage reduced moisture content in pellets to an estimated 6 – 9 %, before pellets were coated in a combination of fish and vegetable oils (to add energy to the feed and delay sinking of the pellets when fed to fish). Screening for environmental pathogens occurred at three different processing stages: (1) the raw ingredients prior to mixing; (2) the uncoated pellets following extrusion and drying; and (3) the coated pellets prior to packaging. Stored samples were unavailable as the finished products were transported in plastic bags to the fish feeding research station immediately after pellet production was complete.

Samples of the raw ingredient pellet 'component 4' (500 g) required 2 minutes soaking in 250 ml sterile PBS to adequately soften it for subsequent homogenisation for 2 minutes using a hand blender. The binder and pellet 'component 3' (500 g each) were dry ground for 2 minutes, with no added liquid. Insect meal, pellet 'components 1' and '2', and the additives were already in a fine powder form meaning that further homogenisation was unnecessary. For each ingredient, replicate samples of 10 g each (*n* = 4) were vortexed for 60 s in 20 ml PBS, and subsequently 20 µl plated onto selective media. A sub-sample (500 g) from each of the two batches of the oil-coated pellets (BP90015101 and BP90015102) was soaked in sterile PBS, homogenised, sampled and plated out onto selective media as described above. After approximately six months storage at the fish feeding research station, the two batches of oil-coated feed pellets were used in a caged fish (salmon) feeding trial and were sampled for microbiological contamination on the same day. From each batch, 200 g was soaked for 2 minutes in 100 ml sterile PBS for subsequent homogenisation for 2 minutes using a hand blender. From each homogenised sample, 10 g (*n* = 4) was vortexed for 60 s in 10 ml PBS, and subsequently 20 µl plated onto selective media and incubated.

3.3.2 Production optimisation of C. frigida larvae

3.3.2.1 Laboratory culture

Colonies of *C. frigida* were cultured from wild larvae collected from Dunbar, Scotland (56°02.7684'N, 2°3036.5112'W) in a climate controlled cabinet (Reftech B.V., Netherlands) at 25 °C ± 2 °C, a relative humidity of 60 % and a 12 h photoperiod. Rearing containers were comprised of 5 L plastic containers (Addis Ltd., UK), with paper towelling secured over an air hole (10 cm x 10 cm) in the lid to enable gas exchange. Larvae in each container were fed a 2 kg mixture of fresh, finely mixed (0.5 cm^2) seaweed (*Laminaria digitata* (Hudson) (70 %) and *Fucus serratus* (L.) (30 %)) harvested from the same coastal location as the larvae. Newly eclosed adults were collected twice daily using attraction to a light box. Flies were anaesthetised with CO_2 for 10 s, sexed and stored at 4 °C in ventilated 150 ml plastic Erlenmeyer flasks, sustained by cotton wool soaked in 50 % glucose solution. Flies were utilised in experiments within 72 h of emergence.

3.3.2.2 Optimising feed substrate: diet preparation

Feed substrate for the larvae was comprised of the fronds and stipes of both *F. serratus* (80 %) and *L. digitata* (20 %). The exception was the commercially sourced seaweed powder, which was available only from *Laminaria* spp. (100 %). Nine experimental diets were prepared (Table 3.3). Seaweed biomass for all diet treatments (with the exception of the seaweed powder trial) was collected from Dunbar, Scotland (56°02.7684'N, 2°3036.5112'W) and utilised within 48 h. The seaweed was washed to remove all epiphytic flora and fauna. Batches of seaweed were immediately frozen at -20 °C for 12 h, and defrosted for 24 h, and then either finely or coarsely minced, and stored at 4 °C until use. Mincing was undertaken using a Buffalo meat mincer (Model K335), employing two alternate mincing attachments to produce fine (0.5 cm^2) or coarse (0.8 cm^2) seaweed particle sizes. The seaweed powder was comprised of *Laminaria* spp. (JustIngredients Ltd., UK) and the seaweed particle size was 425 µm.

Table 3.3. The experimental diets on which *C. frigida* larvae were reared.

Diet ID	Fresh	Frozen-thawed	Finely minced (0.5 cm^2)	Coarsely minced (0.8 cm^2)	Intact	Autoclaved (15 mins at 121 °C)	Dried	Finely milled (425 µm)	Rehydrated with tap water
A	✓		✓						
B	✓			✓					
C		✓	✓						
D		✓		✓					
E	✓		✓			✓			
F		✓	✓			✓			
G	✓				✓				
H		✓			✓				
I							✓	✓	✓

3.3.2.3 Optimising feed substrate: experimental design

Four replicate mesocosms (1.75 L boxes; Stewart Sealfresh, Plastichousewares, UK) were established for each of the nine treatments, each containing 500 g of thoroughly mixed seaweed substrate (400 g of *F. serratus* and 100 g of *L. digitata*). The average depth of the feed substrates was 6 – 8 cm, with the exception of fresh, intact seaweed

(12 cm depth). Each replicate mesocosm with the seaweed powder treatment contained 250 g of the powder and 500 ml of tap water. Excess media was then removed until the substrate weight equalled 500 g, at an average depth of 5.3 cm. A hole was cut in the lid of each mesocosm, over which netting of mesh diameter 0.2 mm was secured to enable gas exchange, and a temperature logger was placed within the substrate of each mesocosm. Twenty adult *C. frigida* were added to each replicate mesocosm (10 females and 10 males), and all mesocosms transferred to a climate controlled cabinet (Reftech B.V., Netherlands) at 25 °C ± 2° C, 60 % relative humidity and a 12 h photoperiod. After 48 h, all flies were removed using attraction to a light box, and the developing larvae were allowed to feed *ad libutum.* Larval harvesting from each mesocosm began when third instar larvae were first observed (judged to be the maximum harvestable larval size), which involved removing and counting all live individual larvae in each mesocosm. Following anaesthetisation with CO_2 (for 10 s), the total weight of larvae from each replicate mesocosm was measured. Larvae were removed from all treatments on day 6, except treatments containing autoclaved seaweed. Compared with other treatments, larvae reared on autoclaved seaweed developed more slowly and third instar larvae appeared, and were removed on, day 8.

Of the nine diets tested in the preceding experiment, frozen-thawed and finely minced seaweed was identified as the optimal feed substrate in terms of producing the highest larval yield compared with all other experimental feeds, and one of the highest outputs of total larval mass (comparable to fresh, coarsely minced seaweed). This experimental diet was therefore selected as the seaweed substrate in which to manipulate larval rearing densities in order to maximise larval mass and yield.

3.3.2.4 Optimising rearing density: experimental design

Eight colonies of *C. frigida* were established as per the initial laboratory culture, with the exception that within each 5 L container, eggs were laid on 2 kg of frozen-thawed and finely minced seaweed (1.6 kg of *F. serratus* and 0.4 kg of *L. digitata*). Adult *C. frigida* were removed 48 h after introduction to the culture boxes, using attraction to a light box. Four days after establishment of the cultures, first instar larvae were removed from the culturing substrate, rinsed in water, and sieved through a mesh (diameter, 710 µm). Only individuals sufficiently small to pass through the mesh were retained in order to

ensure uniformity of larval size. The larvae were transferred using forceps to the experimental mesocosms: 20 to each low density treatment mesocosm, 40 to each mid density treatment mesocosm, and 60 to each high density treatment mesocosm. Mesocosms were comprised of ventilated 150 ml plastic Erlenmeyer flasks containing 50 g of frozen-thawed and finely minced seaweed (40 g of *F. serratus* and 10 g of *L. digitata*), each containing a temperature logger to record temperature at an hourly rate. Three density levels of larvae were thus allowed to feed *ad libutum* on a fixed volume of feed substrate: 0.4 (low density), 0.8 (medium density) and 1.2 (high density) larvae g^{-1} of seaweed. Larvae were harvested from each replicate mesocosm when pre-pupae or third instar larvae were first observed. At the point of harvest all live larvae were removed and counted, and following 10 s anaesthetising with CO_2, the total larval mass from each mesocosm was recorded.

3.3.3 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics v. 21 (SPSS Inc. Chicago, IL, USA). Total larval biomass data from both the feed substrate and larval density experiments did not conform to normal distribution despite transformation and therefore were analysed using a Kruskal-Wallis test at a significance level of 0.05. Post hoc analysis of total larval mass generated by the substrate treatment took the form of step-down analysis of homogenous subsets. Total larval biomass data based on manipulating rearing densities was subjected to step-down analysis in the form of pairwise comparisons. Data from both experiments of larval yields, and of density-dependent mortality rates as part of the rearing density trial, were normally distributed and analysed by one-way Analysis of Variance (ANOVA) at a significance level of 0.05, followed by Tukey post hoc testing.

3.4 Results

3.4.1 Microbiological safety of seaweed-fed BSFL throughout the production chain

3.4.1.1 Microbial contamination during seaweed harvesting and processing

Seawater at the seaweed harvesting site contained concentrations of *E. coli* and enterococci that were indicative of 'excellent' water quality according to the EU Bathing Water Directive (< 250 CFU 100 ml^{-1} for *E. coli* and < 100 CFU 100 ml^{-1} for intestinal

enterococci) (Table 3.4). *E. coli* was associated with freshly harvested *L. digitata* (< 10 CFU g^{-1}), *P. palmata* (< 30 CFU g^{-1}) and *U. lactuca* (< 10 CFU g^{-1}) (Table 3.4). However, these FIOs were below detectable levels on all seaweed species following washing in tap water, overnight dehumidification, and in the processed seaweed powder after two days storage.

3.4.1.2 Microbial contamination of BSF larvae during rearing and processing

E. coli, enterococci, *E. coli* O157, *Salmonella* spp., *Listeria* spp. and *Vibrio* spp. were all below detectable levels in the seaweed powder fed to BSFL at the start of both feeding trials. The larval feed substrate used during Trial 1 contained low levels of *E. coli* (< 10 CFU g^{-1}) (Table 3.4), whilst both dry components of the larval feed contained low levels of *Listeria* spp. (not exceeding 28 CFU g^{-1}) and dry 'component 2' contained relatively high levels of enterococci during Trial 2 (~4 log CFU g^{-1}). Wet components of the larvae feed contained relatively low levels of *Listeria* spp., with the highest concentration (193 CFU g^{-1}) occurring in wet 'component 1' during Trial 1. The concentrations of enterococci and *Listeria* spp. in the final feed mixture were extremely low (< 10 CFU g^{-1} and 31 CFU g^{-1}, respectively). At the point of harvest, larvae produced during both feeding trials were associated with low levels of enterococci (Table 3.4). Concentrations of enterococci in the larval frass however, were typically 5 – 6 log CFU g^{-1} higher than in the larvae, and frass produced in Trial 2 was also associated with a low concentration of *E. coli* (20 CFU g^{-1}). FIOs and pathogenic bacteria were below detectable levels in the larvae meal and lipid products immediately after processing of the larvae.

3.4.1.3 Microbial contamination during feed pellet production and storage

Following shipment to, and approximately two months storage at, the feed pellet production facility, the BSFL meal became contaminated with relatively high concentrations of enterococci and *Listeria* spp. (both ~3 log CFU g^{-1}) (Table 3.4). Several additional commercial raw ingredients mixed with the larvae meal introduced low levels of enterococci (< 10 CFU g^{-1} in all cases) and *Listeria* spp. (detected at a maximum of 65 CFU g^{-1}) to the pellet formulations. Although the extrusion and drying treatments (during which temperatures exceeded 109 °C) ensured the production of initially sterile pellets, subsequent oil application reintroduced very low concentrations of enterococci

(< 10 CFU g^{-1}). After packaging, transport to, and approximately 6 months storage at the research station where the caged fish feeding trial was undertaken, enterococci contamination levels on feed pellets remained relatively stable (Table 3.4).

Table 3.4. Microbiological contamination of raw materials and finished feed pellets used for caged Atlantic salmon. Only ingredients and finished products where bacteria were screened are included (non-detectable concentrations are denoted by '–'). All concentration expressed as either CFU 100 ml^{-1} or CFU g^{-1}. [a]Larvae is expressed per larvae.

Phase	Substrate	Sampling target	*E. coli*			Enterococci			*Listeria* spp.		
			mean	SE	*n*	mean	SE	*n*	mean	SE	*n*
Seaweed harvesting and processing											
	Seawater		<10	-	4	<10	-	4			
	Fresh seaweed	*Laminaria digitata*	<10	-	4	-		-			
		Palmaria palmata	26.78	26.78	4	-		-			
		Ulva lactuca	11.0	6.11	4	-		-			
Larvae rearing and processing											
Trial 1	Raw feed materials	Dry component 2	< 10	-	10	62.0	36.08	10	-		-
		Wet component 1	-		-	-		-	193.1	60.6	10
		Final feed mixture	< 10	-	10	-		-	-		-
	Harvest	Larvae[a]	-		-	< 10	-	10	-		-
		Frass	-		-	1.6×10^6	273648	10	-		-
Trial 2	Raw feed materials	Dry component 1	-		-	-		-	< 10	-	10
		Dry component 2	-		-	1.7×10^4	6302	10	28.0	10.8	10
		Wet component 1	-		-	-		-	78.7	30.0	10
		Wet component 2	-		-	-		-	97.0	35.2	10
		Final feed mixture	-		-	-		-	31.1	18.0	10
	Harvest	Larvae[a]	-		-	29.7	7.6	10	-		-
		Frass	20.0	9.7	10	8.2×10^5	351137	10	-		-

Feed pellet production										
	Raw feed materials	Larvae meal	-	-	3.7×10^3	652.8	4	8.2×10^3	3702.7	4
		Pellet component 2	-	-	< 10	-	4	65.0	12.1	4
		Pellet component 3	-	-	-		-	< 10	-	4
		Pellet component 4	-	-	< 10	-	4	38.5	37.5	4
		Binder	-	-	< 10	-	4	< 10	-	4
		Oil mix	-	-	< 10	-	4	-		-
	Coated in oil	BP90015101	-	-	< 10	-	4	-		-
		BP90015102	-	-	< 10	-	4	-		-
	Stored feed pellets	BP90015101	-	-	< 10	-	4	-		-
		BP90015102	-	-	< 10	-	4	-		-

3.4.2 Production optimisation of C. frigida larvae

3.4.2.1 Optimising feed substrate

The pre-processing of seaweed significantly affected the total larval biomass produced by the time of larvae harvest ($P < 0.001$) (Fig. 3.2a). After six days of feeding and growth, there was no significant difference in larval biomass between the seaweed frozen-thawed and finely minced (diet C), and the coarsely minced fresh (diet B) seaweed treatments (24.3 g ± 1.1 SE and 27.1 g ± 0.7 SE respectively). Both treatments generated significantly greater biomass than fresh finely minced seaweed (diet A) and frozen-thawed, coarsely minced seaweed (diet D), which were not significantly different from each other in terms of biomass output. The total biomass of larvae reared on intact seaweed (diets G and H) and previously sterilised seaweed (diets E and F) (regardless of fresh or previously frozen status) were not significantly different to each other, but were significantly lower than the total biomass of larvae reared on non-sterilised seaweed substrate (regardless of size of feed particle, or fresh or previously frozen status (diets A, B, C and D). Seaweed powder (diet I) failed to produce a single live larvae of sufficient mass to harvest: thick mould developed over the surface of the seaweed powder in three of the replicate mesocosms within three days of the start of the feed trial, at which time the remaining replicate mesocosm without mould contained five dead first instar larvae.

Dietary composition affected the number of larvae produced ($P < 0.001$) (Fig. 3.2b). Compared with all other feed substrates, the greatest number of larvae were harvested from frozen-thawed and finely minced seaweed (diet C) (1006 larvae ± 167 SE, $P < 0.001$ in all cases). The comparable yields of larvae generated by rearing on fresh finely minced seaweed (diet A) (387 larvae ± 42 SE), fresh coarsely minced seaweed (diet B) (516 larvae ± 43 SE) and frozen-thawed coarsely minced seaweed (diet D) (483 larvae ± 81 SE) were significantly greater than the number of larvae harvested from sterilised, intact and powdered seaweed (diets E – I), regardless of fresh or previously frozen status of the substrate ($P < 0.05$). A greater number of larvae developed on intact seaweed (diets G and H) than on seaweed powder (diet I) (which failed to produce any live larvae), although the low yields produced on sterilised seaweed substrate (diets E and F) were not significantly different to that of seaweed powder.

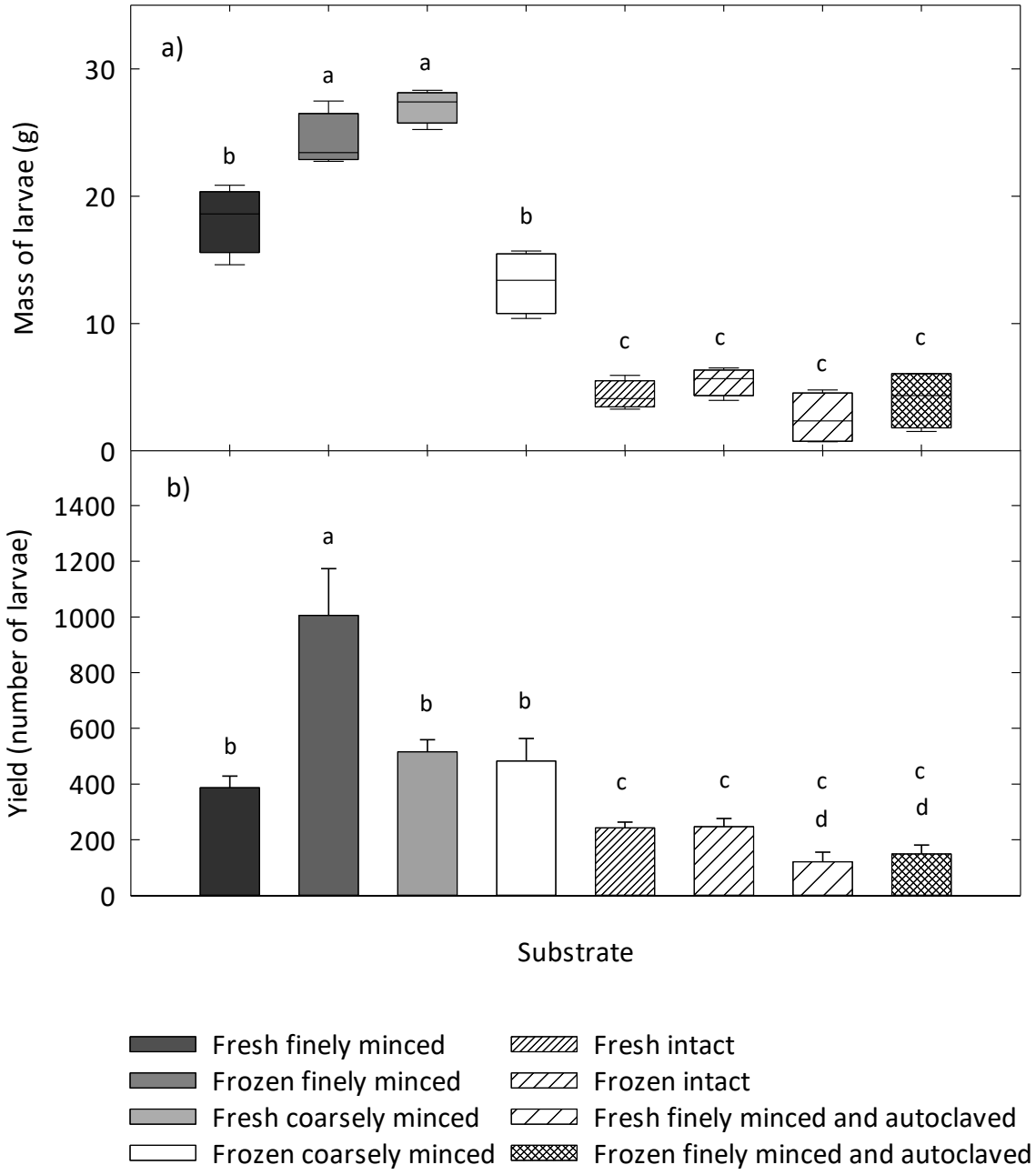


Figure 3.2. Total mass (a) and yield (b) of *Coelopa frigida* larvae reared on experimental diets. Boxes that do not share a letter are significantly different from each other (Kruskal-Wallis, $P < 0.001$; step-down analysis), and bars that do not share a letter are significantly different from each other (one-way ANOVA, $P < 0.001$; Tukey's test, $P < 0.05$). Total mass data are expressed as median values of four replicate mesocosms per treatment ± the 90th and 10th percentiles, and total yield data are expressed as mean values of four replicate mesocosms per treatment + SE.

3.4.2.2 Optimising rearing density

The appearance of third instar larvae in the frozen-thawed and finely minced substrate of all three density treatments began four days after the first instar larvae had been introduced into the mesocosms. Larval harvesting therefore occurred eight days after the eggs were initially laid in the colony cultures. The percentage of larvae surviving to pre-pupation on frozen-thawed and finely minced feeding substrate, from an initially low rearing density (69 % ± 8 SE), did not differ significantly from the number of larvae surviving to harvest on the same substrate type from the mid-density (81 % ± 6 SE) or high density (73 % ± 4 SE) treatments. The initial density at which *C. frigida* larvae were reared on frozen-thawed and finely minced seaweed from first instar development stage affected the total biomass of larvae harvested at pre-pupation stage ($P < 0.05$) (Fig. 3.3a). Following a growth period of eight days on optimal feeding substrate, four of which were at variable larval densities from first instar stage (< 710 μm size) onwards, a high initial rearing density of 1.2 larvae g^{-1} of seaweed failed to produce significantly greater total mean larval mass (0.86 g ± 0.07 SE) than a mid-density of 0.8 larvae g^{-1} (0.76 g ± 0.04 SE). A low initial rearing density of 0.4 larvae g^{-1} of feed substrate generated a lower total larval mass (0.3 g ± 0.03 SE) than that produced by a high initial rearing density ($P < 0.05$), but did not significantly differ from the biomass produced by a mid-level rearing density. The density at which *C. frigida* larvae were reared from four day old first instar larvae on the substrate had a significant effect on the number of larvae surviving to pre-pupation after four days feeding ($P < 0.001$) (Fig. 3.3b). Larval yields generated by low (13.8 ± 1.5 SE), mid (32.5 ± 2.3 SE) and high (43.8 ± 2.6 SE) initial rearing densities were all significantly different from each other ($P < 0.05$).

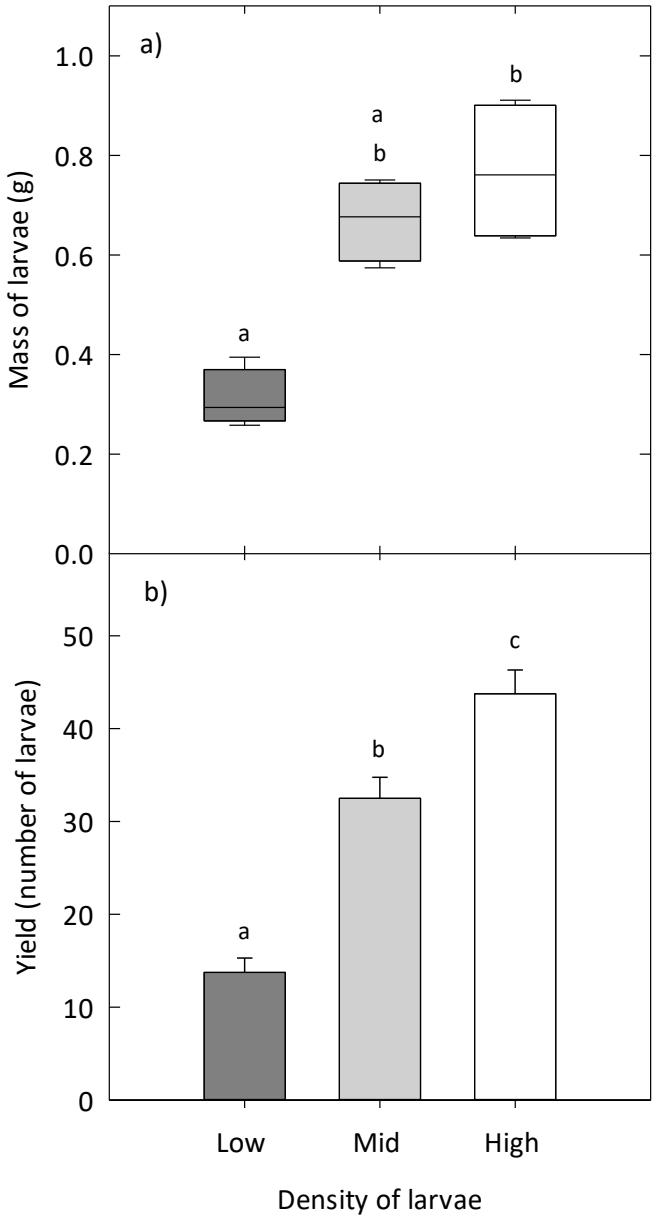


Figure 3.3. Total mass (a) and yield (b) of *Coelopa frigida* larvae established on frozen-thawed and finely minced seaweed at low (0.4 larvae g^{-1} of seaweed), medium (0.8 larvae g^{-1} of seaweed) and high (1.2 larvae g^{-1} of seaweed) initial rearing densities of first instar larvae (< 710 µm size), after a four day growth period. Boxes that do not share a letter are significantly different from each other (Kruskal-Wallis, $P < 0.05$; step-down analysis, $P < 0.05$), and bars that do not share a letter are significantly different from each other (one-way ANOVA, $P < 0.001$; Tukey's test, $P < 0.05$). Total mass data are expressed as median values of four replicate mesocosms per treatment ± the 90th and 10th percentiles, and total yield data are expressed as mean values of four replicate mesocosms per treatment + SE.

3.5 Discussion

The 'excellent' water quality of the seaweed harvesting site in Ireland likely contributed to the low levels of FIO contamination of the seaweed species utilised in the feed production trial. Multiple seaweed species, including *Laminaria* spp. and *A. nodosum*, produce antimicrobial exudates active against food spoilage bacteria such as *E. coli*, which may have contributed to inhibition or die-off of FIOs colonising the living seaweeds harvested for this study (Pina-Perez *et. al.,* 2017). Competitive interactions with natural bacterial biofilms on seaweed surfaces may also have influenced the attachment and survival of epiphytic and planktonic extra-enteric bacteria (Egan *et. al.,* 2013). The absence of FIOs from any seaweed species following subsequent processing stages suggests that the production environment, in terms of handling by personnel and contact with processing surfaces and equipment, was of a good hygienic standard.

In this study, *E. coli* was detected on all three classes of freshly harvested seaweed from a site offering 'excellent' water quality; however, microbial contamination of coastal waters, and thus of seaweeds, will vary both temporally and spatially (Quilliam *et. al.,* 2011b). *E. coli* colonising the harvested seaweeds did not survive subsequent processing, but there is evidence that heat stress during the drying process could induce a viable but non-culturable (VBNC) state in FIO and pathogenic bacterial cells, leading to overestimation of the effectiveness of desiccation as a potential antimicrobial treatment (Zhao *et. al.,* 2017). Thermal challenge studies for FIOs or pathogens attached to seaweed are scarce, although desiccation of *Ulva reticulata* at 28 °C increased abundance of epiphytic *E. coli* and *Vibrio parahaemolyticus* within seven days (Vairappan and Susuki, 2000). Validation of the temperature-time combination applied during the drying treatment (a CCP) to verify good manufacturing practice (GMP) may therefore be warranted to ensure microbial safety of seaweed powder. FIOs however, should not be considered indicators of pathogen presence, since pathogen survival does not necessarily mirror that of FIOs (Castro-Ibanez *et. al.,* 2016; Syamaladevi *et. al.,* 2016). Heat treatment parameters should therefore be defined for individual microorganisms.

At the larval rearing stage of the production chain, no microbiological hazards were detected in the seaweed powder, indicating that good hygienic practices (GHP) applied during packaging, distribution and personnel handling enabled safe storage of this product for at least one year. However, culturing of samples without a pre-enrichment step may have failed to detect bacterial cells present in a VBNC state or at very low concentrations (Li *et. al.,* 2014; Wu, 2008). Enterococcaceae, as detected in association with the BSFL, are found in various insects, including flies and mealworm larvae (Grabowski and Klein, 2017; Wynants *et. al.,* 2018b). During production of mealworms, enterococci abundance increased in the substrate, which was interpreted as a product of growth of enterococci in larval digestive tracts followed by excretion in high concentrations (Wynants *et. al.,* 2018b). Starvation of the BSFL on day nine of both trials may have substantially reduced the microbial load present in the larvae gut, and produced a high load of viable enterococci cells in the frass (Osimani *et. al.,* 2018a). Although BSFL possess high levels of gut antibacterials active against Gram-positive bacteria (Vogel *et. al.,* 2018), BSFL have also been shown to exert no suppressive influence on enterococci in their substrate (Choi *et. al.,* 2012; Lalander *et. al.,* 2015). Enterococci remain one of the most abundant bacteria in mealworm larvae at harvesting stage, and possible dominance of enterococci in the BSFL digestive tracts may explain their detection at the same stage, even at low levels.

Environmental contamination with enterococci from feed and containers, and possibly personnel handling, may all have contributed to the natural autochthonous microbes known to be associated with BSFL (Fraqueza and Patarata, 2017; Jeon *et. al.,* 2011; Zheng *et. al.,* 2013). Enterococci concentrations in frass at the point of larvae harvest far exceeded the initial levels in raw feed ingredients, suggesting conditions in the larvae substrate (temperature, pH, moisture levels, unlimited nutrients) during rearing may have encouraged growth of enterococci. The primary route of larvae exposure to microbes potentially hazardous to human consumers further along the feed and food chain is likely to be the feed substrate. Therefore, incoming raw feed materials are potential CCPs, particularly since they were not subject in this feed trial to further sterilising treatment before consumption by the larvae.

Several commercial feed ingredients with which the seaweed powder was mixed introduced *Listeria* spp., the only potentially pathogenic genus of bacteria detected during the feed trial. Incoming raw materials for industrial insect rearing present a major vulnerability to maintaining GHP as they represent potential points of entry for microbial hazards (Fraqueza and Patarata, 2017). It was assumed that these externally acquired feed materials were subject to quality control checks post-processing at their respective production facilities, suggesting that subsequent contact with various environments and handling may have introduced this microbial contamination (Buchanan *et. al.,* 2017). *Listeria* spp. are found throughout the environment, often occurring in animal feed, and are almost ubiquitous in food processing environments, detection of which is used by the food industry as indicative of conditions that might facilitate the presence, growth and persistence of *Listeria monocytogenes* (Korsak and Szuplewska, 2016; Orsi and Wiedmann, 2016). However, the level of *Listeria* spp. contamination of the raw feed materials for BSFL fell well below the estimated > 1000 CFU infective dose for humans required for *L. monocytogenes* (Schmid-Hempel and Frank, 2007). Importantly, *Listeria* spp. were not detected in the larvae or their frass. However, although *Listeria* spp. and *Salmonella* spp. are typically not detected by direct culturing in a range of insects reared for feed and food (Vandeweyer *et. al.,* 2017b), molecular analysis identified *Listeria* spp. in mealworm larvae that plating on selective media had failed to detect (Garofalo *et. al.,* 2017). Physico-chemical changes to the larvae, and the heat treatment applied during processing, removed enterococci from larvae and would have killed any undetected *L. monocytogenes* cells (NicAogain and O'Byrne, 2016).

Screening of the larvae meal, and several other raw pellet ingredients, at the feed pellet production facility revealed that contamination with relatively high levels of enterococci and *Listeria* spp. had occurred during packaging, distribution or storage between stages in the feed production chain. The production of fish meal pellets typically involves a heating stage followed by a cooling stage, and colonisation of the cooling feed by opportunistic bacteria should be highlighted as a potential CCP (Saucier, 2016). However, the levels of enterococci detected in the finished product did not exceed microbiological quality standards for insect PAPs (Reg (EC) 893/2017; EC, 2017a).

Seaweed powder can be successfully incorporated into the diet of BSFL, meeting both fish nutritional requirements (Liland *et. al.,* 2017) and insect PAP hygiene standards. Gauging whether seaweed powder, or any other form of seaweed processed to extend its shelf-life, can be utilised to effectively rear *C. frigida* larvae for commercial exploitation requires laboratory-scale validation in the first instance. The commercial production of Coelopidae is still in the early stages of feasibility assessment, however it has been demonstrated that seaweed can be frozen at -20 °C and then thawed prior to use as *C. frigida* feed substrate without any negative impacts on output of larval biomass, numbers of larvae or developmental rate. Freezing for long-term storage not only extends the shelf life of fresh seaweed which would otherwise rapidly perish, it also retards the inactivation of nutritionally beneficial bioactive compounds, e.g. antioxidants, antimicrobials and prebiotic polysaccharides (Makkar *et. al.,* 2016; Evans and Critchley, 2014).

Long term storage is particularly important for seaweed feed given the seasonal variation in *L. digitata* and *F. serratus* biomass (Bartsch *et. al.,* 2008; Knight and Parke, 1950; Schiener, *et. al.,* 2015; Schmid *et. al.,* 2014). However, storage of fresh seaweed in freezers on a scale sufficient to mass-rear Coelopidae year-round may require excessive storage capacity, whereas removal of ~90 % moisture content from seaweed by high temperature drying can decrease 10 kg of wet biomass to 1 kg of dry biomass (Sudhakar *et. al.,* 2018). Industrial drying of seaweed for animal feed in order to produce powder with a long shelf life is typical, and low drying temperatures of ~40 °C, as utilised in this commercial seaweed-fed BSFL trial, retain beneficial micronutrients (Evans and Critchley, 2014). The temperature at which the commercially available seaweed powder procured for this study was manufactured is unknown, but desiccation may have killed many seaweed-associated microbes, so whether or not powdered *Laminaria* spp. had been combined with powdered *Fucus* spp., microbial deficiency was likely to be the greatest contributory factor in the marked failure of egg-to-first instar development. Inoculation of the seaweed powder with natural seaweed associated bacteria has been shown to enable successful rearing of *C. frigida* larvae from eggs (Cullen *et. al.,* 1987), and would benefit from the fact that seaweed powder is already commercially available

to insect producers, though the feasibility of the approach at an industrial scale is yet to be tested.

The higher larval yield generated by frozen-thawed and finely minced seaweed compared with fresh, finely minced seaweed may indicate more rapid decay of thawed seaweed and thus greater feed surface availability. Coarse grinding (0.8 cm^2) had no such effect, suggesting an optimal feed particle size for frozen-thawed seaweed of at least 0.5 cm^2. However, rearing of *C. frigida* on even coarsely ground seaweed provided clear production advantages to rearing the larvae on intact seaweed, which itself provided no appreciable benefits compared with sterile seaweed. It can be inferred that freezing and mincing seaweed contributed to the release of seaweed cell exudates, which increased food availability for microbial communities and thus the larvae (Egan *et. al.,* 2013). The lower biomass and yield of *C. frigida* reared on intact seaweed may also reflect the slower development noted in the wild where wrack piles decay more gradually than achieved in this study (Dobson, 1974a). The diverse natural bacterial communities expected to be present on the surfaces of the freshly harvested seaweed used in this study (Egan *et. al.,* 2013) were evidently not damaged by freezing at - 20 °C to any extent that disadvantaged the growth or survival of *C. frigida*. However, a laboratory diet of *Laminaria* spp. and *Fucus* spp. produced different Carbon (C) and Nitrogen (N) stable isotope ratios in *C. frigida* adults compared with those reared in the wild (Edward *et. al.,* 2008), suggesting laboratory diets either lack other genera such as red seaweed, or provide a different seaweed associated bacterial assemblage. Therefore, identifying optimal dietary mixtures of seaweed species as Coelopidae feed may prove important in developing mass cultivation of the larvae.

Although the largest number of *C. frigida* larvae were harvested from the high density treatment, the total mass of larvae reared at high density was no better than that produced from the medium density treatment. This could be due to larvae reared at high density reaching third instar stage at a smaller average size due to food scarcity (Agnew *et. al.,* 2002; Leggett *et. al.,* 1996). Alternatively, or additionally, the proportion of the population which had actually attained the prepupae stage of development by day four may have varied between treatments. Food scarcity and thus slower growth and a developmental delay in individuals reared at high density may explain their lower

total mass (Araujo *et. al.,* 2012). Rearing density did not influence mortality from the first instar stage onwards, but testing a wider range of densities may show a more pronounced effect (Couret *et. al.,* 2014; Butlin *et. al.,* 1984), as *C. frigida* yield can decrease as initial rearing density increases (Burnet and Thompson, 1960). Notably, it is unknown at what life stage the larvae in each treatment died during the study, but a recalculation of average density at harvest in the high density treatment based on recorded mortality (0.97 ± 0.05 larvae g^{-1}) still exceeded the 0.8 larvae g^{-1} threshold. The commercial implication of this is that an optimal rearing density of 0.8 larvae g^{-1} of feed from first instar exists when fed frozen-thawed and finely minced seaweed. Exceeding this threshold rearing density provides an appreciable increase in the harvestable yield, but with no concurrent improvement in total harvested mass.

Upscaling production of *C. frigida* will require improved understanding of the role of other environmental parameters in their growth and development. The distribution of *C. frigida* larvae in their feed substrate is modulated directly or indirectly by temperature (Phillips *et. al.,* 1995) or humidity. Temperature can interact with food availability and larval density to affect developmental rates between dipteran life stages (Couret *et. al.,* 2014), and insects are also sensitive to humidity, which can affect egg eclosion success and development times (Addo-Bediako *et. al.,* 2001; Holmes *et. al.,* 2012). Both parameters may modulate larval biomass, yield, development rate and survival responses to the diet and rearing density identified as optimal in this study. In addition, there is a paucity of data relating to environmental pathogens potentially acquired by *C. frigida* larvae from seaweed (see Chapter 2). *C. frigida* larvae reared on *F. serratus* and *L. digitata* can accumulate arsenic (As) and cadmium (Cd) at concentrations exceeding levels in the substrates and current EU limits (Reg (EC) 32/2002; EC, 2002) (Biancarosa *et. al.,* 2018a). An evaluation of the safety of Coelopidae as animal feed is therefore warranted, subsequent to a more comprehensive production trial of this species.

3.6 Conclusions

Fish feed pellets formulated from seaweed-fed BSFL are not likely to be sources of important foodborne pathogens to human consumers at the end of the food chain.

However, other pathogenic bacteria may have been present in this novel feed and food chain which were not identified in this study. The persistent detection of *Listeria* spp. reflects the widespread occurrence of these potentially pathogenic bacteria in food and feed production environments and the importance of GHP. Crucially, however, bacterial contamination of finished larvae meal and pellets did not originate specifically from either the seaweed, or from the larvae reared on the seaweed, indicating that processing techniques (desiccation and heat) provided sufficient sterilisation of products. As the seaweed and insect farming industries mature, CCPs will emerge which are specific to each insect species, their substrate, the life stage at harvest and processing methods. As an alternative aquafeed to BSFL, the exceptional growth performance of *C. frigida* larvae on frozen- thawed seaweed demonstrates that seaweed storage by freezing does not impair nutritional quality of the seaweed microbiota for the larvae. Output of larval biomass does not benefit from increasing the rearing density from first instars beyond 0.8 larvae g^{-1} of feed. However, the optimal density on the optimal substrate identified in this study may vary depending on rearing temperature and humidity, which further research has yet to optimise.

Chapter 4 | Microbiological safety of seaweed as feed for black soldier fly (*Hermetia illucens*) larvae.

4.1 Abstract

Black soldier fly larvae (BSFL; *Hermetia illucens*) protein as a sustainable ingredient in aquafeed has been permitted in the EU since 2017. Dietary inclusion of seaweed can enrich BSFL in marine omega-3 fatty acids necessary for the health of marine carnivorous fish and of their human consumers. Seaweed is an abundant and renewable animal feed permitted in the EU, but harvested from coastal environments it is susceptible to colonisation by human pathogenic bacteria either naturally present in seawater or emanating from municipal or agricultural sources of faecal contamination. Yet there are no bacteriological water quality standards for seaweed harvesting sites, or for seaweed meal products. Additionally, the industry practice of low temperature seaweed drying for retention of nutritional properties may benefit bacterial survival in dried seaweed meal. The hygiene of insect feed is a key determinant of their safety in the human food chain, yet current generic bacteriological criteria for farmed insects may not reflect seaweed-specific bacteriological hazards. The risk of transference of *E. coli*, chromosomally *lux*-marked (Tn5 *lux*CDABE) *E. coli* O157:H7, *Listeria monocytogenes* and *Vibrio parahaemolyticus* along the seaweed-fed BSFL production chain was evaluated by simulating wastewater contamination of pre-harvest seaweed, which was subsequently processed and fed to BSFL. Attachment by all four bacteria to the seaweed proved resistant to removal by subsequent washing of the seaweed by hand. Low concentrations of *E. coli* and *E. coli* O157:H7 were present in stored dried seaweed powder despite die-off to below the level of detection, or induction into a viable but nonculturable (VBNC) state, in the seaweed following drying at 50 °C. Metabolically active VBNC *E. coli* O157:H7 cells were also detected in the stored powder following 60 °C drying. *V. parahaemolyticus* were below the level of detection in stored seaweed after drying at ≥ 50 °C, but *L. monocytogenes* remained detectable, and grew, in seaweed dried at ≤ 60 °C. BSFL were contaminated by the four bacteria introduced via their feed. BSFL gut antimicrobial activity or direct digestion reduced larval loads of commensal and pathogenic *E. coli* only. Seaweed washing, drying and storage conditions, and powdered seaweed insect feed represent critical control points during production where good hygiene and manufacturing processes could provide targeted control of pathogens. Significant reductions in BSFL bacterial loads during post-harvest

processing into feed constituents would be required for this novel ingredient to be an acceptable aquafeed component.

4.2 Introduction

Seaweed meal is a recognised animal feed substrate in the EU (Reg (EC) 68/2013; EC, 2013a), and can provide a source of proteins, polyunsaturated fatty acids, minerals, vitamins and antioxidants with proven nutritional and bioactive value as a dietary supplement for livestock, aquaculture species and, most recently, insects (Rajauria, 2015; Liland *et. al.,* 2017). The concept of insect protein as a sustainable animal feed ingredient has garnered increasing acceptance across Europe, and is now permitted in aquafeed within the EU (Reg (EC) 893/2017; EC, 2017a). Recent innovative efforts to combine these two ingredients in feed for farmed carnivorous fish has seen the mass production of seaweed-fed black soldier fly larvae (BSFL), *Hermetia illucens* (L.) (Diptera: Stratiomyidae) (Belghit *et. al.,* 2018). Seaweed-fed larvae became enriched with omega-3 fatty acids but also bioaccumulated heavy metals from the seaweed, highlighting the fact that seaweed can be a key source of undesirable substances in mass-produced insects (Belghit *et. al.,* 2018; Van Raamsdonk *et. al.,* 2017). A growing number of studies have isolated human pathogenic bacteria, including *Vibrio* spp. which occur naturally in brackish and estuarine environments, and allochthonous extra-enteric shiga toxin-producing *E. coli* (STEC), from seaweed (Elbashir *et. al.,* 2018; Mahmud *et. al.,* 2007 and 2008; Ishii *et. al.,* 2006). Such pathogens have been associated with gastroentiritis outbreaks, and infections can prove fatal in vulnerable individuals (Parveen and Tamplin, 2013; Byrne *et. al.,* 2015). Critical control points (CCPs) during the production of seaweed-fed BSFL that facilitate contamination, persistence or growth of microbial hazards must be identified in order to guarantee safety of this novel animal feed if it is to enter the human food chain (Reg (EC) 183/2005; EC, 2005).

In Europe, seaweed for animal feed, including for insects, is typically wild harvested from coastal marine waters (Makkar *et. al.,* 2016), although beach-cast seaweed represents a potential additional source of this organic material. Yet, the coastal zone often functions as a downstream receiving water body for terrestrial sources of extra-enteric human pathogenic bacteria and faecal indicator organisms (FIOs), as well as

being a source of autochthonous microbes (e.g. *Vibrio* spp.) which pose a risk to human health (Cho *et. al.,* 2016; Elbashir *et. al.,* 2018). However, only a handful of studies have examined either the potential for seaweed in the natural environment to be colonised by, and function as, a reservoir for such bacteria (Ishii *et. al.,* 2006; Chun *et. al.,* 2017; Mahmud *et. al.,* 2007 and 2008), or have addressed control of microbial risks posed by seaweed through processing interventions, e.g. washing and drying (del Olmo *et. al.,* 2018; Vairappan and Suzuki, 2000; Pawlik *et. al.,* 2003; Hyun *et. al.,* 2018). There are no microbiological standards for seaweed meal in the EU, and those for insect processed animal proteins (PAPs) are limited to maximum levels of *Clostridium perfringens*, *Salmonella* spp. and Enterobacteriaceae (Reg (EC) 893/2017; EC, 2017a). Thus the full range of potential microbiological hazards associated with seaweed entering the feed and food chain are not necessarily controlled by existing industrial practices, or accounted for by current feed hygiene regulations.

Typical post-harvest processing of seaweed for animal feed involves (i) washing to remove visible epiphytic flora and fauna; (ii) hot air drying to reduce bulk and water activity (a_w) to < 0.5 to prevent bacterial growth and inhibit degrading biochemical changes in order to enable long term storage at ambient temperature of an otherwise highly perishable material; and (iii) milling, packaging and storage at room temperature for up to one year (Bonazzi and Dumoulin, 2011; Radulovich *et. al.,* 2015; McHugh, 2003). Water activity (a unit-less parameter ranging from 0 (no water) to 1 (pure water)) essentially quantifies the relative humidity of a feed or food matrix, and is one of the key predictors of microbial survival in feed or food (Pittia and Antonello, 2016; Roos *et. al.,* 2018). Drying (and subsequent storage of dried seaweed) can adversely affect the nutritional content of seaweed by causing chemical changes such as protein denaturation and lipid oxidation, and the magnitude of such modifications is positively correlated with drying temperature (Stevant *et. al.,* 2018; Lage-Yusty *et. al.,* 2014). In addition, antioxidant activity in brown seaweeds for example has been shown to reduce with increasing drying temperature from 25 °C to 75 °C (Moreira *et. al.,* 2016; Gupta *et. al.,* 2011). However, trade-offs are incurred, for example to sufficiently desiccate brown seaweed at 25 °C required an extended drying time which led to greater nutritional losses than did drying at 35 – 40 °C which required a shorter drying time (Gupta *et. al.,*

2011): as a_w decreases, the rate of water transfer slows and the drying period must be prolonged (Bonazzi and Dumoulin, 2011).

Recent interest in using seaweed as a minimally-processed ready-to-eat dried food, has promoted food safety concerns. Assessments of the hygiene efficacy of washing and air drying red, green and brown seaweeds at 46 - 48 °C demonstrated a failure to eradicate Enterobacteriaceae or coliforms (del Olmo *et. al.,* 2018). Other studies have demonstrated that washing seaweed can fail to remove *V. parahaemolyticus*, whilst *E. coli* can continue replicating on seaweed during the desiccation and storage process (Mahmud *et. al.,* 2008; Vairappan and Suzuki, 2000; Pawlik *et. al.,* 2003). Although standardised processing methods in the feed and food industries are key to uniformity of product quality and guaranteed feed safety, such a system is currently lacking in the seaweed industry. The potential for growth and inactivation of bacterial contaminants in seaweed meal is determined by the intrinsic nature of the relationship between the seaweed and the microbe; however, the environmental conditions not only during manufacture of seaweed into feed, but also via subsequent biological processing by BSFL will also be important for the persistence of bacteria (Ross, 2008).

Microbiological hazards that could be associated with seaweed-fed BSFL may arise from passive contamination or active accumulation of pathogens or FIOs by the larvae from their feed substrate, combined with the autochthonous microbiota of BSFL and bacterial transference from the feed processing environment (FPE) (Van Raamsdonk *et. al.,* 2017). BSFL feeding and activity can reduce concentrations of *E. coli* and *E. coli* O157:H7 in animal manures (Liu *et. al.,* 2008; Erickson *et. al.,* 2004), either due to bacterial inactivation following larval digestion, or via antimicrobial action in the gastro-intestinal tract (GIT) (Jeon *et. al.,* 2011; De Smet *et. al.,* 2018; Choi *et. al.,* 2012; Park *et. al.,* 2014). During trial production of seaweed-fed BSFL, raw feed materials were contaminated by *Listeria* spp. during handling at, distribution between, or storage in various FPEs, however the low levels of enterococci associated with the larvae at harvest were effectively eradicated by larval protein and lipid extraction processes (Swinscoe *et. al.,* 2019). Exploiting insect protein as animal feed is still in its infancy, but good manufacturing and hygiene processes specific to each insect species, the feed

substrate and the life stage at harvest need considerable development as microbiological hazards and CCPs emerge (Van Raamsdonk *et. al.,* 2017).

The aims of this study therefore, were to (i) determine the capacity of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* to survive in seawater and attach to a combined mixture of submerged brown, red and green seaweeds in a simulation of intertidal seaweed exposure to a wastewater pollution event; (ii) evaluate the effect of typical industrial processing practices (washing, drying and storage) on the survival of these bacteria attached to these seaweeds; (iii) assess the survival dynamics of these bacterial contaminants when introduced to BSFL via a feed supplement of powdered seaweed, and (iv) identify CCPs during production of seaweed feed and its application as a feed supplement for BSFL mass rearing. It was hypothesised that the different bacterial species would attach to fresh seaweed but that their idiosyncratic survival characteristics in the environment would be reflected in distinct patterns of persistence and die-off in seaweed, BSFL feed and BSFL in response to changing physio-chemical and biotic conditions in the simulated intertidal and feed production environments.

4.3 Materials and methods

4.3.1 Microbial safety of processed seaweed (Experiment 1)

A model system of postharvest industrial processing of seaweed was designed, which involved sequential stages of washing, drying, milling and storage. Sampling for microbiological quality was conducted at key stages of the process.

4.3.1.1 Seaweed material

Living, attached intertidal seaweeds of the species *Laminaria digitata* (Hudson) (Phaeophyceae), *Fucus serratus* (L.) (Phaeophyceae), *Palmaria palmata* (L.) (Rhodophyta) and *Ulva lactuca* (L.) (Chlorophyta), together with seawater from the surf zone, were collected at low tide from Elie, Fife, Scotland (56°11.191'N, 2°48.679'W). *Ascophyllum nodosum* (L.) (Phaeophyceae) was gathered from Ganavan Bay, Oban, Scotland (56°26'05.1'N, 5°28'51.3'W) a day later. Seaweed was rinsed in tap water for 3 mins to remove sand and epiphytic flora and fauna. All seaweed and seawater samples were stored at 4 °C and utilised within 24 h. To enumerate the background *E. coli* and

total heterotrophic bacteria (THB) associated with seaweed, 500 g of each species was individually homogenised for 3 mins using a hand blender (Bosch MSM6700GB). Four 10 g replicate samples of the homogenate of each seaweed species were then added to 10 ml of sterile seawater (sterilised by autoclaving) and vortexed for 1 minute. The supernatant was serially diluted using sterile seawater and 50 µl plated onto Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid) to quantify presumptive *E. coli*, or R2A agar (CM0906, Oxoid) to quantify THB. MLGA plates were inverted and incubated at 37 °C for 24 h and R2A plates at 18 °C for 48 h. Seawater samples (*n* = 4) were shaken and 100 ml vacuum-filtrated through a 0.45 µm cellulose nitrate membrane (Sartorius, Goettingen, Germany). The membrane was transferred to MLGA or R2A plates which were incubated as described above. Representative seaweed samples were dried at 80 °C for 24 h such that bacterial concentrations could be expressed as CFU (colony forming units) g^{-1} dry matter content, and microbiological concentrations in seawater were expressed as CFU 100 ml^{-1}.

4.3.1.2 Inoculum preparation

Three bacterial pathogens were used in this study: a non- toxigenic serotype of *E. coli* O157:H7, which had been chromosomally *lux*-marked (Tn5 *lux*CDABE) (Ritchie *et. al.* 2003); *Listeria monocytogenes* (1706/1/2a-3a) isolated from a mushroom production facility; and *Vibrio parahaemolyticus* (V05/002) isolated from a seafood poisoning outbreak. The lux biomarker of the *E. coli* O157:H7 strain produces bioluminescence, the expression of which is a function of cellular energy and therefore cellular metabolism. Bioluminescence therefore provides a proxy measure of the metabolic activity of the *E. coli* O157:H7 population (Quilliam *et. al.,* 2012). An environmental isolate of *E. coli* was isolated from surfzone seawater adjacent to a wastewater outfall at Portobello Beach, Edinburgh (55°57’25.0’N, 3°06’57.8’W).

To produce bacterial cells tolerant to seawater for use in experimental microcosms, a sample of each bacterial species was added to sterile seawater for 3 h at 10 °C. A 100 ml sample (*n* = 4) was membrane filtered and placed on the relevant selective agar plates. Commensal *E. coli* was grown on MLGA; *E. coli* O157:H7 was grown on Sorbitol MacConkey Agar (SMAC) (CM0813, Oxoid) supplemented with cefixime and potassium tellurite (CT) (SR0172, Oxoid); *L. monocytogenes* was grown on Listeria Selective Agar

(Oxford Formulation) (CM0856, Oxoid) supplemented with Modified Listeria Selective Supplement (Oxford) (SR0206, Oxoid) and *V. parahaemolyticus* grown on Cholera Medium TCBS (CM0333, Oxoid). Following incubation at 37 °C for 24 h, colonies of each species were harvested and cultured individually in Luria-Bertani (LB) broth (CM1018, Oxoid), or Alkaline Peptone Water (APW) (CM1028, Oxoid) in the case of *V. parahaemolyticus*, at 37 °C, at 100 rev min^{-1}, for 18 h. Cells were washed three times in Phosphate Buffered Saline (PBS), resuspended in PBS, and stored in a glycerol- PBS mixture at -80 °C prior to use.

4.3.1.3 Simulated microbiological contamination of pre-harvested seaweed

Fresh samples of *L. digitata*, *F. serratus*, *A. nodosum*, *P. palmata* and *U. lactuca* were combined in equal quantities (40 g each) in 500 ml glass jars (*n* = 32). *L. digitata*, *F. serratus* and *A. nodosum* were comprised of approximately 10 % stipe and 90 % frond, whereas *P. palmata* and *U. lactuca* consisted of 100 % frond. The stipes and fronds of *L. digitata*, *F. serratus* and *A. nodosum* were cut into 5 cm lengths to enable accurate weighing of each seaweed species into replicate batches. Eight replicate jars were used for each temperature (room temperature, 40 °C, 50 °C and 60 °C) of which four replicate jars were inoculated with bacterial pathogens, and four non-inoculated jars were used to assess pH and a_w of seaweed.

The frozen stock culture of seawater tolerant cells of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* were revived overnight in LB broth or APW and the resulting bacterial cells washed in PBS (section 4.2.3). Cells of each of the four bacteria resuspended in PBS were added to 1600 ml of non-sterile seawater. The resulting pathogen-seawater cocktail, intended for bacterial inoculation of the seaweed, was mixed to ensure even distribution of cells. The concentration of each bacterial species in this cocktail was determined retrospectively by serial dilution in PBS and plating 50 µl onto selective media as described above (*n* = 4 for each bacterial species) (section 4.2.2). The concentrations of each bacteria in the pathogen-seawater cocktail were as follows: *E. coli* = 6.32 x 10^9 CFU ml^{-1}; *E. coli* O157:H7 = 7.0 x 10^9 CFU ml^{-1}; *L. monocytogenes* = 5.9 x 10^9 CFU ml^{-1}; *V. parahaemolyticus* = 6.8 x 10^9 CFU ml^{-1}. Luminescence (relative light units (RLU)) of *E. coli* O157:H7 in the pathogen-seawater cocktail was immediately measured using a SystemSURE 18172 luminometer (Hygiena

Int., Watford, UK) to quantify relative metabolic activity of the *E. coli* O157:H7 population (4.7 $\log_{10}$ RLU ml^{-1}). Aliquots of 200 ml of the pathogen-seawater cocktail were poured into each of the glass jars containing the seaweed mixtures intended for inoculation (*n* = 16), to completely submerge and inoculate the seaweed. Aliquots of 200 ml of non-inoculated non-sterile seawater was poured into each of the jars (*n* = 16) used for pH and a_w measurements. The screw lids were closed and all jars secured within a temperature controlled rotating incubator for 24 h at 100 revs min^{-1} at 20.5 °C ± 3 °C. This was intended to simulate contamination of intertidal seaweed with FIO and pathogenic bacteria due to a wastewater pollution event.

Determining bacterial concentrations of *E. coli* and the three pathogens in the seawater and attached to the seaweed after 24 h in the rotating incubators was intended to enable assessment of the capacity for FIO and pathogen attachment to seaweed freshly harvested from simulated intertidal waters contaminated with FIOs and pathogens 24 h previously. The luminescence of *E. coli* O157:H7 in the seawater in the inoculated jars was measured, and concentrations of commensal *E. coli* and the three pathogenic bacteria remaining in the seawater were enumerated by serial dilution in PBS and plating 50 µl of each sample onto each of the four selective media (section 4.2.2). The pathogen-seawater cocktail was drained from each batch of seaweed, using a sieve (mesh diameter 1 mm) to strain the seaweed. Bacteria attached to the seaweed were then quantified by removing a 10 g seaweed sample from each of the inoculated jars, homogenising the sample for 3 mins using a hand blender, and vortexing the homogenate in 10 ml of PBS for 1 minute. The luminescence of *E. coli* O157:H7 in the supernatant was immediately measured, and the concentration of all four bacteria suspended in the supernatant was quantified by serial dilution in sterile seawater and 50 µl plated onto selective media. In addition, 5 g of seaweed was removed from each of the non-inoculated jars (*n* = 16), and vortexed for 1 min in 5 ml distilled water to determine the pH. The pH was measured using an HI 2550 Multiparameter bench meter (HANNA instruments, Bedfordshire, UK).

4.3.1.4 Simulated post-harvest seaweed processing

The first stage of industrial post-harvest processing of seaweed involves a washing step after harvesting in order to remove sand and debris. To simulate this, the seaweed from

each jar was transferred into a sieve (mesh diameter 1 mm) and cold tap water was run continuously over it for 1 min. Each seaweed sample was gently stirred using a sterile metal spatula in order to maintain through flow of water through the sieve. The concentration of *E. coli*, *L. monocytogenes* and *V. parahaemoyticus*, and the concentration and luminescence of *E. coli* O157:H7 cells still attached to the seaweed post-washing were quantified by homogenisation and plating (section 4.2.4). The pH of post-washed batches of seaweed from the non-inoculated groups was measured as described above (section 4.2.4). Following the washing step, a 10 g sample of seaweed was taken from each replicate jar of the non-inoculated groups, and double-bagged in a labelled plastic zip-lock bag for water activity (a_w) measurement. Each seaweed sample was finely chopped to approximately 5 mm^2 and the a_w measured using an AquaLab CX-2, calibrated with a saturated solution of potassium sulphate, with the cooled mirror dew point technique in order to provide readings of ± 0.005 accuracy according to the AquaLab Operator's Manual.

The remaining washed seaweed was immediately transferred to individual foil trays measuring 20 cm (l) x 10 cm (w) x 5 cm (d), and spread out evenly to an approximate depth of 4 cm. A temperature logger was placed in the centre of the seaweed mixture of each replicate tray, and 8 trays (4 inoculated replicates and 4 non-inoculated replicates) were placed in a drying oven at either 40 °C, 50 °C or 60 °C, to simulate the lower end of the range employed in hot air convection or oven drying by the seaweed feed industry (Gupta *et. al.,* 2011). The ovens used in the study were thermostatically controlled electric bench top models (Griffin and George Ltd., Middlesex). During drying, the actual temperatures achieved were 41.8 °C ± 0.03, 49.1 °C ± 0.14, and 64.2 °C ± 0.21. In addition, eight uncovered trays of seaweed were placed on the bench top within the same laboratory to provide a "room temperature" treatment (22.7 °C ± 0.04).

Relative luminescence of *E. coli* O157:H7, and bacterial concentrations on seaweed inoculated with the pathogen-seawater cocktail were enumerated during the drying process at 24, 72, 120 and 168 h. Determination of the concentrations of the four bacteria, and of *E. coli* O157:H7 RLU, on the drying seaweed followed the methods described above (section 4.2.4) with the exception of seaweed dried at 50 °C and 60 °C from 72 h onwards which was sufficiently desiccated to be ground to a fine powder

using a pestle and mortar. To determine *E. coli* O157:H7 RLU and bacterial concentrations in this seaweed powder, 2 g of powder was added to 20 ml of PBS (*n* = 4), the homogenate vortexed for 1 min, and bacterial concentration and RLU enumerated as described above (section 4.2.4). The pH of seaweed in the non-inoculated treatments were also recorded during drying at 24, 72, 120 and 168 h. After 72 h and 168 h drying, a 10 g sample of non-inoculated seaweed was taken from each of the non-inoculated treatments for the determination of a_w, following the method described above, with the exception of samples of seaweed dried at 50 °C and 60 °C, which were sufficiently dry to grind to powder prior to a_w analysis.

After 168 h drying, moisture loss from seaweed that had been dried at room temperature and 40 °C was insufficient to enable the seaweed to be ground to a powder prior to storage. Each seaweed mix from these groups was therefore individually homogenised with no added liquid for 3 min using a hand blender to approximately 5 mm^2 fractions. Seaweed dried at 50 °C and 60 °C was sufficiently desiccated to be ground to a fine powder (approximately 0.5 – 1 mm^2) using a pestle and mortar. Seaweed from all trays were transferred to individual enclosed plastic boxes and stored at room temperature. After 72 h storage, bacterial concentrations in seaweed dried at room temperature and 40 °C were quantified as described above (section 4.2.4). Seaweed that had been dried at 50 °C and 60 °C was already in powder form and therefore required no further homogenisation: 2 g of powder was added to 20 ml of PBS, the homogenate vortexed for 1 min, and bacterial concentration enumerated as described above. *E. coli* O157:H7 RLU was measured for all temperature treatments (section 4.2.4). The pH of seaweed in the non-inoculated treatments was also measured again.

4.3.2 Microbial safety of seaweed as BSFL feed (Experiment 2)

A simulation of mass-rearing of BSFL on feed supplemented with pathogen-contaminated seaweed powder was undertaken. Larvae and the feed substrate were sampled throughout the rearing period up to the point of larvae harvest to assess both the microbial load of the feed and the hygienic status of the larvae.

4.3.2.1 Preparation of BSF colonies

Two colonies of BSF were established from larvae sourced online (livefoodsbypost.co.uk and InternetReptile.com) in insect rearing tents each measuring 75 x 75 x 115 cm (BugDorm-2400, bugdorm.com), in a controlled environment facility (CEF) (Reftech B.V., Netherlands) at 30 °C ± 2 °C, a relative humidity of 70 % and a photoperiod of 12 h. One tent contained two 5 L plastic boxes (Addis Ltd., UK) each containing approximately 1000 larvae, which were reared on a 15:3:1 mixture of wheat bran (Harbro Ltd., Aberdeenshire), whey protein (Holland and Barrett International, UK) and fruit and vegetable waste. Feed substrate was supplemented every 2 days to a depth of approximately 12 cm and 200 ml of water was added every 2 days. Holes in the base of the containers enabled drainage of excess liquid to prevent waterlogging and anoxic conditions developing in the feed substrate. Within the tent, cardboard boxes containing shredded newspaper provided dark sheltered conditions for pupation. Once adult flies emerged, sliced fruit was placed on the surface of the feed substrate and water was sprayed into the tent hourly during the day. Corrugated cardboard strips were laid across the feed container above the level of the feed to provide dry crevices in which the female flies laid their eggs. As soon as eggs were observed in a cardboard strip, the strip was transferred to the other insect tent and suspended above a tray containing feed substrate comprised of the same ingredients, in the same proportions, as described above. Feed substrate was supplemented every 3 days to approximately 7 cm and 200 ml of water was added every 2 days. When the larvae hatched they would fall from the cardboard strip into the feed, and were harvested at approximately 1 week old, and used in the experiments.

4.3.2.2 Preparation of seaweed powder and inoculation procedure

Seaweed and seawater was collected at the same time as above (section 4.2.2), and stored at 4 °C prior to use. Background microbiological and physio-chemical status of the seaweeds and seawater were therefore the same as established previously. The seaweeds (stipes and fronds) were separated by species, washed clean of visible epiphytic flora and fauna using tap water, and oven dried in single layers in foil trays (22 x 22 x 6 cm) at 50 °C for 72 h. Each species of dried seaweed was then ground into a fine powder using a pestle and mortar to pass through a sieve of 500 µm mesh diameter.

Composite 400 g mixtures each comprised of 80 g of each seaweed species (*L. digitata, F. serratus, A. nodosum, P. palmata and U. lactuca*), were placed in three separate stomacher bags. The seaweed powder in two bags was inoculated with a cocktail of seawater tolerant cells of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* produced as described above (section 4.2.3). The initial concentration of each bacterial species in the 3 L pathogen-seawater cocktail was: *E. coli* (6.7 x 10^9 CFU ml^{-1}), *E. coli* O157:H7 (7.15 x 10^9 CFU ml^{-1}), *L. monocytogenes* (7.35 x 10^9 CFU ml^{-1}) and *V. parahaemolyticus* (6.4 x 10^9 CFU ml^{-1}). Of the 3 L pathogen-seawater cocktail, 1.5 L was added to each of two of the stomacher bags and the contents of each agitated by hand for 5 mins to ensure thorough mixing. The remaining 400 g of seaweed powder was mixed with 1.5 L of non-inoculated seawater following the method described above.

4.3.2.3 Simulated mass-production of BSFL reared on seaweed supplement

Approximately one week old larvae (*n* = ~700) (mean weight per larvae = 0.0807 g ± 0.004) were removed from the rearing substrate and placed in two empty 5 L plastic boxes (with paper towelling secured over a hole (10 x 10 cm) in the lid to enable gas exchange) for 24 h to allow the larvae to purge their digestive tracts. Twelve 5 L plastic boxes (Addis Ltd., UK) were established in the CEF (29.5 °C ± 0.08), each containing 900 g of feed substrate (765 g wheat bran and 135 g whey protein). Seaweed powder inoculated with the seawater-pathogen cocktail was added to eight replicate boxes of feed substrate (100 g per box). The remaining four boxes of feed received 100 g each of non-inoculated seaweed powder. To each box was added 1.6 L of tap water, and the feed mixture stirred for 5 mins to ensure thorough mixing. Larvae were added to four of the feed boxes (*n* = 80 to each box) containing inoculated seaweed powder. No larvae were added to the remaining four boxes containing inoculated seaweed powder, which represented the control. Larvae (*n* = 80) were added to each of the four boxes containing non-inoculated seaweed powder. A temperature logger was placed in the centre of the feed within each box containing larvae and non-inoculated seaweed powder, and these replicates were used to provide temperature and pH measurements. Lids with paper towelling secured over a hole (10 x 10 cm) in the lid to enable gas exchange were used to seal all of the boxes, which were then placed at equal height in

the CEF. Feed was not replenished during the experiment, although 300 ml of tap water was added to every box (inoculated and non-inoculated groups) on day 3 to maintain feed moisture levels.

Sampling of larvae and substrate began at 24 h, and continued daily for 8 days, when the majority of larvae had become pre-pupae. Larvae from the inoculated substrate were sampled by removing a scoop of substrate (~100 g) with a metal ladle from each box, removing the first three larvae observed in that material, and returning the substrate to the box. Sterile forceps were used to remove the larvae, which were then anaesthetised through 10 s exposure to CO_2. Visibly attached feed and frass were removed from the larvae exoskeletons using forceps, and the combined weight of the three larvae was recorded. For each sample three larvae were homogenised in 1 ml PBS in a 1.5 ml Eppendorf tube using a micro pestle (Anachem Ltd., Bedfordshire, UK), then transferred to a 15 ml Falcon tube (SARSTEDT, Aktlengesellschaft & Co.) and a further 1 ml PBS added. The homogenate was vortexed for 1 min, *E. coli* O157:H7 RLU measured and bacterial concentration enumerated by plating 20 µl onto selective media as described above (section 4.2.4). Inoculated substrate was sampled from the boxes by removing a scoop of substrate (~100 g) with a metal ladle from each box, transferring 10 g of material to a 50 ml Falcon tube containing 10 ml PBS, and returning the remaining substrate to the box. The homogenate was vortexed for 1 min, *E. coli* O157:H7 RLU recorded and bacterial concentration enumerated by plating 20 µl onto selective media as described above (section 4.2.4). To measure pH, substrate in the non-inoculated boxes was sampled as described above (section 4.2.4). Representative feed substrate samples were dried at 80 °C for 24 h such that bacterial concentrations could be expressed as CFU (colony forming units) g^{-1} dry matter content, and bacterial concentrations in larvae were expressed as CFU $larvae^{-1}$.

4.3.3 Statistical analyses

4.3.3.1 Microbial safety of processed seaweed

Friedman's ANOVAs with pairwise comparisons or step-down follow-up analysis were used to compare water activity (a_w) within each treatment, and Kruskal-Wallis analysis examined differences in a_w between treatments at each sampling stage. One-way

ANOVAs were used to determine the survival capacity of each bacteria in seawater, the attachment efficiency of each bacteria to submerged seaweed, differences between bacterial levels in seawater and seaweed, and the metabolic activity of *E. coli* O157:H7 in seawater and attached to seaweed, after 24 h. Tukey post hoc testing was applied to *E. coli* and *V. parahaemolyticus* $\log_{10}$ CFU data. However Levene's tests indicated that *E. coli* O157:H7 and *L. monocytogenes* $\log_{10}$ CFU data violated the assumption of homogeneity of variances, thus Games-Howell post-hoc testing was applied. Changes in concentrations of each bacteria between initial levels in the pathogen-seawater cocktail and concentrations remaining in seawater and attached to seaweed combined after 24 h were examined using independent t-tests. The effect of washing seaweed on bacterial attachment and luminescence of *E. coli* O157:H7 was tested using paired t-tests. The effect of duration of drying at a given temperature and of storage on bacterial concentrations and *E. coli* O157:H7 RLU were tested using Friedman's ANOVA as the data were not normally distributed despite log transformation, followed by pairwise comparisons with adjusted *p*-values or step-down follow-up analysis. Luminescence data for *E. coli* O157:H7 during each drying treatment were not normally distributed despite log transformation, therefore Friedman's ANOVAs were applied to drying seaweed, followed by pairwise comparisons or step-down follow-up analysis. Differences between temperature treatments in bacterial concentrations and *E. coli* O157:H7 RLU on seaweed at each sampling stage during drying were tested using Kruskal-Wallis analysis, with pairwise comparisons or step-down follow-up analysis. Wilcoxon signed-rank tests were applied to test changes in luminescence following 72 h storage of seaweed. A Mauchly's test following a split-plot ANOVA to examine changes in seaweed pH between and within treatments indicated violation of the assumption of sphericity, therefore Greenhouse-Geisser tests were used.

4.3.3.2 Microbial safety of seaweed as BSFL feed

Bacterial concentrations associated with larvae, their substrate and the larvae-free control substrate over time were analysed using split-plot ANOVAs, followed by Bonferroni *post hoc* tests. *E. coli* O157:H7 luminescence data were tested using Friedman's ANOVA and pairwise comparisons or step-down follow-up analysis. Changes in pH of the non-inoculated feed were tested with a repeated measures ANOVA with

Bonferroni *post hoc* testing. All analyses were conducted using SPSS 21.0 software (SPSS Inc. Chicago, IL, USA).

4.4 Results

4.4.1 Background microbiological status of seaweed and seawater

E. coli was not detected on the freshly harvested seaweed utilised in both Experiments 1 and 2, and was present at a very low concentration (< 10 CFU 100 ml^{-1}) in the seawater from which the seaweed was harvested. Total heterotrophic bacteria were present in low abundance on all species of seaweed and in seawater, the highest concentrations being detected on *L. digitata* and in seawater (Table 4.1).

Table 4.1. Concentrations of background total heterotrophic bacteria (THB) associated with seaweed and seawater samples used in both Experiments 1 and 2. Values represent the mean (*n* = 4) ± SE.

	THB (CFU g^{-1} or 100 ml^{-1})
A. nodosum	11.5 ± 3.3
F. serratus	26.3 ± 19.7
L. digitata	126.5 ± 23.0
P. palmata	< 10
U. lactuca	30.5 ± 9.7
Seawater	175.3 ± 33.5

4.4.2 Microbial safety of processed seaweed

The a_w of seaweed dried at 50 °C and 60 °C was significantly lower than that of seaweed dried at room temperature and at 40 °C after 72 h ($P < 0.05$) and after 168 h ($P < 0.05$) (Fig. 4.1). However the a_w of seaweed dried at room temperature and at 40 °C did not significantly change during drying (Fig. 4.1).

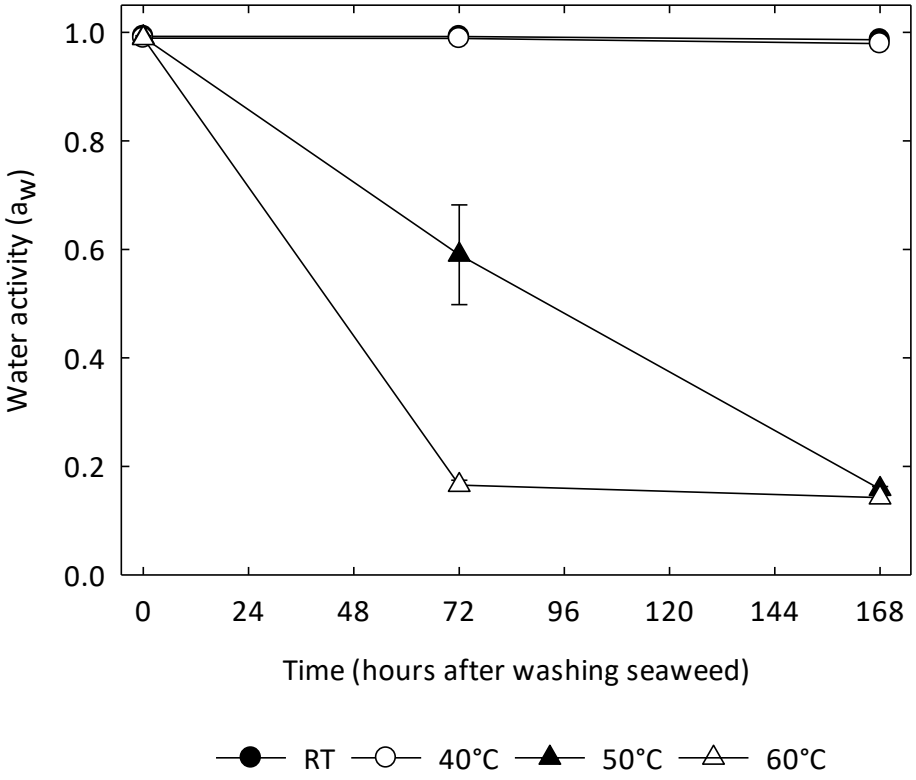


Figure 4.1. Water activity (a_w) in seaweed after washing and drying at RT (room temperature), 40 °C, 50 °C and 60 °C for 72 h and 168 h. Data points are the mean of four replicates ± SE.

After 24 h in a rotating incubator at room temperature (20.5 °C ± 3 °C), concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the seawater had fallen significantly by ~2 $\log_{10}$ CFU ($P < 0.001$ in all cases) (Table 4.2). The level of *E. coli* attachment to seaweed exceeded that of the pathogen-seawater cocktail and of the seawater after 24 h ($P < 0.001$ in both cases) (Table 4.2). *E. coli* O157:H7 colonisation of seaweed was ~2 $\log_{10}$ CFU greater than in the surrounding seawater ($P < 0.001$), but was a similar concentration to that in the pathogen-seawater cocktail (Table 4.2). The concentration of *L. monocytogenes* attached to seaweed did not differ significantly from that in the surrounding seawater or in the pathogen-seawater cocktail. *V. parahaemolyticus* attached to seaweed at a greater concentration (~1 $\log_{10}$ CFU g^{-1}) than remained in the seawater ($P < 0.01$), but colonised seaweed at lower concentrations than had been present in the pathogen-seawater cocktail ($P < 0.001$)

(Table 4.2). The metabolic activity of *E. coli* O157:H7 in seawater fell significantly from 4.7 ± 0.007 in the pathogen-seawater cocktail to 2.83 ± 0.1 over 24 h ($P < 0.001$) and luminescence of *E. coli* O157:H7 attached to seaweed (3.01 ± 0.1) was less than that in the pathogen-seawater cocktail ($P < 0.001$). However, *E. coli* O157:H7 metabolic activity did not differ between seawater and seaweed after 24 h. The combined concentrations of *E. coli* in seawater and seaweed after 24 h exceeded that present in the pathogen-seawater cocktail by 5 $\log_{10}$ CFU ($P < 0.001$), which was also true for *E. coli* O157:H7 (by 4 $\log_{10}$ CFU), *L. monocytogenes* (by 3 $\log_{10}$ CFU) and *V. parahaemolyticus* (by 3 $\log_{10}$ CFU) ($P < 0.001$ in all cases) (Table 4.3).

Table 4.2. The concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the pathogen-seawater cocktail before the addition of seaweed, compared with the seawater and seaweed after 24 h. Data points that do not share a letter are significantly different from each other. Data points are the mean of 16 replicates ± SE.

		After 24 h	
Bacteria	Pathogen-seawater cocktail ($\log_{10}$ CFU ml^{-1})	Seawater ($\log_{10}$ CFU ml^{-1})	Seaweed ($\log_{10}$ CFU g^{-1})
E. coli	6.31 ± 0.1 [a]	4.62 ± 0.1 [b]	6.83 ± 0.05 [c]
E. coli O157:H7	7.0 ± 0.04 [a]	4.51 ± 0.1 [b]	6.8 ± 0.1 [a]
L. monocytogenes	5.88 ± 0.03 [a]	4.06 ± 0.07 [b]	5.01 ± 0.4 [a b]
V. parahaemolyticus	6.8 ± 0.2 [a]	4.4 ± 0.1 [b]	5.3 ± 0.2 [c]

Table 4.3. Growth of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the seawater and seaweed after 24 h combined, compared with initial concentrations of each bacteria in the pathogen-seawater cocktail. Data points that do not share a letter are significantly different from each other. Data points are the mean of 16 replicates ± SE.

Bacteria	Pathogen-seawater cocktail (log_{10} CFU ml^{-1})	Seawater and seaweed after 24 h combined (log_{10} CFU ml^{-1} or CFU g^{-1})
E. coli	6.31 ± 01[a]	11.5 ± 0.12[b]
E. coli O157:H7	7.0 ± 0.04 [a]	11.3 ± 0.14 [b]
L. monocytogenes	5.9 ± 0.03 [a]	9.08 ± 0.4 [b]
V. parahaemolyticus	6.8 ± 0.2 [a]	9.7 ± 0.2 [b]

An increase of <1 log_{10} CFU was detected in the concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* attached to the seaweed after the seaweed was rinsed under cold running tap water compared with concentrations before it was washed ($P < 0.05$ in all cases) (Table 4.4). Washing the seaweed conversely reduced the metabolic activity of *E. coli* O157:H7 attached to the seaweed from 3.01 ± 0.09 log_{10} RLU g^{-1} to 2.5 ± 0.07 log_{10} RLU g^{-1} ($P < 0.001$).

Table 4.4. Concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* attached to seaweed before and after seaweed was washed. Data points that do not share a letter are significantly different from each other. Data points are the mean of 16 replicates ± SE.

Bacteria	Pre-wash (log_{10} CFU g^{-1})	Post-wash (log_{10} CFU g^{-1})
E. coli	6.84 ± 0.05 [a]	7.24 ± 0.08 [b]
E. coli O157:H7	6.8 ± 0.10 [a]	7.21 ± 0.20 [b]
L. monocytogenes	5.01 ± 0.40 [a]	5.83 ± 0.07 [b]
V. parahaemolyticus	5.3 ± 0.20 [a]	5.62 ± 0.20 [b]

From a bacterial concentration of ~7 log_{10} remaining attached to seaweed after it was washed but before it was dried, subsequent desiccation of seaweed at room temperature or 40 °C had no effect on concentrations of *E. coli* attached to the seaweed (Fig. 4.2a). Drying at 50 °C and 60 °C resulted in pronounced *E. coli* die-off to undetectable levels by 168 h and within 24 h respectively ($P < 0.05$) (Fig. 4.2a). Subsequent storage for 72 h did not affect *E. coli* levels in the seaweed, regardless of the temperature at which the seaweed had previously been dried, and although *E. coli* grew during storage from undetectable levels to ~2 log_{10} CFU in seaweed previously dried at 50 °C, this was not a significant increase (Fig. 4.2b). However, there was a significant difference in *E. coli* concentration (~8 log_{10}) between the 60 °C and room temperature treatments after 72 h storage ($P < 0.05$) (Fig. 4.2b).

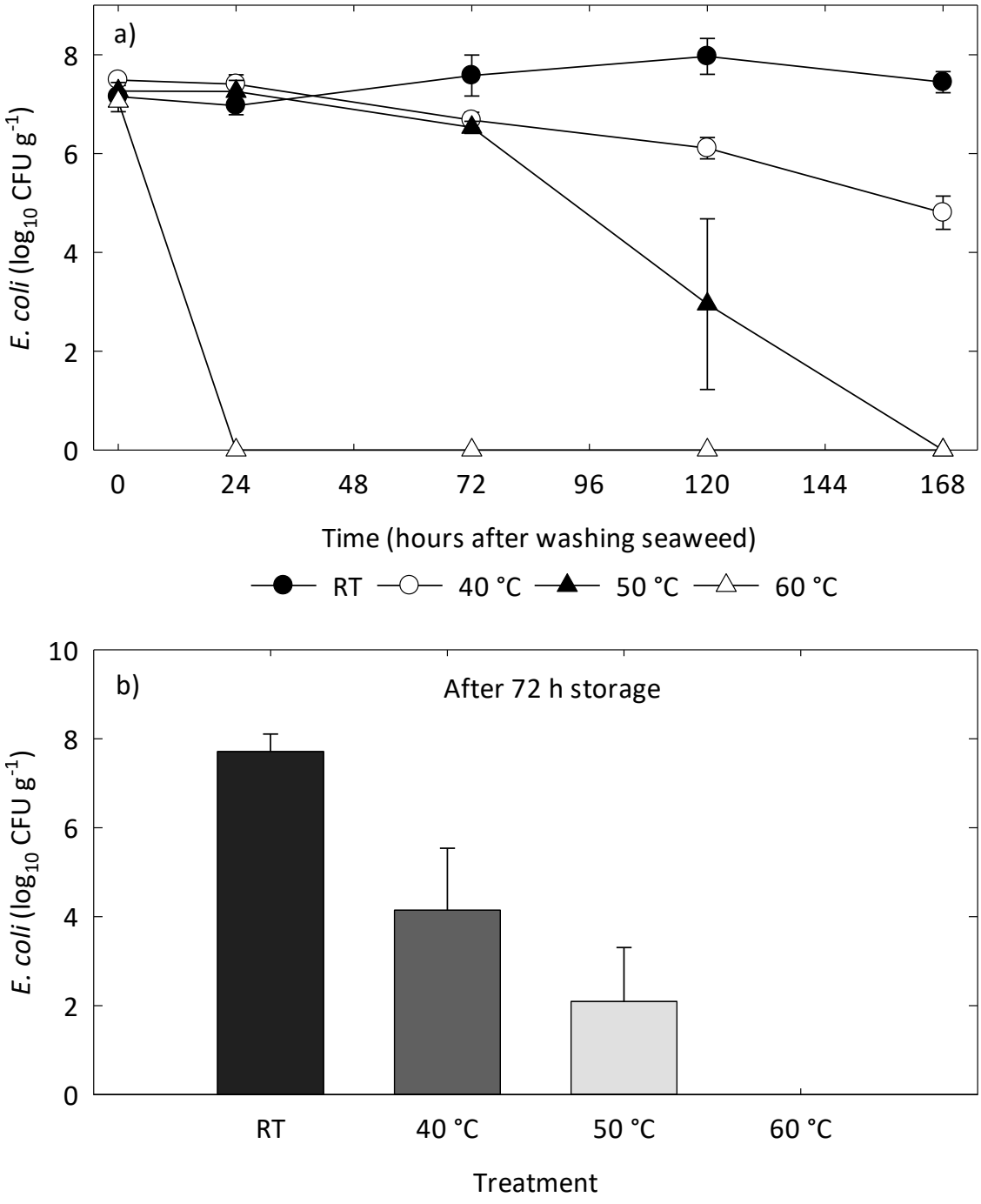


Figure 4.2. *E. coli* survival on seaweed after washing the seaweed (time = 0) and during subsequent drying for 168 h at RT (room temperature), 40 °C, 50 °C and 60 °C (a) (data points are the mean of four replicates ± SE), and during 72 h storage of dried seaweed (b) (data points are the mean of four replicates + SE).

The concentration of *E. coli* O157:H7 attached to seaweed prior to drying was unaffected by drying seaweed at room temperature for 168 h. However, drying seaweed at 40 °C and 50 °C led to similar rates of bacterial die-off to undetectable levels after 168 h ($P < 0.05$), and drying seaweed at 60 °C resulted in rapid die-off of the pathogen by 24 h ($P < 0.05$) (Fig. 4.3a). Storage for 72 h exerted no effect on pathogen levels in seaweed dried at 40 °C or 60 °C, which remained undetectable in both cases, or in seaweed dried at room temperature, which remained ~7 $\log_{10}$ higher by comparison ($P < 0.05$) (Fig. 4.3b). Growth of *E. coli* O157:H7 was detected in stored seaweed which had been dried at 50 °C, though this was not a significant increase. Metabolic activity of *E. coli* O157:H7 populations attached to the seaweed after washing was ~2.5 $\log_{10}$ RLU, but was reduced 1 $\log_{10}$ RLU within the first 24 h of drying at all temperatures, including room temperature ($P < 0.05$ in all cases) (Fig. 4.4a). Metabolic activity of the *E. coli* O157:H7 populations attached to seaweed were unaffected by 72 h storage, irrespective of the previous drying temperature, although higher metabolic activity was recorded in seaweed previously dried at 50 °C compared with that dried at room temperature ($P < 0.05$) (Fig. 4.4b).

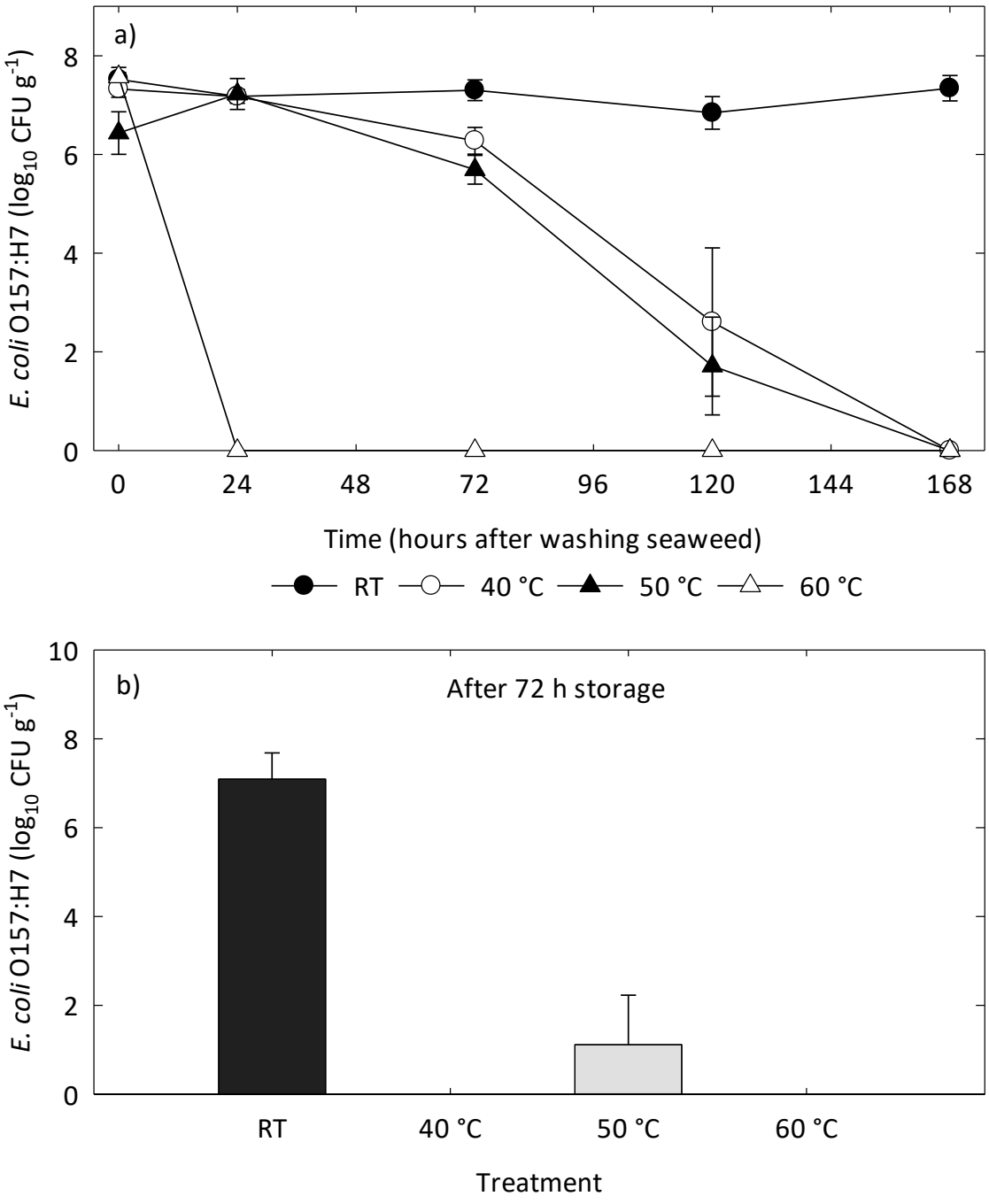


Figure 4.3. *E. coli* O157:H7 survival on seaweed after washing the seaweed (time = 0) and during subsequent drying for 168 h at RT (room temperature), 40 °C, 50 °C and 60 °C (a) (data points are the mean of four replicates ± SE), and during 72 h storage of dried seaweed (b) (data points are the mean of four replicates + SE).

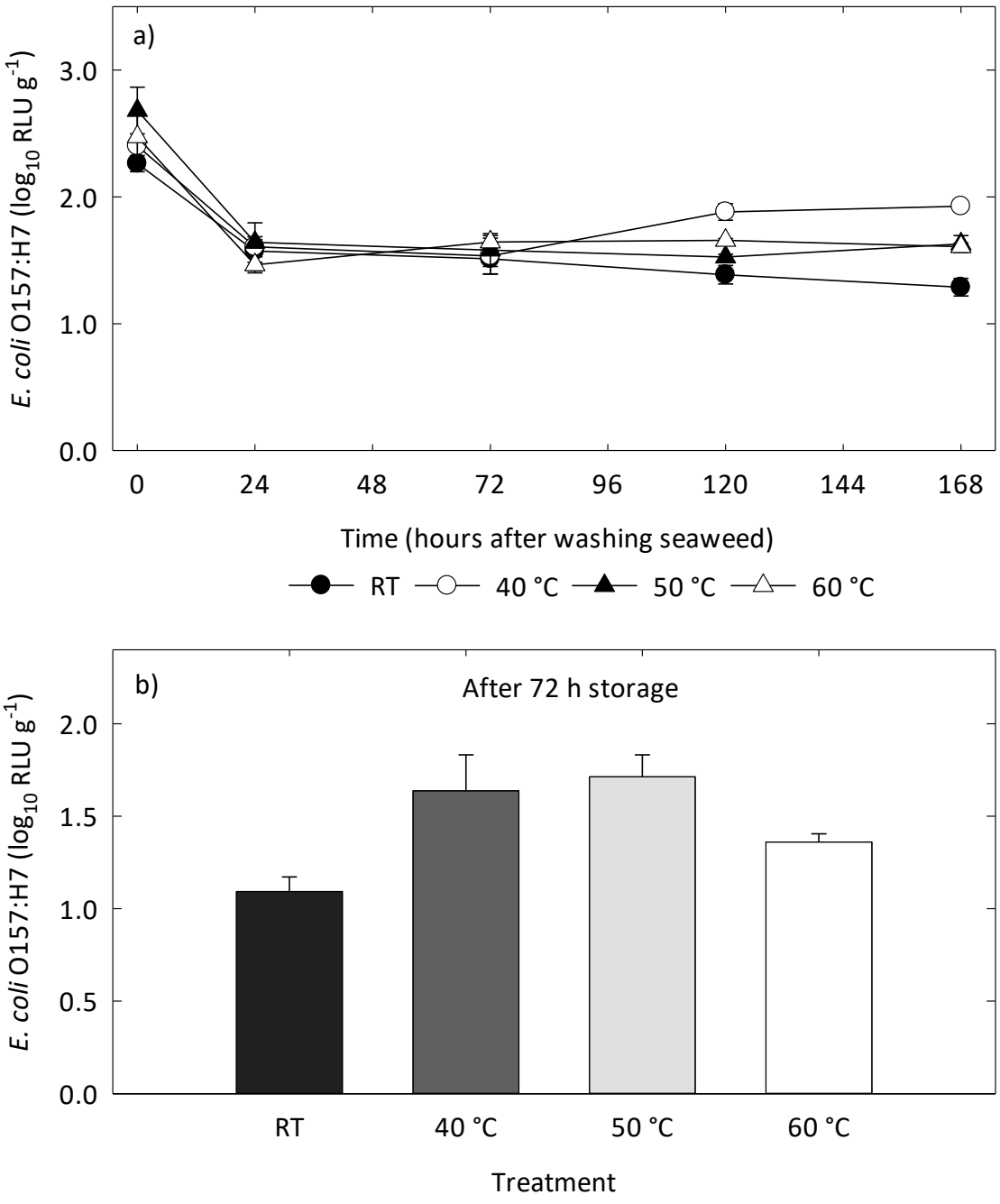


Figure 4.4. Luminescence in RLU of *E. coli* O157:H7 attached to seaweed after washing the seaweed (time = 0) and during subsequent drying for 168 h at RT (room temperature), 40 °C, 50 °C and 60 °C (a) (data points are the mean of four replicates ± SE), and during 72 h storage of dried seaweed (b) (data points are the mean of four replicates + SE).

L. monocytogenes survival on seaweed following washing of the seaweed was significantly reduced by ~5 $\log_{10}$ CFU between 72 h and 120 h by drying at 50 °C ($P < 0.05$), but increased by 2 $\log_{10}$ CFU within the first 72 h on seaweed dried at room temperature ($P < 0.05$) (Fig. 4.5a). *L. monocytogenes* survival on seaweed was unaffected by drying at 40 °C or 60 °C, and there was no significant difference between pathogen loads on seaweed dried at 40 °C, 60 °C or room temperature (Fig. 4.5a). Seaweed associated populations of *L. monocytogenes* were not reduced to undetectable levels by any of the temperature treatments, and the pathogen persisted at ~3 $\log_{10}$ CFU after 168 h of drying at 60 °C (Fig. 4.5a). Storage for 72 h did not alter levels of *L. monocytogenes* attached to the seaweed, regardless of the previous drying temperature; however, the ~6 $\log_{10}$ CFU difference in *L. monocytogenes* concentrations on seaweed previously dried at 50 °C compared with the room temperature treatment was significant ($P < 0.05$) (Fig. 4.5b).

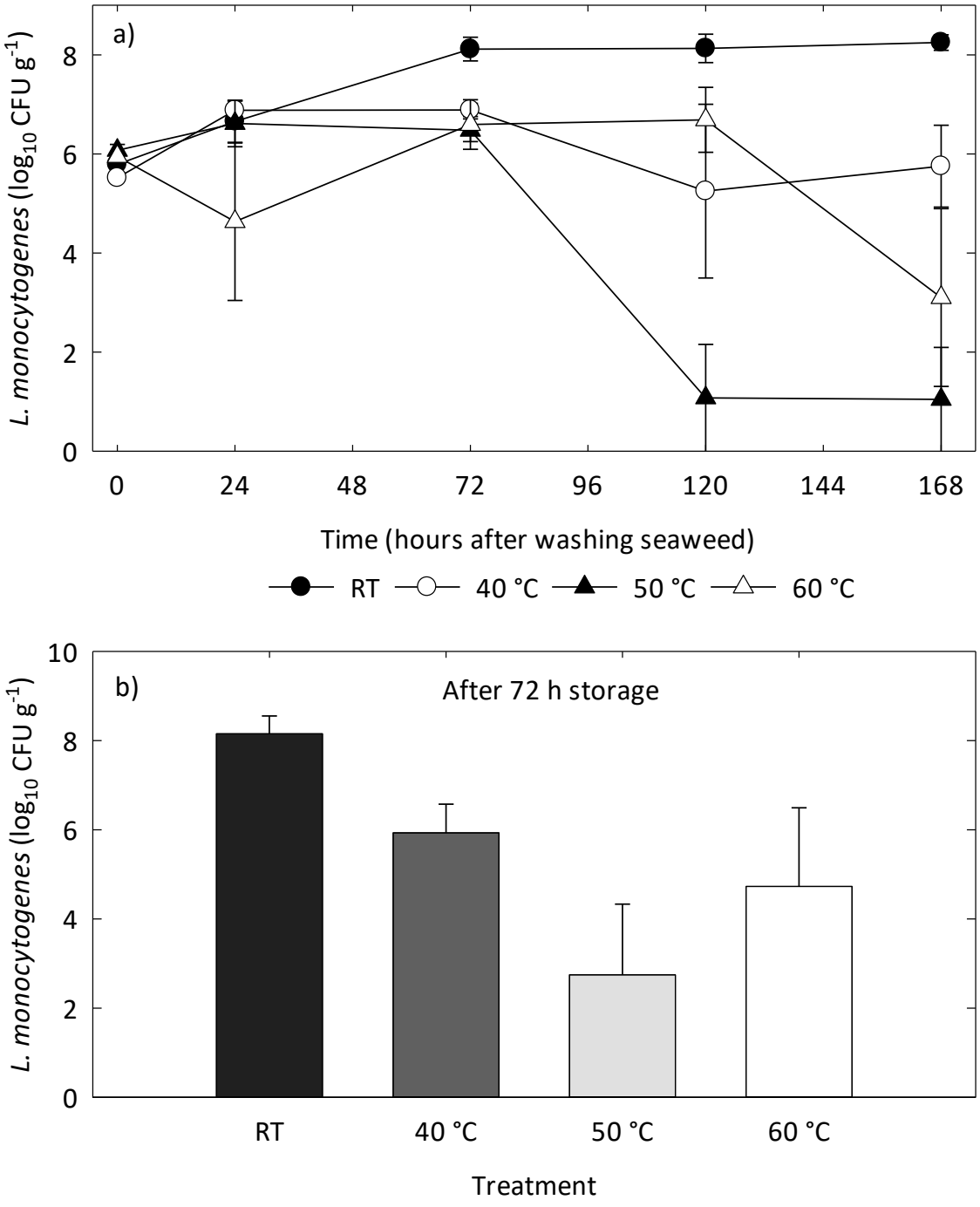


Figure 4.5. *L. monocytogenes* survival on seaweed after washing the seaweed (time = 0) and during subsequent drying for 168 h at RT (room temperature), 40 °C, 50 °C and 60 °C (a) (data points are the mean of four replicates ± SE), and during 72 h storage of dried seaweed (b) (data points are the mean of four replicates + SE).

The *V. parahaemolyticus* concentration of ~6 $\log_{10}$ CFU present on seaweed after it was washed increased by ~2 $\log_{10}$ CFU in seaweed dried at 40 °C in the first 24 h though this increase was not statistically significant, followed by die-off of 7 $\log_{10}$ CFU by 168 h ($P <$ 0.05) (Fig. 4.6a). A significant reduction in the *V. parahaemolyticus* population of 6 $\log_{10}$ CFU to undetectable levels occurred by 168 h at 50 °C ($P <$ 0.05), and in the first 24 h at 60 °C ($P <$ 0.05) (Fig. 4.6a). The *V. parahaemolyticus* population on seaweed dried at room temperature grew ~2 $\log_{10}$ CFU over 168 h ($P <$ 0.05) (Fig. 4.6a). Storage for 72 h did not affect *V. parahaemolyticus* levels in the seaweed, regardless of the temperature at which the seaweed had previously been dried. However, the ~5 - 6 $\log_{10}$ CFU differences in pathogen loads between the 50 °C and 60 °C treatments compared with the room temperature treatment were significant ($P <$ 0.05) (Fig. 4.6b).

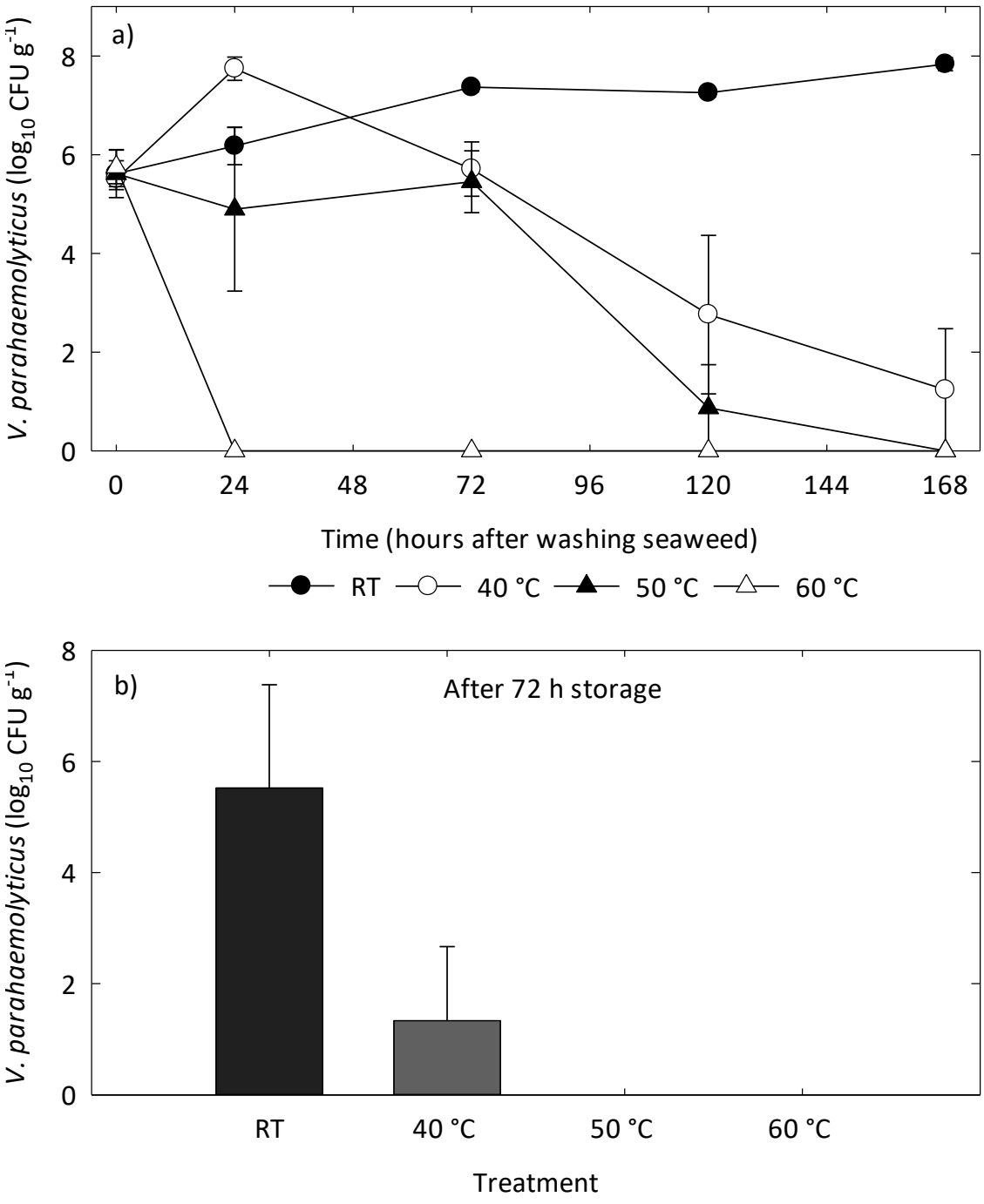
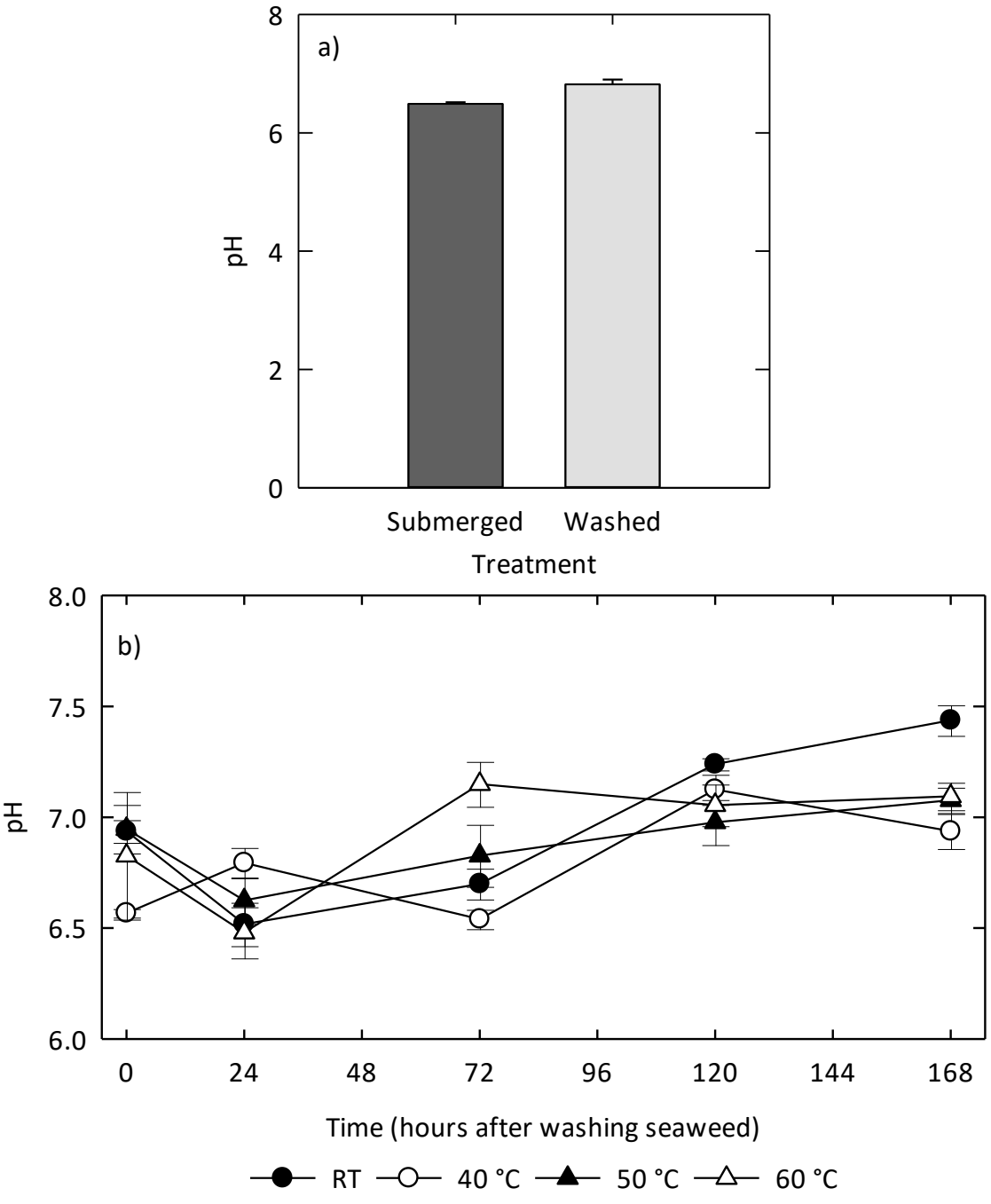


Figure 4.6. *V. parahaemolyticus* survival on seaweed after washing the seaweed (time = 0) and during subsequent drying for 168 h at RT (room temperature), 40 °C, 50 °C and 60 °C (a) (data points are the mean of four replicates ± SE), and during 72 h storage of dried seaweed (b) (data points are the mean of four replicates + SE).

Washing seaweed after 24 h submergence in seawater did not affect seaweed pH (Fig. 4.7a). However, during drying, the pH of the seaweed dried at room temperature, 40 °C and 60 °C increased significantly ($P < 0.05$) (Fig. 4.7b), although after 72 h storage, seaweed dried at all temperatures with the exclusion of the 40 °C treatment became more acidic ($P < 0.05$ in all cases) (Fig. 4.7c).



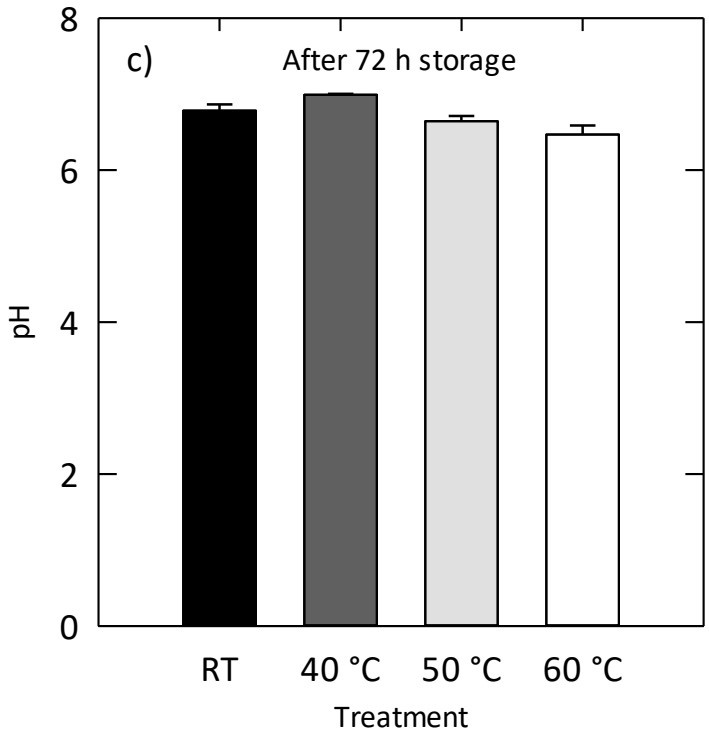


Figure 4.7. The pH of seaweed after 24 h submergence in seawater and subsequent washing (a), during drying at RT (room temperature), 40 °C, 50 °C and 60 °C (b), and after 72 h storage (c). Data points in (a) are the mean of 16 replicates + SE, in (b) are the mean of four replicates ± SE and in (c) are the mean of four replicates + SE.

4.4.3 Microbial safety of seaweed as BSFL feed

The mean weight of individual larvae increased significantly from 0.12 ± 0.01 g on day 3 to 0.26 ± 0.005 g on day 5 ($P < 0.05$), although the onset of pre-pupation from day 6 led to a decline in average weight (Fig. 4.8). Water content in the inoculated substrate in which larvae were present was significantly less than that of the inoculated substrate from which larvae were absent on days 4 and 8 ($P < 0.05$ in both cases), and fell ~10 % in the presence of larvae between days 6 and 8 ($P < 0.05$) (Fig. 4.9). Compared with the bacterial loads in the inoculated feed in which larvae were present, concentrations of *E. coli* in, and on, the larvae were significantly lower throughout the entire sampling period, which was also true of *E. coli* O157:H7 with the exception of day 5, of *L. monocytogenes* with the exception of day 6 and *V. parahaemolyticus* ($P < 0.05$) (Fig. 4.10 a-d). Levels of *E. coli* and *E. coli* O157:H7 associated with larvae fell ~2 $\log_{10}$ CFU

over 8 days (P < 0.05), whereas larval loads of *L. monocytogenes* and *V. parahaemolyticus* did not change over the same time period (Fig. 4.10 a-d).

In the absence of larvae, concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in the feed substrate fell significantly on day 2 (P < 0.05 in all cases) (Fig. 4.10 a- d). Consequently, all four bacteria were 1 – 2 $\log_{10}$ CFU higher in substrate in which larvae were present on days 3 and 4 (P < 0.05 in all cases) (Fig. 4.10 a- d). Metabolic activity of the *E. coli* O157:H7 population in larvae decreased by ~1.5 $\log_{10}$ RLU during the 8 day sampling period (P < 0.05) and ~1 $\log_{10}$ RLU from day 3 onwards in the inoculated substrates regardless of the presence or absence of larvae (P < 0.05 in both cases) (Fig. 4.11). Metabolic activity of *E. coli* O157:H7 in feed in the absence of larvae exceeded that of feed in the presence of larvae on days 4 and 8 (P < 0.05 in both cases) (Fig. 4.11). The pH of non-inoculated feed in the presence of larvae increased from 3.6 ± 0.11 on day 1 to 6.4 ± 0.13 by day 8 (P <0.05) (Fig. 4.12).

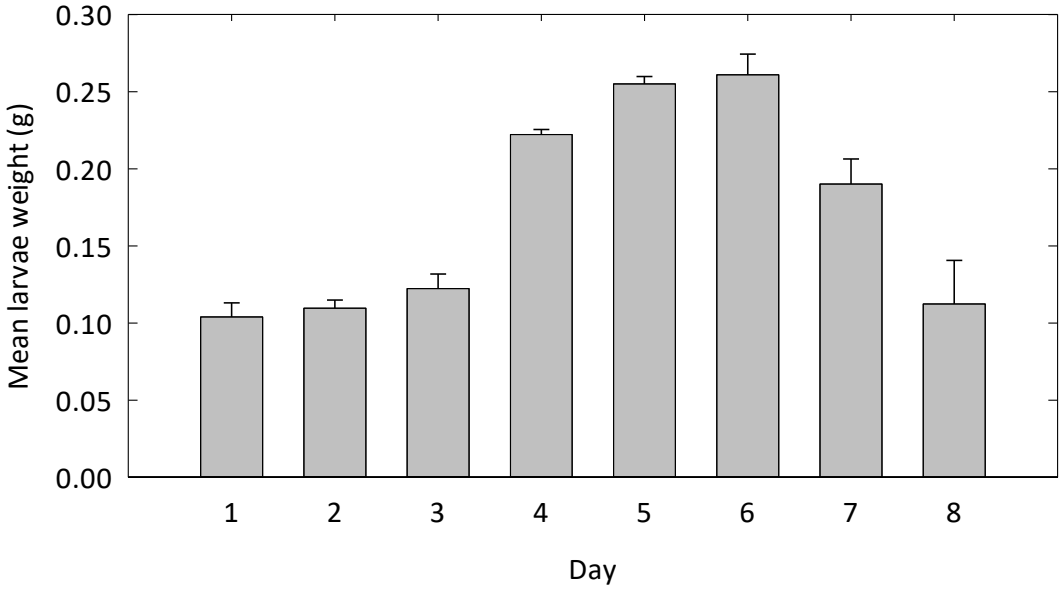


Figure 4.8. Average weight of individual BSF larvae. Data points are the mean of four replicates + SE.

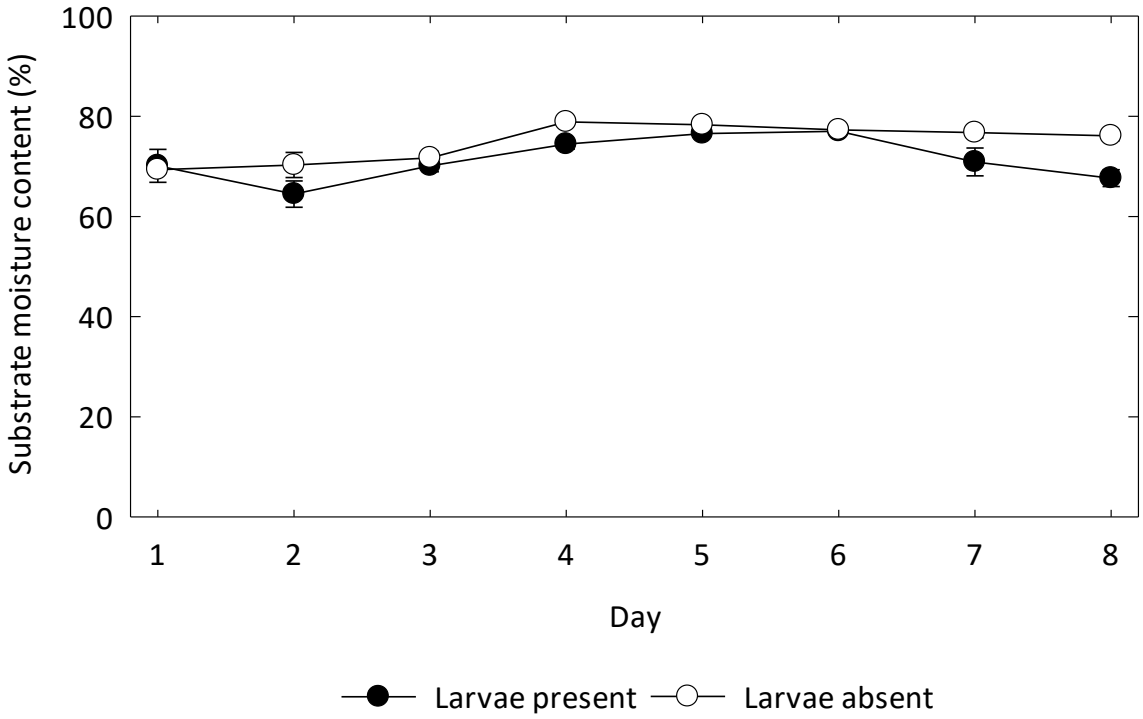


Figure 4.9. Moisture content of feed substrate with and without BSF larvae present. Data points are the mean of four replicates ± SE.

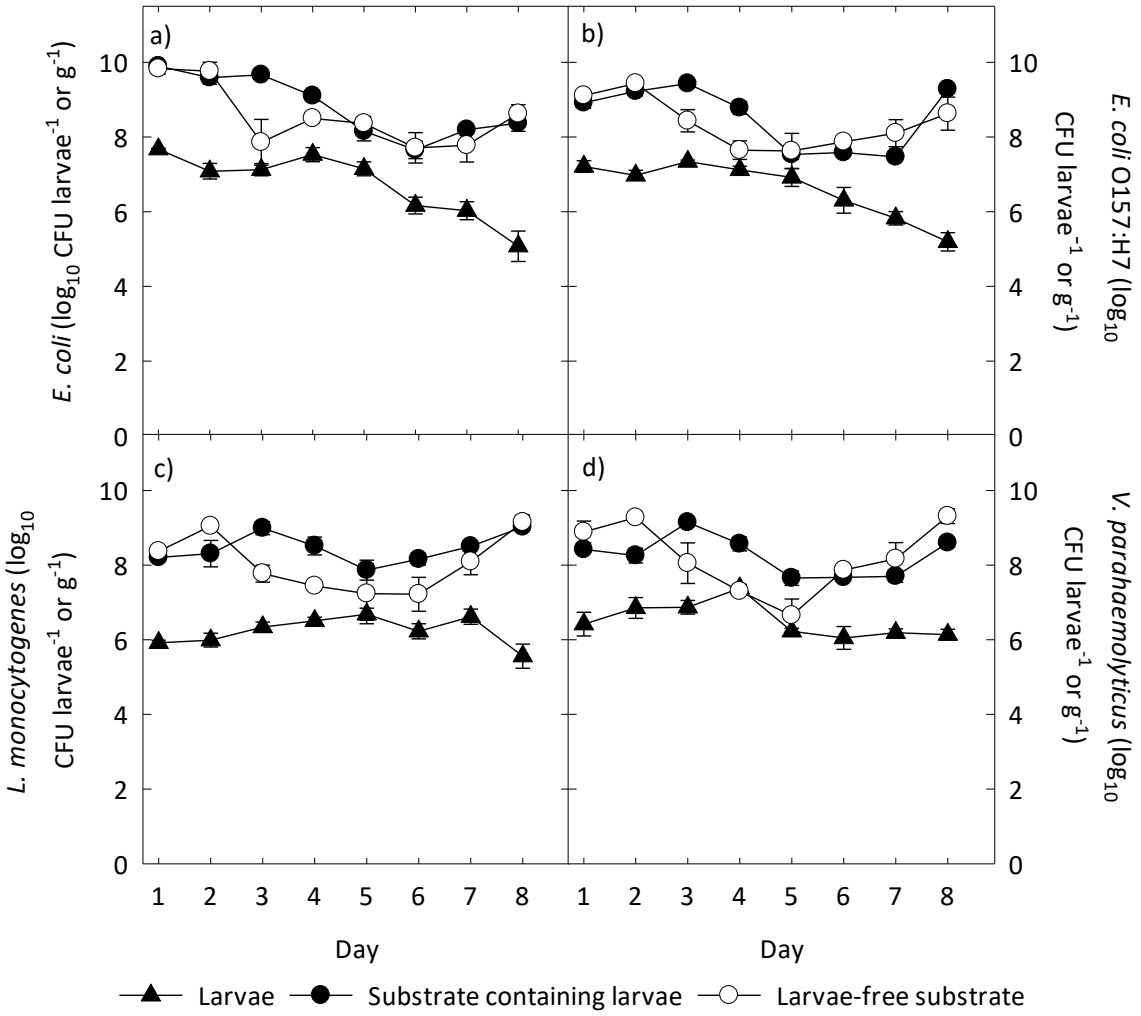


Figure 4.10. Concentration of *E. coli* (a), *E. coli* O157:H7 (b), *L. monocytogenes* (c) and *V. parahaemolyticus* (d) associated with BSF larvae, the substrate containing the BSF larvae and the larvae-free substrate. Data points are the mean of four replicates ± SE.

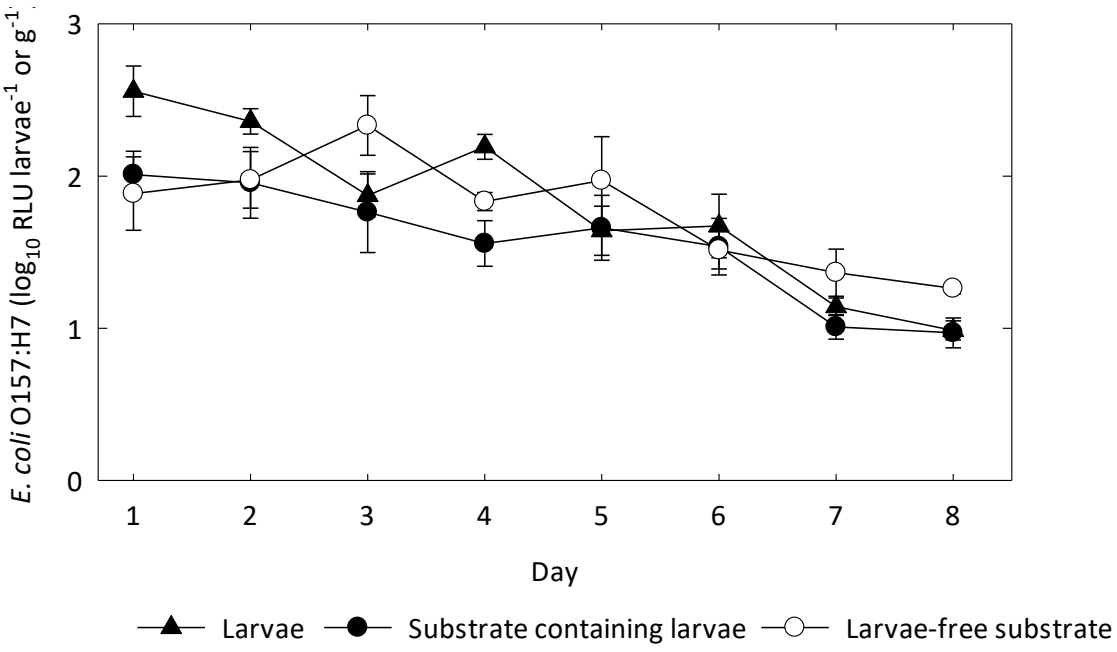


Figure 4.11. Luminescence in RLU of *E. coli* O157:H7 associated with BSF larvae, the substrate containing the BSF larvae and the larvae-free substrate. Data points are the mean of four replicates ± SE.

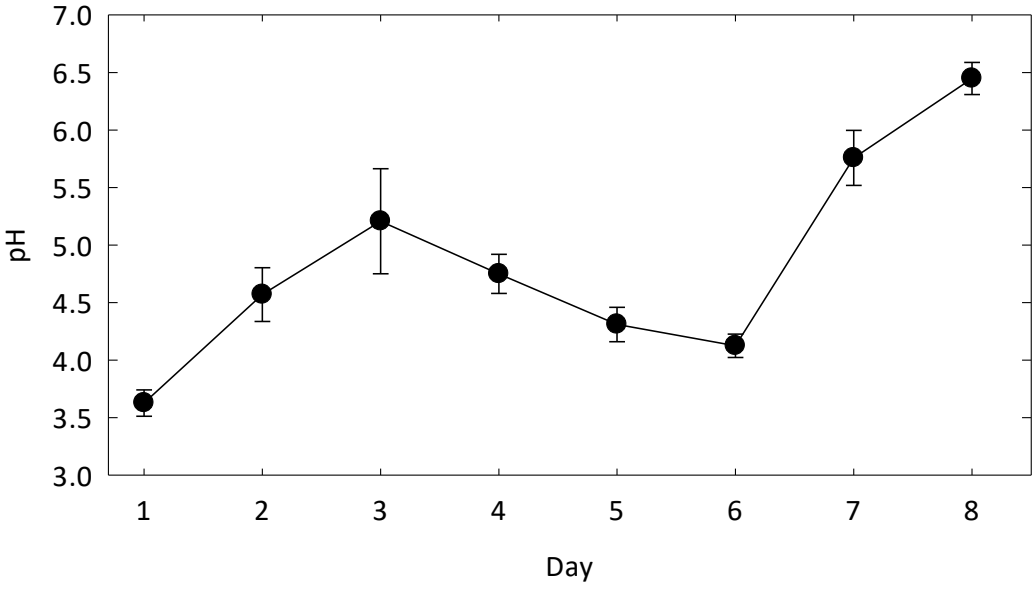


Figure 4.12. The pH of feed substrate in the presence of BSF larvae. Data points are the mean of four replicates ± SE.

4.5 Discussion

4.5.1 Microbial safety of processed seaweed

This study demonstrates that the typical post-harvest processes of washing and drying seaweed intended for animal feed can fail to eradicate, and can even encourage the survival of, FIO and selected human pathogenic bacteria if colonising the seaweed in high concentrations at the point of seaweed harvest. The inadequate hygienic standards achieved by these manufacturing practices consequently produce a dried seaweed feed product in which bacteria hazardous to human health can not only persist during storage but also re-emerge from previously undetectable levels. It is evident that the range of environmental stresses to which bacterial contaminants of seaweed are subjected during the primary stage of seaweed feed manufacturing- desiccation for long term storage- have highly variable effects on bacterial persistence depending on bacterial species and even strain. The findings indicate that the industry objective of maximising the nutritional benefits of seaweed feed by minimising the drying temperature must be balanced against achieving a microbiologically safe product.

E. coli, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* all attached to the submerged senescing seaweed over 24 h, which has not been previously shown for *L. monocytogenes* colonisation in the natural environment. The persistence of *L. monocytogenes* in seawater and its attachment to seaweed likely reflect the biofilm-forming ability of diverse *L. monocytogenes* strains, and the tolerance of this pathogen of osmotic stress in seawater (Kadam *et. al.,* 2013; Bhunia, 2018; Hansen *et. al.,* 2006). The ability of bacteria to switch from planktonic to sessile growth is an adaptation to physicochemical stressors, since aggregates of sessile cells in the extracellular polysaccharide (EPS) matrices formed in biofilms confers protection of cells against hostile conditions such as osmotic stress (Esbelin *et. al.,* 2018; Burgess *et. al.,* 2016). This survival strategy may explain the higher concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and even the halophilic *V. parahaemolyticus* attached to the seaweed compared with the smaller proportion of these populations remaining as planktonic cells after 24 h exposure to seawater. The combined concentrations of each bacteria present in the seawater and attached to seaweed significantly exceeded their

respective inoculant loads introduced 24 h earlier, indicating that *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* populations grew in the seawater and/or in seaweed-associated biofilms. Physically slicing the seaweed would have released leachates onto the seaweed surface and into the seawater, the presence of which is known to encourage *E. coli* survival and growth (Quero *et. al.,* 2015). The release of sugars, such as mannitol and glucose, from the brown, red and green seaweeds may have also facilitated proliferation of *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus*, and maintained the metabolic activity of *E. coli* O157:H7 under otherwise stressful conditions (Quilliam *et. al.,* 2014; Quero *et. al.,* 2015; Van Hal *et. al.,* 2014).

Washing the seaweed under running tap water proved ineffective at reducing levels of attached *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus*, and instead marginally (but significantly) increased the detected concentrations of these bacteria on the seaweed. It is feasible that the unexpected increase in bacterial concentrations on the post-washed seaweed may relate to the washing method employed. Failure of a washing step to reduce FIO contamination of leafy vegetables during produce washing has been linked to insufficient frequency of changing the washing water as well as a high product to wash water ratio (Holvoet *et. al.,* 2012). In this study, rinsing the seaweed contained in a sieve under running tap water resulted in wash water accumulating in the sieve and temporarily submerging the seaweed. This may have redistributed bacterial cells throughout the seaweed rather than removing them. Thus the marginal increase in bacterial loads detected in the washed seaweed may reflect a shift from heterogeneous to more homogenous cell dispersal in the material which was therefore more readily detectable in samples (Buchanan *et. al.,* 2017). The propensity for bacteria to attach to a surface and subsequently form biofilms varies according to the attachment surface (e.g. physicochemical nature or roughness), the surrounding medium (pH, temperature, nutrients, microbial community, osmolarity) and the characteristics of the species or strain, the interacting effects of which are likely to have facilitated attachment by all four bacteria (Goller and Romeo, 2008). *V. parahaemolyticus* in particular has previously been demonstrated to resist removal from seaweed by washing, indicative of the firm attachment of this pathogen

to seaweed surfaces (Mahmud *et. al.,* 2007). Biofilm formation on the seaweed by the four bacteria would have contributed to the inefficiency of the cleaning stage. Increasing the duration of the washing step beyond 1- 2 minutes however is not likely to have improved the effectiveness of bacterial removal, as demonstrated with fresh-cut produce (Pirovani *et. al.,* 2004).

It is assumed that washing fresh-cut food produce lifts bacteria from the product surface and disinfectant present in the wash water kills the suspended cells (Gil *et. al.,* 2009). However, the antimicrobial efficiency of free chlorine (FC) in water is influenced by multiple factors, particularly chlorine concentration, duration of pathogen exposure to the disinfectant, and the bacterial strain/s present (Shen *et. al.,* 2013). FC is present in most disinfected drinking water from large suppliers at concentrations typically within the 0.2 - 1 mg/L range (WHO, 1996). Plain wash water containing no disinfectants normally reduces bacterial loads by < 1 log (Parish *et. al.,* 2003). By contrast, *Salmonella* and *E. coli* O157:H7 in wash water were reduced > 4.5 $\log_{10}$ CFU ml^{-1} by exposure to either > 0.5 mg/L of FC for > 30 seconds or > 1 mg/L of FC for 5 seconds, and if FC concentration and duration of exposure fell below these levels, bacterial survival became strain dependent (Shen *et. al.,* 2013). However, organic matter present in wash water reacts with FC, rapidly neutralising it and reducing its disinfection efficiency (Shen *et. al.,* 2013). Assuming submergence of the seaweed and attached pathogens in 1 mg/L of FC for 1 minute, any associated reduction of bacterial concentrations is likely to have been offset by neutralisation of the FC due to rapid reaction with abundant seaweed exudates, the effect of a high produce to wash water ratio, and the high concentration of each bacteria attached to the seaweed.

During industrial processing of seaweed, bacterial contaminants in the material would be subjected to stressful environmental conditions, primarily lethal or sublethal temperatures, suboptimal a_w and pH, and osmotic pressure (due to salinity increase during moisture loss) (Ross, 2008). Varying degrees of environmental stress can invoke various responses in bacteria, ranging from effects on growth rate, to cell injury inducing a viable but non-culturable state (VBNC) (Wesche and Ryser, 2013; Smelt and Brul, 2014). Bacterial resistance to heat stress in desiccated material is due to (a) low water activity which constrains heat-induced cell damage (e.g. membrane disruption)

and (b) a process whereby cells exposed to a sublethal stress in the production chain can increase their tolerance of a subsequent stress of the same or different nature, and of greater magnitude, encountered further along the processing chain as a result of cross-protection (Lang *et. al.,* 2017; Wesche and Ryser, 2013; Burgess *et. al.,* 2016). During the drying of seaweed at ≥ 50 °C, the feed material was transformed from a high a_w product to a low a_w product, and suboptimal a_w is one of the key controls on microbial growth (Esbelin *et. al.,* 2018).

Desiccation of seaweed at 60 °C led to log-linear inactivation of *E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus* populations to undetectable levels within 24 h, yet had no effect on *L. monocytogenes*. Gram-negative bacteria (*E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus*) are significantly more vulnerable to desiccation than Gram-positive species (*L. monocytogenes*) due to physicochemical differences between the two types of bacterial cell (Burgess *et. al.,* 2016). It is likely that drying at 60 °C exerted a lethal effect on the cells of all bacteria with the exception of *L. monocytogenes*, which is known to tolerate a wide environmental temperature range, as well as desiccation stress which may have contributed to die-off of *E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus* cells (Bhunia, 2018). Application of 72 °C heat for 2 minutes is generally considered to assure sterilisation of food products contaminated with *Listeria* spp. (Smelt and Brul, 2014), though this temperature-time treatment would require validation for seaweed. A metabolically active proportion of the *E. coli* O157:H7 population survived 168 h of 60 °C heat stress presumably as heat tolerant cells in a VBNC state. Enrichment of samples or molecular methods, though not undertaken in this study, may have also revealed the presence of *E. coli* and *V. parahaemolyticus* VBNC cells surviving after 168 h of 60 °C drying, although in the case of *E. coli* non-pathogenic strains are less desiccation tolerant than STEC strains (Hiramatsu *et. al.,* 2005).

A drying temperature of 50 °C generated more complex die-off kinetics amongst *E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus* populations, whereby the onset of cell death or VBNC due to heat stress was delayed, probably as a result of a_w levels remaining permissible to microbial survival and growth. *L. monocytogenes* exhibited poorer heat resistance at 50 °C compared with the 60 °C treatment, most likely a result of the a_w being insufficiently low to protect the bacterial cells from heat damage. The optimum

temperature for growth of *V. parahaemolyticus* is 20 – 37 °C (Bhunia, 2018), and cell die-off of this bacteria at 40 °C may reflect sensitivity of this bacterial species to temperatures > 37 °C. By contrast, the lack of response by *L. monocytogenes* concentrations to this drying temperature may be attributable to the ability of this species to survive at temperatures of up to 45 °C (Bhunia, 2018). Inter-specific competition between the various bacterial species and native seaweed microflora present may have contributed to the different responses of these pathogens to drying at optimum or near-optimum temperatures for growth. However *L. monocytogenes* is a poor competitor unless in low temperature conditions (Carpentier and Cerf, 2011), suggesting other environmental or species-specific factors encouraged *L. monocytogenes* survival at 40 °C. It is also evident that commensal *E. coli* and pathogenic *E. coli* O157:H7 responses to the intrinsic, extrinsic or processing conditions at 40 °C differed due to some factor implicit to the strain (Ross, 2008). A temperature of 40 °C is near-optimum for growth of commensal and pathogenic *E. coli* (Bhunia, 2018). The 40 °C and room temperature treatments did not differ in a_w or pH during the drying period, thus it is evident that 40 °C heat exerted a sublethal stress on *E. coli* O157:H7, as well as *V. parahaemolyticus* cells. Survival of the latter despite 40 °C heat application may be attributable to cell protection from heat stress in biofilm EPS, which this species forms particularly strongly at ~37 °C (Han *et. al.,* 2016). Exposure of halophilic *V. parahaemolyticus* to low salinity stress during washing of the seaweed in freshwater may also have conferred cross-protection from subsequent 40 °C heat stress (Wong *et. al.,* 2004).

The regrowth during room temperature storage of *E. coli* and *E. coli* O157:H7 in seaweed which had previously been dried at 50 °C suggests that bacterial cells which have entered a possible VBNC state can recover culturability in favourable conditions (Orruno *et. al.,* 2017). Since microbial growth could not occur in seaweed at the low a_w recorded at the end of the 50 °C drying period, the relative humidity and temperature of the storage atmosphere must have allowed the dried seaweed powder to absorb water (Hyun *et. al.,* 2018), since the sealed food bags used for storage were not necessarily airtight. Regrowth of *E. coli* O157:H7 in seaweed dried at the lower temperature of 40 °C did not occur under the same conditions, implying that the 40 °C

heat stress inflicted more damage on cells than 50 °C because of the high a_w maintained throughout 40 °C drying. *E. coli*, *E. coli* O157:H7 and *V. parahaemolyticus* cells in seaweed dried at 60 °C may have remained in a VBNC state throughout the 72 h storage period; however, assessment of the growth potential of these bacteria over an extended duration, particularly over the typical 1 year shelf life of dried seaweed powder, was beyond the scope of this study. The extended lag phase of no growth during 72 h storage of *L. monocytogenes* and *V. parahaemolyticus* previously exposed to sublethal 50 °C and 40 °C heat respectively represent a period of injured cells adjusting to a new environment and repairing (Ross, 2008; Smelt and Brul, 2014). It is clear that pathogenic bacteria of importance to animal and public health can both persist and grow in dried and stored seaweed powder despite being subjected to temperature and desiccation stress during manufacture. If bacterial cells shift to a VBNC state in response to processing stress, their potential for prolonged survival in low moisture feed and subsequent growth under favourable conditions further along the processing chain is of concern, and in the context of pathogens with a low infective dose such as *E. coli* O157:H7, a relatively small number of persistent cells can pose a significant health risk (Burgess *et. al.,* 2016; Esbelin *et. al.,* 2018).

Attaining feed safety through desiccation is reliant on achieving a well-controlled and homogenous drying treatment; however, the temperature within convection ovens can vary significantly and this technology does not necessarily guarantee uniform heat dispersion throughout a product (Bonazzi and Dumoulin, 2011; Roos *et. al.,* 2018). In addition, seaweed has a high salt content which was not an intrinsic parameter measured during this processing simulation, yet the presence of salt inhibits bacterial growth due to its disruptive effect on the osmotic balance of cells, whilst also contributing to the lowering of a_w and thus the thermal resistance of bacterial cells (Burgess *et. al.,* 2016; Roos *et. al.,* 2018). In particular, salt is known to increase the thermal tolerance of *L. monocytogenes* (Li *et. al.,* 2017). Determining the response and adaptation of key microbial contaminants during processing of seaweed into animal feed, is therefore complicated by the confounding effects of temperature variability during drying and salt concentration on the survival and inactivation of the feed and food pathogens during processing. It is, however, possible to recommend time-

temperature treatment combinations to control and reduce some key bacterial contaminants in seaweed feed during this CCP. To achieve microbial die-off from high initial concentrations to undetectable levels, with no succeeding regrowth during subsequent storage, using the convection ovens utilised in this study, required a trade-off between low temperature drying and duration of heat application. Although higher drying temperatures achieve shorter drying times (Chenlo *et. al.,* 2018), drying seaweed at a lower temperature retains a higher proportion of nutritional properties within the final seaweed product adding value to animal feed (Sappati *et. al.,* 2018).

4.5.2 Microbial safety of seaweed as BSFL feed

This study shows that BSF larvae can be rapidly contaminated by key human pathogens and an FIO present in their feed in high concentrations, indicating that at the point of harvest a decontamination step would be required to ensure that the larvae products did not pose a risk to animal and human consumers further along the feed and food chain. Any suppressing effect that larval feeding may have been expected to exert on the bacterial populations in their feed through digestion or GIT antibacterial action was overcome due to the high concentrations at which the bacteria were introduced to and persisted in the substrate. The environmental conditions prevailing in the substrate were conducive to the survival of human pathogenic bacteria in the feed in high concentrations, providing a source of larval exoskeleton and GIT recontamination throughout the rearing period.

Concentrations of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* associated with larvae were less than those detected in the inoculated substrate which may be indicative of effective digestion, inactivation, or antimicrobial action on these bacteria in larvae GIT once ingested (Wynants *et. al.,* 2018a). *E. coli* and *E. coli* O157:H7 loads in and on the larvae declined as they developed from 1 week old larvae to prepupae, a pattern not reflected in their feed substrate, or documented for larvae-associated *L. monocytogenes* or *V. parahaemolyticus*. Larval GIT antimicrobial efficacy could vary between bacterial species, and there is evidence that *E. coli* strains could be selectively inactivated in the GIT (Wynants *et. al.,* 2018a; Engel and Moran, 2013). It is therefore likely that ingested cells of these *E. coli* strains were exposed to increasing levels of antimicrobials in the GIT during larval development (De Smet *et. al.,* 2018).

Importantly, neither *E. coli* nor any of the pathogenic bacteria accumulated in the larvae during rearing. By day 8 the pre-harvest pre-pupae load of all four bacteria was as high as ~5 - 6 $\log_{10}$ CFU. This indicates that in this scenario if gut voidance by larvae transitioning into prepupae occurred prior to processing, it would fail to eradicate hazardous bacteria from pre-harvest larvae, and that sterilisation of the larval meal during subsequent processing steps would be essential (Schlüter *et. al.,* 2017).

The larvae grew and developed on the experimental diet of wheat bran and whey protein supplemented with 10 % seaweed powder, with a rapid weight increase occurring between days 3 and 5. It is not known what proportion of feed intake consisted of the seaweed powder as BSFL are thought to prefer a smaller seaweed powder particle size of ~150 µm, however BSFL can consume seaweed powder of 500 – 2000 µm particle size and that utilised in this study fell at the lower end of that size range (Liland *et. al.,* 2017). Since the presence of larvae did not affect bacterial concentrations in the feed substrate for most of the sampling period, neither mechanisms of larval digestion and inactivation of bacterial cells for nutrition, nor GIT antimicrobial action, were sufficient to offset the high bacterial concentrations persisting in the feed. The concentrations at which the four bacteria were introduced to the seaweed powder supplement far exceeded the levels persisting in the stored seaweed powder following drying at 50 °C in Experiment 1. If bacteria were introduced at lower concentrations, larval feeding may have been sufficient to reduce levels of the pathogens and the FIO in both the feed substrate and associated with the larvae (Liu *et. al.,* 2008; Wynants *et. al.,* 2018a). The hydration of the inoculated substrate with potentially 1.6 mg of FC per 1 kg of BSFL feed at the outset of the experiment and 0.32 mg of FC on day 3 (assuming an FC content of 1 mg/L in tap water), is likely to have had a negligible effect on the high pathogen concentrations present in the feed and therefore in the larvae. The warm substrate temperature may have improved the disinfection efficiency of the chlorinated tap water (Delaquis *et. al.,* 2004). However, in the acidic substrate conditions prevailing during addition of water, chlorine may have been largely present in the hypochlorous acid form; this reacts rapidly with organic matter to form combined chlorine compounds which exhibit limited antimicrobial activity (Delaquis *et. al.,* 2004).

In the presence of larvae, levels of *E. coli*, *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* contamination in the substrate were all significantly higher between days 3 and 4, compared with the larvae-free inoculated feed. The pH of substrate in which larvae were present increased over 8 days from acidic to near-neutral, which is associated with the release of ammonia from BSFL feeding and excreting into organic matter (Cickova *et. al.,* 2015; Ma *et. al.,* 2018; Rehman *et. al.,* 2017). The pH of feed, measured only in substrate in which larvae were present, was favourable only to the persistence of *E. coli*, *E. coli* O157:H7 and *L. monocytogenes* on days 3 and 4 (the optimal pH for *V. parahaemolyticus* growth is 8.0 – 8.8), and moisture content was not a limiting factor (Haberbeck *et. al.,* 2017; Bhunia, 2018). It is therefore likely that the presence of the larvae engendered nuanced modifications of the biotic and abiotic conditions in the substrate, the combined effects of which facilitated persistence of *V. parahaemolyticus* at a high concentration, overcoming the inhibiting effect of low pH. Measurement of pH in the larvae-free substrate would be needed to validate this conclusion however.

The introduction of seaweed and insect larvae as novel animal feed ingredients will expand the feed resource base and contribute to future-proofing sustainability of the animal-based feed and food chain, but inadequate control of microbial pathogens in the feed could pose health risks to animals and human consumers at the end of the food chain (Makkar *et. al.,* 2016; Besten *et. al.,* 2017). Understanding opportunities for microbial contamination and growth at critical stages of the farm-to-fork continuum is key to microbiological risk reduction (Membre and Guillou, 2016). As with traditional organic animal feed, quality control of seaweed feed as part of good agricultural practice (GAP) should be seen as the principle means by which the feed industry can control the potential presence of seaweed-associated contaminants in BSF prepupae, since pathogens at high concentrations in the feed lead to high levels of pathogen contamination of larvae (Van der Spiegel *et. al.,* 2013). If pathogenic microbes are introduced to BSFL feed substrate, particularly at high concentrations as a consequence of low temperature drying of the feed during manufacture, the larvae may exhibit insufficient capacity to reduce internal concentrations of pathogens through GIT digestion and antimicrobial action. Pathogen levels in feed are a function of the ability of the specific bacteria to tolerate and adapt to the intrinsic nature of the feed material,

and the physio-chemical stresses incurred during production and processing of the product. Therefore, to ensure adequate control of microbial hazards during key seaweed processing stages, drying temperatures and durations must be validated and standardised prior to distribution of seaweed meal to insect producers as part of good manufacturing practice (GMP) across the industry (Membre and Guillou, 2016).

4.6 Conclusion

Ensuring clean and safe novel animal feed depends on understanding both the specific microbial hazards associated with the novel ingredients, and the response of these bacteria to typical production processes. Persistence in seawater, and rapid colonisation of brown, red and green seaweeds, by some key human pathogens, indicates that water quality at seaweed harvesting sites should be considered a key CCP at the start of the production chain. In the seaweed feed sector, washing and drying seaweed are not intended or expected to remove bacterial contaminants, but as low temperature desiccation is often favoured, attached human bacterial pathogens can persist. Regrowth of *E. coli* O157:H7 in stored dried, powdered seaweed from previously undetectable levels indicates the necessity either for application of a post-desiccation decontamination step or industry-wide adoption of a minimal seaweed drying temperature guaranteed to kill the most heat resistant pathogens. Seaweed washing, drying and storage conditions are thus CCPs that deserve attention by the seaweed industry. High levels of bacteria in seaweed powder fed to BSFL contaminated the larvae and were not eradicated by larval metamorphosis into prepupae. Seaweed feed, like all raw feed materials, will be a key CCP in the management of microbiological hazards in insect production. Identifying temperature-time combinations for drying seaweed which prevent re-emergence of key pathogens in the stored dried product will inform development of robust HACCP guidelines, and GMP and GHP practices therein, for the seaweed feed industry, thus encouraging regulatory and commercial acceptability of seaweed as feed for insects.

Chapter 5 | Seaweed production for feed and food: site selection criteria and prospects for sector growth

5.1 Abstract

Within the last decade, EU policy has encouraged sustainable exploitation of renewable marine resources for feed and food, in order to relieve pressure on traditional bioresources. Various seaweed species have historically been used as animal feed and human food since they can provide high levels of protein, long-chain polyunsaturated fatty acids, and micronutrients, and functional health properties in the form of bioactive compounds. Seaweed is a permitted animal feed in the EU, and compared with terrestrial crops, is naturally abundant, and does not require fertilisers, pesticides or freshwater inputs for growth. However, in the EU, markets and consumers still classify seaweed as a relatively novel product. Therefore, expansion of the seaweed feed and food sectors will incur increasing regulatory scrutiny with regard to the safety and traceability of the products. Seaweed sequesters various undesirable substances from the environment, particularly heavy metals from seawater, and provides an ideal surface for colonisation by potentially pathogenic environmental bacteria. Producers of feed and food materials are ultimately responsible for product safety, which starts with the growing conditions at the harvesting site. Sector growth also relies on producers meeting regulatory and consumer concerns over the environmental sustainability of supply chains. Surveys were conducted with seaweed feed and food producers in Scotland and Ireland, and with regulatory and environmental bodies which advise the sectors, with the aim of identifying the range of factors, including water quality, which influence selection of sites for seaweed harvesting. Perspectives on future prospects for the sectors were also sought. Producers and advisory bodies identified commercial viability and public perception as influential site selection criteria, although site selection is ultimately determined by where a target seaweed species grows. Advisory bodies considered water quality an important factor, but producers considered batch testing of products and avoidance of sites subject to pollution events to be sufficient safety measures. Company relations with local rural communities and other harvesting businesses with whom they share the resource, and public concern over environmental impacts, effectively control harvesting at sustainable levels. Future concerns were associated with climate change effects on seaweed distribution and biomass.

Cultivation of seaweed for the feed market was considered key to overcoming the current bottleneck in the EU supply.

5.2 Introduction

The European Blue Growth initiative highlighted the potential contribution of sustainable aquaculture to economic growth, improved human nutrition, and reduced pressure on wild fish stocks (EC, 2012a). At the same time, the Bioeconomy Strategy recognises that innovative, efficient and sustainable production and conversion of renewable marine biological resources into feed and food can help to address concerns over competing feed and food uses of traditional biomass as well as food insecurity (EC, 2012b). Seaweed has been harvested globally for feed and food for centuries due to the nutritional benefits for livestock and humans including high concentrations of protein, minerals, vitamins and Long Chain Polyunsaturated fatty acids (LC-PUFAs) (Maehre *et. al.,* 2014). In addition, seaweeds have a balanced amino acid profile and depending on species and growth conditions can contain bioactive compounds (Makkar *et. al.,* 2016; Baweja *et. al.,* 2016). Commercially available seaweed biomass can originate from wild naturally growing seaweed (harvested either by hand or specially-designed boats), or commercially cultivated stock (Mac Monagail *et. al.,* 2017). Increasing demand for seaweed in feed, food and other industrial products is predicted to sustain long term growth of the sector (Mac Monagail *et. al.,* 2017). In recent years, the perception of seaweed as a functional feed and food providing human and animal health benefits beyond nutrition (e.g. as a prebiotic) has led to a global resurgence of interest in dietary inclusion of seaweed for livestock and humans (Wells *et. al.,* 2017; Evans and Critchley, 2014). Seaweeds as functional feed and food represent high value markets and will be a driving force in the future development of functional products (Hafting *et. al.,* 2012). As a marine bio-resource requiring only sunlight, nutrients, water and the space abundantly available in many coastal regions of the world, seaweed as a harvestable crop has fewer environmental limits compared with terrestrial food production systems (Forster and Radulovich, 2015). Cultivated seaweed in particular could provide a year-round harvest, offering greater feed and food security than most terrestrial crops (Rajauria, 2015).

Diversification of the feed and food sectors that has been proposed as part of the EU's developing bioeconomy will need to occur within the existing regulatory framework, and market and consumer confidence in innovative raw materials will require adaptation of existing standards or development of new standards, and the sharing of best practice (EC, 2012b). Hazard Analysis and Critical Control Points (HACCP) and ISO 22000 standards for managing safety of seaweed in the food chain are currently being developed for seaweed producers (Hafting *et. al.,* 2015a). Expansion of the seaweed feed and food sectors in the EU, where markets and regulators consider seaweed a relatively novel product, will mean that evidence of seaweed safety and traceability will be subject to increasing scrutiny (Hafting *et. al.,* 2015b). Both wild and cultivated seaweeds can harbour faecal indicator organisms (FIOs) and pathogenic extra-enteric and marine bacteria, as well as sequestering heavy metals (As, Cd, Hg, Pb) from the surrounding seawater (Byappanahalli *et. al.,* 2009; Ishii *et. al.,* 2006; Mahmud *et. al.,* 2007, 2008; Ryan *et. al.,* 2012), which pose a potential health risk to animal and human consumers of the seaweed. The risk of heavy metals in seaweed for animal health is reflected in the strict regulations on maximum allowed levels in seaweed feed (EC, 2002b; EC, 2013b), although interestingly not for seaweed for human food consumption; similar microbiological quality standards specific to seaweed as feed or food have yet to be developed. However, traceability is an increasingly important process for protecting consumers, facilitating market acceptance of innovative products and improving control of safety hazards in the food production chain. Traceability allows regulators to link an identified health hazard in the food chain to its source at any stage in its history of production, processing or distribution (Hafting *et. al.,* 2012; Aung and Chang, 2014). Producers of raw materials are considered ultimately responsible for ensuring the safety of their product (Hafting *et. al.,* 2012), therefore development of the sector will mandate refinement of hygiene standards for seaweed entering the food chain, and enhanced surveillance of production processes and growing conditions in order to protect public health (Aung and Chang, 2014).

To successfully market raw seaweed material for feed and food, supplies must reliably meet demand, comply with the quality standards for the country in which the product is marketed, and manufacturers must address consumer and regulatory concerns over

environmental sustainability, and the quality and ethical procurement of the supply (Hafting *et. al.,* 2015b). A seaweed aquaculture industry at the scale of the well-established fish farming industry is yet to be realised in Europe, despite the existence of standardised and commercially viable cultivation techniques (Rebours *et. al.,* 2014). Currently, approximately 83 % of global seaweed production is for human consumption, which is dominated by cultivation in Asia, and the remainder is utilised in biotechnology, medicine and agriculture, with just 1 % used for animal feed (Craigie, 2011; Rebours *et. al.,* 2014). European seaweed production by contrast is almost entirely dependent on the harvesting of wild stock (Mac Monagail *et. al.,* 2017). In Ireland, the production of *Ascophyllum nodosum* (the species most widely utilised worldwide as animal feed supplement) for feed and food comprises 13 % of EU production (Tabassum *et. al.,* 2017; Evans and Critchley, 2014). Cultivation in Scotland is currently at research-scale only, and the wild harvest is primarily for the alginate and fertiliser industries, though production is growing for the high value food market (Tabassum *et. al.,* 2017; Kenicer *et. al.,* 2000).

The potential for successful expansion of the EU seaweed feed and food markets, particularly in high value products, requires more than a supportive policy environment. The water quality of a site is a key determinant of the hygienic quality of the seaweed growing there, and traceability is more difficult with wild harvested seaweed than with material originating from a cultivation operation (Hafting *et. al.,* 2012; Hafting *et. al.,* 2015b). Yet in the UK and Ireland, due to the small, uncompetitive nature of the seaweed industry, which is largely focused on low volume, high value products (e.g. condiments and cosmetics), there is currently little regulation of water quality. However, non-binding government information advises the harvesting of seaweed from locations free of wastewater or other effluent discharges, such as shellfish harvesting areas (SHAs) (Marine Scotland, 2013). The most stringent regulations apply to seaweed produced organically in the EU which cannot be located in areas where pollutants may contaminate the site (Reg (EC) 710/2009; EC, 2009b). Water quality is just one of a myriad of environmental, legislative, economic and logistical factors which affect, to varying degrees, the suitability of sites for procurement of seaweed for the feed and food markets. Several studies have assessed the suitability of sites for seaweed

cultivation for various applications including food in the EU in terms of physico-chemical, bathymetric, infrastructural, legislative, marine planning and environmental impact factors (Kerrison *et. al.,* 2015; Thomas *et. al.,* 2019; Wood *et. al.,* 2017). Site suitability for wild harvesting has been viewed mainly through the lens of ecological sustainability and its inter-dependence with the local socio-economy (Roberts and Upham, 2012; Mac Monagail *et. al.,* 2017; Angus, 2017). Specific consideration of water quality with regards procurement of seaweed for feed and food has largely focussed on heavy metal pollution as a constraining factor, and nutrient loading (generating increased seaweed biomass) as a positive site characteristic (Roleda *et. al.,* 2019; Wood *et. al.,* 2017).

Key stakeholders in the seaweed feed and food sectors include the commercial harvesters and producers of seaweed products, as well as governmental and non-governmental organisations which provide advice to the industry on topics ranging from regulations to environmental sustainability. The aim of this study was to evaluate the criteria used by these key commercial and advisory stakeholders for identifying seaweed harvesting sites suitable for producing seaweed destined for the EU feed and food markets. Objective 1 was to identify key site selection criteria for seaweed harvesting in the UK and Ireland, and compare the perceptions of organisations which provide advice to the seaweed industry with decision-making by commercial feed and food producers. Objective 2 was to elicit stakeholder views with regard to constraints and opportunities for future growth of the UK and Irish seaweed feed and food sectors.

5.3 Method

The research question required collection of data from individuals and companies who harvest seaweed in the UK and Ireland for the animal feed market, as well as non-commercial governmental and non-governmental organisations with responsibility for providing regulatory, environmental or commercial advice to Scottish and Irish seaweed producers. Internet searches for UK and Irish companies producing seaweed feed, and information from several expert stakeholders representing non-commercial organisations linked to the UK and Irish seaweed sectors, enabled identification of nine feed producers operating in the UK and Ireland that harvest seaweed for animal feed.

The small number of feed producers in existence highlighted the need to broaden the remit of the research, and extend the sample to a sector of the seaweed industry not initially anticipated as relevant to the study i.e. harvesters and producers of edible seaweed for human food. The sample size of producers was therefore increased by including seven UK and Irish producers of seaweed for human food, which were also identified through online searches. Seaweed feed and food producers were located primarily in Scotland, Northern Ireland and Eire. Organisations providing advice to Scottish and Irish seaweed producers (n = 16) were identified through online searches for organisations associated with the seaweed industry. The total population to be sampled was small, and a level of non-response to contact of potential participants was expected (de Vaus, 2002). Thus sampling was strategic, taking the form of a non-random purposive approach whereby all individuals and organisations who met the research criteria were identified and approached (Bryman, 2012). Stakeholders are categorised as producers or advisory bodies from this point onwards.

There is little available published data on the range of factors which guide decision-making about site selection for commercial seaweed harvesting. Therefore, the research strategy needed to be one that facilitated the collation of exploratory data which enabled identification of all possible influential factors, and generated data rather than relying on published data to underpin the research approach (Nardi, 2018). Furthermore, a methodological pluralist approach involving the collection and analysis of both quantitative and qualitative data is of value in a research context involving multi-faceted management of a natural resource in which public, private, commercial and regulatory considerations all play a role (Olsen, 2004). The research aim of associating individual stakeholder groups with specific decision-making criteria and site selection variables, and comparing groups on that basis, was well suited to enumerative induction i.e. quantitative research (Brannen, 2016). A quantitative survey enabled standardisation of questions, and facilitates study replication (Nardi, 2018). A qualitative data collection method was appropriate for the examination of the rationale underlying stakeholders' choice of site criteria, and exploration of opinions about the future of the industry (Nardi, 2018). The outcome of these methodological considerations was mixed methods data collection by means of a structured survey

which formed the primary research strategy, integrated with a semi-structured interview (the secondary strategy), which together generated both quantitative and qualitative data (Brannen, 2016; Olsen, 2004). Data triangulation through a mixed methods approach can facilitate validation of findings, different methods offer different strengths and weaknesses, and qualitative data can provide explanations of, context for and enhancement of quantitative data (Bryman, 2012).

The survey was piloted with an advisory body by means of a self-completion emailed questionnaire, and in response to the feedback, the survey instructions and layout were modified to improve clarity. The structured quantitative survey consisted of a non-exhaustive list of 59 site selection criteria, which a qualitative review of the grey and peer-reviewed literature had indicated could influence selection of locations for harvesting or cultivating seaweed. The criteria were divided into seven thematic areas: (1) Standing stock of target seaweed species; (2) Socio-cultural; (3) Infrastructural; (4) Regulatory; (5) Economic; (6) Environmental; and (7) Physio-chemical. By ticking boxes, participants were asked to identify site selection criteria that were currently influential, from which the five most important criteria were determined. Participants were then asked to identify site selection criteria that may influence future site selection if different from current criteria, from which the five most important future criteria were determined. Space for additional comments enabled respondents to elaborate on whether their selected criteria facilitated or impeded their use of potential harvesting sites and how; this formed the semi-structured interview element of the survey. Thus the qualitative data was generated concurrently with the quantitative data. Participants could also add additional site selection criteria to those listed in the survey; it is necessary that with forced choice questions, all possible options, including unanticipated responses, can be included to avoid biasing responses (de Vaus, 2002). An additional survey section included for producers was comprised of open-ended questions regarding the species of seaweed and markets targeted, and closed-ended questions regarding the methods used for harvesting or cultivation. The survey approach was flexible in that additional questions were sometimes asked of participants in order to clarify answers. The survey questionnaires designed for producers and advisory bodies are shown in Appendices 5.1 and 5.2, respectively.

The purpose of the research and reason for identifying the individual or company as a potential participant was explained to each respondent via email, and the request to anonymously undertake the survey via a subsequent telephone call. Of the nine feed producers, two took part in the survey: three did not respond to emails or return telephone calls, and four declined to participate (Table 5.1). Of the seven food producers, four took part in the survey: three did not respond to emails or return telephone calls (Table 5.1). Despite assurances of anonymity, individuals representing commercial producers frequently chose not to take part, citing concern that their responses could be linked to their companies. Of the 16 advisory bodies, three took part in the survey: six did not respond to emails or return telephone calls, and seven declined to participate due to their stated lack of direct involvement with the seaweed industry (Table 5.1).

Table 5.1. Stakeholder grouping of survey participants.

	Scotland	Ireland	Mode of response
Feed producer			
Company		2	Both face-to-face
Food producer			
Company	3		One by phone; two by email
Self-employed individual	1		Email
Advisory organisation			
Regulatory	1		Face-to-face
Environmental protection	2		One face-to-face; one by email

Given the restricted population available for sampling, mixed modes of administering the survey were selected to maximise the likelihood of recruiting a representative sample (de Vaus, 2002). Surveying was carried out according to participant preference and accessibility, and included either self-administered surveys via an email attachment, telephone-based surveys, or face-to-face surveys. Regardless of the data collection method used, all producers received the same questionnaire survey (Appendix 5.1) and all advisory bodies received the same questionnaire survey (Appendix 5.2). Emailed surveys were the preferred form of contact for more

geographically remote producers and advisory bodies, and although the return of completed self-administered surveys frequently required multiple requests, the process demanded minimal expenditure of time and money (Nardi, 2018). However, respondents who completed the survey electronically and returned it by email attachment tended not to utilise the space provided to elaborate on the choices they selected as influential in site selection. There was no opportunity to probe for explanations for choices from these respondents, or to ascertain if questions or categories had been deliberately or accidentally omitted (de Vaus, 2002). Thus, the self-completion surveys lacked the breadth and depth of responses gained from telephone or face-to-face surveys.

The participant interviewed over the telephone requested to be surveyed by this method for convenience. Sustaining participant engagement with the relatively complex survey by this means of communication proved difficult. Fatigue and the difficulty with remembering choices led to the respondent's answers became increasingly succinct during the progress of the survey, and it was not always possible to ascertain if a question had been clearly understood (de Vaus, 2002; Bryman, 2012). However, the participant did elaborate on their site selection choices when prompted. The responses were recorded by hand in a survey questionnaire by the interviewer. Face-to-face surveying, although more time consuming and expensive to undertake than email or telephone surveys, was the data collection method of choice, and was undertaken by preference unless an alternative method was favoured by the participant or necessary due to inaccessibility of a participant's location. Surveys conducted in person resulted in fewer omitted or misunderstood questions, questions and answers could be clarified, and a rapport between interviewer and respondent produced rich explanatory data during the semi-structured interviews (Nardi, 2018). Each respondent surveyed in person self-completed the survey by hand, including explanations of chosen site selection factors, whilst additional verbal comments and opinions expressed by the participant were recorded by hand by the interviewer, though not verbatim.

Data on seaweed species targeted and methods employed for harvesting the resource by producers were separated according to whether producers represented the feed or food sector, and whether they wild harvested or cultivated the seaweed, and were

tabulated to characterise the commercial profiles of the producers. Factors chosen by respondents as currently influential in site selection were tabulated both to show the range of currently influential criteria, and the factors identified as most important in decision-making, selected by feed producers, food producers and advisory bodies, and to enable visual comparison between the three stakeholder groups. A frequency histogram provided an indication of the numbers of individuals within each of the three stakeholder groups who selected each of the chosen site selection criteria. Respondents' assessments of whether their chosen factors encouraged or discouraged use of a harvesting or cultivation site, and the nature of that influence on decision-making, were extracted from the additional qualitative data provided in the survey, or the comments made to the interviewer. Unless explicitly stated, whether a factor positively or negatively affected site suitability was ascertained by evaluating the respondent's justification for selecting the particular criteria. Future site selection criteria emerged from the tick-box survey but also from the qualitative comments made by respondents, either in the boxes provided in the survey or orally to the interviewer; all data were assessed in the same way as current site criteria and reported in the results (section 5.4.3). The small sample size (n = 9) meant that quantifiable survey responses could not be analysed statistically nor assumed to be representative of all feed or food producers or of advisory bodies in the UK or Ireland.

Data pertaining to perceptions about future constraints and opportunities for future growth of the seaweed industry were extracted from the qualitative data by identifying either explicit or implicit expression of perceptions about the future. These opinions were extracted by applying a content analysis to the text. Themes (e.g. seaweed cultivation, Brexit, market prices) which emerged in the non-verbatim transcripts of verbal comments made by the respondents interviewed in person or via the telephone (n = 5) were identified (Bryman, 2012). Producers' and advisory bodies' perceptions about the future were selected for inclusion in the results (section 5.4.4) based on the frequency of occurrence in the text; repetition of a broad theme (e.g. cultivation) by more than one individual or organisation from the same or any other stakeholder group assured inclusion. For each broad theme included in the results, any ccomments made by individual respondents pertaining to each theme were also included. The data

collated in the survey and semi-structured interviews provide a preliminary and non-generalisable indication of the relative importance to producers and advisory bodies of water quality and other site variables in site selection for procurement of seaweed for feed and food, and viewpoints about future opportunities and constraints for the UK and Irish seaweed industries.

5.4 Results

5.4.1 Profile of stakeholders and commercial operations

A total of nine stakeholders out of an initial shortlist of 32 individuals, companies or organisations responded to the survey (Table 5.1). Hand harvesting of naturally growing seaweed was the dominant method of seaweed collection by both feed and food producers (Table 5.2), although one food producer employed a harvesting boat to trim *A. nodosum* as it was considered a more sustainable method compared with hand cutting which removes more of the plant. One feed producer cultivated seaweed alongside a mussel farm as part of an integrated multi-trophic aquaculture (IMTA) system; however, the majority of seaweed biomass was sourced from wild harvesting. A wide range of seaweed species were harvested for animal feed, including brown, red and green algae, whereas food producers focused solely on brown seaweeds (Table 5.2).

Table 5.2. Targeted seaweed species and method of harvesting by six commercial producers.

Stakeholder		Target species	Methods of harvesting
Feed producer	Wild harvesting (*n* = 2)	*Alaria esculenta* *Ascophyllum nodosum* *Chondrus crispus* *Fucus serratus* *Fucus vesiculosus* *Himanthalia elongate* *Laminaria digitata* *Palmaria palmata* *Pelvetia cannaliculata* *Saccharina latissima* *Ulva intestinalis* *Ulva lactuca*	Hand pick; hand cut with knife/ scissors
	Cultivation (*n* = 1)	*Alaria esculenta* *Laminaria digitata*	Farm or small scale (0-40 x 200m lines); integrated multi-trophic aquaculture (IMTA)
Food producer	Wild harvesting (*n* = 4)	*Alaria esculenta* *Ascophyllum nodosum* *Fucus spiralis* *Fucus vesiculosus* *Laminaria digitata* *Pelvetia cannaliculata*	Hand cut with knife/ scissors; harvesting boat

5.4.2 Current site selection criteria

The most frequently identified site selection criteria by both feed and food producers and advisory bodies related to the available standing stock and physical accessibility of the target seaweed species, as well as statutory permissions for site access and non-statutory advice to limit ecological impacts of harvesting (Fig. 5.1).

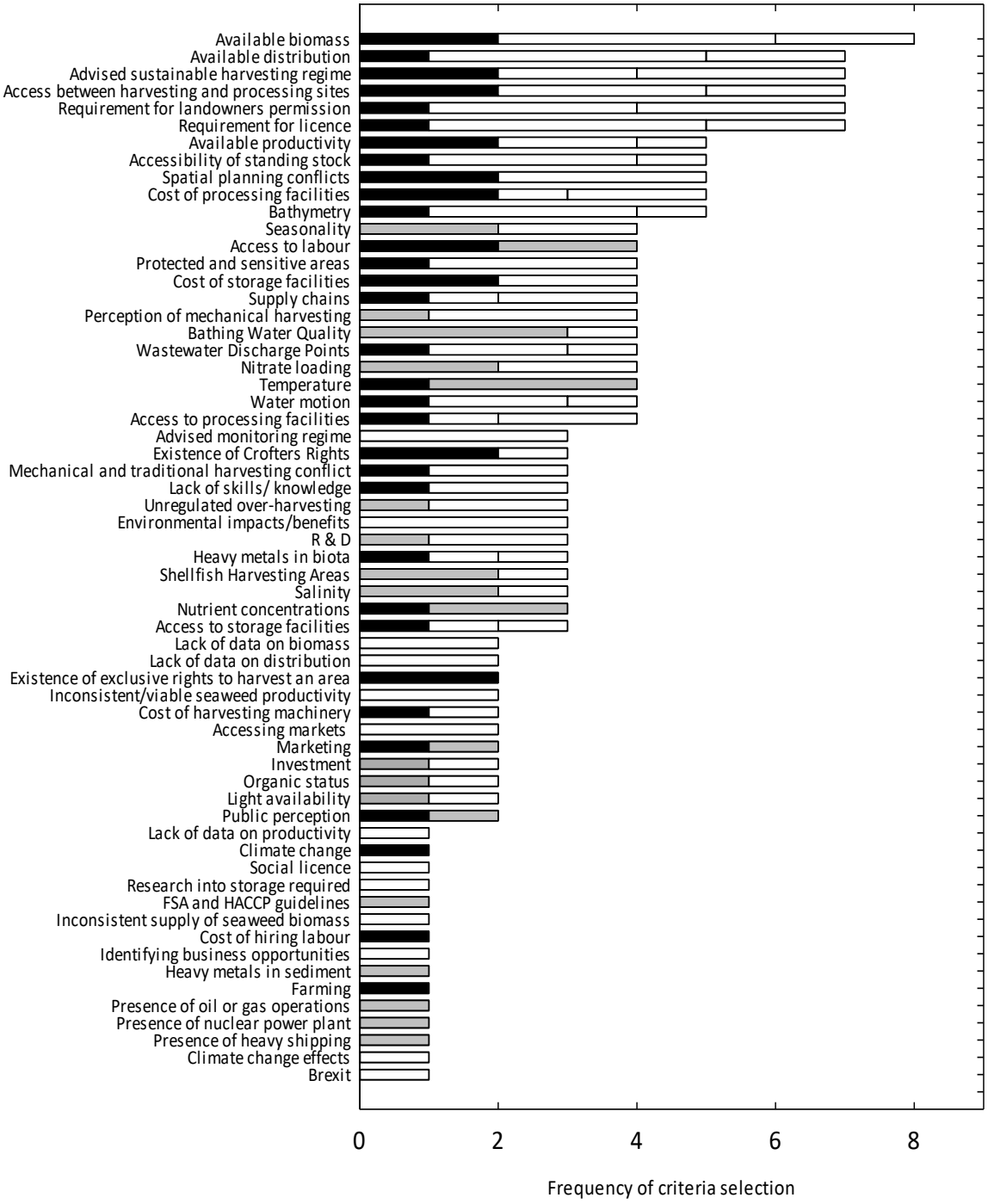


Figure 5.1. Frequency by which stakeholders identified each site selection criteria as currently influential in determining the choice of seaweed harvesting location. Black bars = feed producers. Grey bars = food producers. White bars = advisory bodies.

5.4.2.1 The role of seaweed availability

The availability of the target seaweed species in terms of biomass, productivity and distribution were identified by all stakeholders as key determinants of site selection for seaweed harvesting (Table 5.3). Feed and food producers stated that "You go where the seaweed is", and referred to the location of operations as often relating to the historical use of certain sites. An advisory body believed that inconsistency of seaweed productivity was a significant influence on site selection due to factors such as seaweed disease or fluctuating water temperatures undermining the planning of production (Table 5.3). One food producer highlighted the seasonality of some seaweed species as a key determinant of where cultivation or harvesting is sited, stating that businesses are built around the species identified for commercial exploitation, not the other way around. Seasonality and the limit of one harvest a year was however accepted by producers as a feature of most seaweed species. The commercial response by one producer is to store a dried stock of out of season species during winter. However, one food producer noted that it was not economically rational to target a species that is available for extremely limited periods.

Regulatory and environmental advisory bodies stated that inadequate scientific data on seaweed resources influenced where harvesting occurs (Table 5.3). A food producer stated that improved data on the distribution and availability of natural stocks of commercially important seaweed species would encourage exploration of previously unexploited sites. However, all stakeholders agreed that expert advice on sustainable harvesting regimes was an influence on site selection. A feed producer cited community policing amongst harvesters as a deterrent of unsustainable harvesting in certain locations, attributed to the knowledge amongst harvesters that unsustainable activity by one individual produced a negative public perception of all harvesters. Advisory bodies perceived that monitoring to ensure sustainability of harvesting regimes is a key influence on species and site selection (Table 5.3).

Table 5.3. Factors that can influence site suitability for harvesting and cultivating seaweed as perceived by producers and advisory bodies. Grey boxes = criteria identified by stakeholders as the most important determinants of site suitability.

Site selection criteria	Feed producers	Food producers	Advisory bodies
1) Standing stock of target seaweed species	Available biomass	Available biomass	Available biomass
	Available productivity	Available productivity	Available productivity
	Available distribution	Available distribution	Available distribution
		Seasonality	Seasonality
			Lack of data on biomass
			Lack of data on productivity
			Lack of data on distribution
	Advised sustainable harvesting regime	Advised sustainable harvesting regime	Advised sustainable harvesting regime
			Advised monitoring regime
	Other: Climate change		

2) Socio-cultural	Existence of Crofters Rights/ practices of traditional harvesters		Existence of Crofters Rights/ practices of traditional harvesters
	Conflict between mechanical harvesting and livelihoods of traditional harvesters	Conflict between mechanical harvesting and livelihoods of traditional harvesters	Conflict between mechanical harvesting and livelihoods of traditional harvesters
			Other: Social licence
3) Infrastructural	Accessibility of standing stock	Accessibility of standing stock	Accessibility of standing stock
	Access from harvesting site/s to processing facility/facilities	Access from harvesting site/s to processing facility/facilities	Access from harvesting site/s to processing facility/facilities
	Access to processing facility/facilities	Access to processing facility/facilities	Access to processing facility/facilities
	Access to storage facility/facilities	Access to storage facility/facilities	Access to storage facility/facilities
	Access to labour	Access to labour	
			Research into storage required
	Lack of skills/ knowledge		Lack of skills/ knowledge

4) Regulatory	Difficulty in determining suitable locations that do not conflict with other coastal activities		Difficulty in determining suitable locations that do not conflict with other coastal activities
	Existence of exclusive rights to harvest an area		
	Existence of protected and sensitive areas		Existence of protected and sensitive areas
	Requirement for landowner's permission	Requirement for landowner's permission	Requirement for landowner's permission
	Requirement for Licence	Requirement for Licence	Requirement for Licence
		Unregulated over-harvesting	Unregulated over-harvesting
			Environmental impacts/benefits
		Other: FSS and HACCP guidelines	
5) Economic			Inconsistent supply of seaweed biomass
			Inconsistent/viable seaweed productivity
	Cost of hiring labour		

	Cost of harvesting machinery		Cost of harvesting machinery
	Cost of processing facilities	Cost of processing facilities	Cost of processing facilities
	Cost of storage facilities		Cost of storage facilities
			Accessing markets for low or high value products
	Marketing	Marketing	
		Investment	Investment
		R & D	R & D
	Supply chains	Supply chains	Supply chains
			Other: Identify business opportunities
6) Environmental		Perceived environmental effects of mechanical harvesting preventing investment in mechanical harvesting	Perceived environmental effects of mechanical harvesting preventing investment in mechanical harvesting
		Heavy metals in sediment	
	Heavy metals in biota	Heavy metals in biota	Heavy metals in biota

		Bathing Water Quality	Bathing Water Quality
	Wastewater Discharge Points	Wastewater Discharge Points	Wastewater Discharge Points
		Shellfish Harvesting Areas	Shellfish Harvesting Areas
		Nitrate loading	Nitrate loading
	Other: Farming		
		Other: Organic status	Other: Organic status
	Other: Public perception	Other: Public perception	
		Other: No oil or gas operations in area	
		Other: No nuclear power plant in area	
		Other: No heavy shipping in area	
7) Physio-chemical	Bathymetry	Bathymetry	Bathymetry
	Temperature	Temperature	
		Salinity	Salinity
	Water motion	Water motion	Water motion

	Nutrient concentrations	Nutrient concentrations	
		Light availability	Light availability
			Climate change effects
		Other: Brexit	

5.4.2.2 The role of local communities

Irish feed producers identified the historical Foreshore Rights of individual families as a key influence on the quantities of *A. nodosum* that are harvested (Table 5.3). Producers avoid conflict by paying local harvesters to undertake the harvesting. However, compared with *A. nodosum*, producers harvest much smaller quantities of the red seaweed *P. palmata*, thus harvesting of this species does not impinge on the harvesting activities of local people, enabling producers to harvest *P. palmata* themselves without utilising local harvesters as middlemen. Advisory bodies cite conflict between large and small producers, entrepreneurial businesses and traditional harvesters as a deterrent to harvesting at certain locations (Table 5.3). In Scotland, a food producer attributed the lack of conflict between the Scottish seaweed industry and local communities to the ban on mechanical dredging operations. A regulatory organisation stated that producers seek a 'social licence' to operate from local communities in order to avoid the public perception of large producers as diverting the economic rewards associated with seaweed away from local communities. Social licencing is therefore a preventative measure against mechanical harvesting of certain sites and a control on unsustainable harvesting activities (Table 5.3).

5.4.2.3 The role of available infrastructure

All stakeholders cited ease of access between the seaweed resource, the processing facilities and the storage facilities as a key consideration (Table 5.3). This is due to the often remote location of the standing stock of the target seaweed species, the necessity of processing the seaweed within hours of harvest and the cost of transport between the harvesting sites and processing plant. Although feed producers feel that labour for wild harvesting is easily obtained, in Ireland conflict does arise between local people with Foreshore Rights to seaweed and large producers applying for licences to harvest the same foreshore (Table 5.3). The outcome is that local people refuse to work for these producers. One food producer stated that it is easier to find harvesters willing to collect seaweed by boat than by hand cutting. Feed producers and advisory bodies cite a lack of specialist skills as a challenge for the establishment of seaweed farms.

5.4.2.4 The role of Regulations

Competition between multiple harvesting companies in a region was identified by feed producers and advisory bodies as a potential influence on selecting where to operate (Table 5.3). Regulatory and environmental advisory bodies highlighted the existence of protected and sensitive areas in coastal zones, and the requirement for a marine licence for construction on the seabed, as important determinants of cultivation operations. One feed producer stated that protected areas are potential restrictions on the siting of proposed seaweed farms, and are increasingly acknowledged by the seaweed industry as a potential issue as large international producers take over smaller producers and expand their operations over large stretches of coastline (Table 5.3). All Scottish stakeholders recognised that either the Crown Estate or a private landowner's permission is required to hand-harvest wild seaweed, and that a licence which considers maximum sustainable yield for an area from the Crown Estate is required to harvest natural stocks using a vehicle or vessel. Scottish stakeholders also acknowledged that expectations of environmental sustainability is a key influence on harvesting operations, since producers with permission from the Crown Estate must agree to quotas and rotate harvesting areas to allow for regrowth. Feed producers acknowledged that the existence of Foreshore Rights in Ireland that are historic but not formally documented (and which producers can therefore contest), has led to conflict between producers and communities and can prevent exploitation of certain locations. Regulatory and advisory bodies argued that hand-harvesting for feed should be prohibited for ecological reasons, and that beach-cast or cultivated seaweed are less environmentally damaging sources of seaweed. One food producer cited their adherence to HACCP guidelines together with registration with Food Standards Scotland (FSS) as key to controlling the quality of their products, and a determining factor as to where the seaweed could be sourced (Table 5.3).

5.4.2.5 The role of economic forces

The financial costs to a commercial seaweed business of hiring labour, purchasing harvesting and processing machinery, and establishing storage facilities are significant constraints on expansion of operations, particularly for feed producers due to the volumes of seaweed that require processing, and are recognised as challenges by

advisory bodies (Table 5.3). The lower cost of harvesting seaweed by hand rather than by boat encourages some feed producers to target seaweed species that can be hand-harvested. However, the quantity of biomass that can be harvested by hand is limited, and can constrain the financial return. Therefore, producers that use boats and target seaweed species that can be harvested in larger quantities are at an advantage. The provenance of Scottish and Irish seaweed feed and food products harvested from remote, pristine sites plays a key role in their marketing, thus geographical location is an important consideration for producers (Table 5.3). The cost of haulage from remote harvesting sites and the economies of scale were recognised by both producers and advisory bodies as significant determinants of commercial viability in rural areas.

5.4.2.6 The role of environmental and physico-chemical factors

A food producer stated that the general public's perception of a seaweed business and its products is a crucial factor in developing a sustainable operation from its inception. The negative public perception in Scotland of the environmental damage caused by mechanical harvesting has thus far prevented intensive exploitation of certain sites, specifically kelp beds (Table 5.3). All stakeholders viewed heavy metal concentrations in seaweed as a deterrent to harvesting at certain sites if levels exceeded maximum allowed levels for feed and advised safe levels for food. Feed producers stated that the microbiological quality of the water from which seaweed is harvested or in which seaweed is cultivated is not a major consideration in site selection because regular bacteriological testing of the processed material is used to indicate the hygienic quality of the seaweed product (Table 5.3). One feed producer regularly tested water quality for FIOs, and one food producer regularly tested seaweed batches for spoilage and pathogenic microbes including *Bacillus cereus*, *Listeria* spp., *Salmonella* spp., yeasts and moulds. However, a feed producer argued that water quality monitoring is only a legal requirement for SHAs, or designated Bathing Waters, and as a planning requirement for assessing the potential environmental impacts of hard engineering associated with proposed seaweed farms.

The production objective of seaweed feed producers is to maximise biomass output whilst minimising production costs. However, seaweed destined for human consumption, requires a transparent supply chain of a product sourced from clean

waters, and customers are willing to pay higher prices for products with an organic status. Scottish food producers target remote locations which are free of pollution to site their operations. However, food producers stated that they are not deterred from harvesting at sites not designated as monitored SHAs (Table 5.3). Nitrate loading of sites is not considered a deterrent to harvesting by feed or food producers, and an advisory body believed that nutrient enriched locations could be exploited for growing seaweed (Table 5.3). Feed producers postpone harvesting seaweed from sites that have received slurry run-off until the microbial pollutants have dissipated which typically takes six weeks, and therefore decrease the risk by operating at several different locations (Table 5.3). Both feed producers stated that achieving organic status for seaweed feed does not require a site to meet strict microbiological parameters. Feed producers regarded organic accreditation for produce as placing greater emphasis on sustainable production practices than on product safety. Food producers avoid harvesting seaweed from areas in which oil or gas operations, nuclear power plants and heavy shipping are located because of the public perception of the potential impacts on food safety. Feed and food producers cite physico-chemical factors including bathymetry, temperature, water motion and nutrient concentrations as key determinants of the optimum habitat for, and thus presence of, economically important seaweed species (Table 5.3).

5.4.3 Future site selection criteria

Advisory organisations raised concern that climate change and its environmental effects, e.g. increased water temperatures, will alter the productivity and distribution of commercial seaweed species with the result that scientific data on which the industry depends will become rapidly irrelevant. Advisory bodies identified the future effects of climate change (specifically on water temperature) as a driving force behind the northward shift of Scottish harvesting operations. Due to climate change, one feed producer predicted a commercial shift in targeted seaweed species, and a food producer stated that the company would have to move to be near to the target species if it shifted distribution. One advisory body stated that regulators are increasingly influenced by the power of public perception, which is likely to continue to restrict mechanical harvesting in the future, thus limiting harvesting operations to certain locations. A regulatory organisation cited access to labour as a significant influence on

future harvesting operations due to the challenge of maintaining a full work force for a seasonal harvest. One feed producer stated that although no harvesting licences have so far been allocated in Ireland, in the future harvesters may require them, which could allow companies to more effectively challenge communities over access to certain sites. The need for contingency planning (for example, in the event of an oil spill) encouraged one feed producer to network with other European producers in order to ensure an uninterrupted supply from unaffected international sites.

5.4.4 Seaweed for feed and food: constraints and opportunities

Seaweed cultivation was frequently cited by all stakeholders as an environmentally and economically sustainable alternative to wild harvesting. It was argued that seaweeds could be cultivated in suitable areas where they are not naturally found, together with the potential to share marine space with other activities, e.g. culture of fed aquaculture species in IMTA systems. Seaweed cultivation could also enhance public perceptions of seaweed companies since it is viewed as a more sustainable form of production than wild harvesting. However, seaweed aquaculture farms require skilled labour, such as skippers and hatchery technicians, recruitment of whom may prove a constraint on growth of the sector. One feed company described hand-harvesting of seaweed as having minimal environmental impact; by contrast, advisory organisations stated that seaweed intended for feed should be cultivated due to the environmental risks from wild harvesting compared with the benefits of ecosystem service delivery and fishing gear exclusion associated with cultivation. According to one advisory body, there is likely to be economic value accrued from exploiting the ecosystem services of cultivating seaweed in IMTA systems. The same advisory body suggested that sustainably hand-harvested seaweed should be a small-scale operation and target high value markets, such as food. Yet another regulatory stakeholder described cultivation as not yet economically viable, stating that commercial viability could be achieved in the future through technological improvements and refinements, and by focusing on the bioprospecting potential of seaweed. High value pre-processing such as seaweed biorefining will become increasingly important to accessing multiple markets, since the seaweed waste could be used as feed. A feed producer stated a belief that future business models should be cognisant of the fact that seaweed producers must secure a

guaranteed buyer before committing to production, and investment will occur if demand can be proven.

A key constraint for the growth of the seaweed animal feed sector (identified by one feed producer) is that feed producers sell seaweed in very high quantities at low prices, unlike edible seaweed which is sold in low volume at high prices. A food producer ascribed the high value of edible seaweed, for which there is a growing market, to the ease of use by the consumer, quality assurance, organic and kosher accreditations, and the use of food grade harvesting sites. In the feed sector, demand outstrips supply and the situation has been described as 'a race to the bottom'. Beach cast seaweed was suggested to be a valuable sustainable source of feed biomass by one advisory body. A feed company representative believed that any business established purely to supply the feed market is unlikely to prove economically viable. Access to EU or government funding was identified by an advisory body as key for the establishment of a processing facility by a company. Without such funding, the target seaweed species, and therefore site selection, was dictated by the production costs (as well as access to markets). One food company stated that more research into the potential of previously unexploited seaweed species would help the food sector, since only a narrow range of species are currently utilised in Scotland and Ireland. One feed producer suggested that if microbes hazardous to humans were shown not to travel up the food chain from seaweed, then seaweed could in future be cultivated next to wastewater treatment plants to exploit the nutrients. The feed industry stakeholders stated that it was important to prevent the seaweed industry suffering the same damaging effects of negative public perceptions of environmental pollution experienced by the salmon industry.

5.5 Discussion

5.5.1 Current and future site selection criteria

The key findings of this study indicate a high degree of overlap in criteria selected as influential to locating operations between industry stakeholders and organisations which regulate or advise commercial producers. Currently, the decision-making process by which seaweed feed and food producers identify sites suitable for harvesting is driven by multiple factors, all of which are ultimately associated with the broad themes

of commercial viability and public perception. Water quality at harvesting sites is of greater concern to food than feed producers due to the food safety concerns of human consumers. Industry stakeholders and advisory bodies predicted significant changes to harvesting locations in the future as a result of climate change.

The perception by regulatory and environmental advisory bodies that certain statutory requirements and guidelines regarding water quality, namely monitoring of SHAs and organic standards for seaweed production, are decisive factors for site selection for seaweed harvesting is not shared in practice by feed companies. Classification and monitoring of SHAs in the EU to protect consumer health is based on levels of *E. coli* present in shellfish flesh, and SHAs are correspondingly categorised as A, B or C according to the increasingly stringent post-harvest treatment of the harvested shellfish that is required under the EU Directive (Ventura de Souza *et. al.,* 2018). The bacteriological screening of processed batches of seaweed feed and food products undertaken by some companies was perceived as providing sufficient protection for consumer health and to enable seaweed harvesting to occur outside of SHAs. Bacteriological testing of harvesting waters and seaweed feed and food products are not statutory requirements however, and the limited sample size of industry stakeholders does not necessarily represent widespread production practices. The unique characteristics of the provenance of Irish and Scottish seaweed harvested in pristine, remote sites for the feed and food markets were highlighted as key selling points for consumers. The choice of site by one food producer was governed by that company's adherence to HACCP guidelines and registration with the FSS, and HACCP-certified seafood (which is perceived as 'safer'), has been associated with consumer willingness to pay a premium for products (Alfnes *et. al.,* 2018).

Online company websites show that organic accreditation of products appeal to consumer preferences, and is a marketing strategy employed by several Scottish and Irish seaweed food producers. However, as identified by an industry stakeholder, the guiding vision for organic production practices is environmental and socio-economic sustainability, whilst water quality concerns for organic aquaculture focus on unsustainable and hazardous chemical inputs and outputs (Niggli, 2015; Bergleiter and Meisch, 2015). Although a key principle of the still relatively niche organic aquaculture

market is to promote human health in addition to environmental health, EU consumers have identified the absence of hormones and chemicals rather than a lack of microbial contaminants as the key health advantages of organic aquaculture products (Lembo *et. al.,* 2018). Irish and Scottish feed and food companies do avoid sites contaminated by diffuse agricultural run-off, and feed producers are constrained in site selection by EU heavy metal standards for seaweed feed (Reg (EC) 32/2002; EC, 2002b; Reg (EC) 1275/2013; EC, 2013b). Furthermore, food producers prefer not to utilise sites which produce seaweeds containing high metal concentrations (Commission Recommendation (EU) 464/2018; EU, 2018a), or in areas subject to heavy shipping, oil or gas operations or near nuclear power plants, reflecting the importance of public perceptions of food safety for this sector. The radionuclide ^{99}Tc, for example, is known to accumulate in brown seaweeds, including *F. vesiculosus* and *A. nodosum* (Heldal and Sjotun, 2010). Various edible seaweed species contain high levels of Aluminium and Mercury, however a long term exposure assessment concluded that consuming animals reared on a seaweed supplement containing arsenic posed a negligible threat to human health (Paz *et. al.,* 2019; Mac Monagail *et. al.,* 2018). Ultimately, co-location of harvestable biomass of the target species in SHAs or locations attaining organic standards are considered as advantageous, but not essential, pre-requisite site characteristics by the seaweed feed and food companies surveyed in this study.

All stakeholders recognised that the market demand for a seaweed species and the location of that resource fundamentally determine where seaweed harvesting operations are established. Commercially targeted seaweed species present in sufficient quantities often grow at remote sites, where they may have a long history of exploitation by local coastal communities, as in the west coast and islands of Scotland and in Ireland (Kenicer *et. al.,* 2000; Guiry and Morrison, 2013). However, there is the potential for conflict between local communities, which may be economically dependent on or culturally reliant on local seaweed resources (Mac Monagail *et. al.,* 2017), and seaweed harvesting companies targeting the same resources. Removal of seaweed in excess of its natural renewal rate leads to over-exploitation of seaweed which can reduce total seaweed stands, change intertidal and subtidal community structures, and consequently result in the loss of related ecosystem services (Mineur *et.*

al., 2015). There are currently no regulatory constraints on seaweed harvesting in Scotland, only advice on sustainable harvesting practices recommended by Scottish Natural Heritage, although due to public pressure there has been a recent ban on mechanical dredging of ecologically important kelp beds (Angus, 2017; Scottish Wildlife Trust, 2018). However, wild harvesting businesses tend to self-police sustainable management of their resource (Mac Monagail *et. al.,* 2017). Conflict can stem from actual or perceived unsustainable harvesting practices by commercial organisations, as well as from the incompatibility of current permissions with historical access rights as experienced in Ireland (Mac Monagail *et. al.,* 2017). These issues are bound up in what one regulatory stakeholder described as the concept of 'a social licence to operate', an informal social contract granted by local communities to, in this case, a marine industry to utilise a resource sustainably and with deference to the interests and rights of the public (Kelly *et. al.,* 2017).

Industry stakeholders predicted that commercial responses to northward shifts in species distributions would be either to change the species marketed or move harvesting operations in line with the shifting seaweed stocks. The upper layer of the Atlantic Ocean has warmed at a rate of 0.07 °C per decade since the late 19th century, and by the year 2090 mean global sea surface temperatures are projected to be up to 3.3 °C warmer (Hoegh-Guldberg, *et. al.,* 2014). Optimum temperatures for some commercially important seaweed species may be exceeded causing physiological stress and reduced growth and survival, leading to depleted harvests of natural stocks (Chung *et. al.,* 2017; Rebours *et. al.,* 2014). In the northeast Atlantic, kelps and fucoids dominate the seaweed biomass in the subtidal and intertidal zones respectively (Brodie *et. al.,* 2014). Changes in the distribution of brown seaweeds are predicted, however, warmer temperatures favour production of some species, particularly intertidal fucoids, including *F. vesiculosus* and *A. nodosum* in the west of Scotland where the majority of Scottish seaweed companies operate (Brodie *et. al.,* 2014; Yesson *et. al.,* 2015). Since market demand dictates species choice, and established markets exist for certain species, it is likely that businesses will have to relocate in the short- to medium-term, given a predicted mean rate of range shift amongst seaweeds of 7.3 km y^{-1} (Sorte *et. al.,* 2010). The atmospheric CO_2 concentration has increased from pre-industrial levels of

approximately 280 ppm to 410 in 2019, and is projected under the moderate RCP4.5 scenario to increase to 540 ppm by 2100 (Collins *et. al.,* 2013; Dlugokencky and Tans, 2019; Prather *et. al.,* 2013). Dissolved CO_2 in seawater alters the chemical balance of the water, and as a result the pH of surface water in the North Atlantic has decreased by 0.1 from the pre-industrial period to 1994, and a further reduction of pH 0.15 – 0.5 (depending on emission scenario) is expected by 2100 (Rhein *et. al.,* 2013; Thor and Dupont, 2018). Elevated CO_2 may enhance growth of some seaweed species, whereas increased ocean acidification (OA) may negatively affect other species (Ji *et. al.,* 2016). Therefore, there is an urgency for research to determine the effects of increased atmospheric CO_2 and associated OA on commercially important seaweed species (Chung *et. al.,* 2017).

5.5.2 Seaweed for feed and food: constraints and opportunities

The demand for seaweed, particularly the volumes required for industrial applications such as biofuels, has led in some regions to demand outstripping the supply sourced from harvesting wild stocks, and poses a risk to the long-term future of the seaweed sector (Mac Monagail *et. al.,* 2017). Four key considerations determine whether alternative production in the form of large scale cultivation of seaweed in Scotland and Ireland is practicable: the selected seaweed species, which determines the suitability of a site; environmental impacts; market forces, and the regulatory environment (Roberts and Upham, 2012). Coastal cultivation is the principle mode of seaweed farming utilised worldwide, which provides habitat for other marine species, provides carbon storage, generates employment opportunities, and can reduce the environmental externalities of farming of other aquaculture species (e.g. as a bio-filter in IMTA systems) (Buschmann *et. al.,* 2017). Offshore cultivation of seaweed for animal feed is not yet economically feasible, but seaweed protein for feed can be extracted from a cascading biorefinery system in which seaweed sugars are used for biofuels and chemicals (Van den Burg *et. al.,* 2016; Bikker *et. al.,* 2016). Seaweed cultivation does however, also release significant quantities of particulate organic matter into the marine environment, which could lead to organic enrichment and potential anoxia of benthic zones (Campbell *et. al.,* 2019). Cultivated monocultures of seaweed are also vulnerable

to pathogens of algae, and knowledge of control measures for diseases in European species is lacking (Campbell *et. al.,* 2019).

Though facing significant competition from Asian markets, the markets in the EU for directly edible seaweeds, and hydrocolloids (alginates, agars and carrageenans) used in food processing, are growing, and the role of seaweed, particularly in additive form, in future animal feed markets has great potential due to the increasing demand for raw materials (Van den Burg *et. al.,* 2016). Regulations in the UK and Ireland are yet to be formulated specifically for seaweed, however aquaculture in inshore waters requires a lease from the Crown Estate which advises on site suitability. For example, if cultivation of seaweed destined for food is proposed near sewage outfalls, a Marine Licence which involves an Environmental Impact Assessment, and a Strategic Environmental Assessment are required (Wood *et. al.,* 2017). However seaweed is sourced in the future for feed and food in Scotland and Ireland, the industry is well suited to sustainable ecosystem-based management of the biomass it exploits, and represents a sector with a history of exercising corporate social responsibility, due to the current necessity of sharing resources with rural communities.

5.6 Conclusion

The use of seaweed biomass as a sustainable source of animal and human nutrition directly addresses the challenges and opportunities facing EU food systems outlined in the Blue Growth and Bioeconomy strategies. The potential for UK seaweed as organic and functional food products to command high prices has been proven, and takes advantage of positive public perceptions about sustainably hand-harvested food that has been sourced from remote, pristine locations. Seaweed as functional animal feed has a market demand in the EU, but in order to produce sufficient biomass to compete with the supply of cultivated seaweed from Asia, seaweed for feed will need to be cultivated. Cultivation will also enable control of production standards and growing conditions which will enable UK and Irish sources of seaweed biomass to adhere to increasing consumer and regulatory demand for traceability and safety of feed and food materials in the EU food system. In its current form, hand-harvested seaweed producers

in Scotland and Ireland must achieve a balance between commercial viability and protecting their social licence.

Chapter 6 | General Discussion

Commercial production of insect ingredients in aquafeed represents a revolution in the aquaculture industry. It has the potential to reduce the negative environmental impacts of current feed and food production systems, and establish a blueprint to achieve sustainability through innovation in other food production sectors. Sustainability of the aquaculture sector can be facilitated by harnessing the capacity of insects to convert cheap and abundant seaweed biomass into a functional insect feed to benefit both fish and human health. Regulatory approval of this innovative feed-food production chain rests on gathering evidence that seaweed-specific hazards to human health are not transferred to human consumers. The research presented in this thesis has sought to assess the potential for key environmental bacterial pathogens to enter the seaweed-fed insect production chain from the natural and processing environments and thus pose a possible hazard to human health. This research has also quantified the risk to consumers based on the response of pathogens to processing methods, which will facilitate strategic control of that risk throughout the farm-to-fork continuum.

6.1 Synthesis and implications: The ONE HEALTH approach to sourcing safe seaweed for insect feed

To contribute positively to food and nutrition security (FNS), aquaculture production and aquafeed design must be transformed to be sufficient, sustainable, ethical and safe, and this requires a clean and healthy environment (Jennings *et. al.,* 2016; van Hoof *et. al.,* 2019). The control and prevention of human exposure to foodborne pathogens via introduction of seaweed-fed insects to feed for farmed fish requires not only an understanding of transport and transmission pathways but also the drivers of the global food system (Boqvist *et. al.,* 2018). Projected increases in the demand and production of food, together with complex global supply chains, are expected to lead to a greater incidence of foodborne disease (King *et. al.,* 2017). For example, clinical cases of invasive human listeriosis have increased throughout the EU since 2008, reaching 2480 cases in 2017 including 225 deaths, the highest mortality associated with any zoonotic disease under surveillance in the EU (EFSA, 2018). In the same year, 6073 clinical cases of *E. coli* (STEC) infections, primarily caused by serogroup O157, led to 20 deaths (EFSA, 2018). Within the 'One Health' strategy framework human health risks from infectious

foodborne diseases associated with seaweed-fed insects will arise where seaweed and insects make direct or indirect contact with overlapping human, animal and environmental domains (Destoumieux-Garzon *et. al.,* 2018; Wielinga and Schlundt, 2013) (Fig. 6.1). An additional domain is that of insect vectors of disease agents (Fig. 6.1), of particular concern given that Coelopidae vectors (Chapter 2) are also a candidate aquafeed ingredient.

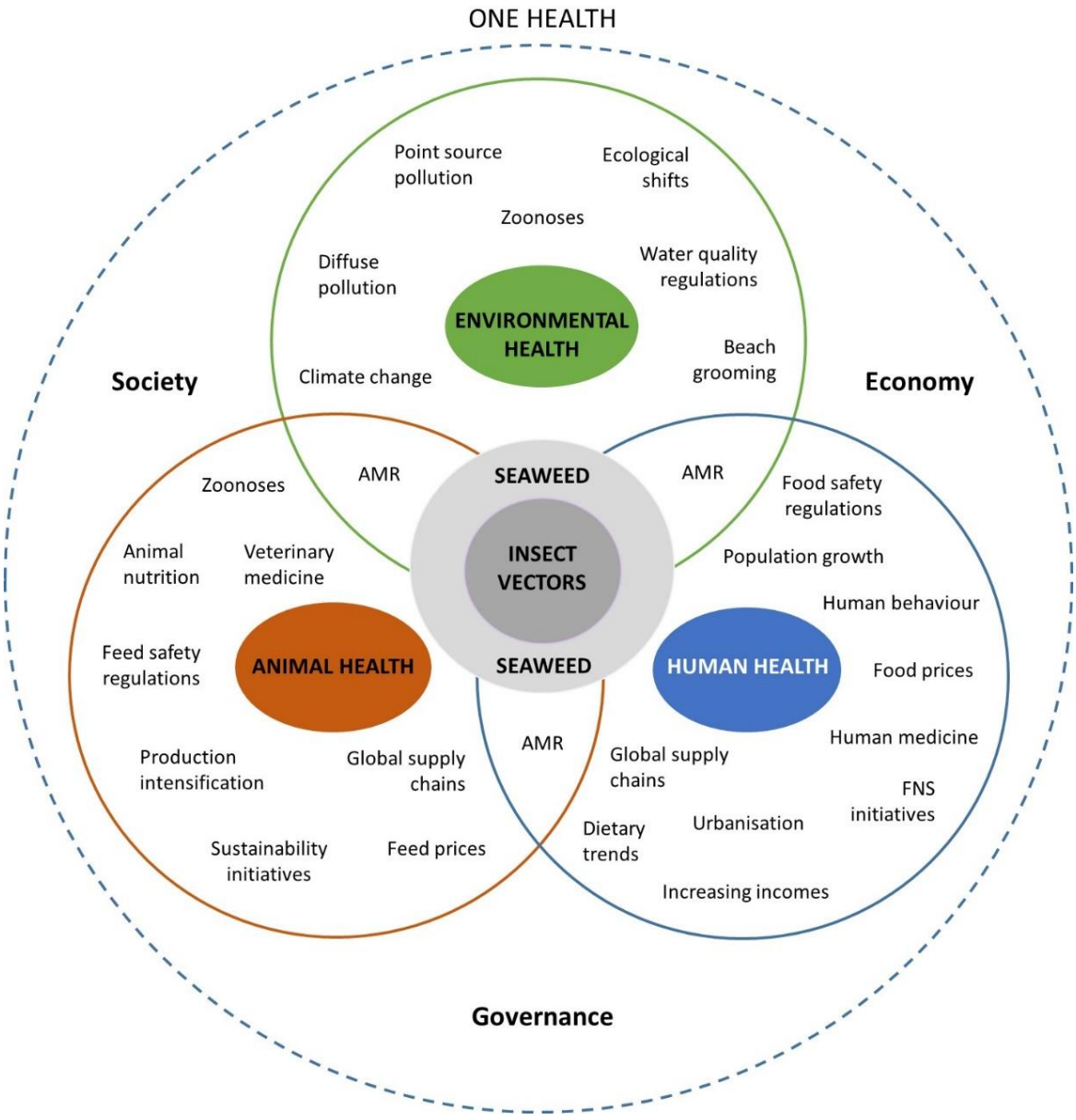


Figure 6.1 The bacteriological safety of seaweed and insects intended as aquafeed is affected by the multifactorial interlinkages between environmental, animal and human health, and the role of insect disease vectors, as captured by the One Health concept. AMR = antimicrobial resistance. FNS = Food and nutrition security.

Risk assessment and management of zoonotic and non-zoonotic foodborne diseases should be grounded in understanding of the ecosystem dynamics that drive the occurrence and recurrence of infectious disease agents, their dispersal pathways and survival in the environment (Destoumieux-Garzon *et. al.,* 2018). The EU Bathing Water Directive (BWD) (Reg (EC) 7/2006; EC, 2006b) has led to EU member states improving the control and treatment of sewage discharged to coastal waters (Quilliam *et. al.,* 2019), and in Chapter 5 seaweed producers stated that they avoid harvesting at locations where wastewater discharge points are sited. However, the 'Programme of Measures' implemented under *River Basin Management Plans* to address complex multiple pollution sources are broadly failing to deliver reductions in diffuse agricultural pollution to EU waterways (Carvalho *et. al.,* 2019). In the coastal zone, reliance by some seaweed harvesters on 'judgement' rather than formal sampling to evaluate microbiological water quality (Chapter 5) reflects an under appreciation of the bacterial hazards typical of the primary production stages of food chains particularly where microbiological legislation and standards are lacking (Kirezieva *et. al.,* 2015). Temporary avoidance of harvesting sites that have received slurry run-off (Chapter 5) similarly reflects a lack of awareness amongst seaweed producers of the potential chronic bacterial risk associated with coastal sands and wrack, which could provide sources of contamination to attached intertidal seaweed long after a pollution event (Solo-Gabrielle *et. al.,* 2015; Ishii *et. al.,* 2006).

Although extra-enteric pathogenic bacteria do not require insect vectors as part of their disease dynamics (Benelli and Duggan, 2018), *C. frigida* are effective bridge hosts of zoonotic *E. coli* O157:H7, although only when their wrack habitat is contaminated with the pathogen (Chapter 2). Management of vector-mediated disease transfer pathways requires appreciation not only of vector and pathogen interactions, but also the prevalence of the pathogen in environmental reservoirs, and the behaviour of susceptible human populations (Benelli and Duggan, 2018; Lloyd-Smith *et. al.,* 2009). *C. frigida* may amplify the pathogen hazard in living intertidal seaweed harvested for feed, by enhancing the spatio-temporal occurrence of wrack and sand as pathogen reservoirs through spatial dispersal of *E. coli* O157:H7 to new Coelopidae vectors (Chapter 2). The Blue Flag beach certification scheme suggests that wrack should be removed via beach

grooming if it has accumulated to nuisance or distasteful levels, and can be considered a hazard if it has started to decay (FEE, 2018). However, grooming can enhance the bacterial reservoir status of seawater (Russell *et. al.,* 2014), as well as encourage Coelopidae to migrate and establish new wrack reservoirs of pathogens. Thus grooming could increase the risk of pathogen attachment to living seaweed. Importantly, harvesters of seaweed for animal feed most often collect living seaweed biomass from relatively remote sites without the same beach management policies as more populated areas (Zielinski *et. al.,* 2019).

Projected warming of ocean and air temperatures will benefit the survival, growth and abundance of pathogens, and increased frequency and magnitude of rainfall will enhance waterborne dispersal of pathogens from catchment sources to coastal areas (Smith *et. al.,* 2015). For example, an increased global sea surface temperature of 1 °C over the past 140 years is one cause of the global increase in human infections by *Vibrio* spp., which are known to colonise seaweeds (Vezzulli *et. al.,* 2015; Mahmud *et. al.,* 2007, 2008). The greatest risk of enhanced bacterial threat under future climate change will be to pre-harvest seaweed in the coastal environment (King *et. al.,* 2017). Increasingly unpredictable distribution and productivity of seaweed in future may also weaken the current role of 'self-policing' by industry (Chapter 5) in upholding sustainable wild harvesting practices (Ostrom, 2009). Northward shift of European kelp and fucoid species and *C. frigida* (Yesson *et. al.,* 2015; Edward *et. al.,* 2007) might suggest that this candidate aquafeed species and bridge host, and seaweed reservoirs (living and decaying), are unlikely to be decoupled in the near future. Therefore, breeding stock of Coelopidae required for commercial production and initially sourced from wild populations may represent a pathway for pathogen transfer into Coelopidae production systems. At the same time however, climatic changes affecting the availability of commercially important species are likely to increase the impetus for cultivation, since creating markets for alternative seaweed species may take time. Offshore cultivation would reduce the contamination threat from catchment sources of extra-enteric pathogens. The influence of social licencing in shaping corporate social responsibility of the seaweed sector (Chapter 5) could also help to develop regulations

and certification of cultivated seaweed feed and thus lead to high quality and safety standards (Mather and Fanning, 2019).

There is also growing global concern about the emergence of multi-antimicrobial resistant (AMR) bacterial pathogens of veterinary and public health importance (Lammie and Hughes, 2016). Antibiotics used to treat humans, livestock and aquaculture species can ultimately end up in agricultural runoff, sewage systems, and coastal waters and sediments, which become reservoirs of AMR bacteria as antibiotics select for resistant bacterial strains (Fresia *et. al.,* 2019; Watts *et. al.,* 2017). For example, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, including *E. coli*, which are resistant to multiple antibiotics of clinical importance including third generation cephalosporins, are rapidly spreading in the environment (Leonard *et. al.,* 2018). Increased threat of zoonoses due to climate change may also result in greater application of veterinary antibiotics. Coastal waters are the interface between terrestrial and aquatic resistomes; in the UK for example, seawater at 11 of 97 bathing waters contained *E. coli* carrying plasmid-borne ESBL $bla_{CTX\text{-}M}$ genes (Leonard *et. al.,* 2018; Watts *et. al.,* 2017). Thus living and detached seaweed may additionally emerge as reservoirs of AMR human pathogens, which poses a serious feed and food safety risk. Synanthropic flies can mechanically vector AMR bacteria including ESBL-producing Enterobacteriaceae, while horizontal transfer of AMR genes between bacterial species can occur in housefly gastrointestinal tracts (GITs) (Onwugamba *et. al.,* 2018; Fukuda *et. al.,* 2016). However the vector competence of Coelopidae for AMR pathogens has yet to be demonstrated.

6.2 Synthesis and implications: recommended application of HACCP, GAP, GMP and GHP to seaweed-fed insect production systems

Hazard Analysis and Critical Control Point (HACCP) guidance can be applied to any part of the feed and food chain with the purpose of identifying bacteriological hazards, opportunities for bacteriological persistence and growth, and ascertaining critical control points (CCPs). At CCPs, controls including Good Agricultural Practices (GAP), Good Hygiene Practices (GHP) and critical limits in manufacturing conditions can be applied to address the hazard (FAO/WHO, 2003). Application of HACCP to aquaculture

(primary) production to prevent or reduce risks to human health from environmental pathogens colonising wild harvested seaweed requires GAP to reduce the overall contamination load in the production environment (Cerf and Donnat, 2011).

V. parahaemolyticus is a leading cause of gastroenteritis associated with consumption of contaminated seafood (Obaidat *et. al.,* 2017). However, the presence of pathogens naturally occurring in seawater cannot be controlled. Mandatory sampling and microbiological criteria for this pathogen, particularly in edible seaweed, would therefore be valuable. In the case of extra-enteric foodborne pathogens, harvesting of seaweed destined for animal feed could be restricted to locations where *E. coli* levels in seaweed meet standards established for shellfish harvesting areas (SHA) (Reg (EC) 2073/2005 (EC, 2005c) and Reg (EC) 854/2004 (EC, 2004b)), i.e. Class A (≤ 230 *E. coli*/100 g of shellfish flesh in 80 % of samples) and Class B (≤ 4600 *E. coli*/100 g of shellfish flesh in 90 % of samples), whilst seaweed harvested for direct human consumption should be restricted to Class A sites only. This would not be unduly restrictive since in Scotland, for example, the vast majority of SHAs densely clustered along the west coast and around the islands met Class A or B standards between 2014 and 2017 (FSS, 2017).

The rigorous post-harvest treatment of shellfish (Reg (EC) 853/2004; EC, 2004a) is intended to reduce bacteriological loads in the product whereas the post-harvest treatment of seaweed intended for insect feed or human food may increase bacteriological risks (Chapter 4). GAP at harvesting sites therefore provides the crucial first line of defence against bacterial contamination of pre-harvest seaweed. The intensity of water quality monitoring by harvesting companies should reflect, (a) land use in adjacent catchment/s, (b) timing of harvesting activities in relation to precipitation events in said catchment/s, (c) degree of wrack accumulation and occurrence of grooming at adjacent beach/es, and (d) appreciation of seaweed vulnerability to pathogen attachment according to seaweed species and season (Bengtsson *et. al.,* 2010). Sourcing the same or different species of seaweed from several harvesting sites (Chapter 5) also presents the added risk of batches harbouring differing levels of initial contamination (Besten *et. al.,* 2017). Subsequent processing and batch sampling will not necessarily take account of this variability in quality.

Environmental pathogens in seaweed freshly harvested as feed for Coelopidae cannot be controlled by an antibacterial treatment as it may affect larval production by impairing the quality or quantity of the natural seaweed microbiota. To mass-produce Coelopidae, fresh seaweed will require a form of storage that halts rapid decay, yet retains the nutritional value of the feed, such as freezing at -20 °C (Chapter 3). Freezing brown seaweed at -20 °C preserves a higher protein content than freezing at -80 °C, however, *L. monocytogenes*, *V. parahaemolyticus* and viable but non-culturable (VBNC) cells of *E. coli* O157:H7 can all survive freezing at -20 °C (Abdollahi *et. al.,* 2019; Archer, 2004; Liu *et. al.,* 2017). Validation of a freezing temperature and duration that balances targeted antibacterial action against pathogens with an optimised nutritional content, or testing of alternative methods such as freeze-drying, are necessary Good Manufacturing Practice (GMP) interventions at this stage. Microbiological criteria for *E. coli* in ready-to-eat (RTE) fresh produce (acceptable if 100 – 1000 CFU g^{-1} in 2 samples and ≤ 100 CFU g^{-1} in the remaining samples) could thereafter be applied to Coelopidae feed (Reg (EC) 2073/2005; EC, 2005c).

Chapter 4 demonstrated pathogen growth in dried, powdered seaweed from previously undetectable levels indicating a loss of control of the a_w during storage, which could be prevented by storing the finished product in vacuum-packed containers as part of GMP. Rehydration of seaweed powder in BSFL feed poses a safety risk as it may contain pathogenic cells surviving at a low infectious dose, or undetectable in a VBNC state, thus seaweed washing activities should be located separately from drying operations (Bhunia, 2018). Verification of GMP efficacy against pathogenic *E. coli* and *V. parahaemolyticus* requires time-temperature treatments that rapidly reduce a_w to < 0.5 in order to enable even relatively low drying temperatures to exert a lethal effect on pathogenic bacterial cells. Maximum limits for *L. monocytogenes* in RTE and dried seaweed could be adopted from the microbiological criteria for *L. monocytogenes* in comparable food products (Reg (EC) 2073/2005; EC, 2005c): 100 CFU g^{-1} in RTE foods able to support growth of *L. monocytogenes* during shelf-life.

Coelopidae and BSFL displayed similar capacity in Chapters 2 and 4 respectively to reduce larval loads of pathogenic *E. coli* even at high concentrations, suggesting that both larval species may effectively clear their digestive systems of this pathogen if

present at relatively low concentrations in their feed. Absence of *L. monocytogenes* in 25 g of insect PAPs (mandatory if intended for food) could be applied to insects as feed, and might indicate efficacy of GHP to prevent *Listeria* spp. contamination from feed production environments (FPEs) and handling of finished products, namely application of antibacterial disinfectants at the manufacturer's recommended concentrations (Larsen *et. al.,* 2014) and hygiene training for personnel. Fly larvae which have been fed seaweed and then processed into insect products will not pose any greater risk to fish or human consumers than fly larvae reared on commercial animal feeds. However, dehydrated seaweed undergoes no decontamination step prior to packaging and distribution and thus, depending on water quality at the harvesting site, poses a potential health risk if utilised as RTE food, or fed to insects that are marketed as minimally processed or RTE feed or food products.

6.3 Limitations

The horizontal transfer of pathogens between individual vectors, or the vertical transmission from one generation of vectors to the next, facilitate the persistence of disease-causing agents in a vector population (Benelli and Duggan, 2018). These mechanisms could have been more fully examined in Coelopidae in Chapter 2 by, for example, exposing *C. frigida* larvae to *E. coli* O157:H7, externally sterilising the eventual pupae, and sampling the emerged adult flies for the pathogen. A firmer basis for assessing the relative capacities of Coelopidae larvae and BSFL for GIT reduction and inactivation of specific bacterial species and strains (Chapters 2 and 4) would have benefited from exposure of larvae to lower pathogen concentrations to avoid overwhelming GIT processes; by exposing Coelopidae larvae to the same range of pathogens as BSFL; by inoculation of seaweed powder for BSFL with pathogenic cells in a VBNC state prior to rehydration; and by 24 hour starvation of prepupae of both species followed by surface sterilisation prior to harvesting. During the 8 day rearing of BSFL on feed supplemented with pathogen-contaminated seaweed, the feed was not replenished at any point and the water was replenished only once, unlike in a commercial insect factory where feed and water are replenished daily. This experimental design probably exerted a negative effect on BSFL growth, survival and development, and may exlpain the increasing difficulty in detecting larvae for sampling,

and the decrease in average individual larval mass, as the experiment progressed. Limited food availability would have restricted feed intake- and thus ingestion of feed-associated pathogens- by the larvae. This may have contributed to the consistently high concentrations of all pathogens remaining in the substrate during the rearing period.

Assessment of bacteriological hazards in Coelopidae larvae (Chapter 2) could have been combined with establishing mass production parameters for the species (Chapter 3). This could have been achieved by inoculating fresh seaweed with a range of pathogens, freezing the seaweed at a range of temperatures, then determining whether the pathogens are transferred from the thawed seaweed to Coelopidae. Furthermore, increasing insect rearing temperatures typically increases growth rate which cannot compensate for a faster developmental rate through successive life stages, ultimately reducing final body size (Harrison *et. al.,* 2012). Thus for *C. frigida* cultured at the density and on the substrate established as optimal for maximising larval biomass, the experimental design would have been improved by also determining the ideal rearing temperature. The measurement of Coelopidae larval biomass during production optimisation would also have been more accurate were the larvae to have been starved for 24 hours prior to weighing in order to exclude GIT contents from the overall biomass.

The direct selective culturing technique utilised in this study for the detection and enumeration of bacteria in seaweed and insects is technically straightforward and enables rapid detection (Jasson *et. al.,* 2010). However, direct culturing is time-consuming and labour-intensive, in complex microbiomes it typically only detects 0.1 % of the microbial community, lacks sensitivity, and can underestimate microbial diversity due to lack of knowledge on the bacterial growth conditions required by all bacterial species and strains present in the sample (Jasson *et. al.,* 2010; Cao *et. al.,* 2017; De Filippis *et. al.,* 2018). The limit of detection or sensitivity of direct culturing means that enumeration of pathogenic bacteria with a low infectious dose such as *E. coli* O157:H7 may require an enrichment step to increase the bacterial concentration to a detectable level (Gill, 2017). As demonstrated in the case of *E. coli* O157:H7 in seaweed subjected to processing stresses, direct culturing cannot distinguish between cells in a VBNC state or bacterial populations falling below the level of detection, potentially leading to false negative results. The metabolic activity of cells of the *lux*-marked *E. coli* O157:H7

serotype used in this study was measurable even when concentrations of the bacteria were below the level of detection or in a VBNC state. Access to similarly *lux*-marked *V. parahaemolyticus* and *L. monocytogenes* in a VBNC state, and extension of the storage period for the powdered seaweed, might have facilitated better assessment of the storage conditions that enable resumption of replication of these pathogens in the low moisture feed during a long shelf life (Gill, 2017). By comparison, culture-independent methods such as metagenomic or high throughput sequencing techniques which sequence the entire DNA or RNA content in a sample can identify all bacterial species and strains present, their relative abundances and can distinguish viable from non-viable cells (Cao *et. al.,* 2017). However, the procedure and data analysis is expensive and requires specialised laboratories, thus molecular techniques of analysis exceeded the scope of this study.

The measurement of salt concentration in seaweed during its' processing (Chapter 4) was not undertaken in this study but would have been valuable. During drying, as seawater in seaweed evaporates, the salt remains attached to the seaweed and increases in concentration. Under a high salt concentration bacterial cells are surrounded in a saline solution which can damage cells by disrupting their osmotic balance (Vogel *et. al.,* 2010; Burgess *et. al.,* 2016). Bacterial responses to osmotic stress can include retardation of bacterial growth and entrance into a VBNC state (Ross, 2008). Osmotic stress can reduce the a_w of the environment immediately surrounding cells meaning that cell tolerance of desiccation and osmotic stress may overlap (Vogel *et. al.,* 2010). For example, a positive correlation exists between the duration of exposure of *L. monocytogenes* to salt, and heat resistance demonstrated by the pathogen (Jorgensen *et. al.,* 1995). *E. coli* grown in a high salt environment prior to desiccation exhibits greater desiccation tolerance because osmotic stress induces cell production of the osmoprotectant sugar trehalose (Welsh and Herbert, 1999). Understanding the cross-protection afforded to seaweed-associated pathogen cells by the salt content of drying seaweed would enable development of better informed GMP for seaweed processing.

Technical challenges with realistically simulating the industrial processes involved in converting fresh seaweed to dry powder, particularly the desiccation treatment, prevented the production of powdered seaweed meal at 40 °C. This was unfortunate

since this drying temperature is increasingly favoured by the seaweed feed industry. The duration of drying time required at 50 °C and 60 °C (168 h) to produce dried seaweed which could be ground to a powder far exceeded the normal 12 h required in seaweed factories using large-scale dehumidification technology. The findings cannot therefore be generalised to commercial settings. The value of the data lies in the identification of the different responses of pathogenic species and strains to processing stresses, not the validation of time-temperature treatments that can be directly and immediately applied to control of the pathogens in an industrial context.

The small sample size achieved for the survey of seaweed industry stakeholders (Chapter 5) could undermine the integrity of the findings as it is not plausible to generalise the results to the wider stakeholder communities beyond the non-representative 6 producers and 3 advisory bodies surveyed (Bryman, 2012). The method by which a survey is administered also affects the quality of the answers by introducing mode effects. For example, both feed producers were interviewed in person which provided an opportunity to collect detailed qualitative data. However, recording comments by hand was challenging, and would have been more accurate, and have facilitated a more rigorous and replicable content analysis, if transcribed from audio recordings (Bryman, 2012). Also, studies of interviewer effects for face-to-face surveys showed that interviewer and respondent sharing socio-demographic characteristics, and an interviewer's physical appearance, skills and attitudes, can exert positive or negative effects on the quality of survey responses particularly on open-ended questions, even on self-administered exercises when interviewers are present (West and Blom, 2017). The desire to express socially desirable attitudes or behaviours may also have influenced participant's answers (Sapsford, 2007) when surveyed in person, particularly with regard to feed producers' emphasis on the role of public perceptions and self-policing by the industry in maintaining sustainable levels of seaweed harvesting. By comparison, the majority of food producers completed the surveys online, which insulated them from the influence of inter-personal interactions with the interviewer. However, the one food producer surveyed by telephone had to memorise lists of options for site selection factors, and the recency effect (the tendency for the last option to be remembered and chosen) may have played a role in their

choices (Lugtig *et. al.,* 2011). Thus mode effects may have biased responses in different ways depending on the principle data collection technique applied to each stakeholder group.

It cannot be assumed that the opinions expressed by respondents are predictors of behaviour or attitudes of the wider population of producers or advisory bodies, since undertaking the survey may have influenced the views voiced by individuals by concentrating their attention on a particular topic during the interview interaction (Sapsford, 2007). At the same time, data bias will have been introduced by non-responders who not only reduced the sample size but may also have differed in important ways from responders; the systematic refusals to participate by large international harvesting companies operating in Scotland and Ireland would not however have been improved by increasing the sample size (de Vaus, 2002). This reflects the difficulty often encountered by researchers in gaining access to companies concerned about potential risks to their image (Bryman, 2012).

In the survey, a small number of similar site selection criteria appeared in more than one category or theme; the option 'Advised sustainable harvesting regime' occurred under the 'Standing stock' theme, 'Unregulated over-harvesting and environmental impacts' occurred under the 'Regulatory' theme, and both of these appeared separately from an 'Environmental' theme. These options were therefore not mutually exclusive, and may have resulted in inadvertent multiple selection, and therefore over-emphasis, of this factor as an influence on site selection (Aidley, 2019). By comparison, excluding from the results section opinions about the industry's future if voiced by only one respondent may have led to omission of viewpoints and concerns which may have emerged as significant were a larger sample size to have been surveyed. The characteristics of the stakeholder groups would also have strongly influenced their survey responses. Feed and food producers selected site suitability criteria based on their practical everyday experience and commercial realities, whereas advisory bodies selected factors based on their perceptions of what commercial operators ought to consider when harvesting. It might therefore be argued that given a larger sample size, survey responses could be divided into those reflecting reality and those reflecting a

hypothetical ideal, as producer practices and advisory bodies' advice cannot be directly compared.

6.4 Future work

Spore-forming bacteria are causative agents of foodborne disease, ubiquitous in the environment and FPE, and highly resistant to processing stresses (Wells-Bennik *et. al.,* 2016). Spore inactivation in food material for example requires application of > 95 °C heat, far below the 40 °C drying temperature currently favoured in the seaweed industry (Gupta *et. al.,* 2010). The potential adherence to seaweed, survival of processing and interaction with insect larvae GITs by these bacteria deserves attention. The microbiota of brown, red and green seaweeds are often dominated by the bacterial genus Bacillus, and can include the foodborne pathogens *B. cereus* and *B. subtilis* (del Olmo *et. al.,* 2018). Heat resistant *B. cereus*, for example, is a typical member of BSFL GIT microbiota, and therefore if ingested in feed, this pathogen may not be inactivated, and may even replicate, in the GIT (Jeon *et. al.,* 2011). Human health hazards from consuming seaweeds also include exposure to microplastics (van der Spiegel *et. al.,* 2013). Polystyrene particles, deposited in coastal waters via waterways and wind from land, can adhere to the surface of *Fucus vesiculosus*, and potentially other seaweed species (Sundbaek *et. al.,* 2018). Ingestion of microplastics poses a health risk to animals and humans not only due to the chemicals and toxic metals utilised in their manufacture but also the pathogenic bacteria that may be absorbed to their surfaces in the environment (Barboza *et. al.,* 2018; Keswani *et. al.,* 2016). The occurrence and control of spore-forming pathogens, and particularly microplastics as a vehicle for bacteria, in insects intended as ingredients in the human food chain are under-researched. There is, therefore, a pressing need to undertake risk assessments of the full range of insect species currently farmed in the EU for feed and direct human food in terms of their individual capacities to introduce, become contaminated with or accumulate spore-forming bacteria or microplastic-associated pathogens from their feed or FPE.

The methods of bacterial detection and enumeration utilised in this study reflect the traditional reliance of food microbiology on culture-dependent techniques; these have their limitations however (section 6.3) which culture-independent molecular

techniques can overcome (De Filippis *et. al.,* 2018). Nucleic acid sequencing methods, namely whole genome sequencing (WGS), next generation sequencing (NGS) and other omics tools, have facilitated the rapid and reliable profiling and monitoring of foodborne pathogens in multiple samples simultaneously (Ercolini, 2013). NGS is emerging as a means to address the major challenges to water quality management posed by the inadequacy of FIOs as indicators of the potential presence of waterborne faecal pathogenic bacteria (Tan *et. al.,* 2015). NGS techniques can link pathogens in water bodies to faecal pollution sources; identify the distribution and relative abundances of human pathogens in the environment in relation to catchment land use, and detect the presence of antimicrobial resistant genes (ARG) in microbiomes in environmental samples, as well as in animal and human digestive tracts (Tan *et. al.,* 2015). NGS tools could improve quantification of the risk that living and decaying seaweed may pose as ARG reservoirs, and enable assessment of ARG transfer throughout the seaweed-fed insect production chain. NGS techniques have also been successfully employed to track fluctuations in the diversity of bacterial contaminants on the surface of vegetables irrigated with wastewater, occurring in response to factors including season and irrigation practices (Cao *et. al.,* 2017). A similar assessment could be undertaken of pre-harvest seaweed from sites subject to anthropogenic pollution in order to better define the relationship between diffuse and point source coastal discharges and the diversity of human pathogens colonising commercially important seaweed species.

Molecular sequencing methods have improved understanding of pathogen survival in foods including in novel pathogen-food combinations, the relative virulence of pathogens (virulence can vary widely between different strains of the same bacterial species) and how they cause disease (the dose-response relationship, a key feature of pathogen risk assessment) (Haddad *et. al.,* 2018). Molecular techniques have been used to demonstrate how microbiomes can vary in composition in different niches within complex food matrices, and to monitor reduction or growth or metabolic activity in bacterial populations in response to processing or storage conditions (Ercolini, 2013). The contribution of the microbial ecology of an FPE and raw feed material to bacterial cross-contamination of insect products could be evaluated using 16S rDNA sequencing

to identify correlations between the microbiota of an FPE, feed ingredients and finished products (Cao *et. al.,* 2017). Microbiotic communities present in feed, food and FPE can inhibit or encourage growth of specific pathogens in feed and food throughout the farm-to-fork continuum (den Besten *et. al.,* 2018). *L. monocytogenes* biofilm formation in an FPE for example was encouraged or inhibited depending on the presence of other protagonistic or antagonistic (respectively) bacterial species existing in the same bacterial community (Fox *et. al.,* 2014). Understanding of the microbiota of an FPE also provides data crucial for verifying the efficacy of factory hygiene measures against, for example, persistent *L. monocytogenes* (Cocolin *et. al.,* 2018). By contrast, the research presented in this study quantified the behaviour of each selected pathogen in seaweed and BSFL in isolation from the wider seaweed- or insect-associated microbiomes. The application of genomic techniques to this research may have facilitated improved understanding of the behaviour of *E. coli* O157:H7, *L. monocytogenes* and *V. parahaemolyticus* in relation to the wider seaweed, insect and FPE bacterial ecosystems, e.g. through competitive interactions (den Besten *et. al.,* 2018).

One of the most important contributions NGS could make to the assessment of the bacteriological safety of seaweed-fed insects intended as feed or food is in the detection of pathogenic bacterial cells that cannot be directly cultured in the laboratory either because their existence is not known (e.g. insects can introduce previously undocumented hazards) or are known and are viable but cannot be cultured (i.e. may be present in a VBNC state) (Cao *et. al*., 2017). This could prove particularly valuable in assessing the bacteriological risks associated with rearing BSFL and other insects on a wide range of waste organic streams considered by regulators and consumers as more controversial than seaweed, such as human and animal manures. An important area of future food safety research concerns the use of molecular biomarkers which are measurable cellular compounds in a foodborne pathogen which can enable prediction of a pathogen's behaviour (den Besten *et. al.,* 2018). The absence or presence of a genetic element in *B. subtilis* which confers heat resistance to this spore-forming bacteria, and may also apply to *B. cereus*, represents a potential biomarker (Berendsen *et. al.,* 2015) which could be traced throughout a seaweed-fed BSFL production chain. Improved dose-response data for foodborne pathogens emerging from the use of

molecular techniques allows regulators to prioritise existing and emerging hazards, and to establish bacteriological standards, surveillance and import controls (Haddad *et. al.,* 2018). Complex global food supply chains can challenge source attribution of foodborne disease outbreaks, and WGS can be used to predict the geographic location of pathogens using databases of sequenced isolates from historical outbreaks (Ronholm *et. al.,* 2016).

Current UK food safety standards take the precautionary, preventative approach which is at the heart of EU food standards. However, no explicit intention to adhere to these regulations and guidance exists in the recent draft Agricultural Bill (Lang and Millstone, 2019; House of Commons, 2018). Harmonisation of international practices and guidelines for production and processing by applying scientifically-based HACCP principles (Cole *et. al.,* 2018) will be particularly important in facilitating UK and EU cross-border trade post-Brexit. In the survey (Chapter 5), a single advisory body to the Scottish seaweed industry highlighted Brexit as a factor likely to influence decisions regarding sourcing of seaweed for feed and food markets. Furthermore, only one feed producer regularly monitored seawater faecal indicator organism (FIO) levels at their harvesting sites, and only one food producer stated that they batch-tested final products for multiple microbial hazards including *Listeria* spp. However, it would be inappropriate to infer industry-wide opinions, or to generalise about commercial feed and food safety practices, based on a sample of 9 individuals. Widening survey participation to other EU member states and to the wider seaweed cultivation industry, may reveal a broader range of attitudes and approaches to seaweed feed and food safety, possibly related to the role of public perception of the seaweed industry in other countries. The concepts of potentially exploiting seaweed biomass produced at effluent release sites or as part of integrated multi-trophic aquaculture (IMTA) for animal feed were proposed in the survey by a feed producer and an advisory body respectively. Sourcing seaweed feed from such locations would require proof that contamination risks are no greater than those associated with seaweed feed currently marketed.

6.5 Concluding remarks

The long term FNS vision of the EU aquaculture sector is to meet consumer demand for a variety of food choices, ethically sourced and produced, which attain high safety and nutritional requirements, at an affordable price. The assessment of the human health risk associated with introducing innovative ingredients to aquafeed, as presented in this thesis, reflects the onus placed by the public on regulators and producers (particularly since the BSE crisis in the 1990s) to take greater responsibility for ensuring safety of the feed and food chain. Seaweed as feed substrate for insects intended as aquafeed poses no greater risk to public health than seaweed as feed for traditional livestock. Food safety systems will never require products to be entirely free of bacterial contamination, as the expense would lead to unacceptable food price increases. Food safety failures can lead to avoidance of certain products due to real or perceived health risks. Innovative feed and food ingredients are particularly vulnerable to public perception and cautious markets. However, until seaweed cultivation in the EU becomes competitive, food safety threats associated with seaweed are most likely to emerge from imported seaweed, since non-harmonised feed and food laws and standards between countries can challenge food safety in global supply chains.

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APPENDICES

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Seaweed-fed black soldier fly (*Hermetia illucens*) larvae as feed for salmon aquaculture: assessing the risks of pathogen transfer

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Abstract

Sustainable ingredients for animal feed are becoming scarcer. Insects have emerged as a promising protein and lipid ingredient for fish feed, and black soldier fly (BSF; *Hermetia illucens*) larvae in particular have great potential to efficiently convert organic matter into high value protein and fat. Seaweeds are a sustainable source of organic matter and complex carbohydrates, but can also provide marine long chain polyunsaturated fatty acids for fly larvae, and therefore could offer a commercially attractive alternative to traditional aquafeeds. However, pathogenic bacteria and faecal indicator organisms (FIOs) readily attach to seaweeds, therefore before this novel BSF larvae feed ingredient is advocated, microbiological risk assessments are warranted to ensure animal and public health protection from farm-to-fork. In this study, screening of raw materials and finished products during formulation of experimental insect meal fish feed was undertaken to evaluate the potential for the introduction of selected bacterial pathogens and FIOs via seaweed substrate to BSF larvae, and subsequent survival during multiple manufacturing processing stages. Processed seaweed powder was found to be a microbiologically safe feed substrate for BSF larvae. Low levels of FIOs were associated with larvae at the point of harvest, although larvae meal and extracted lipids were free of FIOs immediately after processing. During handling, distribution and storage the larvae meal and other externally sourced raw feed ingredients for larvae rearing and feed pellet formation became contaminated with FIOs and *Listeria* spp. FIOs were also present, albeit at very low levels, in the finished feed pellets. Processing treatments provided effective decontamination, and FIO and pathogen concentrations in finished products never exceeded microbiological quality standards for insect processed animal proteins. Microbiological contamination of raw materials and finished products during packaging and distribution, or originating from production environments, were identified as critical control points, requiring assessment to ensure good hygiene practices.

Keywords: feed hygiene, food safety, HACCP, microbiological safety, food production chain

1. Introduction

The commercial production of insects to supply a protein source for the aquaculture feed industry has been permitted in the European Union (EU) since July 2017 (Reg (EC) 893/2017; EC, 2017). Insect meal offers a partial replacement for fishmeal and plant ingredients common in farmed fish diets, and the larvae of Diptera (fly) species in particular offer an amino acid profile similar to that of fishmeal, which can partially replace protein in fish diets with no nutritional deficiencies (Barroso *et al*., 2014; Lock *et al*., 2016). Carnivorous marine fish, such as salmon, obtain essential omega-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) from their marine diets, whilst oily fish are the primary source of n-3 LC-PUFA for humans (Jensen *et al*., 2012). In order for farmed terrestrial insects, such as the black soldier fly (BSF; *Hermetia illucens*) to become a source of essential fatty acids, they must be reared on a feed containing marine PUFAs (Barroso *et al*., 2017). Current EU regulations designed to safeguard human and livestock health state that as farmed insects are technically 'farmed animals' (Reg (EC) 1069/2009; EC, 2009a), their feed substrate must be limited to high grade commercial feed or former foodstuffs that are still safe for human consumption (Reg (EC) 767/2009; EC, 2009b). However, there are no prohibitions on the use of seaweed as a feed material for farmed animals, with the caveat that maximum levels of undesirable substances such as arsenic, cadmium and lead are not exceeded (Reg (EC) 32/2002; EC, 2002). Seaweed can provide a source of marine PUFAs with a proven ability to enrich fly larvae with n-3 LC-PUFA (Liland *et al*., 2017), is a sustainable source of organic matter and complex carbohydrates that insects can efficiently convert into high quality protein, and can offer a commercially attractive alternative to traditional aquafeeds (Sprague *et al*., 2016; Surendra *et al*., 2016).

Feed material not included on the list of prohibited materials cannot be assumed to be safe for animal and ultimately human consumers (Reg (EC) 767/2009; EC, 2009b), and EC Regulation 183/2005 on the hygiene of feed requires that feed manufacturers ensure feed safety from farm to fork (EC, 2005a). During this seaweed-insect aquafeed production chain, however, there is the potential for environmental microbiological contaminants to be introduced, therefore regulatory and commercial acceptance necessitates a microbiological safety assessment of the entire feed production chain (EFSA, 2015).

A variety of red, green and brown species of seaweed are currently wild harvested for animal feed in the EU, although the cultivation sector is still fairly small-scale (Kraan, 2013; Makkar *et al*., 2016). Seaweeds, whether wild harvested or cultivated, are vulnerable to surface colonization by human and fish pathogenic bacteria naturally present in seawater, e.g. *Vibrio* spp., or allochthonous bacteria such as *Listeria* spp. or toxigenic *E. coli* such as O157*,* from sewage, livestock or wild animals (Linke, *et. al.,* 2014; Orruno *et. al.,* 2017). Non-marine bacteria may survive for significant periods in various extra-enteric environmental matrices, including seawater, and thus pose a risk of attachment to the surface of seaweeds (Lothigius *et. al.,* 2010; Mahmud *et. al.,* 2007). However, relatively few studies have described the contamination dynamics of seaweeds by human pathogenic bacteria, or faecal indicator organisms (FIOs) such as *E. coli* (Ishii *et al*., 2006; Mahmud *et al*., 2007; Quilliam *et al*., 2014).

The code of practice on good animal feeding (CAC/RCP 54-2004) identifies good agricultural practices (GAP) at the feed production stage as key to the control of potential environmental contaminants in raw materials (Codex Alimentarius, 2004). Regulations on undesirable substances in seaweed as food and feed have established maximum residue levels of specific chemicals (Reg (EC) 32/2002 and 396/2005; EC, 2002, 2005b). However, defined microbiological standards for seaweed as feed have yet to be determined and the EU seaweed harvesting industry currently lacks standardisation of processing techniques, particularly with regard to drying approaches. This has implications for potential survival of pathogens on seaweed throughout subsequent utilisation, particularly if not subjected to further adequate microbial inactivation treatment after drying (Garcia-Vaquero and Hayes, 2016). Microbiological hazards associated with dried seaweed feed may therefore present animal and human health risks further along the feed and food chain.

Commercial scale insect production in Europe for aquaculture feed is still in its infancy, and there are no globally standardised farming methods in this sector (Van Huis *et al*., 2015). However, all insect producers operate within the EU regulatory framework, which requires adherence to good manufacturing practices (GMP) and good hygiene practices (GHP) during primary and secondary production, which should control microbiological hazards, implemented according to a hazard analysis and critical control points (HACCP) system (Reg (EC) 183/2005; EC, 2005a). HACCP guidance enables operators to identify hazards that may compromise feed safety and public health, and the critical control points (CCPs) during production, processing, packing, storage or distribution where control is necessary to prevent, remove or mitigate a hazard, according to acceptable critical limits for a specific hazard. Action is taken if monitoring of CCPs indicates loss of control of a hazard. Currently, microbiological quality standards for insect processed animal proteins (PAPs) requires sampling of products for *Clostridium perfringens* (absent in 1 g of the product), *Salmonella* spp. (absent in 25 g) and Enterobacteriaceae (unsatisfactory if in excess of 300 colony forming units (CFU) in 1 g) (Reg (EC) 893/2017; EC, 2017).

There is a paucity of data on microbiological hazards specifically related to mass production of the key commercial insect species bred for feed in a controlled environment (Awoniyi *et al*., 2004) and most studies focus primarily on insects produced for human consumption (Klunder *et al*., 2012). A key commercial insect species currently mass produced in the EU for animal feeds is the terrestrial BSF. In contrast to many species of Diptera that have been implicated as vectors of human pathogens (Forster *et al*., 2007; Pava-Ripoll *et al*., 2012), BSF larvae are capable of reducing *E. coli* and *Salmonella* when fed on manures (Erickson *et al*., 2004; Lalander *et al*., 2013, 2015; Liu *et al*., 2008; Zheng *et al*., 2013), and can even produce antimicrobial substances active against Gram-positive and Gram-negative bacteria (Park *et al*., 2014). Studies have shown that feed substrate influences BSF larval gut bacteria and a microbiological safety assessment of the species as food identified small samples of dried and powdered BSF as containing *Bacillus cereus* (Grabowski and Klein, 2017; Jeon *et al*., 2011). Evaluation of the antibacterial effectiveness of high hydrostatic pressure treatment of BSF larvae intended as feed achieved control of yeasts and moulds, but aerobic mesophilic bacteria proved more resistant (Kashiri *et al*., 2018). Following contamination of larvae with *E. coli* O157:H7 through inoculation of their feed, the same

hydrostatic treatment was capable of reducing pathogen concentrations in larvae to safe levels. To date however, the majority of safety assessments of BSF as a feed material focus on mycotoxins and heavy metals. Mycobiotic diversity in BSF larval digestive tracts was shown to differ according to whether larvae were fed chicken feed or vegetable waste, and larvae fed commercial feed inoculated with mycotoxins above maximum allowable limits (Reg (EC) 1881/2006; EC, 2006a) excreted and did not accumulate mycotoxins (Boccazzi *et al*., 2017; Camenzuli *et al*., 2018). BSF prepupae displayed a propensity to accumulate cadmium above maximum allowable EU limits when fed substrate containing even low concentrations, can accumulate lead from their feed, and larvae fed substrate contaminated with heavy metals, mycotoxins and pesticides accumulated only cadmium and lead but at levels exceeding initial concentrations in the feed (Diener *et al*., 2015; Purschke *et al*., 2017; Van der Fels-Klerx *et al*., 2016). BSF larvae fed a supplement of seaweed, which contains naturally high levels of heavy metals and arsenic, accumulated lead, mercury, cadmium and arsenic, the latter two at concentrations exceeding maximum allowable limits (Reg (EC) 32/2002; EC, 2002) (Biancarosa *et al*., 2018). Veterinary medicine, pesticides, mycotoxins and cadmium have also been detected in BSF larvae reared on brewers grain, fish feed waste and yeast, despite larvae being washed and dried at 60- 80 °C (Charlton *et al*., 2015).

The considerable literature available on control of foodborne pathogens in production and processing environments identifies environmental contamination during both primary (harvesting or rearing) and secondary (processing) production, e.g. biofilm formation in processing facilities, as sources of often persistent pathogens in raw materials and finished products (Larsen *et al*., 2014; Phillips, 2016). Two potential sources of microbiological contamination of insect meal are the autochthonous microflora inherent to insects, and bacteria introduced from the external environment during various production stages and subsequently transmitted throughout the production chain (EFSA, 2015). The insect species, the production and processing methods, general environment, feed substrate and stage of insect harvest could all be expected to contribute to the microbiological risk profile of fish feed containing BSF larvae meal (Raamsdonk *et al*., 2017; Van der Spiegel *et al*., 2013). Although it can be assumed that industrial protein and lipid extraction methods decontaminate raw insect materials, CCPs still need to be determined for all production and processing stages (Schlüter *et al*., 2017).

The overarching aim of this study was to identify CCPs within the seaweed and BSF production chains. Various species of seaweed were freshly harvested and milled, the resulting powder fed as a supplement to BSF larvae, and the resulting larvae meal incorporated into feed pellets which were subsequently fed to caged Atlantic salmon in a seawater feeding trial. A microbiological safety assessment was simultaneously conducted to identify potential bacterial hazards related to the utilisation of seaweed as a feed substrate for BSF larvae. This study focused on a production chain that began in Ireland, where seaweed was harvested and processed, before being exported to the Netherlands where the processed seaweed was used as a feed ingredient for rearing insect larvae, and finally this insect meal was exported to Norway where it was used in caged fish-feeding trials. Our objectives were to screen all raw materials and finished products, before and after processing, distribution and storage, at each of the

companies involved in this feed production chain, for the presence of important foodborne pathogenic bacteria and indicators of faecal contamination, i.e. enterococci, *E. coli*, *Salmonella* spp., *Listeria* spp., *Vibrio* spp. and *E. coli* O157. These selected FIOs and pathogenic bacteria were intended to be representative of the range potentially present in the nearshore marine environment, in raw feed materials entering the production chain at various stages, and within factory environments.

2. Materials and Methods

The production companies involved in this project were visited between October 2016 and August 2017. Samples were cultured on the following selective media: membrane lactose glucuronide agar (MLGA, CM1031, Oxoid, Basingstoke, UK) to quantify presumptive *E. coli;* Slanetz and Bartley Medium (CM0377; Oxoid) to quantify presumptive intestinal enterococci; sorbitol MacConkey agar (SMAC, CM0813; Oxoid) supplemented with cefixime and potassium tellurite (CT, SR0172; Oxoid) for isolation of *E. coli* O157; xylose lysine deoxycholate agar (XLD, CM0469; Oxoid) for determining *Salmonella* spp.; *Listeria* selective agar (Oxford formulation, CM0856; Oxoid) supplemented with modified *Listeria* selective supplement (SR0206; Oxoid) for quantifying *Listeria* spp.; and cholera TCBS medium (CM0333; Oxoid) to quantify *Vibrio* spp. All plates were incubated at 37 °C for 24 h (except Slanetz and Bartley plates for enterococci which were incubated at 44 °C). Bacterial CFU/g were normalised by obtaining the dry weights (80 °C for 24 h) of each substrate.

Seaweed harvesting and processing

The first phase in this production chain took place at a commercial seaweed harvesting facility in the Republic of Ireland (Figure 1). Fresh, attached seaweed was hand harvested from the rocky intertidal shoreline of Finavarra beach, County Clare, Ireland at low tide in October 2016. The seaweed species collected were *Laminaria digitata*, *Fucus serratus*, *Ascophyllum nodosum*, *Palmaria palmata* and *Ulva lactuca.* Fresh seaweed was hand washed in cold, potable water to remove sand and visible epiphytic flora and fauna, laid in plastic trays and dehumidified overnight for 12 h at 40 ± 4 °C to achieve a moisture content of < 12 %. Each seaweed species was separately milled to produce a powder of particle size 0.5 – 1 mm, and the individual powders were subsequently combined in equal proportions. This dried seaweed powder mixture was packaged in plastic bags and transported to the BSF larvae rearing facility within two days.

Long term records indicated extremely low FIO levels at two neighbouring bathing water quality monitoring locations (Bishops Quarter Beach and Traught) of comparable adjacent land use conditions to Finavarra (EPA, 2017). Coupled with the likelihood of highly dilute pathogen concentrations, if present in the seawater; the lack of necessary equipment for enrichment of samples before culturing in the improvised laboratory within the seaweed factory; and the potential for environmental stress, such as high salinity, to induce a viable but non-culturable state (VBNC) in the pathogenic bacteria and thus produce false negative results (Ramirez- Castillo *et. al.,* 2015), seawater and seaweed were screened for FIOs only (*E. coli* and enterococci). To assess the level of background FIOs in the harvesting water, four replicate 100 ml samples of seawater were collected and vacuum-filtrated through a 0.45 µm cellulose nitrate membrane

(Sartorius cellulose nitrate membrane filter; Sartorius, Göttingen, Germany), and transferred to selective media. Microbiological screening (focused solely on *E. coli* and enterococci as FIOs) of seaweed took place after: (1) harvesting; (2) washing; (3) drying; and (4) transportation of the samples used for the feeding trial. At least 2 kg of four of the seaweed species *L. digitata*, *F. serratus*, *A. nodosum*, *P. palmata*, and approximately 100 g of *U. lactuca* were harvested from a wide stretch of the intertidal zone. A 500 g sample of each species (50 g of *U. lactuca*) was cut into 2 cm pieces, and individually homogenised (with no added liquid) for 2 minutes using a hand blender (MSM6700GB; Bosch, Stuttgart, Germany). Samples of 10 g (n = 4) were taken from each homogenised batch of each seaweed species, and vortexed in 10 ml of sterile seawater for 60 s, and 20 µl of the supernatant plated onto selective media, inverted and incubated. The remaining 1.5 kg of each seaweed species (50 g of *U. lactuca*) was washed and the seaweeds processed and prepared for microbiological testing as above. The remaining intact 1 kg of each species (25 g of *U. lactuca*) underwent overnight dehumidification followed by processing and microbiological testing as above. Finally, the milled seaweed powders were microbiologically tested as described above.

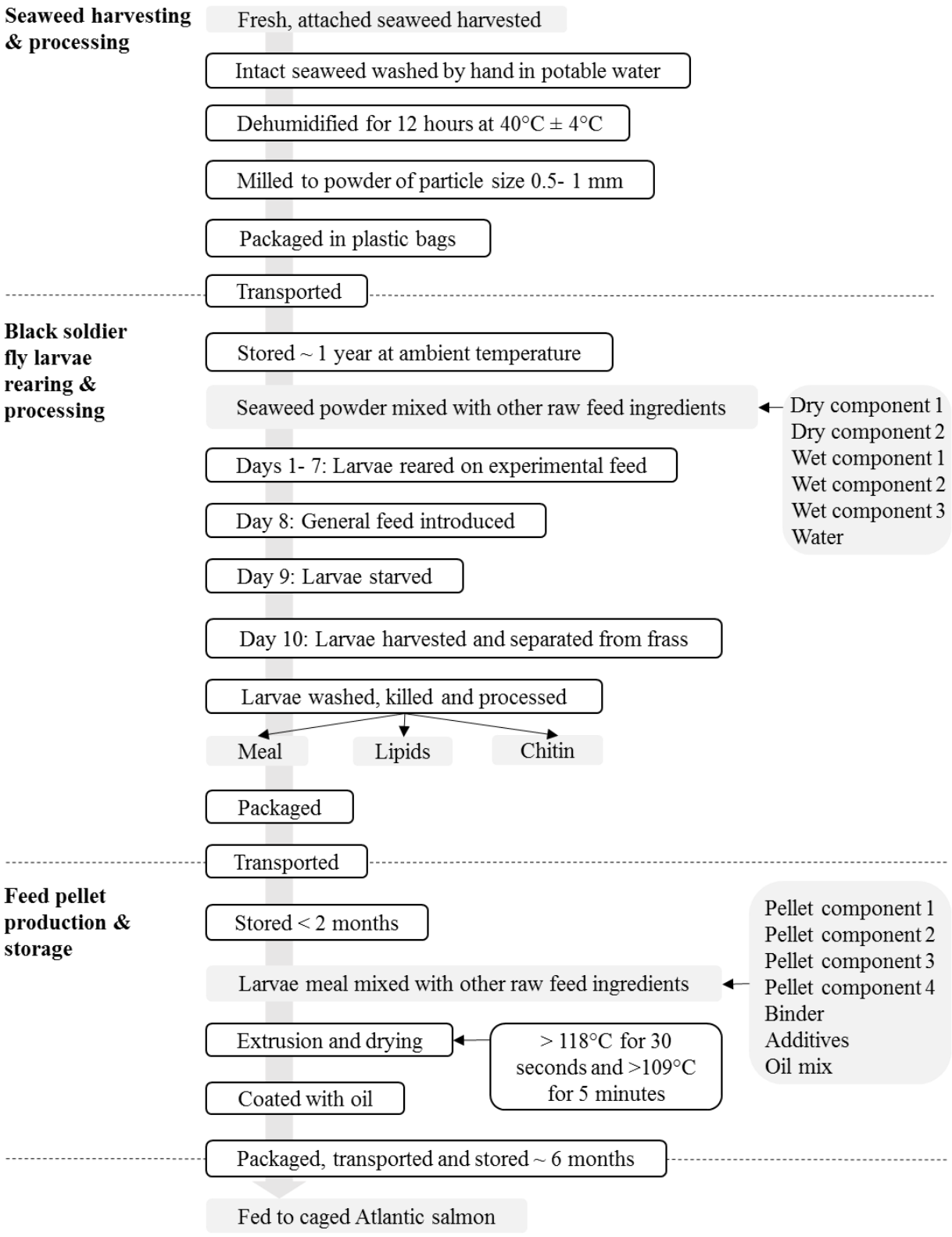

Figure 1. Schematic of production process of fish feed pellets manufactured using seaweed-fed black soldier fly larvae. Grey boxes = raw materials and finished products. White boxes = processing, packaging, distribution and storage.

BSF larvae rearing and processing

The microbiological safety assessment of larvae meal production was conducted at a BSF rearing and processing facility in the Netherlands during 2016. Environmental parameters during larval rearing and processing, physico-chemical conditions of raw materials and finished products, and Quality Control records of each facility were not disclosed. However, environmental swabs and sampling of final products were regularly undertaken according to Regulation (EC) 893/2017 (EC, 2017). The seaweed powder mixture had been stored for approximately one year in plastic bags at ambient temperature at the BSF production facility before being used in the feeding trial. The sequential stages of BSF fly breeding, egg laying, larvae rearing, larvae harvesting, washing, and killing, and the final processing of larvae into fish meal products are depicted in Figure 1. Environmental conditions within the adult breeding rooms, larvae rearing facility and during washing and processing were not disclosed, and the composition of the BSF larval feed is categorised as either dry or wet components to protect the commercial interests of the company.

The seaweed powder was divided between two separate feeding trials, although the feed recipe differed between Trials 1 and 2 due to limited availability of seaweed powder after Trial 1 (Table S1); however, the feeding regime was the same for both trials (Table S2). On day eight, larvae received 'general' feed containing the same ingredients provided on the preceding seven days, with the exclusion of the seaweed powder, since pilot trials showed that any seaweed powder remaining in larval digestive tracts at the time of harvest congested the larvae processing machinery. No new feed was provided on day nine in order to starve larvae and thus encourage them to void their digestive tracts, and larvae were harvested on day ten. Adult BSF used to produce larvae for Trial 1 died before they could be screened for pathogens, and the larvae produced in Trial 2 were not processed due to the success of Trial 1. Therefore, a microbiological safety evaluation of one entire production chain from adult breed stock to processed larvae products was not possible. The materials screened for bacteria were as follows: the five ingredients (with the exception of potable water) of which the larvae feed was comprised, adult flies which produced the larvae utilised in the feeding trial (Trial 2 only), whole larvae and their frass (mixed with waste feed), and finally the meal, lipids and chitin produced by processing of the larvae (Trial 1 only). Stored samples were unavailable, as the finished product from Trial 1 was transported to the feed pellet production facility immediately after processing of larvae.

Each sample of adult flies and larvae consisted of three individuals, and 10 samples of each were taken. Three flies or larvae were added to a 1.5 ml Eppendorf tube containing 100 µl phosphate buffered saline (PBS), the contents were homogenised using a micropestle, and a further 100 µl of PBS added. Each tube was vortexed for 60 s, and 10 µl plated onto selective media and incubated. With the exception of chitin, all other samples were pre-processed into a fine powder or a thick liquid, and would therefore not have benefitted from further homogenisation. Chitin samples (500 g) were homogenised for 2 min, with no added liquid, using a hand blender. From each 500 g sample of all materials, including chitin, 10 g (n = 10) were vortexed in 10 ml PBS for 60 s, and 50 µl plated onto selective media.

Feed pellet production and storage

A pathogen safety assessment of fish feed pellets manufactured using the larvae meal was undertaken at a commercial fish feed company in Norway in 2017. All raw ingredients (including larvae meal) were utilised within 6 months of receipt. The principle processing stages were the grinding and mixing of raw ingredients, the production of feed pellets from this mixture through high temperature and pressure extrusion followed by drying, and the coating of pellets in oil (Figure 1). Eight raw ingredients were mixed to produce four batches of pellets, and two batches (BP90015101 and BP90015102) were used in this study, which contained the same raw ingredients sourced from the same containers. The primary drying stage reduced moisture content in pellets to an estimated 6 – 9 %, before pellets were coated in a combination of fish and vegetable oils (to add energy to the feed and delay sinking of the pellets when fed to fish). Screening for environmental pathogens occurred at three different processing stages: (1) the raw ingredients prior to mixing; (2) the uncoated pellets following extrusion and drying; and (3) the coated pellets prior to packaging. Stored samples were unavailable as the finished products were transported in plastic bags to the fish feeding research station immediately after pellet production was complete.

Samples of the raw ingredient pellet component 4 (500 g) required 2 minutes soaking in 250 ml sterile PBS to adequately soften it for subsequent homogenisation for 2 minutes using a hand blender. The binder and pellet component 3 (500 g each) were dry ground for 2 minutes, with no added liquid. Insect meal, pellet components 1 and 2, and the additives were already in a fine powder form meaning that further homogenisation was unnecessary. For each ingredient, replicate samples of 10 g each (n = 4) were vortexed for 60 s in 20 ml PBS, and subsequently 20 µl plated onto selective media. A sub-sample (500 g) from each of the two batches of the oil-coated pellets (BP90015101 and BP90015102) was soaked in sterile PBS, homogenised and plated out onto selective media as described above. After approximately six months storage at the fish feeding research station, the two batches of oil-coated feed pellets were used in a caged fish feeding trial and were sampled for microbiological contamination on the same day. From each batch, 200 g was soaked for 2 minutes in 100 ml sterile PBS for subsequent homogenisation for 2 minutes using a hand blender. From each homogenised sample, 10 g (n = 4) was vortexed for 60 s in 10 ml PBS, and subsequently 20 µl plated onto selective media and incubated.

3. Results

Microbial contamination during seaweed harvesting and processing

Seawater at the seaweed harvesting site contained concentrations of *E. coli* and enterococci that were indicative of 'excellent' water quality according to the EU Bathing Water Directive (< 250 CFU/ 100 ml for *E. coli* and < 100 CFU/ 100 ml for enterococci) (Table 1). *E. coli* was found to be associated with freshly harvested *L. digitata* (<10 CFU/g), *P. palmata* (26.78 ± 26.78 CFU/g) and *U. lactuca* (10.57 ± 6.11 CFU/g) (Table 1). The target bacteria were below detectable levels on all seaweed species following washing in tap water, overnight dehumidification, and in the processed seaweed powder after two days storage.

Microbial contamination of BSF larvae during rearing and processing

E. coli, enterococci, *E. coli* O157, *Salmonella* spp., *Listeria* spp. and *Vibrio* spp. were all below detectable levels in the seaweed powder fed to BSF larvae at the start of both feeding trials. The larval feed substrate used during Trial 1 contained low levels of *E. coli* (< 10 CFU/g) (Table 1), whilst both dry components of the larval feed contained low levels of *Listeria* spp. (not exceeding 28.0 CFU/g) and dry component 2 contained relatively high levels of enterococci during Trial 2 (~ 4 log CFU/g). Wet components of the larvae feed contained relatively low levels of *Listeria* spp. the highest concentration (193.1 CFU/g) occurring in wet component 1 during Trial 1. The concentrations of enterococci and *Listeria* spp. in the final feed mixture were extremely low (< 10 CFU/g and 31.1 CFU/g, respectively). At the point of harvest, larvae produced during both feeding trials were associated with extremely low levels of enterococci (Table 1). Concentrations of enterococci in the larval frass however were typically 5- 6 log CFU/g higher than in the larvae, and frass produced in Trial 2 was also associated with a low concentration of *E. coli* (19.05 CFU/g). FIOs and pathogenic bacteria were below detectable levels in the larvae meal and lipid products immediately after processing of the larvae.

Microbial contamination during feed pellet production and storage

Following shipment to, and approximately two months storage at, the feed pellet production facility, the BSF larvae meal was found to be contaminated with relatively high concentrations of enterococci and *Listeria* spp. (both ~ 3 log CFU/g) (Table 1). Several additional commercial raw ingredients mixed with the larvae meal introduced low levels of enterococci (< 10 CFU/g in all cases) and *Listeria* spp. (detected at a maximum of 65.03 CFU/g) to the pellet formulations. Although the extrusion and drying treatments (during which temperatures exceeded 109 °C) ensured production of initially sterile pellets, subsequent oil application reintroduced very low concentrations of enterococci (< 10 CFU/g). After packaging, transport to, and approximately 6 months storage at the research station where the caged fish feeding trial was undertaken, enterococci contamination levels on feed pellets remained relatively stable (Table 1).

Table 1. Microbiological contamination of raw materials and finished products during the production chain from freshly harvested seaweed to provision of feed pellets containing black soldier fly larvae meal for feeding caged Atlantic salmon. Only ingredients and finished products in which any of the bacteria screened for were detected are included. Amongst these materials and products, any bacteria screened for but producing a non-detectable result are denoted by '-'.

Phase of production	Substrate	Sampling target	*E. coli* (CFU 100/ml or CFU/g)			Enterococci (CFU 100/ml or CFU/g)			*Listeria* spp. (CFU/g)		
			mean	SE	n	mean	SE	n	mean	SE	n
Seaweed harvesting and processing											
	Seawater		<10	-	4	<10	-	4			
	Fresh seaweed	*Laminaria digitata*	<10	-	4	-		-			
		Palmaria palmata	26.78	26.78	4	-		-			
		Ulva lactuca	10.57	6.11	4	-		-			
Larvae rearing and processing											
Trial 1	Raw feed	Dry component 2	< 10	-	10	62.0	36.08	10	-		-
	materials	Wet component 1	-		-	-		-	193.1	60.6	10
		Final feed mixture	< 10	-	10	-		-	-		-
	Harvest	Larvae[1]	-		-	< 10	-	10	-		-
		Frass	-		-	1.6×10^6	273,648.8	10	-		-
Trial 2	Raw feed	Dry component 1	-		-	-		-	< 10	-	10
	materials	Dry component 2	-		-	1.7×10^4	6,302.3	10	28.0	10.8	10

		Wet component 1	-		-	-		-	78.7	30.0	10
		Wet component 2	-		-	-		-	97.0	35.2	10
		Final feed mixture	-		-	-		-	31.1	18.0	10
	Harvest	Larvae[1]	-		-	29.7	7.6	10	-		-
		Frass	19.05	9.7	10	8.2×10^5	351,137	10	-		-
Feed pellet production											
	Raw feed	Larvae meal	-		-	3.7×10^3	652.8	4	8.2×10^3	3702.7	4
	materials	SPC	-		-	< 10	-	4	65.03	12.1	4
		Wheat gluten	-		-	-		-	< 10	-	4
		PPC- 55	-		-	< 10	-	4	38.5	37.5	4
		Binder	-		-	< 10	-	4	< 10	-	4
		Oil mix	-		-	< 10	-	4	-		-
	Coated in oil	BP90015101	-		-	< 10	-	4	-		-
		BP90015102	-		-	< 10	-	4	-		-
	Stored feed pellets	BP90015101	-		-	< 10	-	4	-		-
		BP90015102	-		-	< 10	-	4	-		-

[1] Larvae CFU/g is expressed per larvae.

4. Discussion

The 'excellent' water quality of the seaweed harvesting site likely contributed to the low levels of FIO contamination of brown, red and green species of seaweed utilised in the feed production trial. Multiple seaweed species, including *Laminaria* spp. and *A. nodosum*, produce antimicrobial exudates active against food spoilage bacteria such as *E. coli*, and such a mechanism may have contributed to inhibition or die-off of FIOs colonising the living seaweeds harvested for this study (Pina-Perez *et al*., 2017). Competitive interactions with natural bacterial biofilms on seaweed surfaces may also have influenced the attachment and survival of epiphytic and planktonic extra-enteric bacteria (Egan *et al*., 2013). Microbial cell density in biofilms associated with living *Laminaria hyperborea*, for example, peak during retardation of kelp growth in winter (Bengtsson *et al*., 2010). Seaweeds for this study were harvested in October, during the non-growth period, which may have coincided with optimal inhibition, by native biofilms, of FIO colonisation. The absence of FIOs from any seaweed species following subsequent processing stages suggests that the production environment, in terms of handling by personnel and contact with processing surfaces and equipment, were of a good hygienic standard.

Processing practices for the seaweed industry typically involve drying immediately after harvest to prevent rapid decay, and the moisture content achieved in the dried seaweed in this study surpassed the advised 15 % target for long term storage (McHugh, 2003; Nitschke and Stengel, 2016). However, the drying temperature of 40 °C used in this study to retain nutritious properties for animal health (Makkar *et al*., 2016) fall far below the approximate 700-800 °C traditionally used by the seaweed industry (McHugh, 2003). Importantly, water content is not correlated with water activity (a_w) (a thermodynamic property which varies with temperature) in food matrices, and thermal resistance of microorganisms, such as *E. coli*, increases when the a_w of low moisture foods decreases (Syamaladevi *et al*., 2016). Thermal challenge studies for FIOs or pathogens attached to seaweed are scarce, although desiccation of *Ulva reticulata* at 28 °C increased abundance of epiphytic *E. coli* and *V. parahaemolyticus* within seven days (Vairappan and Susuki, 2000).

In this study, *E. coli* was detected on all three classes of freshly harvested seaweed from a site offering 'excellent' water quality; however, microbial contamination of coastal waters, and thus of seaweeds, will vary both temporally and spatially (Quilliam *et al*., 2011). *E. coli* found colonising the harvested seaweeds did not survive subsequent processing, but there is evidence that heat stress during the drying process could induce a VBNC state in FIO and pathogenic bacterial cells, leading to overestimation of the effectiveness of desiccation as a potential antimicrobial treatment (Zhao *et al*., 2017). Validation of the temperature-time combination applied during the drying treatment (a CCP) to verify GMP may therefore be warranted to ensure microbial safety of seaweed powder. FIOs however, should not be considered indicators of pathogen presence, since pathogen survival does not necessarily mirror that of FIOs (Castro-Ibanez *et al*., 2016; Syamaladevi *et al*., 2016). Heat treatment parameters should therefore be defined for individual microorganisms.

At the larval rearing stage of the production chain, no microbiological hazards were detected in the seaweed powder, indicating that GHP applied during packaging,

distribution and personnel handling enabled safe storage of this product for at least one year. However, methodological limitations due to restricted access to laboratory equipment was imposed by sampling in-situ within the production facility (as at the other factories involved in the trial). Therefore, it was not possible to adhere to the multi-stage preparation of samples for isolation and detection of foodborne pathogens as outlined by official reference methods (e.g. ISO culture methods). Nonselective pre-enrichment of dried seaweed samples to revive and rehydrate cells induced to enter a VBNC state or injured by dehydration, changes in a_w and osmotic shock incurred during drying, followed by selective enrichment to increase the concentration of the target bacteria to that of sufficient concentration for the sensitivity of the selective culture media, may have enabled detection of pathogens which may have been present in the seaweed at very low concentrations (Lee *et. al.,* 2015; Li *et. al.,* 2014; Wu., 2008).

Several commercial dry and wet feed ingredients with which the seaweed powder was mixed contained various microbial contaminants and introduced *Listeria* spp., the only potentially pathogenic genus of bacteria detected during the feed trial, although the results were presumptive as confirmatory tests were not undertaken. Antimicrobial agents involved in the two-step enrichment process for growth and detection of *Listeria* spp. are particularly important given that the slow growing bacteria is susceptible to being outgrown by competitive microorganisms in a culture (Law *et. al.,* 2015). Therefore, the levels of *Listeria* spp. recorded may have been underestimated. Incoming raw materials for industrial insect rearing present a major vulnerability to maintaining GHP as they represent potential points of entry for microbial hazards (Fraqueza and Patarata, 2017). It was assumed that these externally acquired feed materials were subject to quality control checks post-processing, although not necessarily prior to packaging at their respective production facilities, suggesting that subsequent contact with various environments and handling may have introduced this microbial contamination (Buchanan *et al*., 2017).

Listeria spp. are found throughout the environment, often occurring in animal feed, and are almost ubiquitous in food processing environments, detection of which is used by the food industry as indicative of conditions that might facilitate the presence, growth and persistence of *Listeria monocytogenes* (Korsak and Szuplewska, 2016; Orsi and Wiedmann, 2016). The persistence of *L. monocytogenes* in food production environments is often attributed to biofilm formation by persistent strains (Buchanan *et al*., 2017). Although potentially underestimated due to lack of sample enrichment, the level of *Listeria* spp. contamination of raw materials used in the larvae feed substrate detected in this study falls well below the estimated > 1000 CFU infective dose for humans required for *L. monocytogenes*, which is the species of greatest concern to feed and food producers (Schmid-Hempel and Frank, 2007). Furthermore, *Listeria* spp. were not detected in the larvae or their frass. However, although *Listeria* spp. and *Salmonella* spp. are typically not detected by direct culturing in a range of insects reared for feed and food (Osimani *et al*., 2018; Vandeweyer *et al*., 2017b), molecular analysis identified *Listeria* spp. in mealworm larvae which plating on selective media had failed to detect (Garofalo *et al*., 2017). Physico-chemical changes to the larvae, and the heat treatment applied during processing, would have killed any undetected *L. monocytogenes* cells (NicAogain and O'Byrne, 2016). Processing steps effectively

sterilised the finished larvae products, as evidenced by the removal of enterococci, which had been present in the pre-processed larvae.

The extremely low levels of enterococci associated with harvested BSF larvae indicates that the larvae were not contaminated to the same degree as their frass, despite the fact that the frass, with which the larvae were in physical contact, contained far higher concentrations of enterococci. Enterococcaceae are associated with various insects, including flies and mealworm larvae (Grabowski and Klein, 2017; Wynants *et al*., 2018). This contrast in contamination levels may reflect underestimation of enterococci populations, as well as non-detection of the target pathogens, in the larvae due to omission of an enrichment step. Starvation of the larvae on day nine of both trials may have substantially reduced the microbial load present in the larvae, since apart from the exoskeleton and mouthparts, the insect gut contains many of the microbes associated with insects, which might explain the sizeable difference in contamination levels between the larvae and their frass (Osimani *et al*., 2018). Although 24 h starvation of mealworm larvae at 30 °C (comparable to BSF rearing temperature) whilst in contact with their frass produced no significant difference in larval bacterial loads compared with non-starved mealworms, results may vary between insect species (Wynants *et. al.,* 2017), between batches of a specific species produced by a company, as well as for the same insect produced by different companies (Vandeweyer *et. al.* 2017a), emphasising the necessity of developing HACCP systems specific to BSF and form of production. Although BSF larvae possess notably high levels of antibacterials active against both Gram-positive and Gram-negative bacteria (Vogel *et. al.,* 2018), this mechanism of bacterial inactivation in the gut may have been insufficient to offset enterococci growth in uneaten feed and frass. Moreover, BSF larvae have been shown to reduce pathogenic bacteria in manure, but to simultaneously exert far less effect on coliforms and no suppressive influence on enterococci in the same substrate, possibly reflecting antibacterial effects of the larvae on Gram-negative as opposed to Gram-positive bacteria (Choi *et al*., 2012; Lalander *et al*., 2015). During production of mealworms, enterococci abundance increased in the substrate, which was interpreted as a product of growth of enterococci in larval digestive tracts followed by excretion in high concentrations (Wynants *et al*., 2018). Enterococci remained one of the most abundant bacteria in the mealworm larvae at harvesting stage, and possible dominance of enterococci in the BSF digestive tracts may explain their detection, even at low levels.

The larvae were also found to be free of *Listeria* spp. which was detected at low concentrations in several of their feed ingredients, although again, *Listeria* spp. may have been present but undetected in the larvae due to methodological limitations. Environmental contamination with enterococci from feed and containers, and possibly personnel handling, may all have contributed to the natural autochthonous microbes known to be associated with BSF larvae (Fraqueza and Patarata, 2017; Jeon *et. al.,* 2011; Pava-Ripoll *et al*., 2012; Zheng *et. al.,* 2013). Larvae and frass were screened for microbiological hazards only after 10 days exposure to the feed substrate, thus it is unknown whether the previous growth of enterococci populations in the substrate during the feeding period accounts for the relatively high concentrations detected in the frass at harvest. Enterococci concentrations in frass at the point of larvae harvest far exceeded the initial levels in raw feed ingredients, suggesting conditions in the larvae substrate (temperature, pH range, moisture levels, unlimited nutrients) during rearing

may have encouraged growth of enterococci. The substrate on which mealworms were reared contained the same or higher total viable aerobic counts than associated with the larvae, which was attributed to the presence of frass and favourable environmental conditions (Wynants *et al*., 2018). Microbes in feed material, combined with possible contamination emanating from handling during rearing, were cited as the likely cause of microbial differences between batches of fresh mealworms grown within the same production environment, in the absence of any difference in rearing parameters within the feed substrate for each batch (Vandeweyer *et al*., 2017b). The primary route of larvae exposure to microbes potentially hazardous to human consumers further along the feed and food chain is likely to be the feed substrate. Therefore, incoming raw feed materials are potential CCPs, particularly since they were not subject in this feed trial to further sterilising treatment before consumption by the larvae.

Screening of the larvae meal, and several other raw pellet ingredients, at the feed pellet production facility revealed that contamination with relatively high levels of enterococci and *Listeria* spp. had occurred during packaging, distribution or storage between stages in the feed production chain. Other pathogens may have been present in these materials, which an enrichment step may have revealed. However, the temperature challenge during extrusion and drying which eradicated enterococci and *Listeria* spp. would have been capable of removing any pathogens undetected due to the methodological limitations. The production of fish meal pellets typically involves a heating stage followed by a cooling stage, and colonisation of the cooling feed by opportunistic bacteria should be highlighted as a potential CCP (Saucier, 2016). Whether enterococci persisted in the pellets for approximately six months prior to being fed to the fish, or were reintroduced during packaging, distribution or storage, the levels detected in the finished product did not exceed microbiological quality standards for insect PAPs (Reg (EC) 893/2017; EC, 2017).

5. Conclusions

An assessment of the microbiological risk to consumers posed by the use of seaweed feed to produce BSF PAPs for aquafeed has demonstrated that seaweed-fed BSF larvae are not likely to be sources of some important foodborne pathogens- *E. coli* O157, *Salmonella* spp., *Listeria* spp. or *Vibrio* spp.- in this feed and food production chain. However, the lack of an enrichment step prior to culturing of samples may have led to failure to detect these pathogens if present at low levels in the larvae or other feed materials, or resulted in underestimation of bacterial contamination levels. Other pathogenic bacteria may also be present in this novel feed and food chain which were not identified in this study. The persistent detection of *Listeria* spp. reflects the widespread occurrence of these potentially pathogenic bacteria in food and feed production environments. Crucially, however, bacterial contamination of finished larvae meal and pellets did not originate specifically from either the seaweed, or from the larvae reared on the seaweed, indicating that processing techniques (desiccation and heat) provided sufficient sterilisation of products. HACCP systems are specific to the products being manufactured and the processing techniques. Therefore, as the seaweed and insect farming industries mature, and innovative feed substrates for insects are explored, CCPs will emerge which are specific to each insect species, their substrate, the life stage at harvest and processing methods. Current flexibility in seaweed processing temperatures may pose a safety risk depending on water quality at

harvesting locations, but will be a key theme for development of GAP guidelines for seaweed harvesting.

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Supplementary Material

Table S1. The varying proportions of seaweed powder supplement added to other raw feed materials, and the seaweed-free general feed recipe, constituting the substrate provided for black soldier fly larvae during feeding Trials 1 and 2.

	Ingredients	Quantity (kg)
Trial 1 Recipe	Seaweed powder	47
	Dry component 1	7.5
	Dry component 2	7.5
	Wet component 1	157
	Wet component 2	78
	Wet component 3	78
	Water	50
		Total: 425
		Proportion (%)
General feed	Dry component 1	10
	Wet component 1	45
	Wet component 2	22.5
	Wet component 3	22.5
		Quantity (kg)
Trial 2 recipe	Seaweed powder	15
	Dry component 1	7.5
	Dry component 2	7.5
	Wet component 1	157
	Wet component 2	78
	Wet component 3	78
	Water	15
		Total: 358
		Proportion (%)
General feed	Dry component 1	10
	Wet component 1	45
	Wet component 2	22.5
	Wet component 3	22.5

Table S2. Feeding regime for rearing black soldier fly larvae on seaweed- supplemented feed during feeding Trials 1 and 2. One scoop was equivalent to ~0.9 kg of feed

Day	Number of scoops of feed
1	4
2	4
3	9
4	9
5	9
6	6
7	6
8	3 + 3kg of General Feed
9	0 + 1 L water
10	0

APPENDIX 5.1 | Survey documentation for feed and food producers

Identify whether you wild harvest and/ or cultivate seaweed	Harvest:
	Cultivate:
List the seaweed species which you harvest and/ or cultivate	
List the animal feed and/or human food markets you supply (domestic and international)	
Identify the method/s by which you harvest seaweed	Hand gather beach-cast seaweed
	Mechanically gather beach-cast seaweed
	Hand pick:
	Hand cut with knife:
	Hand cut with sickle:
	Mechanical hedge cutter:
	Other:
Identify the method/s by which you cultivate seaweed	Farm or small scale (0-40 x 200m lines):
	Medium scale (41-80 x 200m lines):

	Extensive (>80 x 200m lines):				
	Integrated multi-trophic aquaculture (IMTA):				
	Off-bottom monoline:				
	Raft system:				
	Single longline ropes:				
	Seedling production in hatchery:				
	Other:				
Factors	**Select factors that influence current site selection (from the 59 options listed in column 1)**	**Identify the 5 most important current factors (from the 59 options listed in column 1)**	**Select factors that might influence future site selection (if different from current) (from the 59 options listed in column 1)**	**Identify the 5 most important future factors (if different from current) (from the 59 options listed in column 1)**	**Additional comments**
a) Standing stock of target seaweed species					
Available biomass					

Available productivity					
Available distribution					
Seasonality					
Lack of data on biomass					
Lack of data on productivity					
Lack of data on distribution					
Advised sustainable harvesting regime					
Advised monitoring regime					
Other					
b) Socio-cultural					
Existence of Crofters Rights/ practices of traditional harvesters					
Conflict between mechanical harvesting and livelihoods of traditional harvesters					

Other					
c) Infrastructural					
Accessibility of standing stock					
Access from harvesting site/s to processing facility/facilities					
Access to processing facility/facilities					
Access to storage facility/facilities					
Access to labour					
Research into storage required					
Lack of skills/ knowledge					
Other					
d) Regulatory					
Difficulty in determining suitable locations that do not conflict with other coastal activities					

Existence of exclusive rights to harvest an area					
Existence of protected and sensitive areas (e.g. SACs, SPAs, PMFs, MPAs, seal haul-out sites, archaeological features (wrecks) and Historic Marine Protected Areas (HMPAs), Ramsar sites, Natura 2000, Scottish Priority Marine Features, Important Plant Areas for marine seaweeds, Sites of Special Scientific Interest, areas protected under the EU Habitats Directive)					
Requirement for landowner's permission					
Requirement for Licence					
Unregulated over-harvesting					
Environmental impacts					
Other					

e) Economic					
Inconsistent supply of seaweed biomass					
Inconsistent seaweed productivity					
Cost of hiring labour					
Cost of harvesting machinery					
Investment in mechanical harvesting undermining local employment of harvesters					
Cost of processing facilities					
Cost of storage facilities					
Accessing markets for low or high value products					
Marketing					
Risk					
Investment					
R & D					
Supply chains					

Other					
f) Environmental					
Perceived environmental effects of mechanical harvesting preventing investment in mechanical harvesting					
Heavy metals in sediment					
Heavy metals in biota					
Bathing Water Quality					
Wastewater Discharge Points					
Shellfish Harvesting Areas					
Nitrate loading					
Other					
g) Physio-chemical					
Bathymetry					
Temperature					

Salinity					
Water motion					
Nutrient concentrations					
Light availability					
Climate change effects					
Other					

APPENDIX 5.2 | Survey documentation for advisory bodies

Factors	Select factors that influence current site selection (from the 59 options listed in column 1)	Identify the 5 most important current factors (from the 59 options listed in column 1)	Select factors that might influence future site selection (if different from current) (from the 59 options listed in column 1)	Identify the 5 most important future factors (if different from current) (from the 59 options listed in column 1)	Additional comments
a) Standing stock of target seaweed species					
Available biomass					
Available productivity					
Available distribution					
Seasonality					
Lack of data on biomass					
Lack of data on productivity					
Lack of data on distribution					
Advised sustainable harvesting regime					

Advised monitoring regime					
Other					
b) Socio-cultural					
Existence of Crofters Rights/ practices of traditional harvesters					
Conflict between mechanical harvesting and livelihoods of traditional harvesters					
Other					
c) Infrastructural					
Accessibility of standing stock					

Access from harvesting site/s to processing facility/facilities					
Access to processing facility/facilities					
Access to storage facility/facilities					
Access to labour					
Research into storage required					
Lack of skills/ knowledge					
Other					
d) Regulatory					
Difficulty in determining suitable locations that do not conflict with other coastal activities					
Existence of exclusive rights to harvest an area					
Existence of protected and sensitive areas (e.g. SACs, SPAs, PMFs, MPAs, seal haul-out sites, archaeological features (wrecks) and Historic Marine Protected Areas (HMPAs), Ramsar					

sites, Natura 2000, Scottish Priority Marine Features, Important Plant Areas for marine seaweeds, Sites of Special Scientific Interest, areas protected under the EU Habitats Directive)					
Requirement for landowner's permission					
Requirement for Licence					
Unregulated over-harvesting					
Environmental impacts					
Other					
e) Economic					
Inconsistent supply of seaweed biomass					
Inconsistent seaweed productivity					
Cost of hiring labour					
Cost of harvesting machinery					

Investment in mechanical harvesting undermining local employment of harvesters					
Cost of processing facilities					
Cost of storage facilities					
Accessing markets for low or high value products					
Marketing					
Risk					
Investment					
R & D					
Supply chains					
Other					
f) Environmental					
Perceived environmental effects of mechanical harvesting preventing investment in mechanical harvesting					
Heavy metals in sediment					
Heavy metals in biota					

Bathing Water Quality					
Wastewater Discharge Points					
Shellfish Harvesting Areas					
Nitrate loading					
Other					
g) Physio-chemical					
Bathymetry					
Temperature					
Salinity					
Water motion					
Nutrient concentrations					
Light availability					
Climate change effects					
Other					