An Investigation of Harmful Jellyfish Mitigation Measures: From Sting Management to Jellyfish Forecasting

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Declaration

I, Jasmine L. Headlam, certify that this thesis is all my own work and I have not obtained a degree in this University, or elsewhere, based on this work.

Signature: 

Date: 10/07/2020

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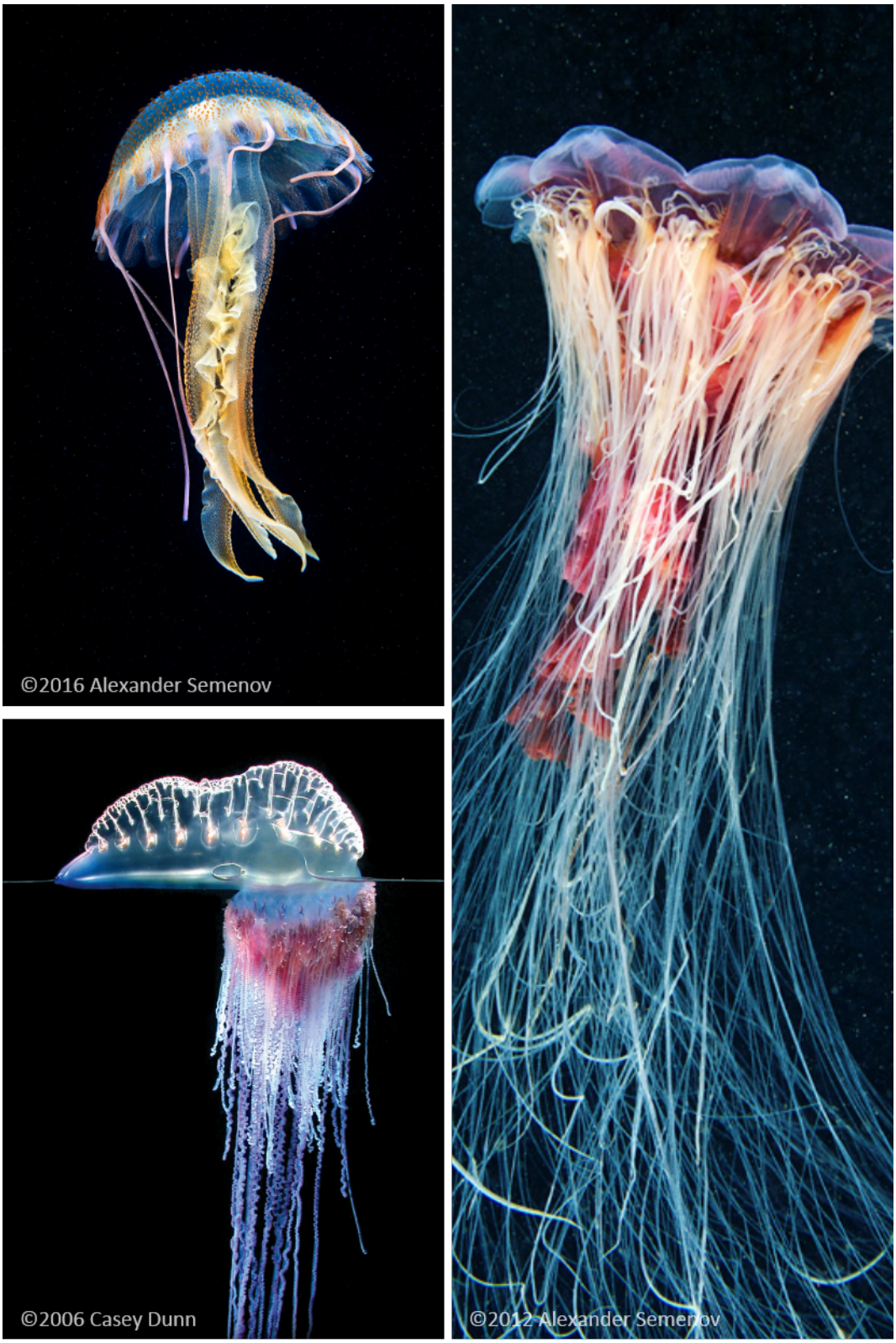
In loving memory of Jim Kennedy.



Chapter 1 – General Introduction

1. General Introduction

1.1. Phylum Cnidaria

Jellyfish belong to the phylum Cnidaria (Figure 1.1). The phylum Cnidaria comprises two clades (groups of organisms that are classified in the same taxon and share a common ancestor): Anthozoa (7,500 species) and Medusozoa (3,786 species) (Daly & Daly, 2007; Mapstone, 2015). The class Anthozoa comprises all members of the clade Anthozoa; the Medusozoa contains four classes: Cubozoa (36 species), Scyphozoa (200 species including lion's mane jellyfish *Cyanea capillata* and mauve stinger *Pelagia noctiluca*), Hydrozoa (3,500 species including Portuguese man of war *Physalia physalis*), and Staurozoa (50 species) (Daly & Daly, 2007; Mapstone, 2015). Cnidaria was formerly grouped with ctenophores in the phylum Coelenterata, but increasing awareness of their differences caused them to be separated into two distinct phyla (Daly & Daly, 2007). The phylum Cnidaria contains approximately 10,000 species, 100 of which are known to be dangerous to humans (Williamson, Fenner, Burnett, & Rifkin, 1996).

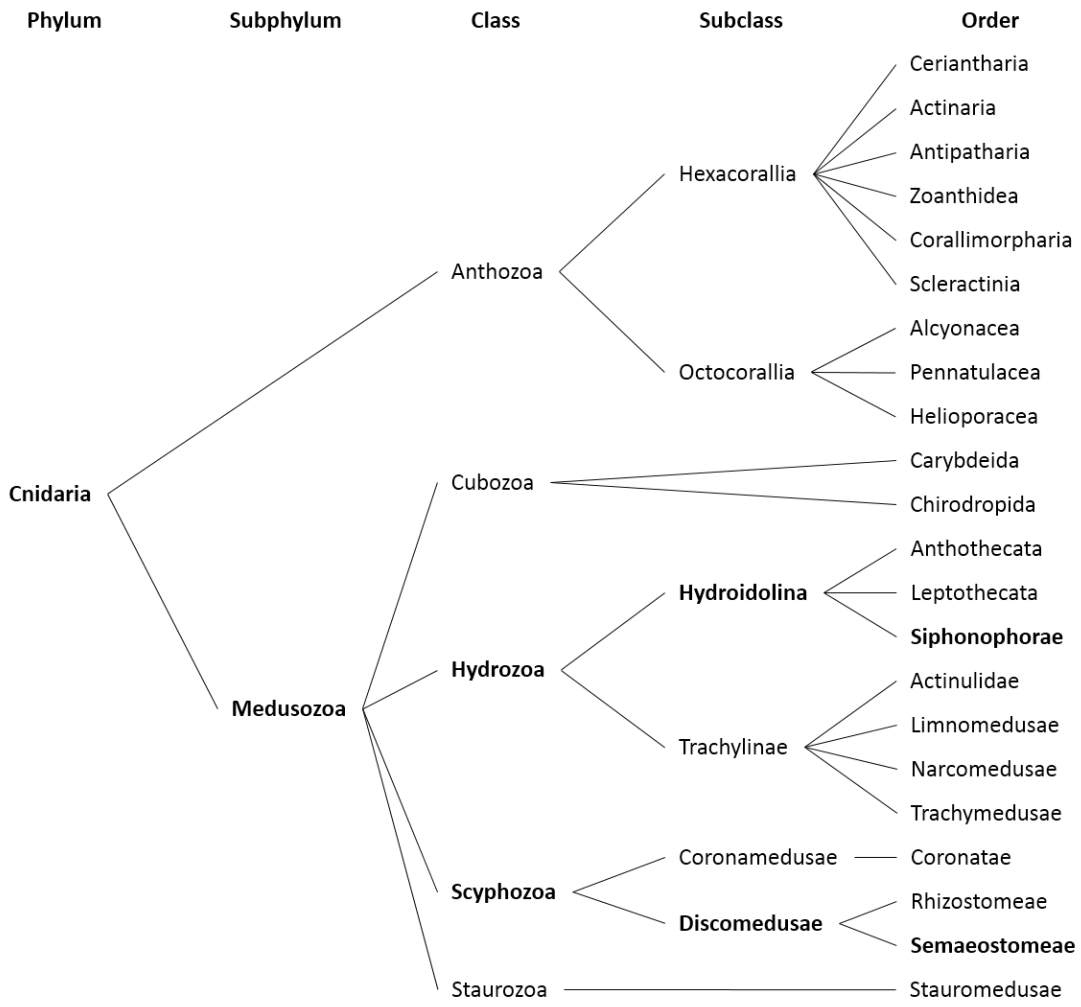


Figure 1.1. Classifications of Cnidaria adapted from Collins (2009). The species discussed in this thesis belong to the orders highlighted in bold.

In this study, the term "jellyfish" refers exclusively to pelagic cnidarians, hydrozoans and scyphozoans, which possess long tentacles armed with nematocysts (i.e. venomous capsules). Most jellyfishes are radially symmetrical, (the sail of *Ph. physalis* is bilaterally symmetrical and the polyps are radially symmetrical), with tentacles encircling a mouth at one end of the body (Barnes, Calow, & Olive, 1993; Hickman, Roberts, Larson, I'Anson, & Eisenhour, 2006). The mouth is the only opening to an internal space for digestion, called the "gastrovascular cavity" (Barnes et al., 1993; Hickman et al., 2006). Two morphotypes occur in jellyfish lifecycles: a pelagic "medusa" and a benthic "polyp" which may be colonial (Barnes et al., 1993; Hickman et al., 2006) (Figure 1.2). Polyps are tubular: one end, the oral end, has a mouth and the other end, the aboral end, has a basal disc that attaches the animal

to the substratum (Barnes et al., 1993; Hickman et al., 2006). Medusae resemble an inverted umbrella, thus structures on the outer side of the umbrella are associated with the exumbrella (the aboral surface) while the subumbrella refers to the area on the underside of the umbrella (the oral surface) (Barnes et al., 1993). A tube called the manubrium hangs from the subumbrella. At the distal end of the manubrium is the mouth which leads to the gastrovascular cavity (Hickman et al., 2006). Jellyfishes are generally carnivorous (Hickman et al., 2006), feeding on plankton, other jellyfishes and arthropods (Arai, 1997; Purcell, 1997, 2003), and are important predators of fish eggs and larvae (Purcell, 1985). Jellyfishes are diploblastic, i.e. they possess two cell layers: the outer epidermis, or ectoderm, and the inner gastrodermis, or endoderm (Barnes et al., 1993; Hickman et al., 2006). The mesoglea lies between these two layers (Hickman et al., 2006). Jellyfishes have a nerve net where the neurons and their processes are spread among the epithelial cells of the epidermis and the gastrodermis (Watanabe, Fujisawa, & Holstein, 2009) and they possess sense organs which include statocysts (organs of equilibrium) and ocelli (photosensitive organs (Hickman et al., 2006).

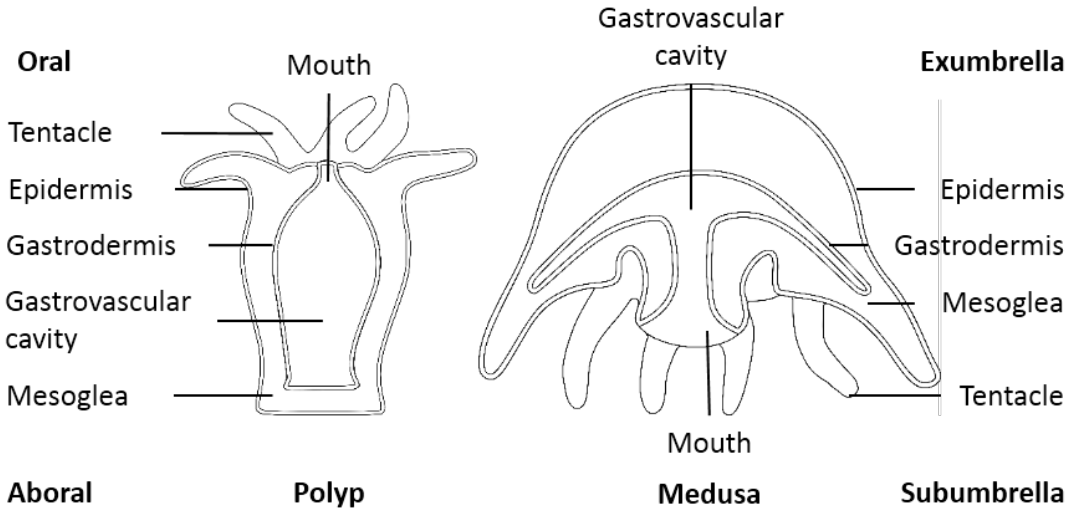


Figure 1.2. The two body forms of cnidarians after Barnes et al. (1993).

A typical jellyfish life cycle alternates between two stages: an asexually reproducing polyp stage and a sexually reproducing medusa stage (Barnes et al., 1993; Hickman et al., 2006). In the life cycle of a scyphozoan jellyfish like *C. capillata*

(Figure 1.3), adult medusae reproduce sexually and the resultant zygote develops into a motile, ciliated planula larva (Hickman et al., 2006). The planula settles on a hard surface and metamorphoses into a scyphistoma, a scyphozoan polyp (Hickman et al., 2006). Scyphistomae reproduce asexually by a process called strobilation (Hickman et al., 2006). In strobilation, scyphistomae, or strobilae, produce saucer-like buds called ephyrae which break loose and grow into mature medusae (Hickman et al., 2006). Hydrozoa also possess a medusa and a polyp stage, however, some hydrozoans, for example by-the-wind-sailor (*Velella velella*) and *Ph. physalis*, spend their entire life as a colony of polyps at the ocean surface attached to a float rather than a hard surface (Hickman et al., 2006).

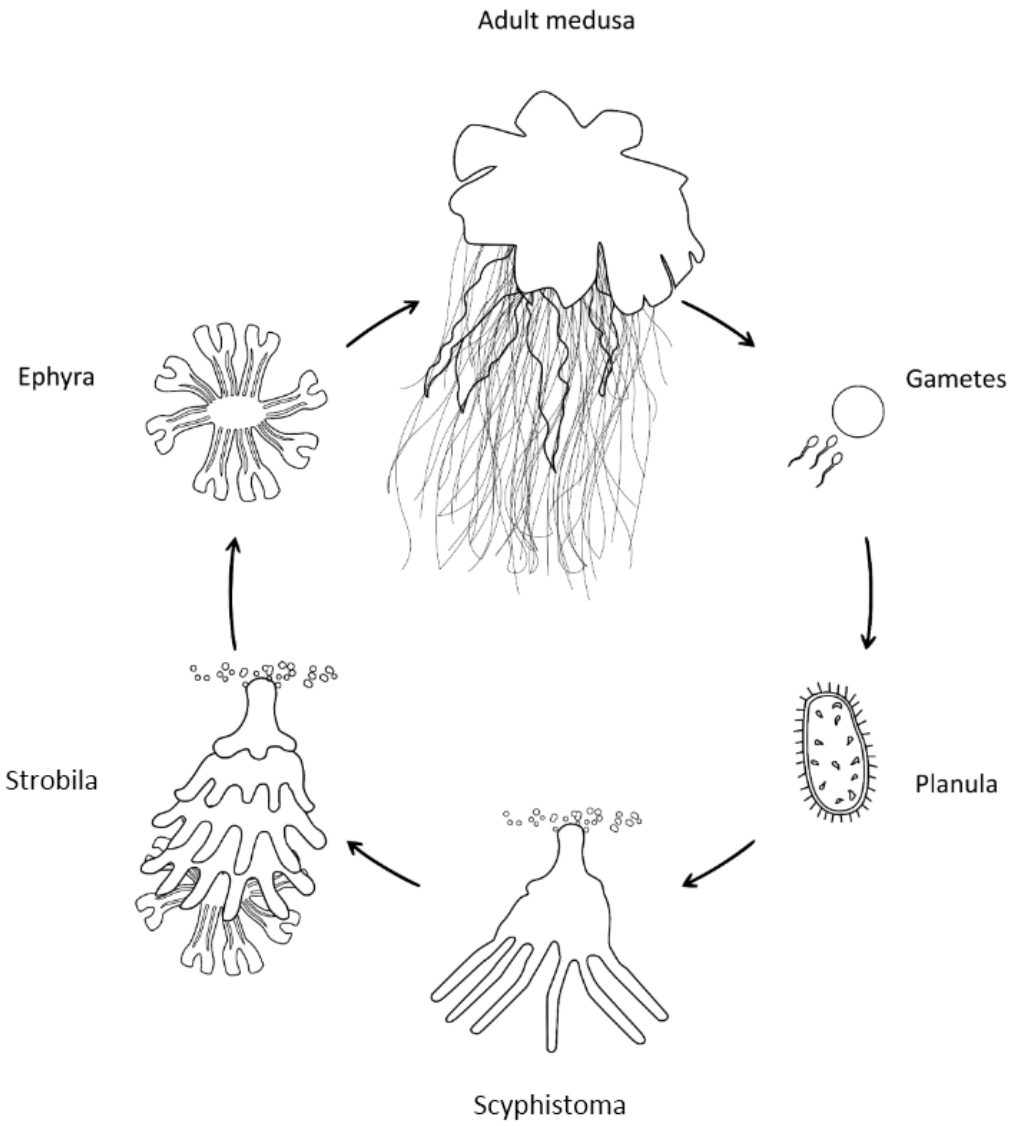


Figure 1.3. Typical *C. capillata* life cycle showing alternation of medusa (sexual) and polyp (asexual) stages.

Jellyfish have a seasonal life cycle and often increase in number (i.e. bloom) every year during spring or summer when planktonic food is in abundance (Mills, 2001). The ability to bloom is intrinsic to their life cycles (Purcell, Uye, & Lo, 2007). Despite a lack of evidence, it has been suggested that some jellyfish are increasing in abundance as a result of anthropogenic ecological disturbances such as climate change, eutrophication, over-harvesting of fish stocks and the introduction of non-native species (Arai, 2001; Graham & Bayha, 2007; Hay, 2006; Oguz, 2005; Purcell, 2005). However, most available evidence suggests that jellyfish fluctuate periodically with natural climate cycles and that any increases are localized and not global (Brotz,

Cheung, Kleisner, Pakhomov, & Pauly, 2012; Condon et al., 2013; Mills, 2001; Purcell et al., 2007). Nevertheless, reports of human problems with jellyfish, such as jellyfish stinging swimmers and interfering with fishing, aquaculture and power plant operations, have increased in recent decades (Purcell et al., 2007).

1.2. Cnidae

Within the epidermal layer of all cnidarians are microscopic everting structures called cnidae. Cnidae are highly specialised, secretory, subcellular organelles (Hyman, 1940; Weill, 1934; Yanagita & Wada, 1959) used for defence and prey capture (Carré & Carré, 1980; Tardent & Holstein, 1982; Watson, 1988). There are three distinct types of cnidae: penetrant nematocysts (exclusive cnida type in scyphozoans and cubozoans) (Rifkin, 1991), volvent (entangling) spirocysts (Weill, 1934) and glutinant (sticky) ptychocysts (Mariscal, Conklin, & Bigger, 1977) (found exclusively in anthozoans) (Rifkin, 1988; Watson & Wood, 1988; Williamson et al., 1996). Nematocysts consist of a capsule and an eversible, helically-folded tubule immersed in venom (Mariscal, 1974a; Tibballs, 2006; Williamson et al., 1996) (Figure 1.4). Upon chemical or mechanical stimulation of a sensory structure (called cnidocil) (Pantin, 1942; Williamson et al., 1996), the capsule operculum opens and the shaft emerges and pierces the tissue of the prey or victim before the barbed tubule emerges and completely everts (Yanagihara, Kuroiwa, Oliver, & Kunkel, 2002). Tubules turn inside out so that the venom is excreted on the outside of the tubule (Cegolon, Heymann, Lange, & Mastrangelo, 2013). Some tubules are hollow and discharge venom through the terminal opening (Kramp, 1961; Tibballs, 2006; Williamson et al., 1996). The entire process takes less than 3 µs and the tubule discharges at a speed of 2 metres per second or an acceleration of 40,000 G (Holstein & Tardent, 1984). Nematocysts can function even when separated from the tentacle or if the jellyfish is dead (Haddad Junior, Silveira, & Migotto, 2010; Lotan, Fishman, & Zlotkin, 1996; Tibballs, 2006).

Initially, nematocysts were divided into sixteen categories based on observations made under the light microscope (LM) (Weill, 1934). Additional nematocyst types were identified by Carlgren (1940, 1945) and Cutress (1955) and

since the advent of high resolution LMs and scanning electron microscopes (SEM), over thirty different types of nematocysts have been identified (Bouillon, Boero, & Gravier—Bonnet, 1986; Carlgren, 1940, 1945; Cutress, 1955; Mariscal, 1974b; Östman, 1983, 1997a; Östman & Hydman, 1997; Werner, 1965; Williamson et al., 1996).

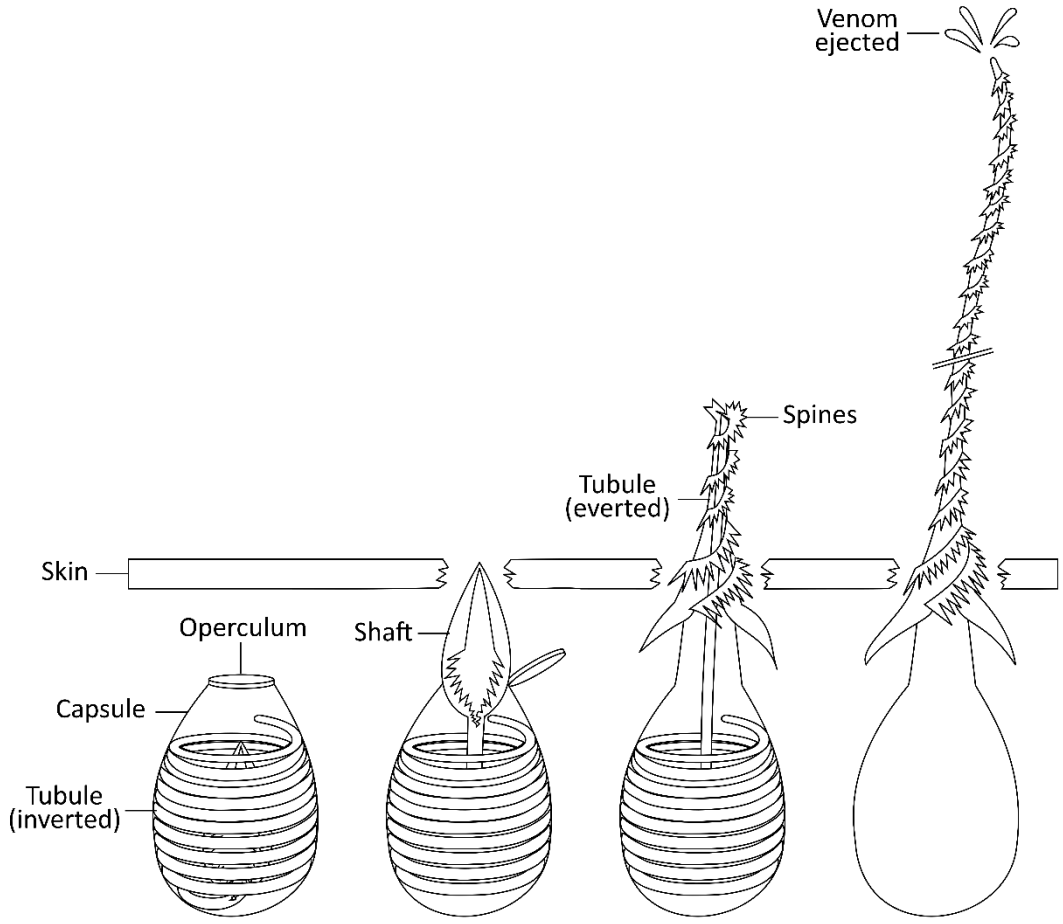


Figure 1.4. Diagram of a nematocyst capsule ejecting its tubule and releasing venom from inside the capsule. Based on an image by Byron Inouye.

1.3. Jellyfish Envenomation of Humans

Stings due to jellyfish are some of the most common envenomations encountered by humans in the marine environment (Lee, Chang, Choi, & Park, 1998). Although all jellyfish are capable of envenomation, most are harmless to humans. For

example, this might be because the nematocysts do not have tubules long enough to inject venom deep into the epidermis (Barnes, 1960; Tibballs, 2006) or because the nematocysts do not produce venom harmful to humans (Barnes, 1966). Harmful jellyfish include *Physalia physalis* which have long tubules to inject venom nearly 1 mm into the epidermis (Yanagihara et al., 2002) and large jellyfish like *Cyanea capillata* (up to 1 m bell diameter) which are able to release large amounts of venom (Cegolon et al., 2013).

Skin contact with tentacles results in a local and immediate reaction due to the penetration of the tubule and the activity of various venom components (Cegolon et al., 2013), such as phospholipase A2 (Nevalainen et al., 2004). This reaction occurs within minutes to hours of the sting and manifests as painful lesions which are linear, urticarial and erythematous (Figure 1.5) (Lakkis, Maalouf, & Mahmassani, 2015; Menahem & Shvartzman, 1994). In severe cases, often from box jellyfishes (class Cubozoa), these lesions can become vesicular, haemorrhagic, necrotic or even ulcerative (Lakkis et al., 2015). Some jellyfish stings, e.g. from *Pe. noctiluca* (Figure 1.5B), can leave scars and hyperpigmentation which remains for some years after the sting (Mariottini, Giacco, & Pane, 2008).

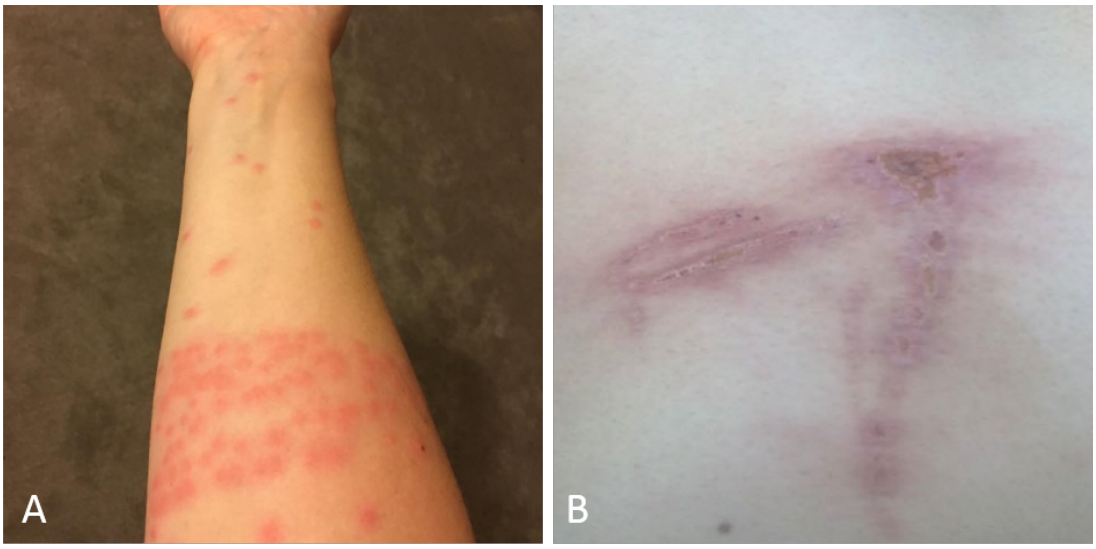


Figure 1.5. Examples of jellyfish envenomation in Ireland. A) *Cyanea capillata* sting on the forearm of an adult female. B) *Pelagia noctiluca* sting on the ribs of an adult male.

In addition to the above reactions, nematocysts can also cause systemic reactions if the tubules are long enough to inject venom into the circulatory system (Tibballs, 2006). These reactions may be gastrointestinal, cardiac, muscular or neurological (Cegolon et al., 2013) due to the direct action of the venom on the liver, kidneys, heart or nervous system (Lakkis et al., 2015). Irukandji syndrome is a serious systemic reaction caused by certain box jellyfish species (Gershwin et al., 2013), including *Carukia barnesi* (Ramasamy, Isbister, Seymour, & Hodgson, 2005), however, *C. capillata* have also been known to cause similar symptoms. Symptoms develop 20 – 60 minutes after the sting and include "back pain, nausea, abdominal cramps, sweating, hypertension, tachycardia and a feeling of impending doom" (Little, Pereira, Carrette, & Seymour, 2006). Inside the body, these symptoms are marked by catecholamine excess and cytokine storm causing hypertension and tachycardia followed by hypotension, bradycardia, cardiac failure, pulmonary oedema and/or cerebral haemorrhage (Yanagihara, Wilcox, Smith, & Surrett, 2016). In severe cases, death may occur as a result of cerebral haemorrhage (Yanagihara, Wilcox, Smith, et al., 2016).

Stings by the Australian box jellyfish (*Chironex fleckeri*) are the most medically significant (Lakkis et al., 2015). These stings result initially in painful violaceous skin lesions, and within minutes, death as a result of cardiovascular collapse (Yanagihara & Shohet, 2012).

Considering the risk associated with the presence of jellyfishes in coastal water or stranded on the shore, high abundance of jellyfishes sometimes leads to beach closures. In the Mediterranean, closures due to *Pe. noctiluca* (Bastian, 2011) and *Ph. physalis* (Labadie et al., 2012) are not uncommon; in Ireland, beach closures have been enforced due to *C. capillata* (pers. comm. T. K. Doyle) and *Ph. physalis* (pers. comm. T. K. Doyle). Beach closures are often a source of significant economic loss for coastal communities. Therefore, authorities in tourist areas have taken mitigation measures to minimise jellyfish encounters (e.g. anti-jellyfish nets and jellyfish forecasting systems) (Canepa et al., 2016; Piraino et al., 2016).

1.4. Jellyfish Envenomation of Fish

Interference with aquaculture, particularly Atlantic salmon (*Salmo salar*), is one of the most frequently reported problems associated with large jellyfish blooms (Purcell et al., 2007). Jellyfishes may damage fish either indirectly or directly. Indirect damage occurs through hypoxia and subsequent suffocation when they block the exchange of water between the pen and surrounding water column (Båmstedt, Fosså, Martinussen, & Fosshagen, 1998; Lucas, Gelcich, & Uye, 2014). Direct damage occurs by jellyfish stinging the skin and gills of the fish when small gelatinous zooplankton such as *Muggiaea atlantica* (Hydrozoa, Siphonophorae) (Fossa, Flood, Olsen, & Jensen, 2003) enter the pens intact (Figure 1.6) or when large jellyfish such as *Aurelia aurita* (Scyphozoa, Semaeostomeae) (Yasuda, 1988), *C. capillata* (Bruno & Ellis, 1985), and *Pe. noctiluca* (Doyle et al., 2008) pass through the mesh of pens becoming broken up into smaller pieces (Baxter, Sturt, et al., 2011; Mitchell, Baxter, Holland, & Rodger, 2012). The smaller pieces still possess nematocysts, which, if drawn in by the fish during ventilation (Figure 1.6), cause severe damage to the gills (Rodger, Henry, & Mitchell, 2011). Gill damage leads to respiratory and osmoregulatory distress, reduced feeding and sometimes death (Baxter, Rodger, McAllen, & Doyle, 2011; Baxter, Sturt, et al., 2011; Bruno & Ellis, 1985; Rodger et al., 2011). In Ireland, gill damage is one of the most serious causes of mortality with average losses of 12% (Rodger, 2007). Despite this, knowledge of the species-specific effects of jellyfish on fish remains poor.

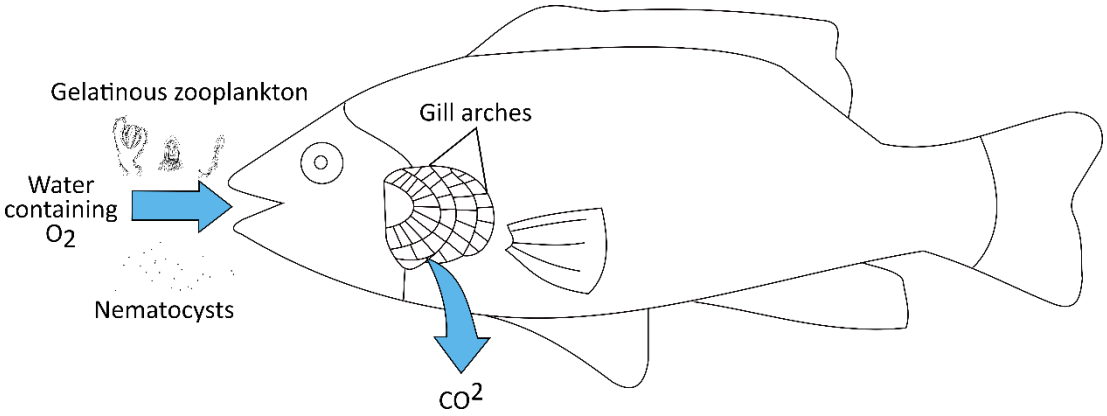


Figure 1.6. Diagram of a fish inhaling water containing O_2 into its mouth and exhaling CO_2 out of its gills. If small gelatinous zooplankton and/or nematocysts from large jellyfish are inhaled, they pass over the gills and cause significant damage.

In addition, there are limited mitigation measures in place to protect fishes from jellyfishes. Potential mitigation methods to minimise the impact of jellyfishes have been tested including early-warning systems of impending jellyfish blooms (Lucas et al., 2014; Rodger et al., 2011), reduction or complete cessation of feeding (Hay & Murray, 2008), site relocation (Rodger et al., 2011), bubble curtains (Haberlin, 2018) and net-cleaning to reduce biofouling (Baxter et al., 2012). However, none have proven sufficiently effective and more research is required in the area of mitigation (Rodger et al., 2011). In particular, there is a requirement for research to be undertaken into medicines and remedies as previous remedies have been associated with gill damage, for example, formalin (Speare, Arsenault, MacNair, & Powell, 1997), hydrogen peroxide (Kiemer & Black, 1997), and chloramine – T (Powell, Haman, Wright, & Perry, 1998; Sanchez, Speare, & Johnson, 1997).

1.5. Aims and Objectives

The research presented within this thesis describes investigations on various aspects of jellyfish envenomation throughout the Irish and Celtic Seas in the Northeast Atlantic Ocean. This thesis aimed to address the impacts of jellyfish on aquaculture and public safety in Ireland and demonstrate how their negative impacts might be mitigated in the future. The specific objectives addressed in this study are:

1.5.1. Chapter 2 – Jellyfish Forecasting

- To use a Lagrangian particle-tracking model informed by *Ph. physalis* stranding data to provide insights on the origin and drift trajectories of these organisms prior to stranding.
- To validate the use of a Lagrangian particle-tracking model by forecasting offshore observations of *Ph. physalis* from where they were observed at sea and comparing results to stranding data, with the aim of providing a forecasting model of jellyfish strandings.

1.5.2. Chapter 3 – Sting Management

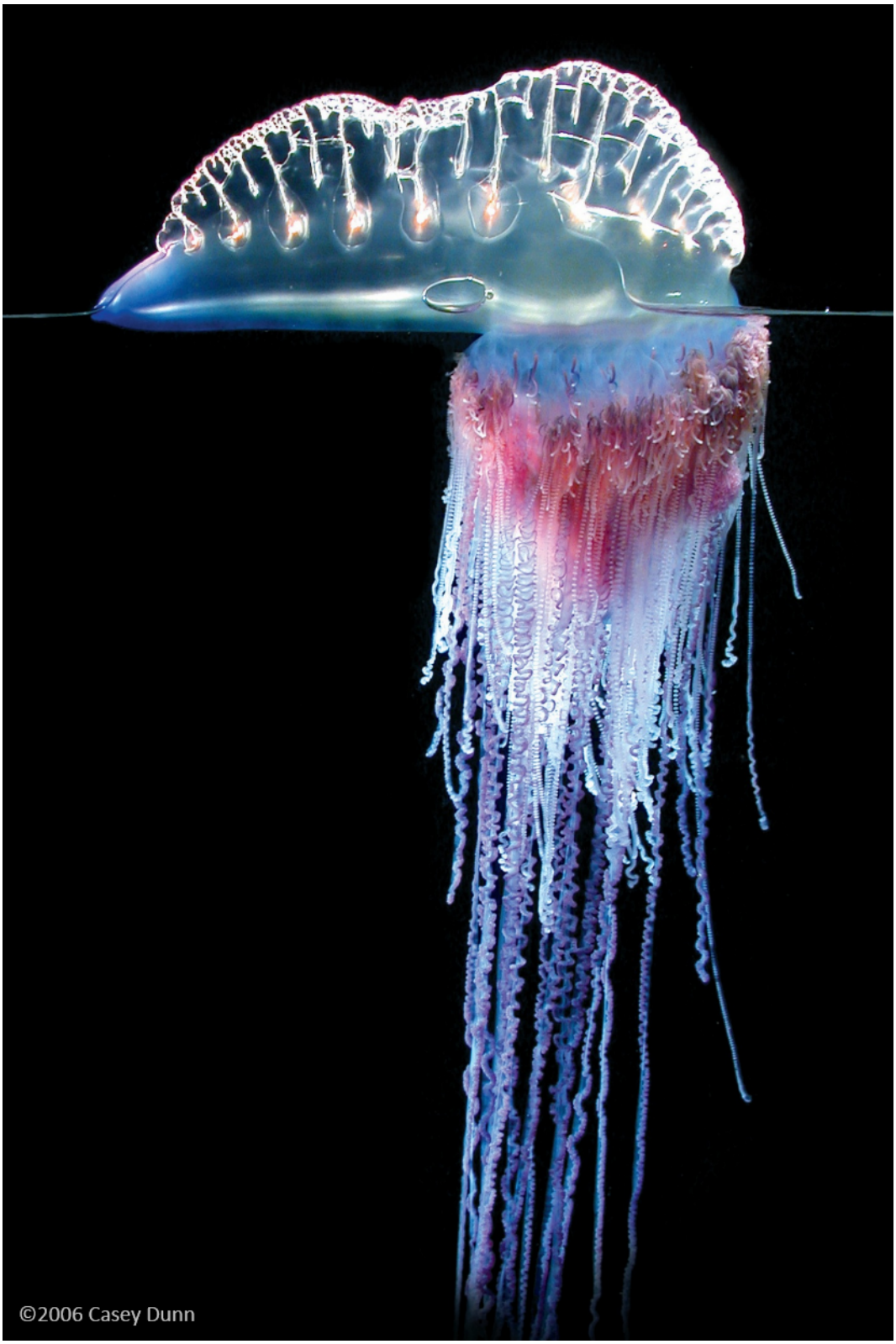
- To re-evaluate first aid recommendations for jellyfish stings using *in vitro* and *ex vivo* envenomation models.
- To compare the results of *in vitro* and *ex vivo* envenomation models to further evaluate whether *in vitro* based studies are clinically relevant.
- To compare the results for three species (*C. capillata*, *Ph. physalis* and *Pe. noctiluca*) to determine species-specific responses and whether there is a universally applicable first aid solution.

1.5.3. Chapter 4 – Nematocyst Analysis

- To compare the abundance and size of different nematocyst types between three tentacle regions and the total complement of nematocysts for *C. capillata* and *Pe. noctiluca*.
- To provide initial estimates for the total number of nematocysts possessed by each species.

1.5.4. Chapter 5 – Fish Envenomation Therapeutic

- To explore the haemolytic activity of *C. capillata* venom in *S. salar.*
- To take tentative steps toward the development of an effective jellyfish envenomation therapeutic.



Chapter 2 - Jellyfish Forecasting

2. Insights on the Origin and Drift Trajectories of Portuguese Man of War (*Physalia physalis*) Over the Celtic Sea Shelf Area

This chapter has been submitted for peer-review publication: Headlam, J. L., Lyons, K., Kenny, J., Lenihan, E. S., Quigley, D. T. G., Helps, W., Dugon, M. M., Doyle, T. K. (2020). Insights on the origin and drift trajectories of Portuguese man of war (*Physalia physalis*) over the Celtic Sea shelf area. Submitted to *Estuarine, Coastal and Shelf Science*

Abstract

Many marine animals are difficult to study because they are widely dispersed across oceans and are not captured by traditional sampling methodologies such as fishery surveys. A case in point is the Portuguese man of war (*Physalia physalis*) (Linnæus, 1758) which, despite being pleustonic and remarkably conspicuous, is one of the least studied and understood gelatinous zooplankton species, especially in terms of its ecology. During August to October 2016, the Irish coastline experienced the largest mass stranding of *Ph. physalis* in over 150 years. At the same time, *Ph. physalis* were recorded offshore in the Porcupine Seabight. Here we used these stranded and offshore observations of *Ph. physalis* to inform a Lagrangian particle-tracking model forced by wind to 1) hindcast the backwards drift of this species for three months to determine their likely origin and provide some insights on likely pathways to Irish shores and, 2) forecast the drift of this species towards the Irish coastline. Hindcasting stranded *Ph. physalis* from the Irish coastline suggested that they most likely originated from an extensive source area located over the European basin but ultimately from the North Atlantic Current. Our forecast model indicated that particles released from the Porcupine Seabight stranded on Irish shores, in fact, stranding patterns were 82% similar to actual strandings. Both models combined suggested that the Porcupine Seabight was an important source area, but that many *Ph. physalis* likely originated from further south and took a more tortuous trajectory towards Ireland determined by wind. This study also highlights the value of collecting

routine beach stranding data and opportunistic offshore visual observations to inform future coastal and shelf modelling studies.

2.1. Introduction

Many animals are difficult to study because of their rarity, elusiveness or episodic nature (e.g. giant squid and cicadas) (Kubodera & Mori, 2005; Lloyd & Dybas, 1966). Other animals are simply difficult to study because of the environment in which they live (e.g. deep-sea and polar landscapes) (Hoving et al., 2014; Kooyman, 1967) or because of their migratory behaviour (Righton et al., 2016). Today enormous advances in electronic animal tracking technologies (e.g. satellite tags, acoustic tags and camera traps) (Hussey et al., 2015; Karanth, Nichols, Kumar, & Hines, 2006; Klaassen et al., 2014) and molecular techniques (e.g. meta-barcoding and stable isotope analysis) (Inger & Bearhop, 2008; Lamb et al., 2017) are revolutionising how we study animal behaviour and ecology for many of these species. However, despite these technological advances, many marine animals remain elusive and avoid our best efforts to study them. A case in point is the oceanic siphonophore, the Portuguese man of war (*Physalia physalis*). Despite being pleustonic (surface dwelling) and remarkably conspicuous (vividly coloured and highly venomous), it is one of the least studied and understood gelatinous zooplankton species, especially in terms of its ecology. Most of the literature on *Ph. physalis* refers to envenomation (Burnett, Fenner, Kokelj, & Williamson, 1994; Labadie et al., 2012; Wilcox, Headlam, Doyle, & Yanagihara, 2017) or toxinology (Edwards & Hessinger, 2000; Stillway & Lane, 1971; Tamkun & Hessinger, 1981).

The Portuguese man of war is a buoyant colony of numerous polyps, or zooids, as opposed to a singular multicellular organism (Totton & Mackie, 1960). Each zooid is specialized for a specific function (Munro, Vue, Behringer, & Dunn, 2019). For example, the pneumatophore is a gas-filled float used as a sail to catch the wind and gastrozooids are feeding polyps used for the ingestion and digestion of food (Munro et al., 2019). Unlike medusae which propel themselves by creating vortices in the water (Dabiri, Colin, Costello, & Gharib, 2005), *Ph. physalis* align their pneumatophores with the wind and drift passively in the same direction that the

wind is blowing (Iosilevskii & Weihs, 2009). Wind plays a crucial role in their dispersion and distribution, even at wind speeds as low as 5 ms^{-1} (Ferrer & Pastor, 2017; Iosilevskii & Weihs, 2009; Prieto, Macías, Peliz, & Ruiz, 2015). This effect applies to other organisms or pieces of debris drifting close to the surface (e.g. fish eggs and microplastics) (Myksvoll, Sandvik, Skarðhamar, & Sundby, 2012; Simionato, Berasategui, Meccia, Acha, & Mianzan, 2008; Zhang, 2017). For *Ph. physalis,* the effect is most significant near coastlines where intense winds push them towards beaches over relatively short distances (Prieto et al., 2015).

In the Northeast Atlantic, beach strandings of *Ph. physalis* occur intermittently and are therefore difficult to predict or observe, especially over large areas. They are known to strand in UK and Irish waters (Wilson, 1947) and in the Bay of Biscay (Labadie et al., 2012). In these areas, there is mounting concern for the emerging health risk linked to *Ph. physalis* strandings. In 2010 and 2011, an increase in the numbers of stings along the Aquitaine coast coincided with a marked increase in the number of *Ph. physalis* strandings in the region (Labadie et al., 2012). Even in areas where they are not commonly known to strand, *Ph. physalis* have stranded in large numbers. For example, in 2010, the Mediterranean basin experienced a mass stranding of more than 100,000 colonies (Prieto et al., 2015).

Often mass stranding events are discovered in isolation of offshore observations i.e. the animals are only observed after they have stranded. Very few stranding studies have gathered data on the animals offshore, before or during a stranding event, which makes it very difficult to determine their likely origin. For example, a previous study by Wilson (1947) that described a mass stranding event of *Ph. physalis* in UK and Irish waters in 1945 – 46, stated that stranded "swarms" of *Ph. physalis* were likely to have "come from the Azores-mid-Atlantic region rather than the Canaries-Gibraltar" region. Therefore, given the opportunity presented by a mass stranding event of *Ph. physalis* along the Irish coastline in 2016, combined with detailed offshore observations of *Ph. physalis* made before and during the observed stranding event, this study set out to use a Lagrangian particle-tracking model informed by the stranding data, to predict their origin and hindcast the most likely route traversed by the organisms prior to stranding. Specifically, we tested the

hypothesis that *Ph. physalis* originated from a south westerly direction (i.e. Azores-mid-Atlantic region) rather than a southerly direction (Canaries-Gibraltar region) (Wilson, 1947). Additionally, we validate the use of our model by simulating the offshore observations forwards through time from where they were observed offshore. Lastly, we analyse wind speed and wind direction to determine their influence on this stranding event.

2.2. Materials and Methods

2.2.1. Coastal observations

To provide a historical context for the 2016 mass stranding event, *Ph. physalis* strandings were compiled from several different sources including a literature review (for 1834 – 1968), personal communication (1989 – 2016) and social media (2008 – 2016) (Table A.2.1). The unique event of August – October 2016 was carefully monitored by analysing *Ph. physalis* strandings from the database of the "Big Jellyfish Hunt" (BJH) Facebook page (https://www.facebook.com/ecojel/) established in 2008. Facebook messages containing positive identifications of *Ph. physalis* were compiled and the dates of observation, locations (longitude and latitude) and abundance were recorded. In addition, the authors carried out regular beach surveys along the west and southwest coast during September and October 2016 and communicated with BirdWatch Ireland of Cape Clear Island (southwest of Ireland) for records of *Ph. physalis* strandings. All strandings were aggregated at beach level and converted into a cumulative density value (indiv.100m^{-1}) by using Google Maps to measure the length of the beach where the strandings were reported.

2.2.2. Model Simulation

Ocean simulations were conducted using an implementation of the Regional Ocean Modelling System (ROMS) (Shchepetkin & McWilliams, 2005) for the Northeast Atlantic Ocean (Figure 2.1A) with a horizontal resolution ranging from 1.1 km in Irish coastal waters to 3.5 km in the south of the domain with 40 vertical (terrain-following) levels downscaled from the CMEMS global analysis and forecast product GLOBAL ANALYSIS FORECAST PHY 001 024 (available at

http://marine.copernicus.eu/). The ROMS model includes atmospheric forcing from the European Center for Medium-range Weather Forecast (ECMWF) and tidal forcing from the TPX0 global tidal model (OSU Topex/Poseidon Global Inverse Solution). A limitation of the dataset used in our simulation is that the model output was at 3-hourly resolution instead of the 1-hourly resolution required to properly resolve tidal currents.

Particle-tracking simulations were performed using the free modelling tool *Ichthyop* (version 3.3, available at http://www.ichthyop.org/) (Lett et al., 2008) forced by the ROMS described above. Each *Ph. physalis* colony was simulated as a particle floating at a depth of 0 m. Particles were not allowed to grow or die but were passive drifters advected by the joint effects of surface currents (computed by the ROMS model) and wind drag. We treated a strip of coastal water 30 km directly adjacent to the observed stranding locations as a pre-defined geographical zone. Within this geographical zone, 5 particles were released randomly at the surface every 6 hours (00:00, 06:00, 12:00 and 18:00) for the period 31/10/2016 until 01/08/2016, four days before the first actual *Ph. physalis* stranding. These particles were tracked backwards in time under a Runge Kutta 4^{th} order advection scheme (Lett et al., 2008) with a turbulent dissipation rate of $1x10^{-9}$ m^3s^{-3} (Monin & Ozmidov, 1981) until 01/08/2016, before too large a proportion of particles drifted outside of the ROMS domain. The model output provided a time and location (longitude and latitude) for each particle. These were used to calculate the sum of the distance (km) that each particle drifted for each day of the simulation (i.e. km day^{-1}).

2.2.3. Wind Effect

Wind data were downloaded from the ERA-interim reanalysis provided by ECMWF (freely available at http://apps.ecmwf.int/datasets/, data downloaded on 16/01/2020) for a domain that covers Irish waters in the Northeast Atlantic. Each particle drifted in the wind direction at 4.5% of the wind velocity according to Ferrer and Pastor (2017) but previous studies indicate that 10% is more appropriate for simulating the drift of *Ph. physalis* (Iosilevskii & Weihs, 2009; Prieto et al., 2015);

however, experiments showed no significant differences in particle dispersion. When backtracking the particles, the wind direction was reversed.

To analyse the effect of the wind on the dispersion of *Ph. physalis*, daily mean drift (km day^{-1}) and daily mean wind speed (ms^{-1}) were compared for significant differences for each month of the simulation and daily mean drift (km day^{-1}) was modelled using a Generalized Additive Model (GAM) (Hastie & Tibshirani, 1990). The fit of the model was evaluated by analysing the total variability explained. One-way ANOVA and Tukey's Post Hoc Test were conducted in IBM SPSS Statistics (version 26). All GAM analyses were conducted in R using the "mgcv" package (R Core Team, 2018).

2.2.4. Model Validation

Results from the particle-tracking model were compared to offshore observations of *Ph. physalis* collected from the *Oceanic Endeavour* traversing over the Celtic Sea (~49.5°N – 50°N, 11°W – 13°W) during 04/08/2016 – 21/09/2016, i.e. results were filtered to calculate the proportion of particles which moved through the Porcupine Seabight. All observations (1681 minutes of observations) were made from the port side of the bridge during daylight hours (09:00 – 21:00). Sample periods were between 30 min and 3.5 h long. Location (longitude and latitude), time, depth, speed (knots), wind direction, wind force (Beaufort scale), sea state, swell, visibility and glare were recorded every hour. Approximate sighting distance was up to 100 m dependent on weather conditions (Table A.2.2).

The area of the Porcupine Seabight where the observations were made was treated as an offshore geographical zone (~49.5°N – 50°N, 11°W – 13°W). Within this geographical zone, one particle was released randomly at the surface every 6 hours (00:00, 06:00, 12:00 and 18:00) for the period 04/08/2016 – 21/09/2016. These particles were tracked forwards through time until 31/10/2016. When particles reached the coastline, they were simulated to strand and the time and location (longitude and latitude) were recorded; these stranding data were then compared to observed strandings.

Strandings were treated as a binary response (0 = absence, 1 = presence). The Simple Matching Coefficient (SMC) was used to investigate the similarity between the daily occurrence of actual strandings and strandings predicted by the particle-tracking model from the onset (04/08/2016) until termination (31/10/2016). The SMC compared the number of matches within datasets and was chosen as other methods discard mutual absences which are informative here (Gower, 1971). SMC is defined as:

$$S\ (\%) = \frac{p + n}{t} \times 100$$

Where p is the number of days where both coastal observations and the particle tracking model recorded strandings, n is where both recorded no strandings and t is the total number of possible days.

2.3. Results

2.3.1. Coastal observations

During 05/08/2016 – 18/10/2016, the BJH Facebook page received a total of 90 records of *Ph. physalis* observations in the form of private messages, public visitor posts or comments made to posts published by the page administrators. Overall, >1,100 individual strandings of *Ph. physalis* were recorded. The authors made a total of 12 beach surveys during 22/08/2016 – 26/10/2016 and a total of 42 individual strandings were recorded from Derrynane, Co. Kerry, (*N* = 8) on 26/09/2016, Youghal, Co. Cork, (*N* = 7) and Ardmore, Co. Waterford, (*N* = 27) on 07/10/2016. BirdWatch Ireland provided a further 13 records of *Ph. physalis* equating 728 individual strandings. All strandings were aggregated at beach level and converted to a cumulative density value (indiv.100m^{-1}) (Figure 2.1B). Strandings were most concentrated along the southwest coast of Ireland (Figure 2.1B).

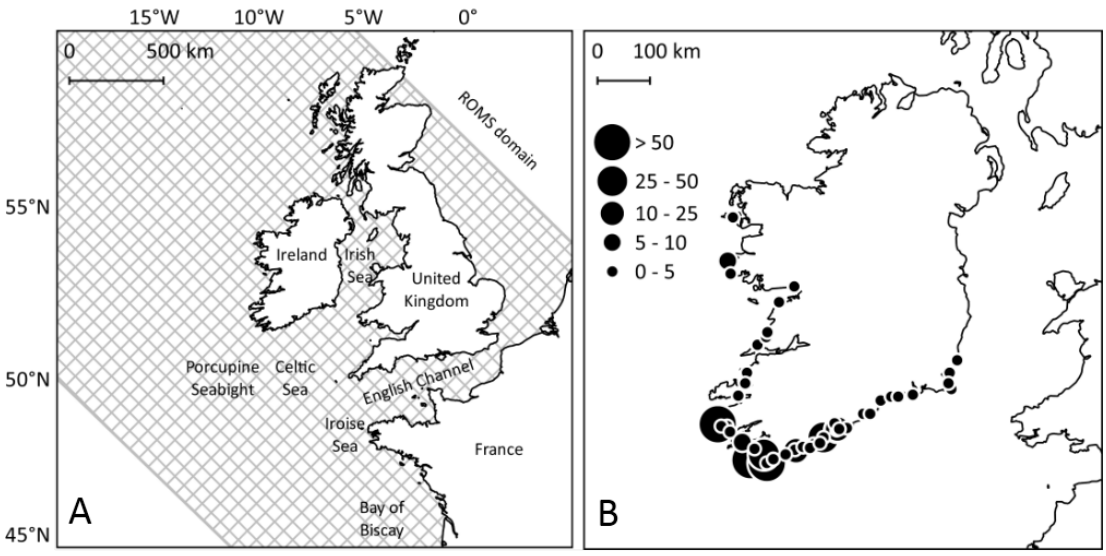


Figure 2.1. Map showing study area and coastal observations. A) Grid represents the Regional Ocean Modelling System (ROMS) Northeast Atlantic Ocean domain. B) Observed strandings of *Ph. physalis* during 05/08/2016 – 26/10/2016. Cumulative density (indiv.100m^{-1}) of observed *Ph. physalis* strandings aggregated at beach level.

Prior to the 2016 event, the Facebook page had only received two reports of *Ph. physalis* strandings, one in 2012 and another in 2015. Historically, the most recent notable *Ph. physalis* mass stranding event occurred in 1968 when a total of 446 colonies were found stranded on Cape Clear Island, an island southwest of Ireland, between 10/10/1968 – 31/10/1968 (Sharrock, 1969) (Table A.2.1). The author noted that the largest stranding of 234 colonies followed a period of persistent south easterly winds and coincided with the stranding of tens of thousands of by-the-wind sailor (*Velella velella*), another highly modified hydrozoan (Sharrock, 1969). The previous year, 200 colonies of *Ph. physalis* were found stranded on the same island (Sharrock, 1968b, 1968a). The oldest record of *Ph. physalis* stranding in Ireland dates back to 1834 (Thompson, 1835) (Table A.2.1). Therefore, the mass stranding event of 2016 is only the third such recorded event in the past 150 years and is the largest of three stranding events.

2.3.2. Particle-Tracking Model Simulation

The coastal observations (Figure 2.1B) were used to inform the particle-tracking model to determine a geographical zone for the release of particles. Five

particles were released from random locations within this zone every 6 hours during 31/10/2016 – 01/08/2016. Particles were tracked backwards through time until 01/08/2016.

In total, 1,825 trajectories of particles were obtained in the particle-tracking simulation, starting from the coastal geographical zone (Figure 2.2). Plotting the predicted distribution of particles indicated that particles released during the first four weeks of the simulation (31/10/2016 – 01/10/2016) moved west into the Celtic Sea before changing direction and moving southeast towards the southwest coast of the UK, west again and southeast through the Celtic Sea towards the western entrance of the English Channel and the Iroise Sea, north of the Bay of Biscay. For the next eight weeks, all particles moved in a narrow band (~250 km wide) southwest through the Celtic Sea until the model was terminated on 01/08/2016. Overall, 13% of particles moved through the small area where offshore observations of *Ph. physalis* were made (~49.5°N – 50°N, 11°W – 13°W) and, in total, 28% of particles moved outside of the ROMS domain.

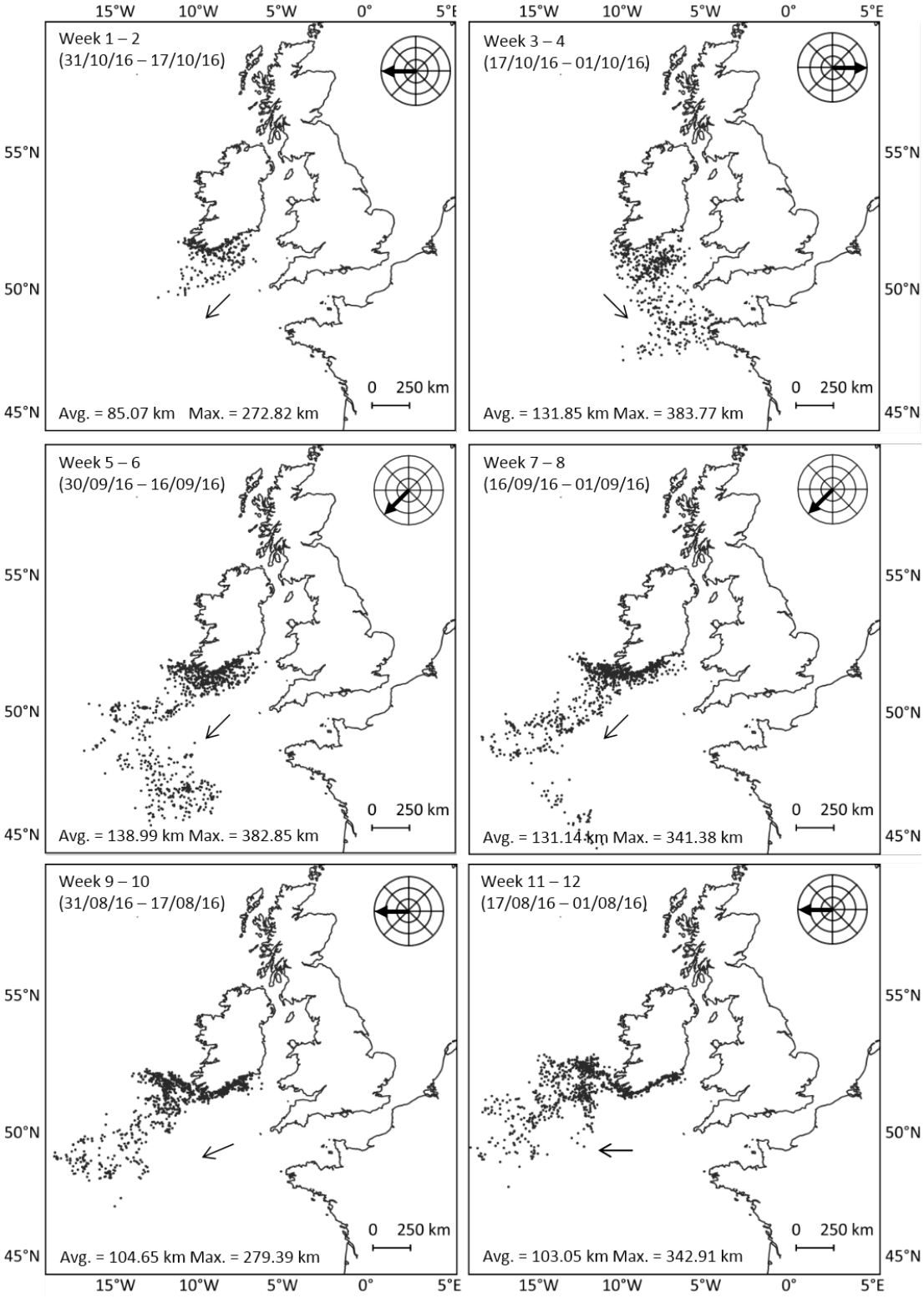


Figure 2.2. Hindcast particle-tracking model. Hindcast model wind direction is the reverse of the forecast model wind direction. Dates move backward in time from left to right and top to bottom. Five particles were released every 6 hours for 31/10/2016 – 01/08/2016 within a coastal geographical zone. Particles were tracked backwards

through time until 01/08/2016. Maps show particle distributions and travelled distances by the end of each fortnight (14 days). Black open arrows represent the main direction of particle drift. Wind roses represent prevailing wind direction for each fortnight.

2.3.3. Wind Effect

In the hindcast particle-tracking model, daily mean drift (km day^{-1}) was significantly lower in August (Figure 2.3) when compared to September (Tukey's Test, $P < 0.0001$; Figure 2.3) and October (Tukey's Test, $P < 0.005$; Figure 2.3). However, there were no significant differences between September and October for daily mean drift (km day^{-1}) (Tukey's Test, $P = 0.249$) or August, September and October for daily mean wind speed (ms^{-1}) (One-way ANOVA, $P = 0.066$).

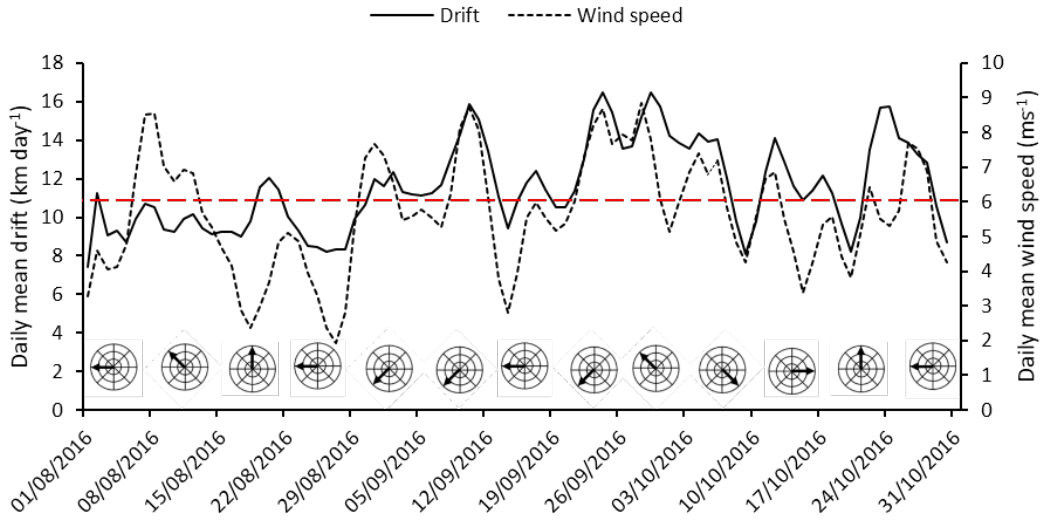


Figure 2.3. Comparison of three-day moving mean drift (km day^{-1}) and daily mean wind speed (ms^{-1}) for 01/08/2016 – 31/10/2016. Daily mean drift was obtained from the hindcast particle-tracking model. Red dashed line represents 6 ms^{-1} wind speed. Wind roses represent prevailing wind direction for each week.

A GAM analysis was completed to examine the effect of daily mean wind speed (ms^{-1}) on daily mean drift (km day^{-1}) (Figure 2.4). Generalized additive model analysis revealed that wind explained 45% of variability in drift. Wind speeds above

6 ms^{-1} had an increasingly positive effect on the drift of particles, with a maximum positive effect at 10 ms^{-1}.

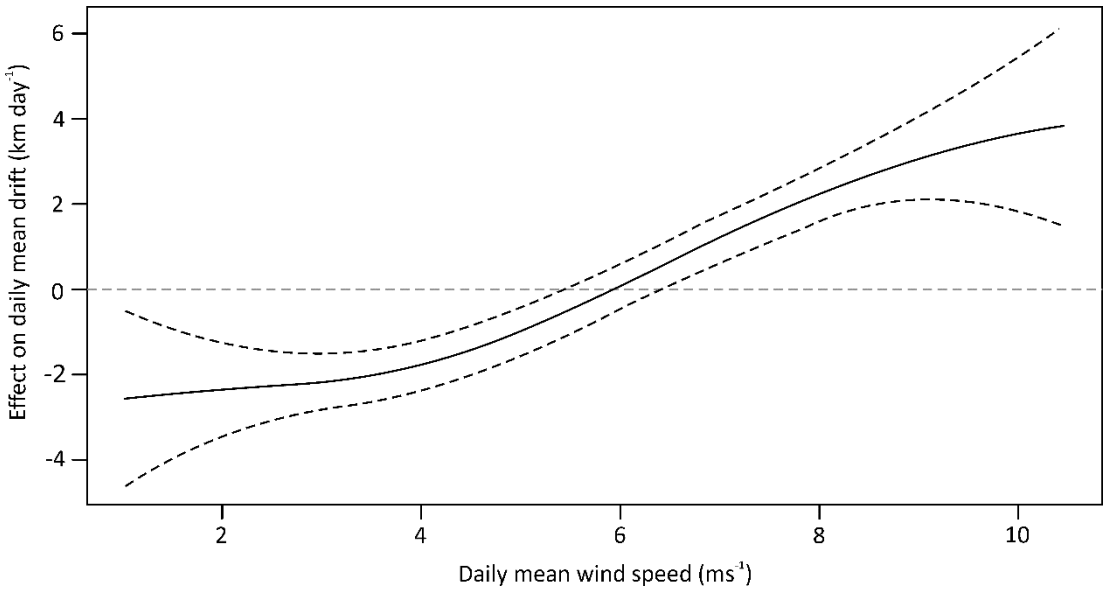


Figure 2.4. Plot from GAM showing the effect of daily mean wind speed (ms^{-1}) on daily mean drift (km day^{-1}). Positive and negative values on the y-axis indicate increasing and decreasing effect, respectively.

In August, a smaller proportion of days had a mean wind speed greater than 6 ms^{-1} (32.25%) when compared to September (60%) and October (46.67%).

2.3.4. Model Validation

Particles released from the offshore geographical zone (~49.5°N – 50°N, 11°W – 13°W) during 04/08/2016 – 21/09/2016 (Figure 2.5) drifted east through the Celtic Sea towards the southwest coast of the UK and northeast towards the southern entrance of the Irish Sea. The first particles stranded on the south coast of Ireland on 12/09/2016. The first particles to strand in the UK stranded on the southwest coast in Cornwall on 19/09/2016. Particles continued to drift northeast from the offshore geographical zone towards the south to southeast coast of Ireland and the southwest coast of the UK until 01/10/2016 when particles drifted west and ~68% of particles stranded along the southwest to southeast coast of Ireland. Several particles drifted further west and stranded in Ireland as far north as counties Galway (~53.4°N, 9.8°W) and Mayo (~54°N, 10.1°W) before the model was terminated on 31/10/2016.

The SMC analysis indicated that strandings predicted by the particle-tracking model matched the coastal observations of strandings by a similarity of 82%.

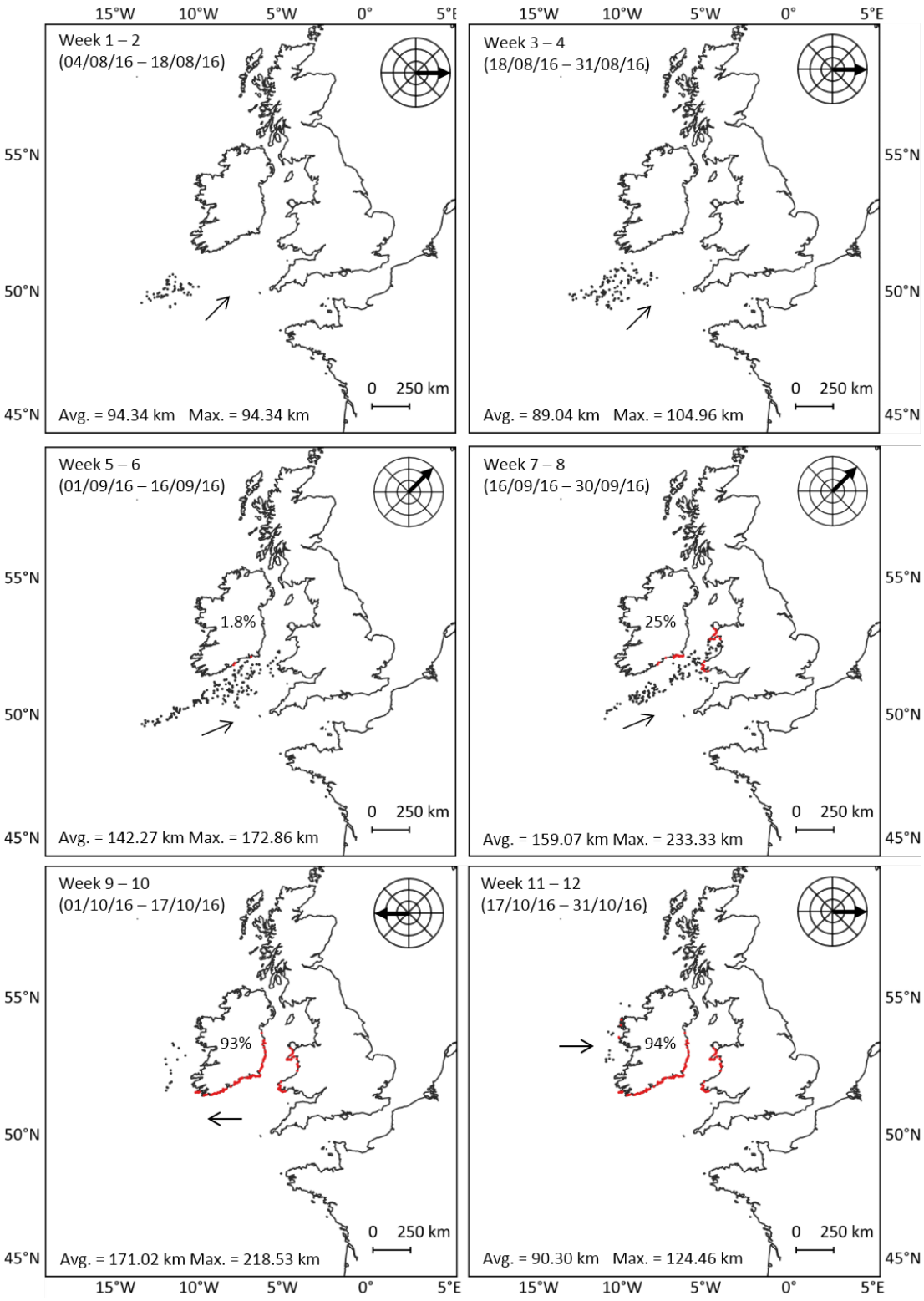


Figure 2.5. Forward particle-tracking model. Dates move forward in time from left to right and top to bottom. One particle was released every 6 hours for 04/08/2016 –

21/09/2016 within an offshore geographical zone (~49.5°N – 50°N, 11°W – 13°W) (*N* = 192 particles in total). Particles were tracked forwards through time until 31/10/2016. Maps show particle distributions and travelled distances by the end of each fortnight (14 days). Percentages represent proportion of particles stranded (red particles). Black open arrows represent the main direction of particle drift. Wind roses represent prevailing wind direction for each fortnight.

2.4. Discussion

Understanding the origin of *Physalia physalis* has been a challenge for coastal communities from Portugal to France for the last decade as mass stranding events can result in serious envenomation and beach closures (Ferrer et al., 2015; Luis Ferrer & Pastor, 2017; Labadie et al., 2012; Prieto et al., 2015). While historically, *Ph. physalis* have not been a beach management issue in Ireland, since 2016, there have been mass stranding events documented every year (>500 every single year, unpublished data from the Big Jellyfish Hunt Facebook page). While only few serious envenomations were reported during this period, several beach closures were enforced during peak stranding events (pers. comm. T. K. Doyle). Within this context, our particle-tracking model and offshore observations of *Ph. physalis* are particularly relevant.

Most importantly, our hindcast model identified an extensive *Ph. physalis* source area located over the European basin, southwest and west of the Celtic Sea shelf (Figure 2.6). This is loosely in agreement with Wilson (1947) who stated that they likely "come from the Azores-mid-Atlantic region rather than the Canaries-Gibraltar" region. However, our results are much more in agreement with the recent modelling of *Ph. physalis* by Ferrer and Pastor (2017) that clearly identified the North Atlantic Current (NAC) as the main source area for strandings on Basque shores. While our source area is located slightly further east than the main arm of the NAC (Figure 2.6), it makes sense that the NAC is the ultimate source of *Ph. physalis* that strand on Irish and UK shores. For example, juvenile and sub adult sea turtles (*Caretta caretta*) are carried to European shores via the NAC (Hays & Marsh, 1997; Monzón-Argüello et al., 2012). Genetic studies have confirmed that these sea turtles have

mostly originated from the east coast of America (Monzón-Argüello et al., 2012). Furthermore, studies on tropical drift seed strandings in Europe have always identified the Gulf Stream and the NAC as the most likely transport mechanism for carrying these seeds northeast towards Europe (Darwin, 1859; Nelson, 1978; Quigley & Gainey, 2018). Unfortunately, our model domain only includes a small part of the NAC and therefore we cannot model beyond our identified source area.

More regionally, our hindcast model provides a much more nuanced and detailed picture of how *Ph. physalis* may reach Irish shores from this wider source area. Our results suggest that groups of *Ph. physalis* may take very different pathways to Ireland depending on their original starting point within the source area, the time of year when they started their 'final journey' and the direction and strength of winds experienced during transit. For example, the model suggests that in late July/early August, *Ph. physalis* entered the source area from the NAC but were deflected south within the Portugal Current as wind conditions were light and the dominant surface currents determined their direction (Figure 2.6A). However, towards the end of August/early September, individuals from the source area drifted directly towards Ireland via the Porcupine Seabight and through the area where we made offshore observations (Figure 2.6B). Some would eventually strand ashore in early September. Drift speeds of *Ph. physalis* during this time were calculated to be almost 16 km day^{-1} (Figure 2.3). In September, a different pattern emerged as a large strip of *Ph. physalis*, ~1,000 km in length and oriented in a NW-SE direction (Figure 2.6C), started to drift onto the Celtic Sea shelf. Individuals at the northern extremity of the strip would arrive on Irish coastlines in late September, whereas those located at the southern end of the strip would move close to Brittany and the English Channel. We then observed a significant change in wind direction, from W-SW to SE (Figure 2.3) that was matched closely by a corresponding directional change for most *Ph. physalis* (from SW to SE) (Figure 2.2). These individuals then moved inexorably towards Ireland where they eventually stranded throughout October. Clearly, these modelled pathways highlight the varied and often meandering pathways that individual *Ph. physalis* may take before they strand on a beach. However, importantly, they also stress the importance of wind. For example, our analysis of wind data showed a

higher proportion of windier days in September (60%) and October (47%), when *Ph. physalis* followed the wind direction, compared with August (32%), when *Ph. physalis* followed current direction. Previous studies on *Ph. physalis* (Ferrer & Pastor, 2017; Iosilevskii & Weihs, 2009; Prieto et al., 2015) all corroborate this inference that wind is the main mechanism controlling their drift.

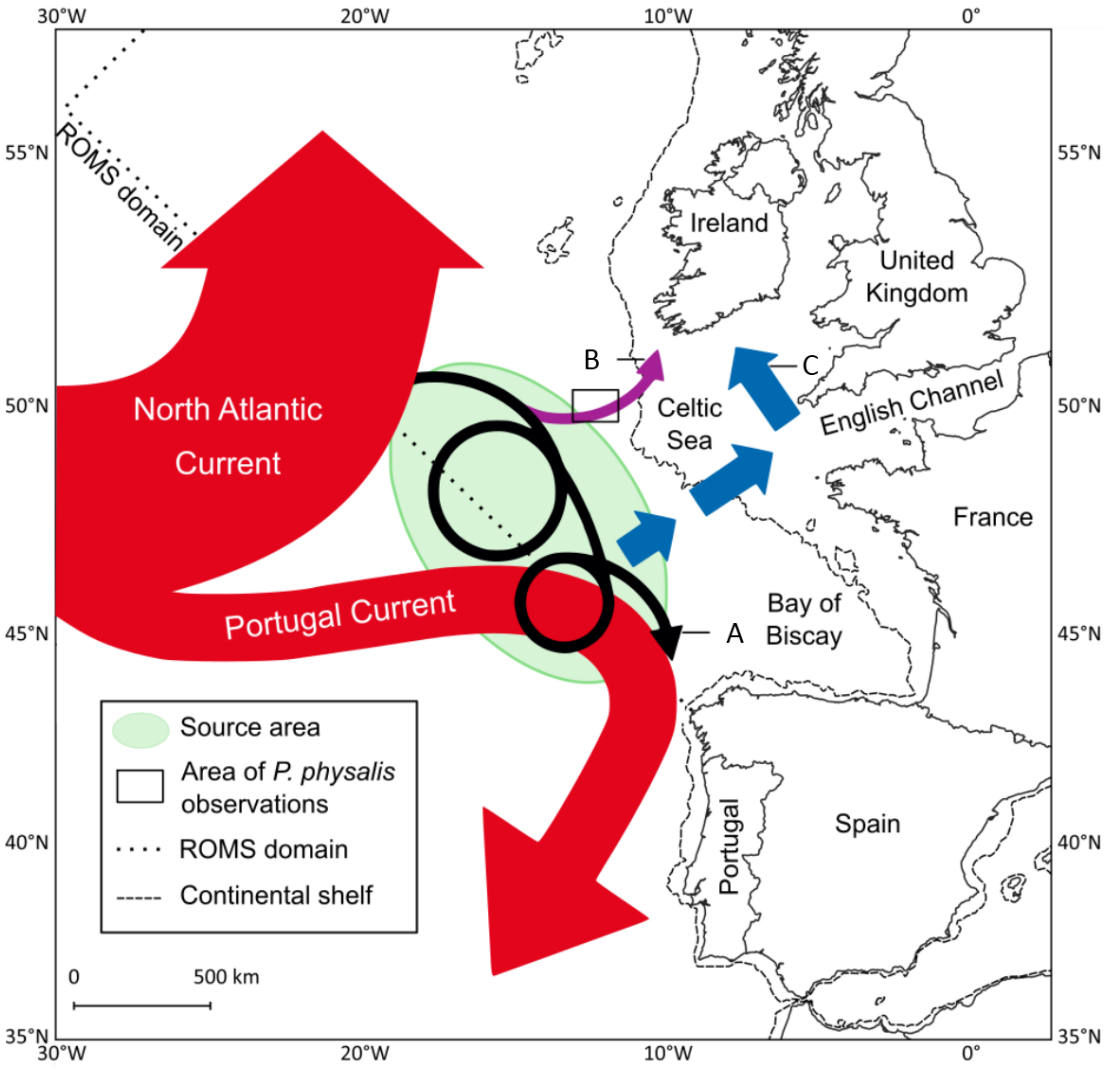


Figure 2.6. Schematic drawing of likely drift patterns for *Ph. physalis* before stranding in Ireland. Stranded *Ph. physalis* originated from an extensive source area (green), but ultimately from the North Atlantic Current (NAC) (red arrow). A) In late July/early August, *Ph. physalis* (black arrow) were deflected south from the NAC with the Portugal Current (PC) (red arrow). B) In late August/early September, *Ph. physalis* (purple arrow) from the source area drifted directly towards Ireland through the area where offshore observations of *Ph. physalis* were made (black box). C) During

September – October, *Ph. physalis* (blue arrows) drifted onto the Celtic Sea shelf (dashed line) towards France and the English Channel and into the Celtic Sea.

Fortuitously, offshore observations of *Ph. physalis* were made prior to, and during the mass stranding event, therefore the forecast model was well informed. Previous studies on cetacean strandings in the Bay of Biscay have also shown how informative it is to have such prior offshore observations (Peltier et al., 2012). In our study, *Ph. physalis* were confirmed from the Porcupine Seabight during the months of August – September but we cannot say for certain that these individuals stranded in Ireland. Regardless, most particles released from the Porcupine Seabight stranded on Irish shores, therefore confirming the Porcupine Seabight as an important source area. Furthermore, the model output had an 82% similarity to observed strandings on Irish shores and even simulated particles at the very northern limit of observed strandings (Figure 2.1B, Figure 2.5). However, our model also predicted some strandings in Dublin where we observed none. Nevertheless, when the model outputs are combined, it seems unlikely that this single location over the Porcupine Seabight caused the entire mass stranding event. For example, the hindcast model of 1,825 particles showed that only 13% of particles passed through the Porcupine Seabight prior to stranding which indicates that the source of *Ph. physalis* to the Irish coast came from a much wider area (Figure 2.6). The combination of both models has greatly improved our understanding of the 2016 *Ph. physalis* stranding event. Overall, *Ph. physalis* originated from the NAC (or its southern extension, the Portugal Current) and were advected towards Ireland by strong winds. The Porcupine Seabight was an important source area but many *Ph. physalis* likely originated from further south and took a more tortuous pathway towards Ireland.

From a historical perspective, the 2016 *Ph. physalis* mass stranding event documented here is only the third such event ever documented along the Irish coastline since 1834 (Thompson, 1835) (Table A.2.1). Only in 1945 – 1946 (Wilson, 1947) and 1967 – 1968 (Sharrock, 1968a, 1969) does there appear to have been anything comparable with the strandings of 2016. The records collected by Wilson (1947) were likely the earliest and most complete set of records of *Ph. physalis* strandings for Ireland and the UK. More than 500 *Ph. physalis* were recorded

stranded or observed offshore during the summer and autumn of 1945 and early 1946 (Wilson, 1947). The second previous event was in 1967 – 1968 when almost 650 *Ph. physalis* were recorded off the south coast of Ireland along several beaches on Cape Clear Island (Sharrock, 1968a). Given the conspicuous nature of these animals, it is very unlikely that previous events of this scale (>500 colonies stranding) have been missed, and certainly not since 2003 which saw a renewed interest in jellyfish research in Ireland and the UK with the commencement of several large projects (Blackett, Licandro, Coombs, & Lucas, 2014; Doyle, Houghton, Buckley, Hays, & Davenport, 2007; Fleming, Harrod, Newton, & Houghton, 2015; Houghton, Doyle, & Davenport, 2006; Lynam, Hay, & Brierley, 2005; Pikesley, Godley, Ranger, Richardson, & Witt, 2014).

Given the limited information on species which disperse into the open ocean, our findings highlight the importance of the routine collection of biological data. While we were opportunistic here in collecting coastal and offshore observations of *Ph. physalis,* our results highlight how important such datasets are. For example, if routine monitoring programmes, either through social media or offshore visual observations are introduced, such information can be incorporated into future particle-tracking models to refine the prediction of mass stranding events. In Malta, a particle-tracking model has been launched through which users can forecast the dispersal of the mauve stinger (*Pelagia noctiluca*) or the fried egg jellyfish (*Cotylorhiza tuberculata*) observed offshore by providing information on the location, extent and density of the bloom (https://www.facebook.com/Jellyrisk/) (Deidun, 2014). The model highlights which areas of the coastline are more likely to be impacted by strandings. In the future, our forecast model may be combined with dedicated offshore observations on-board ships of opportunity (Doyle et al., 2007) or fisheries by-catch data (Bastian, Lilley, Beggs, Hays, & Doyle, 2014) to provide an early-warning tool for *Ph. physalis* as well as other species. The ROMS model used in this study provides forecasts for 3 – 6 days and is used to forecast the expected trajectory of harmful algal blooms (HAB) in Irish waters (https://www.marine.ie/Home/site-area/data-services/interactive-maps/weekly-hab-bulletin). Considering the highly venomous nature of *Ph. physalis*, such a tool

would be welcomed by local authorities in Ireland, the UK and Brittany, to alert swimmers of likely strandings.

Chapter 3 – Sting Management

3. Evaluation of Sting Management Protocols for *Cyanea capillata, Physalia physalis* and *Pelagia noctiluca*

This chapter has been published as part of two peer-reviewed publications: Doyle, T. K., Headlam, J. L., Wilcox, C. L., MacLoughlin, E., & Yanagihara, A. A. (2017). Evaluation of *Cyanea capillata* sting management protocols using ex vivo and in vitro envenomation models. *Toxins, 9*(7). Wilcox, C. L., Headlam, J. L., Doyle, T. K., & Yanagihara, A. A. (2017). Assessing the efficacy of first-aid measures in *Physalia* sp. envenomation, using solution- and blood agarose-based models, *Toxins*, *9*(5).

Author contributions: *Cyanea capillata* studies were funded by Angel A. Yanagihara (A.A.Y) and Thomas K. Doyle (T.K.D), *Physalia physalis* studies were funded by grants obtained by A.A.Y., *Pelagia noctiluca* studies were funded by grants obtained by T.K.D.; T.K.D. and A.A.Y. conceived and designed *C. capillata* experiments, which were performed in the laboratory of T.K.D. by T.K.D., **Jasmine L. Headlam (J.L.H)** and A.A.Y. and *C. capillata* cnidae discharge experiments were conducted on-site at the Point Defiance Zoo and Aquarium in Tacoma, WA, USA, by Christie L. Wilcox (C.L.W); A.A.Y. and C.L.W. conceived and designed *Ph. physalis* experiments, which were performed in the laboratory of A.A.Y. by C.L.W. and technical staff trained by A.A.Y., on-site in Deland, Florida by C.L.W. in the laboratory of David Kersetter of Nova Southeastern University, and on-site in the laboratory of T.K.D. by **J.L.H.** and T.K.D; T.K.D., **J.L.H.,** and A.A.Y. conceived and designed *Pe. noctiluca* experiments, which were performed in the laboratory of T.K.D. by **J.L.H.**; Eoin MacLoughlin (E.M), A.A.Y., **J.L.H**. and T.K.D. performed *C. capillata* husbandry and collections in Ireland; C.L.W. and other A.A.Y. lab staff performed field collections and *Ph. physalis* husbandry in Hawaii, while **J.L.H.** and T.K.D. did the same in Ireland; **J.L.H.** performed *Pe. noctiluca* husbandry and collection in Ireland; C.L.W. and **J.L.H.** analysed *C. capillata* data, C.L.W. analysed *Ph. physalis* data and **J.L.H.** analysed *Pe. noctiluca* data; C.L.W., T.K.D. and A.A.Y. wrote and edited the *C. capillata* manuscript with contributions from **J.L.H.** and E.M. and C.L.W. and A.A.Y. wrote and edited the *Ph. physalis* manuscript with contributions from T.K.D. and **J.L.H**.

Abstract

There has been much scientific debate about the most appropriate management for jellyfish stings, particularly regarding the use of vinegar, because most current recommendations recommend against vinegar. Current recommended protocols lack rigorous scientific support. In this study, we sought to evaluate sting management protocols for three medically problematic species: *Cyanea capillata*, *Pelagia noctiluca* and *Physalia physalis*. We used previously described envenomation models that allow for direct measurements of venom activity. We found that seawater rinsing, the most commonly recommended method of tentacle removal for these species, and ethanol, induced increases in venom load. However, rinsing with vinegar or Sting No More® spray did not. Temperature treatments also affected venom activity. Forty minutes of hot pack treatment reduced lysis of red blood cells, a direct representation of venom activity, by over 90% in *C. capillata*. Ice pack treatment had no effect on *C. capillata* sting severity but, after 24 hours, *Ph. physalis* stings treated with ice packs for 40 minutes were 13% worse than those left at room temperature and more than 100-fold worse than those treated with hot packs for the same time. These results indicate that sting management protocols for these species need to be revised immediately to include rinsing with vinegar and the use of hot-pack treatment.

3.1. Introduction

Cnidarian envenomations are a matter of concern owing to their impact on several human activities and on public health. Notably, jellyfish outbreaks can affect economic activities, such as bathing, fishing and more generally tourism, with serious consequences to the economy of some coastal areas. Envenomation involves the triggering and discharge of hundreds to thousands of specialized cells, called cnidocytes or nematocytes. Certain cnidocytes contain penetrant cnidae; these microscopic venom-injecting capsules are called nematocysts. Upon contact, nematocysts can deliver a potently toxic chemical cocktail into unsuspecting prey or human victims (Halstead, 1988). Jellyfish envenomation can range from mild to life

threatening, depending on the species involved, and the amount of venom delivered (Cegolon et al., 2013; Tibballs, 2006; Williamson et al., 1996).

Jellyfish of the genus *Cyanea* are widely distributed in temperate, boreal and polar waters of the Pacific and Atlantic Oceans (Barz & Hirche, 2007; Dawson, 2005; Dong, Liu, & Keesing, 2010; Doyle et al., 2007; Kolbasova et al., 2015). While all *Cyanea* species are known to be venomous, lion's mane jellyfish (*Cyanea capillata*) have been medically problematic for at least 100 years (Lord & Wilks, 1918). They are a large jellyfish (up to 1 m bell diameter) with eight groups of ~100 tentacles each located on the subumbrella side of the jellyfish (Russell, 1970). In Irish and UK waters, lion's mane jellyfish can be encountered from June until late September (Doyle et al., 2007). They are one of the least abundant jellyfish in Irish and UK waters, typically occurring as single individuals rather than in blooms or aggregations (Bastian et al., 2011; Doyle et al., 2007). Despite being one of the least abundant jellyfish, relatively high densities of lion's mane jellyfish have been recorded close to high population centres (e.g. Dublin Bay), making stings a frequent problem. Furthermore, open-ocean swimming is very popular in the UK and Ireland, and many swimming clubs and events are held in areas where lion's mane jellyfish are known to be abundant, and therefore stings are a recurrent concern. Over the past 10 years, there have been several beach closures due to lion's mane jellyfish and at other times signs have been put in place warning bathers that the water is not safe to swim because of the lion's mane jellyfish. Indeed, during a previous study in these areas, 51% of bathers (N = 77) said that they had been badly stung, and three said they required treatment at a hospital (unpublished data). While no central database exists in Ireland documenting the numbers of sting incidents requiring medical attention, it is likely between 10 and 100 persons per year (pers. comm. T. K. Doyle).

Cyanea stings, though not generally considered fatal, can cause severe local reactions, including extreme pain and oedema, as well as systemic symptoms and clinical signs (Burnett, 2001; Burnett & Calton, 1987; Šuput, 2009; Tibballs, 2006; Tønseth, 2007; Williamson et al., 1996). Envenomation involving large specimens can be particularly dangerous, as the thousands of almost invisibly thin tentacles can extend to several meters long. Initial dermal contact may result in itching or localized

pain that may radiate to other areas of the body, potentially progressing to severe pain within 20 minutes or more. Weakness, vertigo, nausea, headache, muscle cramps, lacrimation and perspiration may also occur. In very severe stings, there may be difficulty breathing and pain on respiration, tachycardia, muscle spasms and stiffness of back and joints. The skin may become red with urticarial weals, local oedema, blisters and weeping of the skin, which may progress to ulceration and secondary infection. In some cases, stings can result in Irukandji-like syndrome (i.e. symptoms include back "pain, nausea, abdominal cramps, sweating, hypertension, tachycardia and a feeling of impending doom" and usually develop 20 – 60 minutes after a sting (Little et al., 2006)).

Mauve stingers (*Pelagia noctiluca*) are small pelagic jellyfish, 3 to 12 cm in diameter, whose colour varies from pink, mauve or light brown (Morabito, Marino, La Spada, Pane, & Mariottini, 2015). They have a wide distribution in tropical areas as well as in colder areas, such as the North Atlantic and North Pacific Ocean (Mariottini et al., 2008). They are commonly encountered on European and Mediterranean coasts during spring and summer months (Morabito et al., 2015). Mauve stingers are considered the most venomous Mediterranean jellyfish because of their widespread occurrence and painful sting (Mariottini et al., 2008; Mariottini & Pane, 2010). The last few decades have seen recurrent outbreaks and subsequent human health problems. Dramatic immediate reactions have been observed after *Pe. noctiluca* stings including erythema, oedema and vesicular topical lesions (Kokelj & Burnett, 1988; Scarpa, Kokelj, Del Negro, & Tubaro, 1987) which persist for 1 – 2 weeks (Kokelj & Burnett, 1988); systemic complications are considerably more infrequent (Kokelj & Burnett, 1990). Indeed, a review of sting reports from the eastern Mediterranean region (Vlachos & Kontos, 1991), following a *Pe. noctiluca* bloom in the late 1970s to early 1980s (Mariottini et al., 2008; Mariottini & Pane, 2010), suggests that fewer than 8% of sting victims presented with serious systemic symptoms such as "dizziness, vomiting, hypotension and diarrhoea"; most of the symptoms were local dermal reactions including "redness, pain, itching, burning and vesicles" (Mariottini et al., 2008; Mariottini & Pane, 2010).

The marine siphonophore Portuguese man of war (*Physalis physalis*) is a dangerous marine organism commonly found in tropical oceans. It consists of an above-water float (pneumatophore), various specialized feeding and reproductive structures and long, stinging tentacles used for capturing prey. This marine stinger is often found in large groups or "armadas" that can be blown ashore by strong winds. While deaths from *Physalia* stings are rare (Burnett & Gable, 1989; Stein, Marraccini, Rothschild, & Burnett, 1989), stings can be excruciating and lead to systemic complications, including headache, vomiting, abdominal pain and diarrhoea (Burnett, Fenner, Kokelj, & Williamson, 1994; Cazorla-Perfetti et al., 2012; Cegolon et al., 2013; Halstead, 1988; Tibballs, 2006; Virga, Bechara, da Silveira, & Morandini, 2013; Williamson et al., 1996). Their nematocysts have long tubules, which can deliver venom nearly 1 mm into tissues and remain embedded, likely causing secondary immunological reactions (Yanagihara et al., 2002). A recent study has described a marked increase in *Ph. physalis* stings along the French Atlantic coasts (Labadie et al., 2012), further stressing the urgency of standardized, evidence-based care for *Ph. physalis* stings.

Because studies have shown that physiological responses to all cnidarian envenomation are dose-dependent, and only a small portion of available cnidae discharge upon initial contact (Yanagihara *et al.*, 2016), one of the key first steps in first aid is to ensure the safe removal of adherent tentacles and undischarged cnidae (which are capable of firing for at least two weeks after their separation from the tentacle (Lane & Dodge, 1958)). Thus, a critical initial first-aid goal is to remove stinging material without increasing the amount of venom injected or the number of cnidae discharged into the skin, and thus rinse solutions that irreversibly inhibit cnidae discharge are preferred. Rinses that trigger functional cnidae discharge to effect further impalement of the epidermis with venom deposition cause more harm than good, potentially turning a mild or moderate sting into a severe one. In a somewhat counter-intuitive manner, however, certain rinses such as sea water which may appear at first consideration to be "inert" and represent a "do no harm" type of approach, simply allow the tentacles, as well as dissociated intact cnidae, to be moved further on the surface of the skin at which point the tentacle cnidae or

isolated cnidae sting, thus "spreading" the sting area and increasing the "venom load". Further, another potential area of unexpected contradiction is the finding that while some rinses induce discharge of cnidae along an isolated tentacle on a microscope slide, the discharged cnidae are not functionally capable of impaling skin and/or the venom is immediately inactivated. These two findings caused us to carefully re-examine the extant literature. The prevailing premise has been that microscope slide assays of tentacle responses to potential rinse solutions directly correlate to venom load and thus utility in sting management. The finding that this is not the case (Yanagihara & Wilcox, 2017) is pivotal in efforts to develop optimal sting management protocols.

Despite the dearth of studies evaluating the effects of potential interventions, most authorities currently recommend that *C. capillata, Pe. noctiluca* and *Ph. physalis* tentacles be removed by rinsing with seawater/saline (Berling & Isbister, 2015; Burnett, 2009; Cegolon et al., 2013; Fenner & Fitzpatrick, 1986; Montgomery, Seys, & Mees, 2016; Tønseth, 2007) and that the sting site should be treated with either hot water immersion/heat (Berling & Isbister, 2015; Burnett, 2009; Marino et al., 2007; Montgomery et al., 2016; Morabito et al., 2017) or cold packs/ice (Cegolon et al., 2013; Exton, Fenner, & Williamson, 1989; Mariottini et al., 2008; Montgomery et al., 2016; Tibballs, 2006).

Currently, sting management protocols suffer from a lack of rigorous evidence-based support. For example, a recent literature review (Hoffmann et al., 2017) found very few studies evaluating recommended sting protocols for species found in German waters (including *C. capillata*), and those that were identified were classified level 4 or less on the evidence classification scale, as described by the Oxford Centre for Evidence-Based Medicine (CEBM) (Centre for Evidence-Based Medicine, 2009). Previous reviews have similarly found scant evidence supporting first aid methods for *C. capillata* and *Pe. noctiluca* stings (Cegolon et al., 2013; Ward, Darracq, Tomaszewski, & Clark, 2012). For *C. capillata* specifically, only two studies have been conducted which evaluate the efficacy of potential first aids (Exton et al., 1989; Fenner & Fitzpatrick, 1986). The only study examining potential removal methods was conducted more than 30 years ago, relied solely on *in vitro* examination

of nematocyst discharge in response to potential rinse solutions, and did not include quantitative results, raw data images, or statistical comparisons between treatments (Fenner & Fitzpatrick, 1986). Recent work has demonstrated that nematocyst discharge *in vitro* has limited (if any) correlation to sting severity as measured by direct functional assays (Yanagihara & Wilcox, 2017) or human clinical trials (DeClerck et al., 2016). Similarly, the only study supporting the use of cold packs for pain relief was uncontrolled and contained no statistical analysis (Exton et al., 1989). For, *Pe. noctiluca,* only one study has evaluated the efficacy of potential rinse solutions (Morabito, Marino, Dossena, & La Spada, 2014) but the study relied solely on *in vitro* examination of nematocyst discharge in response to potential rinse solutions, did not include any raw data images and only evaluated the discharge of one type of nematocyst. Similarly, there appears to be no evidence supporting the use of seawater and baking soda for deactivating cnidae and cold packs for pain relief, even though these are widely recommended for *Pe. noctiluca* stings (De Donno et al., 2014).

The few investigations on potential rinse solutions for *Physalia* stings have also been fraught with inconsistent and sometimes contradictory results. Initial studies reported that alcohols cause massive cnidae discharge, while discharge is absent or inhibited in the presence of weak acetic acid solutions (~5%, in distilled water), or household vinegar (Burnett, Rubinstein, & Calton, 1983), a finding confirmed in another hydromedusan species, *Olindia sambaquiensis* (Mianzan, Fenner, Cornelius, & Ramírez, 2001). Some reduction in pain was also noted with vinegar application in *Physalis* stings in a prospective controlled clinical trial (N = 20) (Turner, Sullivan, & Pennefather, 1980). However, microscopic examination in another study (Exton, 1988) found moderate cnidae discharge in the presence of vinegar (an average of "2" out of "5", with 5 being "maximal" discharge), and a more recent paper (Birsa, Verity, & Lee, 2010) observed what they described as a relatively high degree of discharge among the data observed (nematocysts per 1 mm of linear tentacle; roughly double the discharge observed after exposure to 1:10 dilution of 70% ethanol) from *Ph. physalis* tentacles with the application of dilute (1:10) 5% acetic acid. Because of the variability in laboratory-based results, it was suggested in

the 1980s that vinegar use for *Physalia* stings be discontinued (Exton, 1988; Fenner, Williamson, Burnett, & Rifkin, 1993). This suggestion has become standard; medical doctors warn of vinegar's danger, even calling its use "forbidden" in the case of *Physalia* stings (Kajfasz, 2015), and national and international organizations often question or warn against the use of vinegar if *Physalia* is implicated (e.g., Australian Resuscitation Council, 2010; National Health Service, 2016). The recommendation has stuck despite the fact that the combined evidence from the highest-quality studies supports vinegar as the best course of first aid (Ward et al., 2012).

In the clinical literature, medical databases and lay-level advice articles, species-specific recommendations are often not given; instead, general recommendations are made for all "jellyfish" (sometimes linked by geographic area) (Auerbach, 1991; Markenson et al., 2010; Toxbase, 2016.; Weinstein, Dart, Staples, & White, 2009), all scyphozoans species (sometimes lumped as "sea nettles") (Auerbach, 1991; McGoldrick & Marx, 1992), or all non-tropical and/or non-cubozoan species (Australian Resuscitation Council, 2010; Fenner, 2000; Gershwin, 2017). For these reasons evidence-based research utilizing direct activity assays are urgently needed to systematically evaluate medically relevant species.

The purpose of this study was to re-evaluate first aid recommendations for potential rinse solutions as well as popular folk remedies (such as urine and lemon juice) for three cnidarian species using a combination of *in vitro* solution-based methods, or cnidae discharge tests, as well as *ex vivo* envenomation models which evaluate functional venom activity (Yanagihara, Wilcox, King, et al., 2016). We compared the results for two scyphozoans, *C. capillata* and *Pe. noctiluca,* and one hydrozoan, *Ph. physalis,* to look for species-specific responses, as well as determine whether there is likely to be a universally applicable first-aid solution. Lastly, we examined whether the results of solution-based methods that evaluate cnidae discharge correlate to functional measures of venom load (in a blood agarose-based model) to further evaluate whether solution-based studies are clinically relevant.

3.2. Materials and Methods

The chemicals used in all assays are as follows: acetic acid (Fisher Scientific, Fair Lawn, NJ, USA), baking soda (mixed 3:1 with seawater; ALDI stores Ltd., Atherstone, Warwickshire, UK), copper gluconate (30 mM in 150mM saline; Strem Chemicals, Newburyport, MA, USA), distilled white vinegar (Tesco, produced in the UK for Tesco Stores Ltds., Chestnut, UK for experiments conducted in Ireland; Market Pantry, Target Corporation, Minneapolis, MN, USA for *C. capillata* experiments in the USA; Bakers and Chefs CJ314, SAM's West Inc., Bentonville, AR, USA for *Ph. physalis* experiments in the USA), ethanol (Sigma Aldrich for *Pe. noctiluca* experiments; Pharmco-Aaper, Brookfield, CT, USA for *Ph. physalis* experiments), freshwater (tap water), gelatine (Knox® Gelatine, Kraft Food Groups, Inc., Northfield, IL, USA), isopropanol (91%, Up & Up, Target Corporation, Minneapolis, MN, USA for *C. capillata* experiments; Fisher Scientific for *Ph. physalis* experiments), lemon juice (Tesco, produced in Belgium for Tesco Stores Ltd., Chestnut, UK), magnesium sulphate (50 mM in filtered 150 mM saline; Fisher Scientific), malt vinegar (Heinz Brand, H.J. Heinz Corp., Pittsburgh, PA, USA), seawater (locally collected), sodium chloride (Fisher Scientific) and Sting No More® Spray (contents include vinegar, copper gluconate, urea, and magnesium sulphate; Alatalab Solutions™ LLC, Honolulu, HI, USA). Urine was freshly collected from a willing volunteer.

3.2.1. Animal Collection

For *in vitro* examination, *C. capillata* tentacles were collected just prior to experiments from live animals harvested from Puget Sound and kept in aquaria at the Point Defiance Zoo and Aquarium in Tacoma, WA, USA. Animals had spent approximately one year in captivity prior to experiments. Live *Ph. physalis* were collected from gulfstream waters off Miami's South Beach (25°30'1.74''N 79°28'50.08''W). Animals were netted and bagged in gallon Ziploc bags with ample seawater and kept cool until use. Animals were warmed to room temperature in 0.45 µm filtered seawater before their tentacles were used. For functional assays involving blood cells, live *C. capillata* were collected from Dublin Bay (between the Forty Foot bathing area and Dalkey Island, 53°17'18.25''N 6°6'12.03''W), live *Pe.*

noctiluca were collected from Fanore Beach, Co. Clare (53°7'9.14''N 9°17'21.35''W) and live *Ph. physalis* were collected from several beaches (Derrynane, Co. Kerry, Youghal, Co. Cork and Ardmore, Co. Waterford). Aside from *C. capillata*, most specimens were collected within a few hours of stranding and several were collected from the incoming tide. Animals were placed in ample amounts of seawater, before having their tentacles excised. Tentacles were harvested close to the bell or pneumatophore using a pair of dissecting scissors and were handled with fine forceps prior to their use in experiments; all experiments were conducted within 72 h of collection.

3.2.2. Tentacle Solution Assay (TSA) and *in vitro* Tests

To test for the induction of discharge, freshly cut *C. capillata* and *Ph. physalis* tentacles (1 – 2 cm in length) were placed on clean, dry microscope slides and examined quickly for discharge; any lengths with notable discharge were discarded (Yanagihara, Wilcox, King, et al., 2016). Sixty µl of the test solution was then added to the tentacle. For *C. capillata*, test solutions were seawater, vinegar, urine and isopropanol (Sting No More® Spray was not used during the test as it was not available). For *Ph. physalis*, test solutions were seawater, freshwater, urine, Sting No More® Spray, 30 seconds of pressure, 70% ethanol, >95% ethanol, white vinegar, cider vinegar and malt vinegar. After one minute of incubation for *C. capillata* or ten minutes for *Ph. physalis*, a cover slip was gently placed over the tentacle. Preliminary tests with seawater confirmed that coverslip addition did not induce significant discharge. All photos were taken of the tentacles ten minutes after the various treatments through the coverslip using a compound microscope at 10X and 40X magnification (microscope: OMAX M837ZL Compound Microscope, OMAX, Bucheon, South Korea; camera: OMAX A35140U; photo software: ToupLlte vers. 1.0., ToupTek, Zhejiang, China) for *C. capillata* and at 10X magnification (Olympus model CKX41SF, Olympus Corporation, Tokyo, Japan) for *Ph. physalis.* Counts of discharged and undischarged cnidae were performed in ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA), and statistical analyses were performed using Graphpad Prism ver. 6.0 (GraphPad Software, La Jolla, CA, USA).

To further evaluate *C. capillata* discharge in response to vinegar, tentacles could sting a 2 mm thick slab of 5% gelatine in seawater for five minutes. After tentacles were pulled off using tweezers, adherent cnidae were examined microscopically at 10X magnification. A video was taken as vinegar was applied to an area with both discharged and undischarged cnidae, and one minute of footage was recorded. Video footage was examined to determine whether vinegar application induced any nematocysts in the field of vision to discharge and if so, whether the everting tubules penetrated the gelatine substrate (microscope: AmScope M158C-E Compound Monocular Microscope, AmScope, Irvine, CA, USA; camera: AmScope MD35; photo software: Proscope HR version 1.2.1., Bodelin, Wilsonville, OR, USA).

Due to the small number of live *Pe. noctiluca* specimens no tentacle solution assay (TSA) or *in vitro* experiments were conducted for this species.

3.2.3. Tentacle Blood Agarose Assay (TBAA)

The effects of solutions and temperature treatments on sting severity were evaluated using variations of the Tentacle Blood Agarose Assay (TBAA) *ex vivo* envenomation model outlined in (Yanagihara, Wilcox, King, et al., 2016). For *C. capillata* rinse experiments, a "skin" covered adaptation was used. Briefly, 15 blood agarose rounds were extracted from premade sheep's blood agar plates (20 mm diameter). These rounds were placed on top of a layer of cling film laid over the open end of a glass jar (diameter approx. 45 mm). Sections of the prepared ovine intestine or "skin" were laid over the agar rounds. The lids of the glass jars had a hole approximately 25 mm cut into them and these lids were placed on top of glass jar, cling film, agar and intestine. Fresh tentacles could sting for 3 minutes before the test solutions were applied directly onto the tentacles using a spray bottle. The tentacles remained for another 2 minutes before the skins and tentacles were removed. For *Pe. noctiluca* and *Ph. physalis,* fresh tentacles were incubated in test solutions for 2 minutes and rinsed twice for 2 minutes in seawater prior to 5 minutes of stinging of premade sheep's blood agar plates without a "skin". A glass slide was added as weight to increase stinging. Photos were taken after 40 minutes and 12 hours (*C.*

capillata), 40 minutes, 3 and 24 hours (*Pe. noctiluca*) and 3, 18 and 24 hours (*Ph. physalis*) of incubation at room temperature.

To evaluate temperature treatments for each species, nine blood agar plates were acclimated to room temperature. Two tentacles were added to each blood agar plate and allowed to sting for three minutes for *C. capillata* or five minutes for *Pe. noctiluca* and *Ph. physalis* before they were pulled off using tweezers. Three plates each received (a) no temperature treatment (control); (b) an ice pack for 40 minutes; and (c) a 45°C hot pack for 40 minutes (thus a total of $N = 6$ stings for each condition). Plates were incubated at room temperature. The *C. capillata* experiment was repeated. In the first experiment, photos were taken at 40 minutes and 24 hours. However, for the second experiment, photos were taken at 40 minutes, 3 and 14 hours. In the *Pe. noctiluca* experiment, photos were taken after 40 minutes, 3 and 24 hours. In the *Ph. physalis* experiment, photos were taken after 3, 18 and 24 hours.

3.2.4. Statistical Analyses

For blood agar experiments, the area of the zone of haemolysis was calculated using ImageJ (United States National Institutes of Health, Bethesda, MD, USA). Briefly, the image scale was set using known widths and subsections (50 mm x 15 mm or 15 mm x 7.5 mm for *C. capillata*; 50 mm x 20 mm or 50 mm x 15 mm for *Ph. physalis*) were taken from each replicate for analysis to remove edge effects. Controls were used to set the colour threshold for no haemolysis. The total area of the haemolytic zone was taken directly from the "analyse particles" function for *C. capillata* and *Ph. physalis* or measured manually for *Pe. noctiluca*. Haemolytic zone was evaluated as the area exhibiting >80% haemolysis. Outliers for *Ph. physalis* were defined using the median absolute deviation (MAD) method detailed in (Leys, Ley, Klein, Bernard, & Licata, 2013), with the level of decision set conservatively at three; any replicates which were outliers at all time points were removed. Shapiro-Wilk normality tests were conducted on the single time point datasets; if the data from one third or more of the treatments were not normally distributed, then Kruskal-Wallis tests were used to compare means. Otherwise, one-way ANOVAs were used. Two-way ANOVAs were used for multiple time point datasets. All statistical analyses

and post-hoc multiple comparisons were conducted in GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) for *C. capillata* and *Ph. physalis* and IBM SPSS Statistics version 23 (25.0.0.2) for *Pe. noctiluca.*

3.3. Results

3.3.1. Testing of Potential Rinse Solutions Using the Tentacle Solution Assay (TSA)

To compare with previous investigations, we examined the *in vitro* effects of potential rinse solutions using the Tentacle Solution Assay (TSA). We were able to visualize cnidae in detail and readily calculate percent discharge in response to different test solutions (Yanagihara, Wilcox, King, et al., 2016). Given that these animals live in seawater, the response of cnidae to seawater was used as a baseline. Seawater elicited negligible discharge for both species (Figure 3.1A, Table 3.1). Sting No More Spray® and three different types of vinegar elicited no significant discharge in *Ph. physalis* (Table 3.1). When *C. capillata* tentacles were treated with white vinegar (Figure 3.1B), some discharge of cnidae did occur, but discharge was not equally distributed between cnida types. Cnidae identified as a-isorhiza and eurytele nematocysts (or possibly immature cnidae, based on their size) discharged to some extent (<20% discharge, white arrows), but A-isorhiza, O-isorhiza and birhopaloid nematocysts largely did not (estimated <5% discharge of each). In contrast, the application of urine (Figure 3.1C), isopropanol (Figure 3.1D) and pressure (data not shown) led to ~50% discharge of all cnidae types. Similarly, urine, pressure, freshwater and different concentrations of ethanol elicited significant cnidae discharge in *Ph. physalis* (Table 3.1).

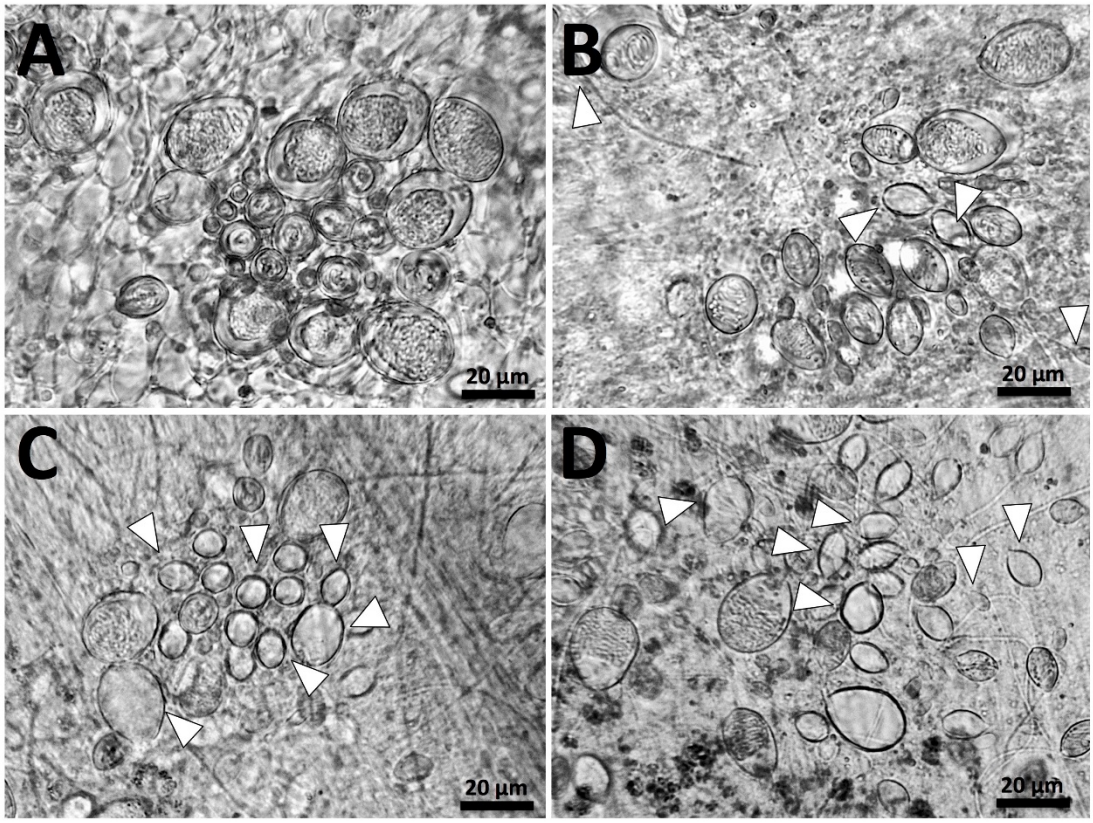


Figure 3.1. *Cyanea capillata* cnidae discharge in response to (A) seawater; (B) vinegar; (C) urine; (D) isopropanol. No discharge can be seen in (A), while (B – D) all contain discharged cnidae (examples indicated with white arrows). However, the discharge in (B) is only partial (<20%) and limited to putative a-isorhiza and eurytele nematocysts, while (C, D) show moderate discharge (~50%) of all cnidae types.

Table 3.1. Discharge of *Ph. physalis* tentacle cnidae in the Tentacle Solution Assay (TSA) (mean ± SE; *N* = between 3 and 6). Asterisk represent degree of discharge significantly greater than seawater (one-way ANOVA with Fisher's LSD post-hoc tests, $P < 0.05$).

First – Aid Solution	Cnidae Discharge (%)
Seawater	00.59 ± 00.26
Freshwater	40.94 ± 02.88*
Urine	42.54 ± 06.88*
Sting No More® Spray	00.00 ± 00.00
Pressure	46.64 ± 02.97*

Alcohols	
70% Ethanol	15.71 ± 03.68*
> 95% Ethanol	33.96 ± 04.35*
Vinegars	
White Vinegar	01.04 ± 00.04
Cider Vinegar	00.31 ± 00.13
Malt Vinegar	00.47 ± 00.38

To further evaluate *C. capillata* discharge in response to vinegar, tentacles were placed upon 5% gelatine and remained in place for five minutes. Tentacles were then pulled off with tweezers, and adherent cnidae were examined microscopically as vinegar was applied to discern cnida type, relative abundances and proportional type-specific discharge. Among the adherent cnidae, smaller size classes were more likely to be discharged (Figure 3.2A, white arrows). While many cnidae did not react to vinegar (e.g. Figure 3.2A, black arrow), specific cnidae types evidenced morphological responses (e.g. Figure 3.2A, grey arrows). However, in all cases, tubules could be seen everting upwards into the vinegar droplet and not penetrating the gelatine below, suggesting that no additional venom delivery into the gelatine in response to vinegar application despite the solution causing cnida discharge.

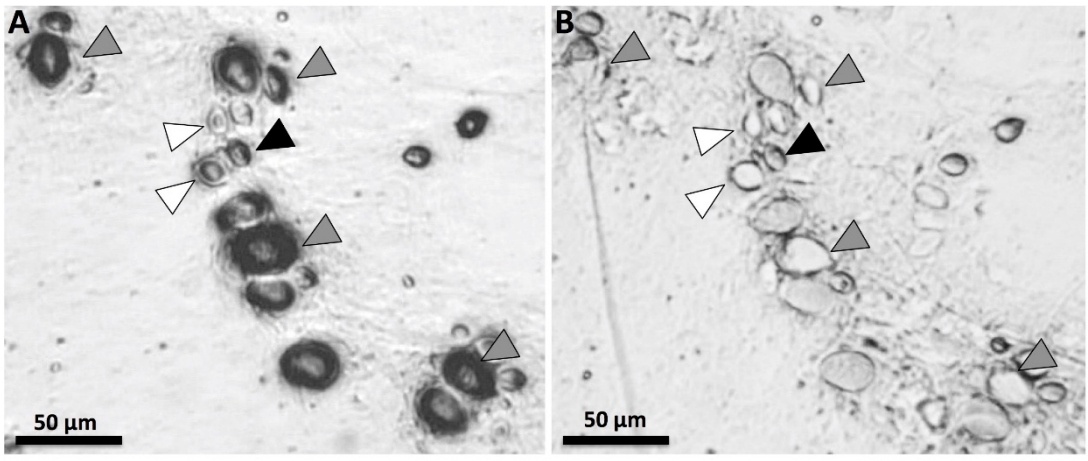


Figure 3.2. Nematocysts remaining on 5% gelatine after a five-minute *C. capillata* sting before (A) and after (B) vinegar application. White arrows indicate discharged, penetrant nematocysts and black arrows indicate non-discharged nematocysts that

did not discharge at any point during the experiment. Grey arrows point to nematocysts that discharged in response to vinegar application.

3.3.2. Testing of First-Aid Measures Using the Tentacle Blood Agarose Assay (TBAA)

The application of potential rinse solutions had a significant impact on haemolytic zone size for all species (one-way ANOVA, $P < 0.0001$; Figure 3.3A for *C. capillata,* one-way ANOVA, $P < 0.05$; Figure 3.3B for *Pe. noctiluca* and Kruskal-Wallis Test, $P < 0.05$; Figure 3.3C for *Ph. physalis*). When compared with simply pulling off tentacles, the application of urine or seawater significantly increased the size of the haemolytic area from *C. capillata* tentacles after 12 hours (Fisher's LSD, $P = 0.035$ and 0.0001, respectively), while the use of vinegar or Sting No More® Spray reduced haemolysis ($P = 0.0061$ and 0.0045, respectively). When compared with seawater, pre-treatment with baking soda, ethanol and urine resulted in significantly larger haemolytic zones after 24 hours in the case of *Pe. noctiluca* (Fisher's LSD, $P = 0.014$, 0.008 and 0.016, respectively). In *Ph. physalis*, vinegar and Sting No More® Spray pre-treatment reduced the haemolytic zone area, particularly when compared with ethanol (Dunn's multiple comparisons test, $P < 0.05$).

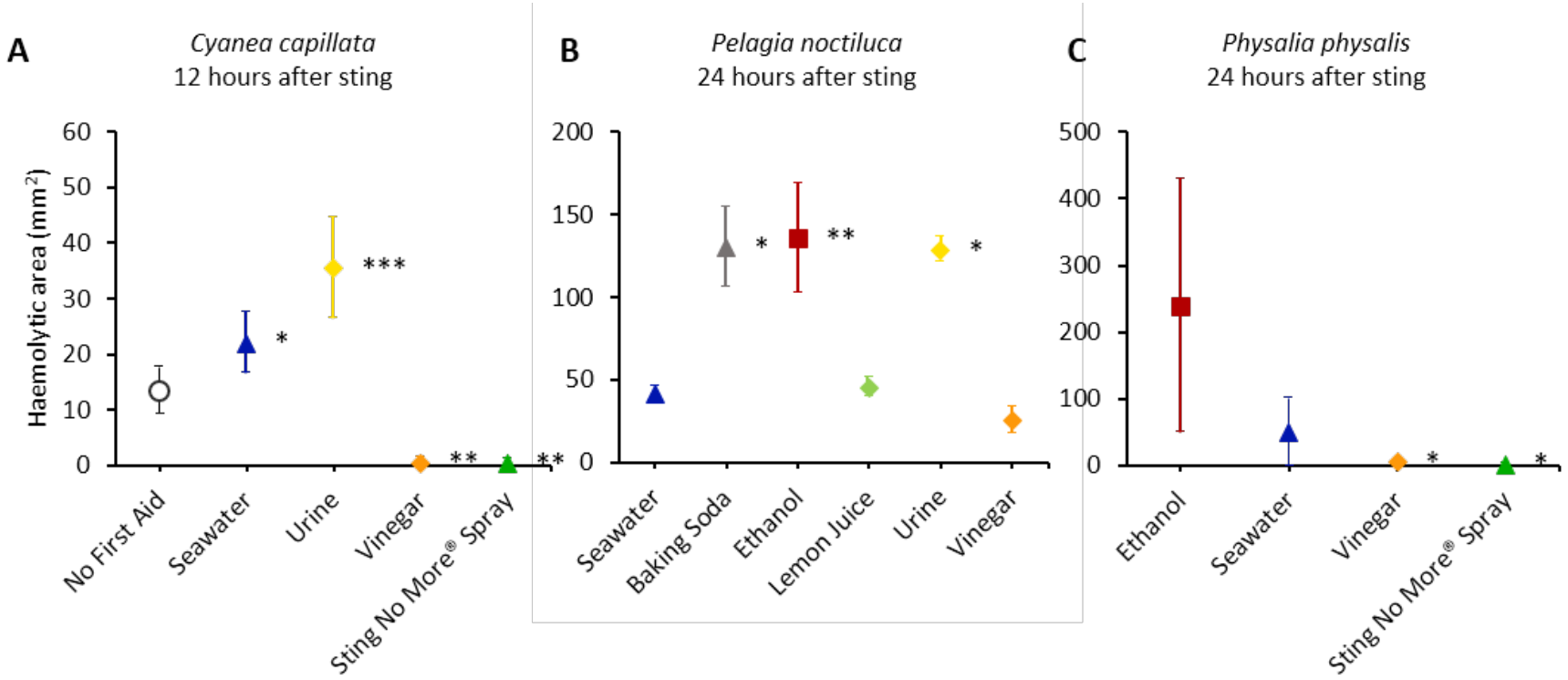


Figure 3.3. Size of venom-induced haemolytic zone over time using the TBAA model: A) when *C. capillata* tentacles were removed by rinsing with seawater, urine, vinegar or Sting No More® Spray (mean ± SE; *N* = 3), or B) when *Pe. noctiluca* tentacles were pre-treated with seawater, baking soda, ethanol, lemon juice, urine or vinegar (mean ± SE; *N* = 6), or C) when *Ph. physalis* tentacles were pre-treated with ethanol, seawater, vinegar or Sting No More® Spray (mean ± SE; *N* = 6). Asterisk represent significant differences from no first aid for *C. capillata*, seawater for *Pe. noctiluca* and ethanol for *Ph. physalis* * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

In *C. capillata* experiment 1, there were significant differences between the treatments after 24 hours (one-way ANOVA, $P < 0.0001$; Figure 3.4A) with hot packs significantly reducing the haemolytic area size when compared with either ice packs (Fisher's LSD, $P < 0.0001$) or no temperature treatment ($P = 0.0004$). All three treatments in the second *C. capillata* experiment (Figure 3.4B) showed much greater haemolysis than the first experiment (Figure 3.4A). In experiment 2, a significant difference was detected at the 14-hour time period between hot and cold treatments (two-way ANOVA with Fisher's LSD, $P = 0.0483$). After 24 hours, hot packs and ice packs reduced the haemolytic area size for *Pe. noctiluca* stings (Figure 3.4C) though the results were not significant. Temperature treatments had significant impacts on the size of the haemolytic zone for *Ph. physalis* 24 hours after the sting (Kruskal Wallis Test, $P < 0.0001$; Figure 3.4D). *Physalia physalis* stings treated with ice packs for 40 minutes were 13% worse than those left at room temperature and more than 100-fold worse than those treated with hot packs for the same length of time (Dunn's multiple comparisons test, $P = 0.3964$ and $P < 0.0001$, respectively).

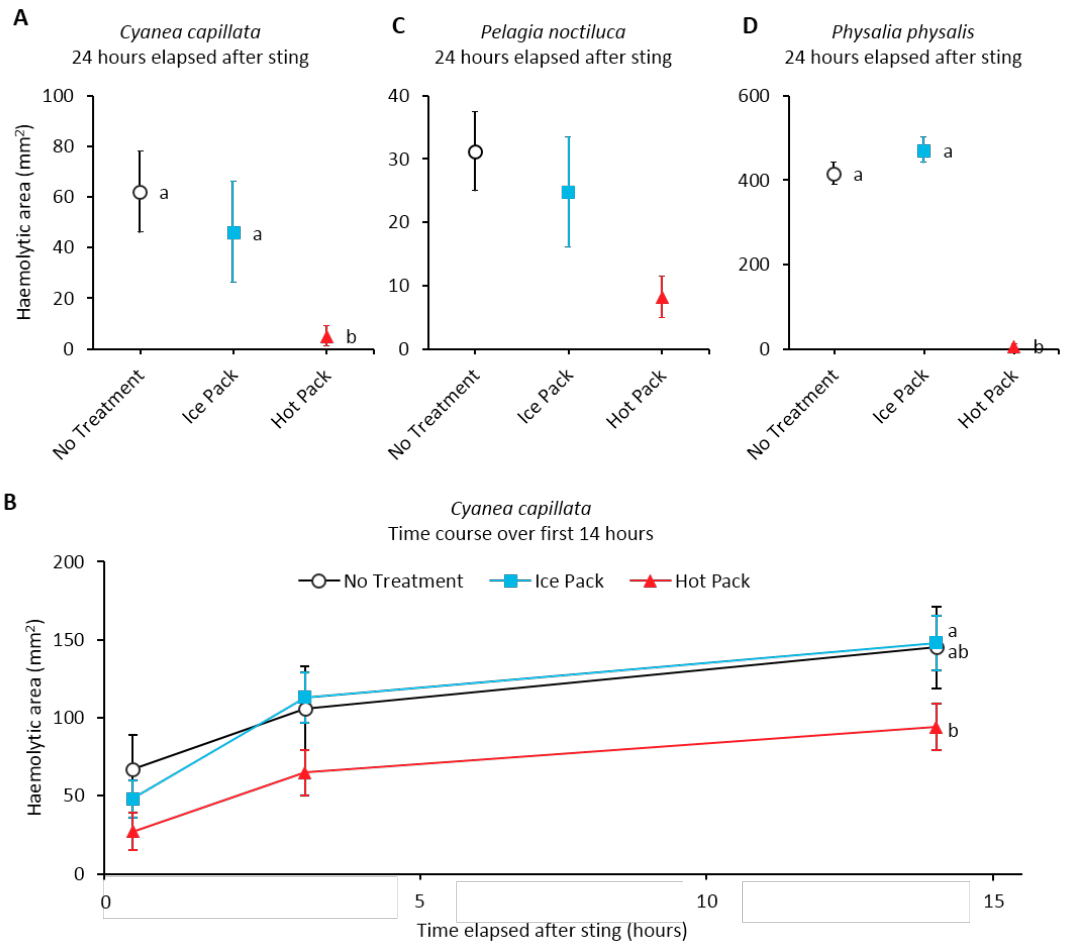


Figure 3.4. Size of venom-induced haemolytic zone over time using the TBAA model when stings were treated with hot packs, ice packs or kept at room temperature for stings of 5% sheep's blood agar. A) *C. capillata* experiment 1 was carried out on the 27th July 2016 and the haemolytic zone was reported after 24 hours (mean ± SE, (mean ± SE; *N* = 6). B) *C. capillata* experiment 2 was carried out on 4th August 2016 and the haemolytic zone was measured at three time periods (40 minutes, 3 hours and 14 hours) (mean ± SE; *N* = 6). For C) *Pe. noctiluca* and D) *Ph. physalis* experiments, the haemolytic zone was measured after 24 hours (mean ± SE; *N* = 6). Different letters denote significant differences among treatment means.

3.4. Discussion

To compare with previous investigations and studies conducted on other species, we examined the *in vitro* effects of potential rinse solutions using the Tentacle Solution Assay (TSA) (Yanagihara, Wilcox, King, et al., 2016). Precise quantification of cnidae

discharge in response to test solutions proved difficult given the complexity of the Cyanea cnidome, which consists of eurytele, birhopaloid and three different isorhiza nematocysts – a-isorhiza, A-isorhiza, and O-isorhiza – each with multiple size classes (Östman & Hydman, 1997) (Figure 3.5). It is essential to distinguish these types when evaluating discharge because studies have demonstrated that different nematocysts can vary not only in their morphology and penetrant abilities but also in their toxic effects (Burnett, Ordonez, & Calton, 1986; Endean & Rifkin, 1975; Helmholz et al., 2011). However, precise quantification of percent discharge for each *C. capillata* nematocyst type and size class was not possible with the microscope and camera available for this study; best efforts were made to identify cnidae types based on Östman & Hydman (1997). Further, it is also likely that some of the cnidae visualized in this study are immature, particularly smaller cnidae (Östman & Hydman, 1997); it was not possible to distinguish immature versus mature cnidae in this study. We were, however, able to visualize *Ph. physalis* cnidae in detail and readily calculate percent discharge in response to different test solutions (Yanagihara, Wilcox, King, et al., 2016).

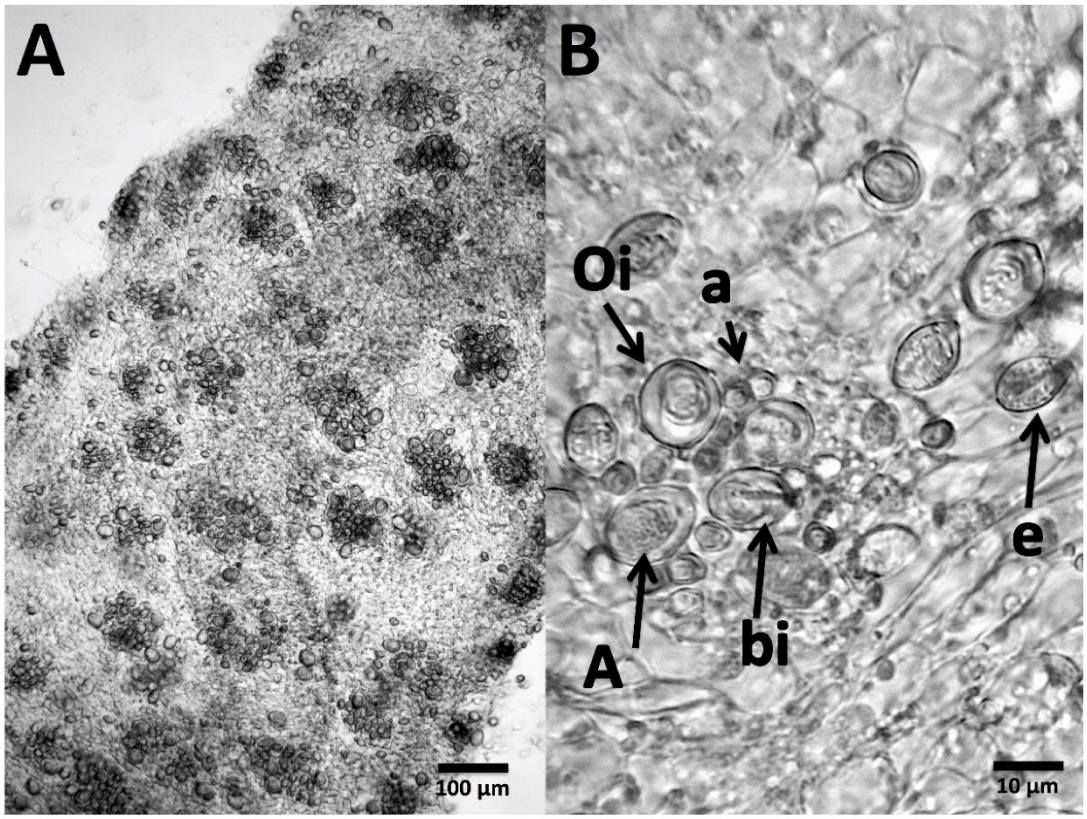


Figure 3.5. Cyanea tentacles viewed microscopically. A) Tentacle viewed at 10× magnification; discrete 'batteries' of cnidae can be seen, each containing a mix of different cnidae types. B) 40× magnification of a typical battery showing the complex and difficult to identify cnidome; cnidae identified according to nematocysts Östman & Hydman (1997). a, a-isorhiza; A, A-isorhiza; e, eurytele; Oi, O-isorhiza; bi, birhopaloid nematocysts.

While the *C. capillata* results are qualitative rather than quantitative, there were clear differences between the different treatments with no response from seawater, mostly <5% firing of cnidae for vinegar (except for a-isorhiza and eurytele nematocysts which had a <20% firing rate) and then ~50% firing rate for all other treatments: urine, isopropanol and pressure.

Since previous studies have found discrepancies between discharge seen *in vitro* and venom activity in functional assays (Yanagihara & Wilcox, 2017), the effects of vinegar on *C. capillata* cnidae discharge were further examined in a stinging model. While recording video, vinegar was added directly to the adherent cnidae to evaluate

whether vinegar application induced any specific morphological response. This was done to address the question as to whether vinegar rinsing could result in functional discharge (i.e. envenomation) if used as a post-sting rinse. It should be noted that while intact cnidae capsules respond to various stimuli, the response can include non-everting rupture, partial eversion discharge, and fully everting discharge in which the tubule productively impales the substrate (prey tissue) as well as eversions in which the tubule non-productively discharges into the surrounding seawater. For this reason, it is noteworthy that among cnidae that discharged in response to vinegar application, all the tubules appeared to evert non-productively upwards into the vinegar droplet and did not impale the gelatine. Thus, no envenomating discharge of cnidae was observed in response to vinegar application, suggesting that the subpopulation of cnidae that are triggered to discharge in response to the application of vinegar are either not penetrant venom-laden nematocysts, or that if they are, this type of capsule rupture does not result in functional venom delivery. Instead, vinegar application may essentially inactivate this subpopulation of cnidae by inducing aberrant capsule rupture rather than authentic trauma inducing discharge.

To further examine solutions with promise based upon the TSA results and authenticate or “ground-truth” the use of TSA in evaluating first-aid solutions, functional activity assays were conducted using live tentacles in *ex vivo* Tentacle Blood Agarose Assays (TBAA) to evaluate whether a subset of rinse solutions tested in the TSA as well as post sting topical hot-, ambient- or ice-pack exposure (for which there is no method for testing using TSAs) led to increases or decreases in haemolytic zone formation. Live red blood cell haemolysis is a direct venom activity assay and a functional metric of various venom constituents including venom cytolysins, proteases and lipases.

Our results support the use of vinegar to rinse away adherent tentacles and reduce their ability to sting. The inhibition of discharge by vinegar was not due to its acidic nature alone, as other solutions with similar pH were unable to prevent discharge. Vinegar and dilute acetic acid have long been used to preserve food and fix tissues (Baker, 1958). Vinegar or ~5% acetic acid causes marked swelling of

collagen by increasing the absorption of water (Baker, 1958). It has long been documented that vinegar exposure "fixes" nematocysts rendering them incapable of functional discharge. This is likely due to the fact that nematocyst capsule walls are comprised of collagen and that acetic acid induced swelling irreversibly alters the structural features required for functional firing (Yanagihara et al., 2002). We found strong evidence against the use of ethanol, freshwater and baking soda as these solutions significantly increased cnidae discharge (Table 3.1, Figure 3.1D) and worsened stings in the TBAA model (Figure 3.3). In addition, our results do not support the use of the most infamous sting treatment: urine (Table 3.1, Figure 3.1C, Figure 3.3A, Figure 3.3B). It is unclear exactly when the use of urine for jellyfish stings became popular (certainly, a scene featuring the treatment method in the sitcom "Friends" aided its spread (Calhoun & Jensen, 1997)), but it has become one of the most persistent myths in toxinology. Urine induced significant discharge in *C. capillata* and *Ph. physalis*, with discharge on par with isopropanol and ethanol in respective cases (Table 3.1, Figure 3.1C). Urine also caused significant increases in haemolysis in the *C. capillata* and *Pe. noctiluca* functional assay (Figure 3.3A, Figure 3.3B), we did not have the opportunity to test *Ph. physalis* using the TBAA.

It may seem surprising that rinsing with vinegar led to significant decreases in *C. capillata* venom activity (Figure 3.3A) given that it induces some cnidae discharge *in vitro* (Figure 3.1B, Figure 3.2B). In our study, we noted that vinegar does not equally induce discharge in all cnidae types and did not elicit as much discharge as other solutions (urine, isopropanol). It is not known how each cnida type contributes to toxicity, but previous studies have suggested that the largest A-isorhiza and O-isorhiza nematocysts disproportionately contribute to haemolysis (Helmholz et al., 2011), and these cnida types were not triggered by vinegar application. Thus, it may be that vinegar does not induce discharge the most toxic types of cnidae, and that any discharge that occurs during the rinsing process does not contribute measurably to haemolytic activity, the functional metric of our venom activity assays. Or, as seen when vinegar was applied to adherent cnidae in our simple gelatine model (Figure 3.2B), it is possible that vinegar application induces agonal, biologically inactive discharge (as suggested by Auerbach (2013)), rendering cnidae incapable of

functionally delivering venom. Upon tentacle contact with skin, a certain percentage of cnidae immediately discharge. However, a great number of cnidae were found to have been transferred to the skin intact. Because seawater does not irreversibly inhibit cnidae discharge, cnidae left at the contact site retain the capacity to discharge and thus as the tentacle rolls along the skin during the seawater rinse, additional undischarged cnidae are transferred beyond the original "sting" site. Finally, the data in this study demonstrate that these residual undischarged cnidae discharge spontaneously over the time course examined, to result in a greater area of a haemolytic zone (Figure 3.3). Urine was even worse than seawater (Figure 3.3), which aligns with our TSA data showing it elicits ~50% discharge of all cnidae types. These results stress the importance of evaluating first aid protocols using functional activity assays rather than solution-only tests, as they add to a growing number of studies that have shown *in vitro* examinations are not necessarily predictive of clinically relevant effects (DeClerck et al., 2016; Yanagihara & Wilcox, 2017).

Lastly, the application of heat reduced haemolysis in the TBAA for all three species (Figure 3.4). The observed reduction of venom activity by heat is in concurrence with similar studies in cubozoans (Yanagihara & Wilcox, 2017; Yanagihara, Wilcox, King, et al., 2016) and that body of clinical literature that demonstrates improved clinical outcomes with heat application as well as the low level of heat thermotolerance of cnidarian venoms (for review see Wilcox & Yanagihara (2016)). Some have suggested that improved outcomes from heat application (in particular the reduction of pain seen in clinical studies (Loten et al., 2006; Nomura et al., 2002; Thomas, Scott, Galanis, & Goto, 2001)) are not the result of reduction in venom activity, but instead, reflect modulation of neurological pain processing (Muirhead, 2002). This is directly disputed by the results of our envenomation modelling, where we demonstrate a direct dampening effect of heat application on venom activity in a model system that lacks any neurons or neural pathways. And as similar results have been achieved across three separate cnidarian classes, these data suggest that cnidarian venoms in general are heat-sensitive and that the sustained application of heat (at least 40 min), in the form of 45 ^{o}C hot packs or hot water immersion, is an effective first aid for reducing damage caused by

injected venom. Indeed recent research has shown that a crude venom extract from *Pe. noctiluca* also exhibits a loss of potency at temperatures higher than 40 ^{o}C (Marino et al., 2007; Morabito et al., 2017). Similarly, biochemical studies have shown marked 45 ^{o}C heat related loss of activity *in vitro* (Baxter & Marr, 1969; Carrette, Seymour, Cullen, Peiera, & Little, 2002; Chung, Ratnapala, Cooke, & Yanagihara, 2001; Cuiping, Pengcheng, Jinhua, Rongfeng, & Huahua, 2011; Endean & Henderson, 1969; Feng et al., 2010; García-Arredondo, Murillo-Esquivel, Rojas, & Sanchez-Rodriguez, 2014; Hernández-Matehuala et al., 2015; Kang et al., 2009; Koyama et al., 2003; Li et al., 2013; Marino et al., 2004; Monastyrnaya, Zykova, Apalikova, Shwets, & Kozlovskaya, 2002; Noguchi et al., 2005; Pereira & Seymour, 2013) which may reflect thermal unfolding or aggregation. Additional research is needed to determine exactly why heat has this direct, negative effect on venom activity; there may be less evolutionary pressure for heat tolerance among cnidarian venom proteins than mammalian proteins. Taken together, the observation that venom protein activities are significantly inhibited at tolerably hot temperatures far below those required to induce mammalian protein biophysical denaturation, (i.e. measurable loss of tertiary or secondary structure) provides the basis for safe first aid reduction of the activity of lytic components.

The application of cold not only failed to reduce haemolysis, it worsened stings in the case of *Ph. physalis* (Figure 3.4D). While it is possible that the application of cold increased discharge of shed cnidae, thus directly increasing venom load, we posit an alternative possibility. These results suggest that while the physical trauma of a sting and initial pain are acute events, venom pathogenic mechanisms may have a protracted kinetic course. The enzyme kinetics have yet to be carefully elucidated but time course studies (data not shown) reveal that the lipase reaction kinetics exhibit substrate to product conversion over 12 hours comparable to other cnidarian lipases (Yue et al., 2017). The effects of initial temperature treatments on such kinetics are not known; however, it is reasonable to believe that cold treatment could enhance venom activity, as previous studies have found that activity is preserved at cold temperatures and abolished at hot ones (for a review see Wilcox & Yanagihara (2016)). Further, anecdotal accounts have noted increases in pain or “reawakening”

(rebound) of the sting upon rewarming after the application of cold (Barnes, 1965; Bennett, 1834). To our knowledge, this is the first quantitative report of both acute and protracted harm from the use of cold packs and warrants immediate revaluation of the use of cold packs in the treatment of cnidarian envenomations. Studies evaluating the possible injurious effects of cold pack application in more lethal species should be conducted expediently.

Because of the great diversity of stinging jellyfish (cubozoans, hydrozoans and scyphomedusae), it has been previously stated that different jellyfish may require different treatments (Burnett, 2001; Fenner, 1997). Building on previous work on box jellyfish (Wilcox & Yanagihara, 2016b; Yanagihara & Wilcox, 2017), this study now shows that jellyfish from three different classes of Cnidaria (Cubozoa, Hydrozoa and Scyphozoa) respond in the same way to the application of vinegar (despite slight differences in response to vinegar in vitro) and heat. This will, therefore, simplify the development of a first aid protocol for jellyfish stings even in countries that have several very different venomous jellyfish species.

3.5. Conclusion

As *C. capillata, Pe. noctiluca* and *Ph. physalis* represent a significant medical burden worldwide, it is important that evidence-based medical treatments be employed when generating first aid management protocols. We found that despite inducing some detectable cnida discharge *in vitro* in *C. capillata,* vinegar was the most effective non-commercial rinse solution for safely removing adherent tentacles and cnidae. The commercial product Sting No More® Spray was equally effective, while the use of seawater, urine, freshwater, ethanol and baking soda exacerbated stings. We also found the application of a 40 minute, 45°C hot pack reduced the activity of successfully injected venom, and thus worked well as a treatment. Because our model does not include metrics for pain or neurological processes, we are able to affirm that heat has a direct effect on venom proteins rather than an indirect, modulating effect on pain sensory systems. In *C. capillata,* heat application reduced the activity of injected venom, while the application of ice had no significant effect. In *Ph. physalis,* ice packs significantly exacerbated stings. The results for *Pe. noctiluca*

were not statistically significant perhaps due to the small sample size (*N* = 6). Thus, we conclude that the best first aid for these species is a two-step protocol of (1) rinsing with vinegar or Sting No More® Spray and (2) 40 minutes or longer treatment with hot packs or hot water immersion (45°C).

Chapter 4 – Nematocyst Analysis

4. A Quantitative and Comparative Analysis of Nematocysts in *Cyanea capillata* and *Pelagia noctiluca*

Abstract

Two of Ireland's most problematic jellyfish are *Cyanea capillata* and *Pelagia noctiluca*. *Cyanea capillata* regularly sting bathers and open water swimmers and *Pe. noctiluca* sting bathers and surfers. When evaluating nematocyst discharge in response to rinse solutions for sting management, it is essential to distinguish nematocysts by type and size as different nematocysts are potentially more toxic than others and ineffective treatments may cause these nematocysts to discharge, thus turning a mild or moderate sting into a severe one. For this reason, an examination of the cnidome is an essential first step to improving sting management. This study compares and quantifies the abundance and size of different nematocyst types along the length of tentacles and between species. In both species, the size of the different nematocyst types did not differ between the base, middle and tip of the tentacles but the abundance of eurytele and medium birhopaloid nematocysts differed in *Pe. noctiluca*. Eurytele nematocysts were more abundant in the middle and medium birhopaloid nematocysts were less abundant at the base of tentacles. When the species were compared, there were significant differences in the abundance and size of all nematocyst types. In *C. capillata*, a-isorhiza nematocysts were significantly more abundant (49%) and larger in size and O-isorhiza (7%) and small birhopaloid nematocysts (7%) were significantly more abundant but smaller in size. In *Pe. noctiluca*, eurytele (23%) and medium birhopaloid (44%) nematocysts were significantly more abundant and larger in size. Overall, per cm of tentacle, *C. capillata* and *Pe. noctiluca* had similar amounts of nematocysts (*N* = 9,900 in *C. capillata* and *N* = 9,820 in *Pe. noctiluca*). However, per animal, *C. capillata* had three orders of magnitude more nematocysts than *Pe. noctiluca* (~238 million in *C. capillata* vs. ~500,000 in *Pe. noctiluca*) due to their much longer and more numerous tentacles. This fact alone may explain why *C. capillata* envenomations are much more severe than *Pe. noctiluca*.

4.1. Introduction

All cnidarians are equipped with highly specialised, secretory, subcellular organelles called cnidae (Hyman, 1940; Weill, 1934; Yanagihara et al., 2002; Yanagita & Wada, 1959). There are three types of cnidae: nematocysts, ptychocysts and spirocysts. Nematocysts are the only cnidae type found on the tentacles of cubozoans (box jellyfish) and scyphozoans (true jellyfish) (Rifkin, 1991), which are the two most venomous cnidarian classes. Nematocysts consist of capsules and eversible tubules immersed in venom (Mariscal, 1974a; Tibballs, 2006; Williamson et al., 1996) which discharge to penetrate tissue and deliver venom (Östman, 2000; Williamson et al., 1996) for defence or prey capture (Östman, 2000; Stachowicz & Lindquist, 2000). Nematocysts vary greatly in their morphology and penetrative abilities. Weill (1934) originally divided them into 16 categories but additional nematocyst types were subsequently identified culminating today in over thirty varieties (Bouillon et al., 1986; Carlgren, 1940, 1945; Cutress, 1955; Mariscal, 1974a; Östman, 1983, 1997b, 2000; Östman & Hydman, 1997; Rifkin, 1996; Werner, 1965). Nematocyst classifications are generally based on Weill (1934) with modifications by Calder (1977); Carlgren (1940); Cutress (1955); Mariscal (1974); Östman and Hydman (1997) and Rifkin (1996).

Tentacles contain between a few thousand and several billion nematocysts (Tibballs, 2006; Williamson et al., 1996). When jellyfish respond to chemical or mechanical stimuli, nematocysts discharge to penetrate tissue and deliver venom. All jellyfish are capable of discharging nematocysts and inflicting stings (Cegolon et al., 2013), but sting severity varies between species and nematocyst types. For example, some of the most problematic jellyfish are the larger jellyfish which are thought to release large amounts of venom (e.g. the lion's mane jellyfish (*Cyanea capillata*)) (Tibballs, 2006; Williamson et al., 1996). In addition, nematocysts with tubules which are long enough to enable the tubule to penetrate deep into the skin (Kitatani, Yamada, Kamio, & Nagai, 2015), nematocysts with rapid discharge velocities (e.g. O-isorhiza and eurytele nematocysts) (Colin & Costello, 2007), or nematocysts with large capsules which are capable of holding more venom (e.g. O-isorhiza and A-

isorhiza nematocysts) (Helmholz et al., 2011) are potentially more toxic than other nematocyst types.

The lion's mane jellyfish and the mauve stinger (*Pelagia noctiluca*) are the two most problematic species in Ireland in terms of sting management (Doyle, Headlam, Wilcox, MacLoughlin, & Yanagihara, 2017). Lion's mane jellyfish have a large bell (up to 1 m in diameter) (Doyle et al., 2017) and eight groups of ~100 tentacles (Doyle et al., 2017; Russell, 1970) up to 7 m in length (pers. comm. T. K. Doyle). *Cyanea capillata* are known for their painful stings characterised by severe local and systemic reactions (Doyle et al., 2017). In Ireland, they regularly sting bathers and open water swimmers and over the last 10 years they have caused several beach closures (Doyle et al., 2017). *Pelagia noctiluca* have a much smaller bell between 6 – 8.5 cm in diameter (Malej, 2004) and eight tentacles approximately two to three times the bell diameter in length (Russell, 1970). Stings by *Pe. noctiluca* are characterized by severe and immediate local reactions whereas systemic reactions are rare (Kokelj & Burnett, 1990). In Ireland, they are also known to sting bathers and surfers along the west coast of Ireland when they occur in large aggregations (pers. comm. T. K. Doyle).

The first step in treating a sting, such as those inflicted by *C. capillata* and *Pe. noctiluca*, is the deactivation of undischarged nematocysts by an appropriate rinse solution. The efficacy of a rinse solution is measured by the number of nematocysts which discharge in response to its application (Doyle et al., 2017; Wilcox et al., 2017). For *C. capillata*, in particular, precise quantification of nematocyst discharge in response to test solutions has been hampered by the complexity of their cnidome (Doyle et al., 2017). Indeed, it is essential to distinguish nematocysts by type and size when evaluating nematocyst discharge as studies suggest that different nematocyst types (e.g. O-isorhiza, A-isorhiza and eurytele) are potentially more toxic than others (Colin & Costello, 2007; Helmholz et al., 2011; Kitatani et al., 2015). An ineffective rinse solution may cause these nematocyst types to discharge, potentially turning a mild or moderate sting into a severe one. Conversely, effective rinse solutions such as vinegar may have been overlooked because they caused nematocysts to discharge. However, by distinguishing the discharged nematocysts by type, it was recently suggested that vinegar did not cause any of the potentially more toxic

nematocysts to discharge and therefore should be recommended as an effective rinse solution (Doyle et al., 2017).

While the cnidome of *C. capillata* has been studied in great detail from the North and Baltic Seas (Östman and Hydman, 1997; Wiebring et al., 2010) and Outer Hebrides (Helmholz et al., 2011), recent studies suggest that there may be regional differences in the abundance and size of different nematocyst types (Wiebring et al., 2010). In addition, traditionally, *C. capillata* nematocysts have been examined *in situ* at the bases and tips of tentacles (Helmholz et al., 2011; Östman & Hydman, 1997). Where nematocysts have been isolated from tentacles, they were not distinguished between the bases and tips even though studies suggest that the abundance and size of nematocysts vary along the length of tentacles (Östman & Hydman, 1997). In addition, more traditional methods have not quantified the total number of nematocysts in *C. capillata* even though this might help explain why their stings are so severe as it has been suggested that larger jellyfish, such as *C. capillata*, possess larger quantities of venom.

Therefore, to provide additional insights into the cnidome of *C. capillata*, in particular, and to aid the improvement of sting management for two of Ireland's most problematic species: *C. capillata* and *Pe. noctiluca,* the aims of this study were to isolate nematocysts from the base, middle and tip of their tentacles and 1) calculate the abundance and size of different nematocyst types along the length of their tentacles, 2) compare the abundance and size of different nematocyst types common to both species and, 3) provide initial estimates of the total number of nematocysts.

4.2. Materials and Methods

4.2.1. Sample Preparation

Sixteen specimens of *C. capillata* (diameter 20 – 40 cm) were collected from Dublin Bay, Ireland (53°19´N, 6°6´W) in August 2019 and 97 specimens of *Pe. noctiluca* (diameter 3 – 10 cm) were collected from St. Finian's Bay, County Kerry, Ireland (51°50´N, 10°20´W) in August 2017. Immediately after collection, marginal tentacles

were excised and separately immersed in 1 mol. trisodium citrate. Samples were stored at 4°C until September 2019.

To compare the abundance and size of nematocysts between the base, middle and tip of *C. capillata* and *Pe. noctiluca* tentacles and between species, for each species, a 1 cm section was taken from the base, middle and tip of 100 tentacles and nematocysts were isolated and concentrated into a pellet following the methods of Yanagihara and Shohet (2012). Briefly, nematocysts were isolated by gently rotating the tentacle sections at 4°C in 1 mol. trisodium citrate for 3 days. After 3 days, tentacle sections were filtered through a 0.25 mm sieve and the filtrate was immediately centrifuged at 3,000 rpm for 10 minutes. Undischarged nematocysts were resuspended in chilled 1 mol. trisodium citrate and washed at 3,000 rpm or 3,500 rpm for *C. capillata* and *Pe. noctiluca*, respectively, until the supernatant was clear, and nematocysts were pelleted. After each wash, and prior to resuspension, the supernatant was examined microscopically for nematocysts in suspension. If nematocysts were in suspension, then the wash was repeated without resuspension. Prior to nematocyst counts, the supernatant was removed, and the volume of each nematocyst pellet (~300 µl) was recorded. There were six pellets in total: three for each species and one for each tentacle section.

4.2.2. Microscopy

For each pellet, the nematocysts were identified according to Östman and Hydman (1997) and Avian et al. (1991) based on their gross morphology, i.e. capsule shape and size, presence or absence of a shaft and orientation of the inverted tubule. Nematocysts were counted using a counting chamber (Neubauer Improved) and an Olympus BX53 upright microscope. A 100 µl sample of each pellet was suspended in 200 µl of a glycerol 75% (v/v) and ethanol 25% (v/v) solution. A 20 µl sample of each suspension was loaded into the counting chamber. The abundance of each nematocyst type was counted in each of the four outer squares (1 mm^2, Figure 4.1). The counting chamber was repeated with 5 aliquots for each nematocyst suspension.

For length and width measurements, a drop of nematocyst suspension was placed on a microscope slide and observed at 40X magnification. The lengths and widths of undischarged nematocysts were measured using an ocular micrometre. In total, 30 length and width measurements were made for each nematocyst type in each nematocyst suspension.

4.2.3. Calculations

The height of the counting chamber is 0.1 mm, so a 1 mm × 1 mm × 0.1 mm chamber has a volume of 10^{-4} ml or 10^{-1} µl. To obtain the total number of each nematocyst type per µl of suspension, the total number of each nematocyst type counted was divided by the number of outer squares counted and multiplied by 10^{1}. However, to compare the abundance of each nematocyst type between the base, middle and tip of tentacles and between species, we needed the abundance of each nematocyst type per cm. To calculate the abundance of each nematocyst type per cm, we needed the abundance of each nematocyst type per pellet. Therefore, the abundance of each nematocyst type per pellet was calculated from the number of nematocysts per µl using the following equation:

$$\text{No. per pellet} = \frac{N_{nematocysts}}{N_{squares}} \times 10 \times DF \times V_{pellet}$$

Where $N_{nematocysts}$ is the total number of each nematocyst type counted, $N_{squares}$ is the total number of outer squares counted, DF is the dilution factor and V_{pellet} is the pellet volume (Figure 4.1).

To calculate the abundance of each nematocyst type per cm, the abundance of each nematocyst type per pellet was divided by the number of 1 cm tentacle sections. To clarify, each ~300 µl pellet contained all the nematocysts isolated from 100 × 1 cm sections. Therefore, to calculate the abundance of each nematocyst type per cm, we divided the abundance of each nematocyst type in a ~300 µl pellet by 100. To calculate the abundance of each nematocyst type per tentacle and per animal, the abundance of each nematocyst type per cm from the base, middle and

tip of *C. capillata* and *Pe. noctiluca* tentacles were combined and the average was multiplied by the average length and number of tentacles, respectively.

4.2.4. Statistical Analysis

Shapiro-Wilk normality tests were conducted on all datasets; if the data were not normally distributed, Kruskal-Wallis tests were used. Otherwise, one-way ANOVAs were used to test for significant differences between the abundance and size of each nematocyst type between the base, middle and tip of *C. capillata* and *Pe. noctiluca* tentacles and between species. Chi-Square tests of independence were used to compare the abundance of each nematocyst type between this study and Östman and Hydman (1997). All statistical analyses were conducted in IBM SPSS Statistics (25.0.0.2).

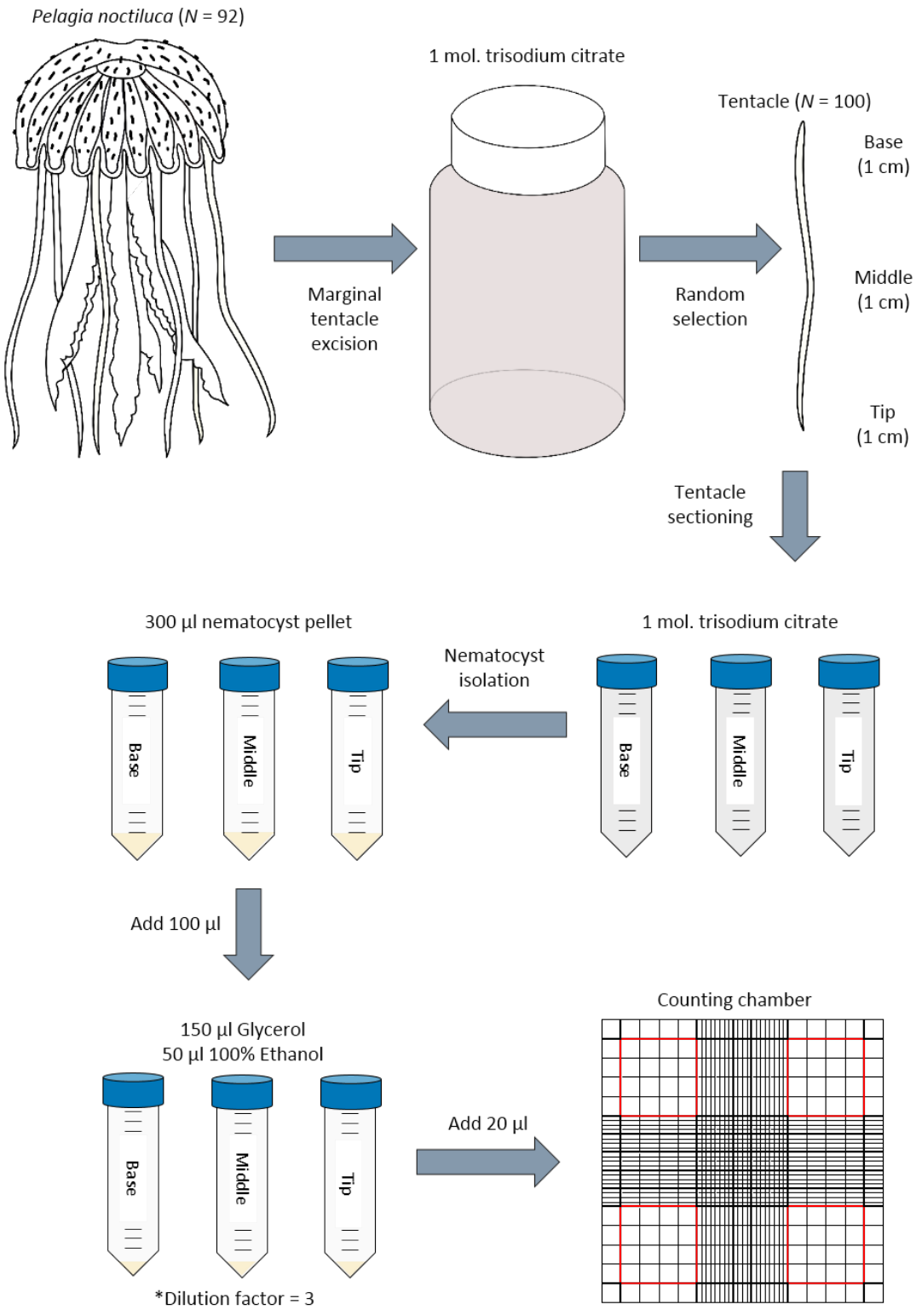


Figure 4.1. A schematic diagram of sample preparation for *C. capillata* and *Pe. noctiluca* prior to microscopy and calculations. Number of nematocysts in a $1mm^2$ square (red) × 10^1 = Number of nematocysts per µl suspension.

4.3. Results

In total, nine nematocyst types were identified in *C. capillata* (Figures 4.2, 4.3) including five isorhiza nematocysts: a-isorhiza (Figure 4.3A), small A-isorhiza (Figure 4.3B), medium A-isorhiza (Figure 4.3C), large ovate A-isorhiza (Figure 4.3D), large spherical A-isorhiza (Figure 4.3E) and O-isorhiza (Figure 4.3F), one eurytele nematocyst (Figure 4.3G) and two birhopaloid nematocysts: small birhopaloid (Figure 4.3H) and medium birhopaloid (Figure 4.3I). Overall a-isorhiza nematocysts were the smallest in size (length × width, 5.2 – 10.9 × 3.1 – 6.0 µm, Figure 4.4) but the most abundant (49% of total nematocysts counted, Table 4.1) while large spherical A-isorhiza nematocysts were the largest in size (17.2 – 32.6 × 15.2 – 24.8 µm, Figure 4.4), but one of the least abundant (4% of total nematocysts counted, Table 4.1). The abundance and size of each nematocyst type did not differ between the base, middle and tip of *C. capillata* tentacles (one-way ANOVA, $P > 0.05$, Figure 4.4).

In total, six nematocyst types were identified in *Pe. noctiluca* (Figures 4.5, 4.6) including three isorhiza nematocysts: a-isorhiza (Figure 4.6A), isorhiza (Figure 4.6B) and O-isorhiza (Figure 4.6C), one eurytele nematocyst (Figure 4.6D) and two birhopaloid nematocysts: small birhopaloid (Figure 4.6E) and medium birhopaloid (Figure 4.6F). Overall, a-isorhiza nematocysts were the smallest in size (length × width, 5.1 – 7.9 × 3.1 – 5.0 µm, Figure 4.7) and O-isorhiza nematocysts were the largest in size (15.2 – 25.8 × 14.2 – 24.8 µm, Figure 4.7), both were some of the least abundant (8% and 4% of total nematocysts counted, Table 4.1). Medium birhopaloid nematocysts (10.3 – 16.8 × 8.1 – 12.0 µm, Figure 4.7) were the most abundant type (41% of total nematocysts counted, Table 4.1).

The abundance of two out of six nematocyst types differed between the base, middle and tip of *Pe. noctiluca* tentacles: eurytele nematocysts were more abundant in the middle of the tentacle (mean ± SE, $2.42 \times 10^3 \pm 2.63 \times 10^2$ nematocysts per cm, Figure 4.7) than at the tip of the tentacle (mean ± SE, $1.74 \times 10^3 \pm 1.37 \times 10^2$ nematocysts per cm, Figure 4.7) (one-way ANOVA, $P = 0.043$, Figure 4.7) and medium birhopaloid nematocysts were less abundant at the base of the tentacle (mean ± SE,

$3.48 \times 10^3 \pm 1.64 \times 10^2$ nematocysts per cm, Figure 4.7) than in the middle (mean ± SE, $4.22 \times 10^3 \pm 1.16 \times 10^2$ nematocysts per cm, Figure 4.7) and at the tip of the tentacle (mean ± SE, $4.24 \times 10^3 \pm 1.50 \times 10^2$ nematocysts per cm, Figure 4.7) (one-way ANOVA, $P = 0.004$, Figure 4.7). While significant differences were found in these two nematocyst types, the abundance of the four other nematocyst types did not differ along the length of the tentacle (one-way ANOVA, $P > 0.05$, Figure 4.7). The size of each nematocyst type did not differ between the base, middle and tip of *Pe. noctiluca* tentacles (one-way ANOVA, $P > 0.05$, Figure 4.7).

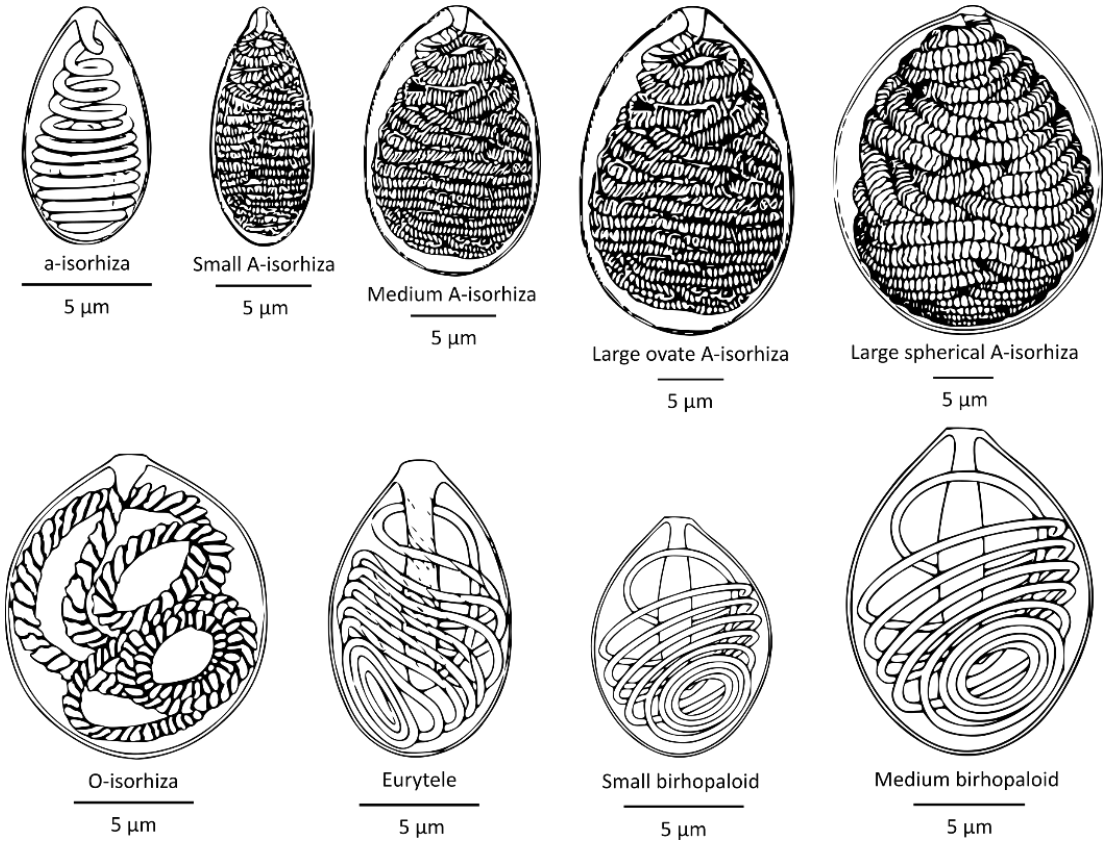


Figure 4.2. Illustrations of *C. capillata* nematocysts adapted from Östman and Hydman (1997).

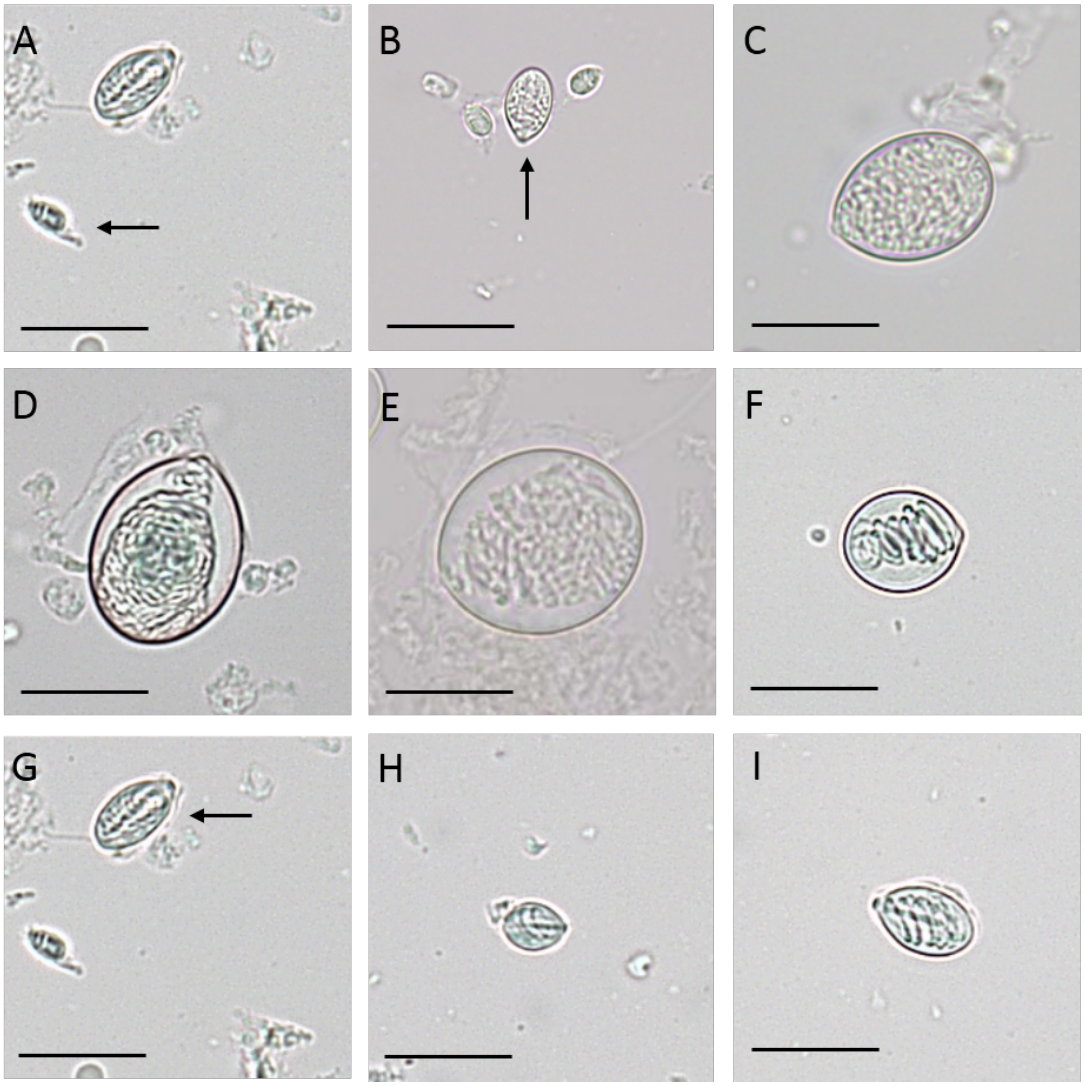


Figure 4.3. Light microscopy of *C. capillata* nematocysts. A) a-isorhiza, B) small A-isorhiza, C) medium A-isorhiza, D) large ovate A-isorhiza, E) large spherical A-isorhiza, F) O-isorhiza, G) eurytele, H) small birhopaloid and I) medium birhopaloid. Scale bar represents 20 µm.

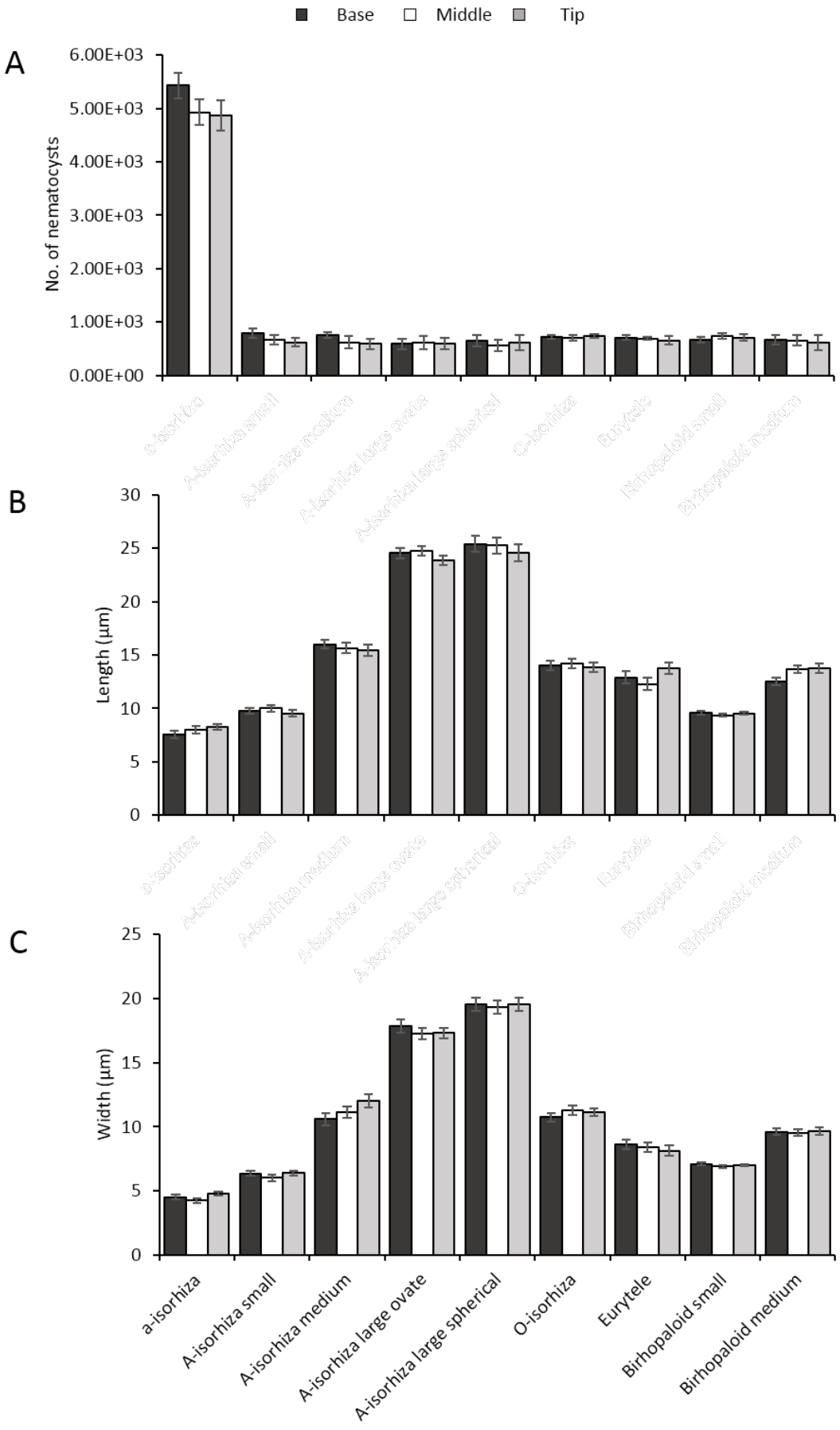

Base
Middle
Tip
A
No. of nematocysts
6.00E+03
5.00E+03
4.00E+03
3.00E+03
2.00E+03
1.00E+03
0.00E+00
B
Length (µm)
30
25
20
15
10
5
0
C
Width (µm)
25
20
15
10
5
0
a-isorhiza
A-isorhiza small
A-isorhiza medium
A-isorhiza large ovate
A-isorhiza large spherical
O-isorhiza
Eurytele
Birhopaloid small
Birhopaloid medium

Figure 4.4. Abundance and size in µm of undischarged nematocysts in the base, middle and tip of *C. capillata* tentacles. (A) Number of nematocysts per cm (mean ± SE), (B) Capsule lengths (mean ± SE; µm) and (C) Capsule widths (mean ± SE; µm).

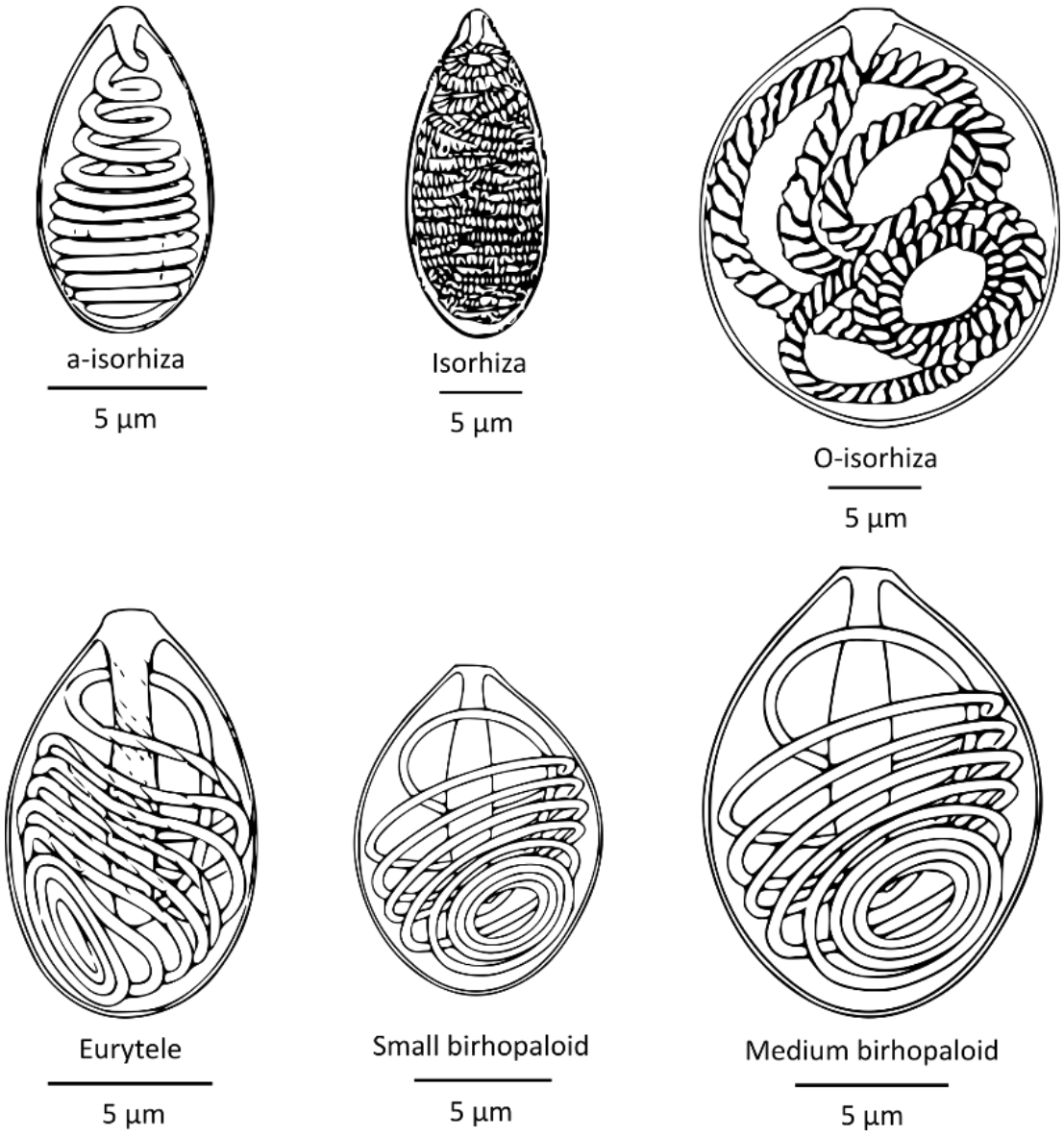


Figure 4.5. Illustrations of *Pe. noctiluca* nematocysts adapted from Östman and Hydman (1997).

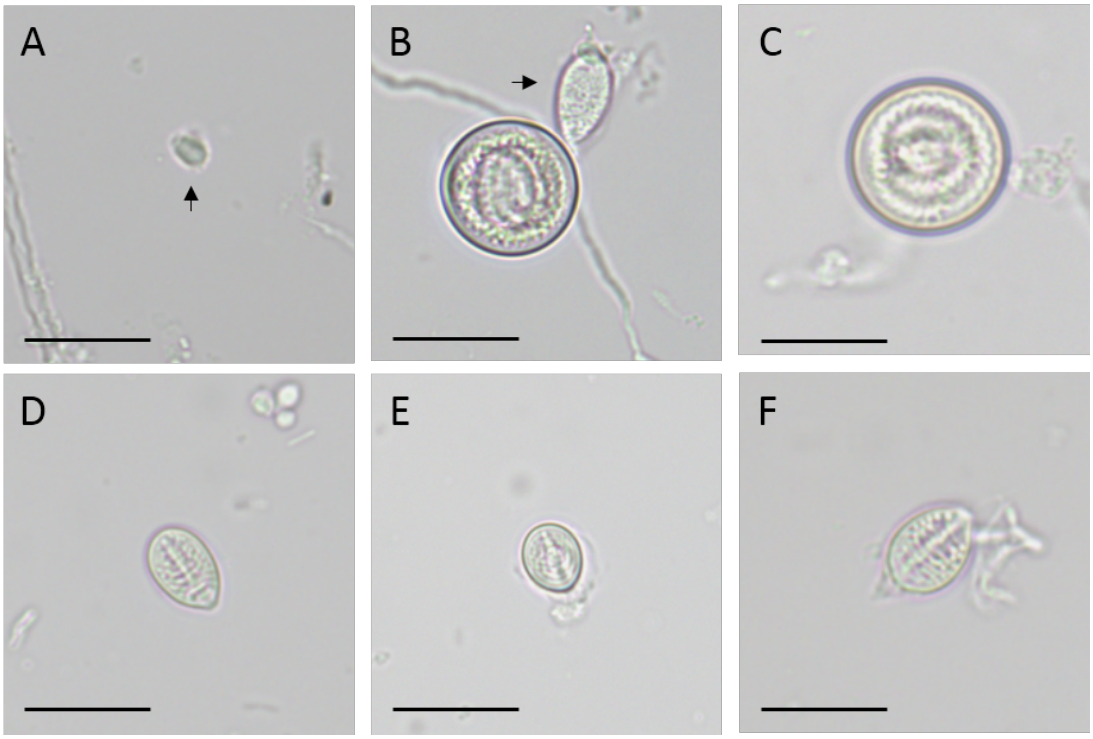


Figure 4.6. Light microscopy of *Pe. noctiluca* nematocysts. A) a-isorhiza, B) isorhiza, C) O-isorhiza, D) eurytele, E) small birhopaloid and F) medium birhopaloid. Scale bar represents 20 µm.

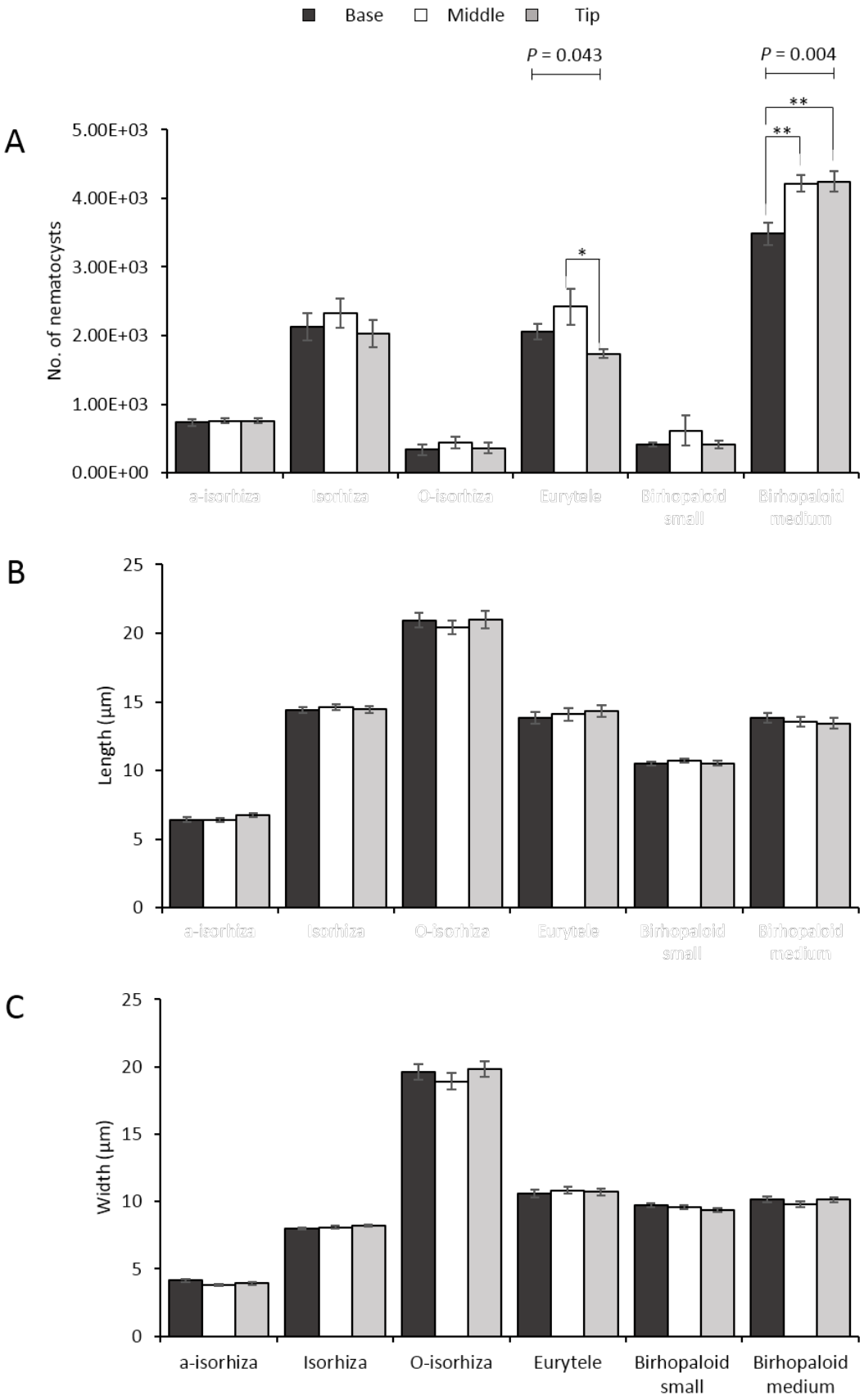

Base
Middle
Tip
A
P = 0.043
P = 0.004
**
**
*
No. of nematocysts
5.00E+03
4.00E+03
3.00E+03
2.00E+03
1.00E+03
0.00E+00
a-isorhiza
Isorhiza
O-isorhiza
Eurytele
Birhopaloid small
Birhopaloid medium
B
Length (µm)
25
20
15
10
5
0
C
Width (µm)

Figure 4.7. Abundance and size in µm of undischarged nematocysts in the base, middle and tip of *Pe. noctiluca* tentacles. (A) Number of nematocysts per cm (mean ± SE), (B) Capsule lengths (mean ± SE; µm) and (C) Capsule widths (mean ± SE; µm). * P < 0.05, ** P < 0.01, *** P < 0.001.

The abundance of all nematocyst types common to both *C. capillata* and *Pe. noctiluca* (i.e. a-isorhiza, O-isorhiza, eurytele, small birhopaloid and medium birhopaloid) differed significantly between the two species (one-way ANOVA, $P < 0.001$). Two types of isorhiza nematocysts (a-isorhiza and O-isorhiza) and small birhopaloid nematocysts were more abundant in *C. capillata* than in *Pe. noctiluca* (mean ± SE, $4.67 \times 10^3 \pm 3.97 \times 10^2$ for a-isorhiza, $6.15 \times 10^2 \pm 4.42 \times 10$ for O-isorhiza and $5.90 \times 10^2 \pm 8.31 \times 10$ for small birhopaloid in *C. capillata* and $7.34 \times 10^2 \pm 4.75 \times 10$ for a-isorhiza, $3.38 \times 10^2 \pm 7.70 \times 10$ for O-isorhiza and $4.10 \times 10^2 \pm 2.70 \times 10$ for small birhopaloid in *Pe. noctiluca*) (one-way ANOVA, $P < 0.001$) and eurytele and medium birhopaloid nematocysts were more abundant in *Pe. noctiluca* than in *C. capillata* ($6.05 \times 10^2 \pm 4.66 \times 10$ for eurytele and $5.91 \times 10^2 \pm 9.48 \times 10$ for medium birhopaloid in *C. capillata* and $2.06 \times 10^3 \pm 1.10 \times 10^2$ for eurytele and $3.48 \times 10^3 \pm 1.64 \times 10^2$ for medium birhopaloid in *Pe. noctiluca*) (one-way ANOVA, $P <0.001$). In addition, the sizes of a-isorhiza, O-isorhiza, eurytele and small birhopaloid nematocysts differed significantly between *C. capillata* and *Pe. noctiluca* (one-way ANOVA, $P <0.05$). In *Pe. noctiluca*, a-isorhiza nematocysts were significantly smaller than in *C. capillata* (length × width, 5.5 – 10.9 × 3.1 – 6.0 µm in *C. capillata* and 5.1 – 8.0 × 3.1 – 5.0 µm in *Pe. noctiluca*) and O-isorhiza, eurytele and small birhopaloid nematocysts were significantly larger than in *C. capillata* (10.1 – 17.9 × 8.1 – 14.0 µm for O-isorhiza, 8.1 – 17.9 × 5.1 – 12.0 µm for eurytele and 8.2 – 11.0 × 6.1 – 8.0 µm for small birhopaloid in *C. capillata* and 15.3 – 25.8 × 14.1 – 24.8 µm for O-isorhiza, 10.1 – 18.0 × 8.3 – 13.0 µm for eurytele and 9.1 – 11.8 × 8.1 – 11.0 µm for small birhopaloid in *Pe. noctiluca*) (one-way ANOVA, $P <0.01$). There were no significant differences in the length of medium birhopaloid nematocysts between *Pe. noctiluca* and *C. capillata*, but the width was significantly larger, meaning the capsules were broader, in *Pe. noctiluca* (width, 7.1 – 12.0 µm in *C. capillata* and 8.1 – 12.0 µm in *Pe. noctiluca*) (one-way ANOVA, $P = 0.025$).

Using the number of nematocysts per centimetre of tentacle, estimates for the total number of nematocysts per tentacle and per animal were made (Table 4.1). *Cyanea capillata* specimens were 20 – 40 cm in diameter and, when contracted, the length of their tentacles ranged from 25 – 45 cm. The number of tentacles on each *C. capillata* specimen were not counted but *C. capillata* typically have eight groups of ~100 tentacles (Doyle et al., 2017; Russell, 1970). *Pelagia noctiluca* specimens were 3 – 10 cm in diameter and, when contracted, the length of their tentacles, eight in total, ranged from 4 – 10 cm. For each species, the number of nematocysts per cm was multiplied by the average length of tentacles to calculate the number of nematocysts per tentacle which was multiplied by the number of tentacles (800 for *C. capillata* and 8 for *Pe. noctiluca*) to calculate the number of nematocysts per animal. Per cm of tentacle, *C. capillata* possess a total of 9,900 nematocysts and *Pe. noctiluca* possess a total of 9,820 nematocysts. Per tentacle, *C. capillata* possess a total of 297,000 nematocysts and *Pe. noctiluca* possess a total of 69,100 nematocysts. Per animal, *C. capillata* possess a total of 238,000,000 nematocysts and *Pe. noctiluca* possess a total of 553,000 nematocysts, therefore, *Pe. noctiluca* possess significantly less nematocysts per tentacle and per animal (one-way ANOVA, $P < 0.001$, Table 4.1).

Table 4.1. Estimation of the average number of nematocysts per cm, per tentacle and per animal for C. capillata and Pe. noctiluca. Asterisk represent significant differences between species * P < 0.05, ** P < 0.01, *** P < 0.001.

Species	Nematocyst type	No. per cm	No. per tentacle	No. per animal
Cyanea capillata	a-isorhiza	4.82×10^3	1.45×10^5	1.16×10^8
	A-isorhiza small	6.54×10^2	1.96×10^4	1.57×10^7
	A-isorhiza medium	6.15×10^2	1.85×10^4	1.48×10^7
	A-isorhiza large ovate	5.84×10^2	1.75×10^4	1.40×10^7
	A-isorhiza large spherical	5.86×10^2	1.76×10^4	1.41×10^7
	O-isorhiza	6.87×10^2	2.06×10^4	1.65×10^7
	Eurytele	6.52×10^2	1.95×10^4	1.56×10^7

	Birhopaloid small	6.81×10^{2}	2.04×10^{4}	1.63×10^{7}
	Birhopaloid medium	6.22×10^{2}	1.86×10^{4}	1.49×10^{7}
	Total	9.90×10^{3}	$2.97 \times 10^{5***}$	$2.38 \times 10^{8***}$
Pelagia	a-isorhiza	7.49×10^{2}	5.27×10^{3}	4.22×10^{4}
noctiluca	Isorhiza	2.16×10^{3}	1.52×10^{4}	1.22×10^{5}
	O-isorhiza	3.81×10^{2}	2.68×10^{3}	2.15×10^{4}
	Eurytele	2.07×10^{3}	1.46×10^{4}	1.17×10^{5}
	Birhopaloid small	4.79×10^{2}	3.37×10^{3}	2.69×10^{4}
	Birhopaloid medium	3.98×10^{3}	2.80×10^{4}	2.24×10^{5}
	Total	9.82×10^{3}	$6.91 \times 10^{4***}$	$5.53 \times 10^{5***}$

4.4. Discussion

In *C. capillata*, we observed five types of isorhiza, one type of eurytele and two types of birhopaloid nematocysts. These results agree with previous studies which found the same nematocyst types in *C. capillata* sampled from the North and Baltic Seas (Östman & Hydman, 1997; Wiebring et al., 2010) and Outer Hebrides (Helmholz et al., 2011). Our measurements closely matched the measurements of Östman and Hydman (1997), however, the abundance of each nematocyst type differed significantly (Chi-Squared test, $\chi^2 = 16.33$, df = 8, $P = 0.04$, Figure 4.8). In this study, a-isorhiza (48.69% of total nematocysts counted, Table 4.1), were most common, followed by O-isorhiza (6.94%), small birhopaloid (6.88%), small A-isorhiza (6.60%), eurytele (6.58%) and medium birhopaloid (6.28%), and medium (6.21%), large spherical (5.92%) and large ovate (5.90%) A-isorhiza nematocysts were rare. In contrast, Östman and Hydman (1997) found that a-isorhiza were most common followed by eurytele, medium and large A-isorhiza and O-isorhiza, and small A-isorhiza, and birhopaloid nematocysts were absent or rare. In other words, eight out of nine nematocyst types differed in their overall abundance to Östman and Hydman (1997).

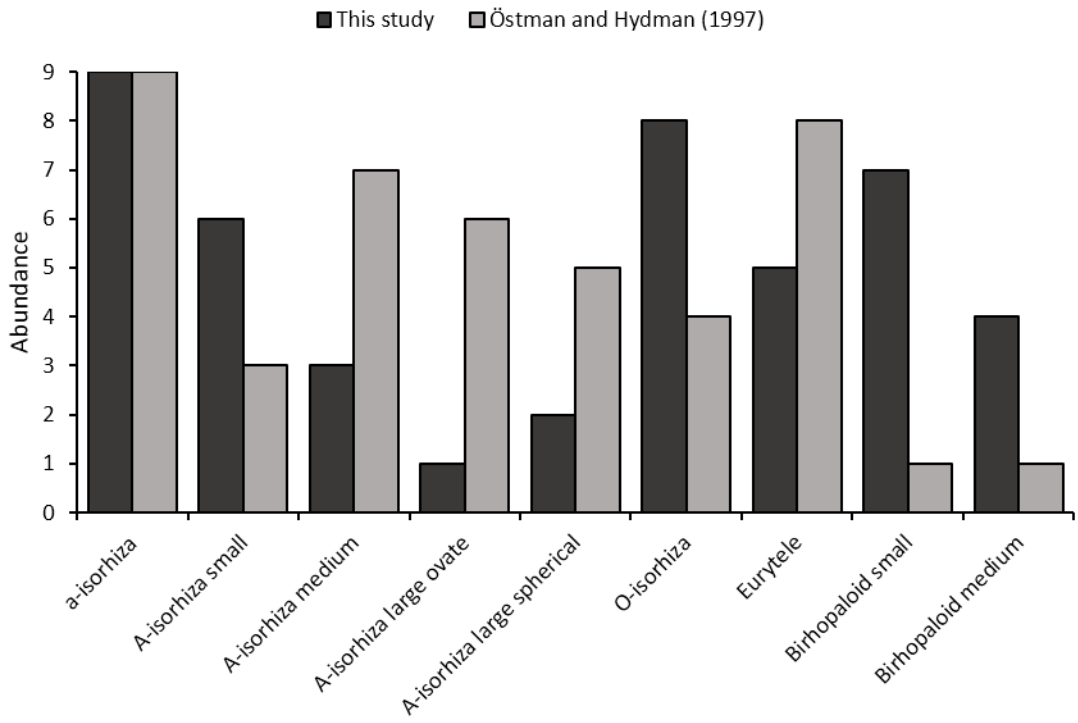


Figure 4.8. Abundance of *C. capillata* nematocysts from this study compared with Östman and Hydman (1997). Nematocyst abundance has been ranked from 1 (low in abundance) to 9 (high in abundance) as Östman & Hydman (1997) did not provide absolute counts.

Where we found no differences between the base, middle and tip of *C. capillata* tentacles, Östman and Hydman (1997) found that, in the tentacle bases, O-isorhiza nematocysts were generally the only nematocyst type present and a-isorhiza nematocysts which, overall were the most abundant type, were absent or rare. However, the most surprising difference between the two studies was observed in the small nematocyst types. For example, some of the most abundant nematocysts in this study were all the small nematocysts (i.e. a-isorhiza, small A-isorhiza and small birhopaloid). In contrast, small A-isorhiza and small birhopaloid nematocysts were absent or rare in Östman and Hydman (1997) and even though a-isorhiza were the most abundant nematocyst type overall, they were absent or rare in the tentacle bases. These differences may be because the samples were prepared using different methods. For example, it may be that the small nematocyst types were underestimated by Östman and Hydman (1997) because they were difficult to

distinguish from the tentacle tissue. However, by isolating the nematocysts from the tentacles, we were able to avoid this issue. The larger nematocysts (i.e. medium, large ovate and large spherical A-isorhiza) were the bottom three least abundant nematocysts in this study but were ranked in the middle in Östman and Hydman (1997). Indeed, the larger nematocysts may have been displaced during the process of preparing our samples, thereby affecting our results. For example, when the tentacles were excised from *C. capillata* and placed in 1 mol. trisodium citrate, some of the larger nematocysts may have sloughed off. However, we also found a high abundance of medium birhopaloid nematocysts, similar in size to medium A-isorhiza nematocysts, which suggests that there was no differential sloughing of the larger nematocyst types and that our results are quite accurate. Because of these significant differences, future studies should compare the enumeration of nematocysts both in nematocyst suspensions and *in situ* (on the tentacles) immediately after sample collection in order to distinguish results from artefacts of the preparation procedure. Additionally, comparable methods should be used to determine whether there are regional differences in the cnidome between *C. capillata* collected in the Irish and North Seas (Östman & Hydman, 1997) as the size and distribution of nematocysts have been shown to vary with abiotic factors such as salinity and temperature (Wiebring et al., 2010).

Previously in *Pe. noctiluca*, Avian et al. (1991) described three types of isorhiza nematocysts, one type of eurytele nematocyst and one unknown type resembling a p-mastigophore nematocyst. Our measurements agreed with Avian et al. (1991) but different nematocysts were identified; we observed the same three types of isorhiza and eurytele nematocysts but also small and medium birhopaloid nematocysts. Birhopaloid nematocysts have never been reported in *Pe. noctiluca*, most likely because the study by Avian et al. (1991) preceded the study by Östman and Hydman (1997) which first identified them. Indeed, more recent studies have also not identified them in *Pe. noctiluca* (Marchini, De Nuccio, Mazzei, & Mariottini, 2004). Marchini et al. (2004) identified nematocysts which were like eurytele nematocysts but smaller in size and these were probably small birhopaloid nematocysts. Even though the capsule shape and pattern of the undischarged tubule of birhopaloid

nematocysts are similar to those of eurytele nematocysts, due to two dilations on their shaft (Östman & Hydman, 1997), birhopaloid nematocysts are regarded as distinctive. This distinction is most obvious upon examination using electron microscopy, however, even under the light microscope birhopaloid nematocysts can be distinguished by their broader, more rounded capsules (Östman & Hydman, 1997). Additionally, the isorhiza nematocysts observed in this study were similar in size and appearance to medium A-isorhiza nematocysts (Östman, 2000; Östman & Hydman, 1997) but were less broad and more narrow in width. However, Avian et al. (1991) described these isorhiza nematocysts as heterotrichous, with two or more kinds of spines, and Östman and Hydman (1997) described A-isorhiza nematocysts as homotrichous, with all the same kind of spines. In addition, a-isorhiza nematocysts were described as atrichous, with no spines, by Avian et al. (1991) but homotrichous by Östman and Hydman (1997). Examination of the spines using an electron microscope is required to determine whether the *Pe. noctiluca* isorhiza and a-isorhiza nematocysts are equivalent to A-isorhiza and a-isorhiza nematocysts in *Cyanea*. Avian et al. (1991) did not provide any images of the unknown type resembling a p-mastigophore nematocyst but described it as “fusiform, flat and slightly curved” with “a smooth, cylindrical shaft from which the tubule emerges”. No nematocysts resembling p-mastigophore nematocysts were observed in this study or in a previous study (Marchini et al., 2004). However, we did observe immature nematocysts resembling those capture by Östman and Hydman (1997) which fit the description of p-mastigophore nematocysts. Similarly, examination of these nematocysts using an electron microscope is required to determine whether they are immature nematocysts as opposed to p-mastigophore nematocysts.

Generally, in *C. capillata* and *Pe. noctiluca*, the abundance and size of each nematocyst type did not differ along the length of their tentacles despite previous studies suggesting that there may be significant differences (Östman & Hydman, 1997). The only significant difference observed in this study was that the abundance of eurytele and medium birhopaloid nematocysts varied along the length of *Pe. noctiluca* tentacles, i.e. eurytele nematocysts were more abundant in the middle and medium birhopaloid nematocysts were less abundant at the base of tentacles. The

primary function of nematocysts is to capture and retain prey, therefore, any significant differences in the abundance and size of different nematocyst types can generally be related to predation mode. For example, eurytele and birhopaloid nematocysts are highly effective penetrant nematocysts which penetrate prey with the full length of their tubule (Colin & Costello, 2007). Therefore, the fact that they occur in high abundance far from the bell where most prey is caught (Corrales-Ugalde, Colin, & Sutherland, 2017) and the fact that *Pe. noctiluca* prey on hard-bodied prey items (Canepa et al., 2014) suggests that their abundance might be an adaptation to increase capture efficiency (Corrales-Ugalde et al., 2017). Additionally, the fact that they were more abundant in *Pe. noctiluca* than they were in *C. capillata* (7% of total nematocysts counted for eurytele and 6% of total nematocysts counted for medium birhopaloid in *C. capillata* and 23% of total nematocysts counted for eurytele and 7% of total nematocysts counted for medium birhopaloid in *Pe. noctiluca*, Table 4.1) suggests that their abundance might also correspond to available prey types. For example, *C. capillata* prey on soft-bodied prey items (Båmstedt, Ishii, & Martlnussen, 1997) whereas *Pe. noctiluca* prey on hard-bodied prey items (Canepa et al., 2014) and require more penetrative nematocysts that can penetrate the exoskeleton (Purcell, 1984). *Cyanea capillata* possessed the most small birhopaloid (7% of total nematocysts counted in *C. capillata* and 5% of total nematocysts counted in *Pe. noctiluca*) and O-isorhiza (7% of total nematocysts counted in *C. capillata* and 4% of total nematocysts counted in *Pe. noctiluca*) nematocysts, which are also penetrant (Colin & Costello, 2007), but a-isorhiza nematocysts (49% of total nematocysts counted) were by far the most abundant and these nematocysts only partially penetrate or adhere and entangle prey, thus, are more suited for the capture of soft-bodied prey items (Purcell, 1984). Additionally, the fact that eurytele and birhopaloid were larger in size in *Pe. noctiluca* than they were in *C. capillata* (8.1 – 17.9 × 5.1 – 12.0 µm for eurytele and 8.2 – 11.0 × 6.1 – 8.0 µm for small birhopaloid in *C. capillata* and 10.1 – 18.0 × 8.3 – 13.0 µm for eurytele and 9.1 – 11.8 × 8.1 – 11.0 µm for small birhopaloid in *Pe. noctiluca*) suggests that they contained longer tubules and more venom and could capture larger prey items (Helmholz et al., 2011; Purcell, 1984). However, *C. capillata* have three orders of magnitude more nematocysts on their tentacles than *Pe. noctiluca* (2.38×10^8

nematocysts in *C. capillata* and 5.53×10^5 nematocysts in *Pe. noctiluca*, Table 4.1), which suggests that *C. capillata* are more suited to capturing large prey items, such as *Aurelia aurita* (Båmstedt et al., 1997).

Eurytele and birhopaloid nematocysts are also associated with inflicting more severe stings because they can penetrate deep into the skin (Kitatani et al., 2015). Even though their tubules are relatively short (0.3 µm for eurytele and 0.15 µm for birhopaloid) in comparison to other nematocysts, such as O-isorhiza (0.8 µm), they discharge at a rapid velocity and penetrate with the full length of their tubule (Colin & Costello, 2007). This impact stimulates pain receptor neurons causing severe pain and inflammation (Kitatani et al., 2015). The fact that they are high in abundance suggests that eurytele and birhopaloid nematocysts contribute to the severity of *Pe. noctiluca* stings which are characterized by severe local reactions (Kokelj & Burnett, 1990). If these are the nematocysts which cause the most damage, sting management and mitigation measures should target these types. Equally as important, treatments should not cause these types to discharge (Doyle et al., 2017). Conversely, a-isorhiza (i.e. the smallest nematocyst type) were by far the most abundant nematocyst type in *C. capillata* (49% of total nematocysts counted, Table 4.1) even though studies suggest that the nematocysts with longer tubules (Kitatani et al., 2015), larger capsules (Helmholz et al., 2011) and rapid discharge velocities (Colin & Costello, 2007) are more toxic and *C. capillata* stings are considerably more severe; therefore, *C. capillata* should presumably possess a higher abundance of the larger and penetrant nematocyst types. While *C. capillata* did possess more penetrant O-isorhiza (7% of total nematocysts counted in *C. capillata* and 4% of total nematocysts counted in *Pe. noctiluca*) and small birhopaloid nematocysts (7% of total nematocysts counted in *C. capillata* and 5% of total nematocysts counted in *Pe. noctiluca*) and large A-isorhiza nematocysts than *Pe. noctiluca*, the severity of *C. capillata* stings is most likely due to the size of the animal itself. Indeed, *C. capillata* have hundreds of tentacles several metres in length and every centimetre is covered in 9,900 nematocysts (Table 4.1). Bearing in mind that only <1% of nematocysts discharge upon initial tentacle contact (Yanagihara, Wilcox, King, et al., 2016), by our estimates, if a person were to encounter a *C. capillata* and contact 20 – 30% of its

tentacles that equates approximately 1.1×10^7 discharged nematocysts. If each individual nematocyst contains approximately 850 pg of venom (Yanagihara & Shohet, 2012), the injured person would receive a venom dose of 9.4 mg. For comparison, this is 100 million times the volume of venom injected by a honeybee (*Apis mellifera*) sting (82.5 pg, Schumacher et al., 1994) and only 7% of the volume of a snake bite (e.g. 63 mg in Russell's viper (*Vipera russelli*), Pe and Cho, 1986). Future studies should aim to accurately quantify the *C. capillata* venom load as this may be helpful in the diagnosis and management of systemic symptoms.

Chapter 5 – Fish Envenomation Therapeutic

5. A Pilot Study on Atlantic Salmon (*Salmo salar*) to Aid the Development of Effective Jellyfish Envenomation Therapeutics

Abstract

Aquaculture records demonstrate that mass jellyfish exposure leads to significant mortality events and recurrent gill damage in marine-farmed salmon. Despite this decades long documented history of negative interactions, no fully effective mitigation measures exist. The demand for effective protective, preventative or therapeutic approaches to address envenomation-associated morbidity and mortality in salmon, prompted us to explore the effects of lion's mane jellyfish (*Cyanea capillata*) in Atlantic salmon (*Salmo salar*) as well as potential mitigating agents to aid in the development of potentially effective solution(s). We present here a reproducible and simple *ex vivo* model system to elucidate both pathogenic effects and mitigation efficacy in freshly drawn *S. salar* blood. Firstly, we exposed whole *S. salar* blood to *C. capillata* venom and prepared time course blood smears over a period of 20 minutes. Secondly, red blood cells (RBCs) were stained and scored with a system that rated the damage between 0 and 4 using a range of parameters associated with the different stages of haemolysis. The results of this pilot study revealed that *C. capillata* venom immediately caused significant haemolysis. Immediately (within 60 seconds) after exposure to *C. capillata* venom, up to 96% of *S. salar* RBCs showed distinct morphological changes and signs of membrane perturbation and rupture including crenulation and a swollen shape. The percentage of RBCs exhibiting morphological aberrations remained constant. Further, after 3 minutes there were abundant RBC nucleated ghosts (41% of RBCs, swollen RBCs utterly devoid of haemoglobin exhibiting swollen diffusely stained nuclei). These results demonstrate that even short-term exposure to jellyfish venom result in immediate and significant damage to exposed tissues (gills, skin, eyes) in marine-farmed fish.

5.1. Introduction

For the past few decades, jellyfish blooms have negatively impacted the salmon aquaculture industry, particularly in Northern Europe, e.g. Scotland, Norway and Ireland (Baxter, Sturt, et al., 2011; Doyle et al., 2008; Rodger et al., 2011), and to a lesser extent in Chile, Australia, Asia and North America (Adams, Ellard, & Nowak, 2004; Haberlin, 2018; Palma, Apablaza, & Soto, 2007; Rodger et al., 2011). They either cause acute mortality events when they occur in large densities, for example, 250,000 Atlantic salmon *(Salmo salar*) were killed by a 26 km^2 bloom of the scyphomedusae *Pelagia noctiluca* in Northern Ireland in 2007 (Doyle et al., 2008; Lucas et al., 2014), or chronic gill damage, which accounts for between 1 – 79% of annual mortalities in Ireland (Rodger et al., 2011). Gill damage occurs when small gelatinous zooplankton, e.g. *Muggiaea atlantica* (Fossa et al., 2003), or large scyphomedusae, e.g. *Pe. noctiluca* or *Cyanea capillata* (Bruno & Ellis, 1985; Doyle et al., 2008), enter salmon pens in one piece or are broken up into smaller fragments as a result of passing through the mesh of the pens (Baxter, Sturt, et al., 2011; Mitchell et al., 2012). The smaller jellyfish tissue fragments still possess intact and active cnidae including penetrant nematocysts, i.e. microscopic stinging capsules, and gill damage comes from the gill filtration of nematocysts during respiration, i.e. fish breathe by taking water in through their mouths and then forcing it out through their gills. During this process, if there are nematocysts in the water, then these are carried past the delicate gill tissues where they potently fire to discharge venom (Baxter, Sturt, et al., 2011; Bosch-belmar, Rabet, Dhaouadi, & Chalghaf, 2016; Rodger et al., 2011). Jellyfish exposure has also been shown to cause lesions on the skin and eyes of fish (Rodger et al., 2011).

Potential mitigation methods to minimise the impact of jellyfish have been tested including reduction or complete cessation of feeding (Hay & Murray, 2008), routine monitoring (Ruane et al., 2013), site relocation (Rodger et al., 2011), bubble curtains (Haberlin, 2018) and net-cleaning to reduce biofouling (Baxter et al., 2012). However, none have proven sufficiently effective and more research is required in the area of mitigation (Rodger et al., 2011). In particular, there is a requirement for research to be undertaken into medicines and remedies (Rodger et al., 2011) as well

as prophylactic agents to prevent cnidae discharge or inhibit potent venom compounds to reduce morbidity and mortality from severe envenomations. However, the development of an effective remedy has been hampered by the significant knowledge gaps in the pathophysiology of fish envenomation. These gaps are largely due to the complexity of jellyfish venom (Rodger et al., 2011).

Jellyfish envenomations involve both physical trauma in the expulsion of hundreds of microns long nematocyst tubules that impale the tissue (Yanagihara et al., 2002) as well as the injection of venom. These venoms are complex mixtures of bioactive compounds including: porins, lipases, proteases, small molecular weight compounds and biologically active lipids (Yanagihara, Wilcox, Smith, et al., 2016), which differ in composition between taxa (e.g. scyphomedusae and cubomedusae (Jouiaei et al., 2015)) and species. Of these, the fastest-acting agent in the venom are porins, or pore-forming proteins (Chung et al., 2001; Yanagihara & Shohet, 2012). Negative-staining electron microscopy of human RBCs exposed to cubozoan porins demonstrates that porins self-assemble to form rivet-like transmembrane pores that perforate RBCs, white blood cells (WBCs) and platelets (Yanagihara & Shohet, 2012). This results in a dose-dependent release of the respective intracellular contents (Yanagihara, Wilcox, Smith, et al., 2016). For example, light microscopy of cubozoan toxin-exposed human RBCs demonstrates several minutes of swelling and potassium loss prior to swelling sufficient to allow release of large tetrameric haemoglobin molecules and RBC ghost formation (Yanagihara & Shohet, 2012). In both piglet and mouse models, high doses of porin and subsequent perforation of RBCs led to systemic hyperkalaemia, rapid, acute cardiopulmonary collapse and pulseless electrical activity (PEA) (Yanagihara & Shohet, 2012); lower doses can invoke Irukandji syndrome (Yanagihara, Wilcox, Smith, et al., 2016). All jellyfish venoms analysed to date contain a close homolog of potent haemolytic porins (haemolysins) (Yanagihara, Wilcox, Smith, et al., 2016). Other haemolysins include lipases, a slower-acting venom component, specifically phospholipase A_2 (PLA_2) enzymes, which degrade the phospholipid bilayer in cell membranes liberating free-fatty acids inducing a massive pro-inflammatory effect (Yanagihara, Wilcox, Smith, et al., 2016).

Haemolysis and PLA_2 activity have been demonstrated in human RBCs exposed to *C. capillata* toxin (Helmholz, Ruhnau, Schütt, & Prange, 2007).

While the pathophysiology of human envenomation is well understood, there have been relatively few research projects in the scientific literature which have investigated the pathophysiology of fish envenomation. Most research to date has addressed gross pathology of the gills (e.g. Baxter et al., 2011; Bosch-belmar et al., 2016; Powell et al., 2018). Yet, the precise sequences of events after exposure to potentially toxic venoms are not fully understood. However, because cell damage in *in vitro* assays serves as an indicator for the potential toxic effects, cell-based approaches are useful tools for venom analysis (Helmholz, Johnston, Ruhnau, & Prange, 2010). For example, a study by Helmholz et al., (2010) exposed rainbow trout gill cells to *C. capillata* and *Aurelia aurita* venom to demonstrate their cytotoxic effects. As a result, there was a significant reduction in gill cell viability. In addition, haemolysis is often used in *in vitro* assays as a test for the effects of venom (Helmholz et al., 2007). Even though *C. capillata* venom has a strong haemolytic activity (Helmholz et al., 2010), haemolytic activity has only been tested in humans, rats and rabbits (Walker, 1977a, 1977b; Walker, Martinez, & Godin, 1977), the haemolytic activity against fish has not been investigated to date.

Therefore, the aim of this pilot study was to use an *ex vivo* assay to explore the effects and the time course of *C. capillata* venom on *S. salar* RBCs with the overarching aim of providing information on whether acute phase haemolysis occurs. Immediate haemolysis suggests the action of potent haemolytic porins thus far found to be the fastest-acting agent in jellyfish venom (Yanagihara, Wilcox, Smith, et al., 2016). *Cyanea capillata* is a large jellyfish with a bell measuring up to 1 m in diameter and has nematocysts localized in the tentacles, oral arms and upper surface of the bell (Doyle et al., 2017). In the past, negative interactions between *C. capillata* and *S. salar* have been reported. For example, *C. capillata* caused the mortality of 90,000 farmed salmon in Ireland (Bruno & Ellis, 1985) and 1,000 salmon in Scotland (Nickell, Davidson, Fox, Miller, & Hays, 2010).

5.1. Materials and Methods

5.1.1. Venom Preparation

Cyanea capillata were collected from Dublin Bay, Ireland (between the Forty Foot bathing area and Dalkey Island, 53°17'18.25''N 6°6'12.03''W) on 26/07/2016. Immediately after collection, marginal tentacles were excised and immersed in 1 Molar. disodium citrate. Nematocysts were isolated from marginal tentacles according to Yanagihara and Shohet (2012). Briefly, nematocysts were isolated by gently rotating the tentacles in 1 Molar disodium citrate at room temperature. Tentacles were filtered through a 250 µm sieve and the filtrate was centrifuged at 3,000 rpm for 15 minutes. Undischarged nematocyst pellets were resuspended in 1 Molar trisodium citrate, then gently diluted with distilled water to a slurry and transferred to a chilled high-pressure cell extrusion French Press pressure cell and subjected to 2,500 psi for several seconds. Venom was expelled and recycled once, then centrifuged at 3,000 rpm for 1 minute Venom was snap frozen in liquid nitrogen and stored at -80°C.

5.1.2. Blood Smears

Fish blood samples were collected from two specimens of *S. salar* at Mannin Bay salmon farm in Drinagh, Errislannan, Clifden, County Galway. Blood samples were drawn from the caudal vein of fish killed for processing into vials containing Acid Citrate Dextrose (ACD), to preserve blood specimens. Analysis of the haemolytic activity of *C. capillata* venom on the RBCs of *S. salar* was tested by adding 100 µl of crude venom to whole fish blood (400 µl) and 100 µl of citrate to a separate control. The venom erythrocyte mixture and the control were incubated at room temperature. At 0, 2, 3, 8, 10 and 20 min, a drop of blood approximately 1 mm in diameter was deposited then quickly spread on a glass microscope by placing another slide at a 45° angle and backing into the drop of blood. Once the drop had briefly spread by capillary action along the edge of the 45° angle slide, the second slide was quickly swiped upwards across the slide to make the smear. There were twelve blood smears in total: one for the control and one for the venom at each

timepoint. The blood smears were air-dried and stained with Wright's Giemsa stain solution for 2 min. The stain was washed off using distilled water and the blood smears were left to air dry.

5.1.3. Microscopy

For field counting, a field of each blood smear was selected at random using the Olympus upright fluorescent BX53 microscope with a 40× objective. To simplify counting, each field was divided into eight equal-sized sub-fields and the total number of cells at each stage of haemolysis were counted and averaged across the sub-fields. The stages of membrane disruption and haemolysis include crenulation, swelling and RBC ghost formation. Red blood cell ghosts are swollen RBCs devoid of haemoglobin with swollen diffusely stained nuclei (i.e. unlike mammalian RBCs, amphibian, fish and bird RBCs contain a nucleus). In this study, the stages of haemolysis were defined as: normal (0), crenulation (1), swollen RBC (2), swollen nuclei (3) and RBC ghost formation (4; Table 5.1, Figure 5.1). Red blood cells were assigned to a stage of haemolysis based on their stain morphology including the shape of the cell, the presence of a crenulated membrane and the shape and size of the nucleus (Table 5.1).

Table 5.1. Geimsa Wright Stained morphology of the different stages of haemolysis of fish red blood cells.

Stage of haemolysis	Cell shape	Membrane appearance	Nucleus appearance
0	Oval	Normal	Oval
1	Oval	Crenulated	Oval
2	Swollen	Normal	Irregular
3	Swollen	Normal	Swollen
4	None	None	Swollen and diffuse

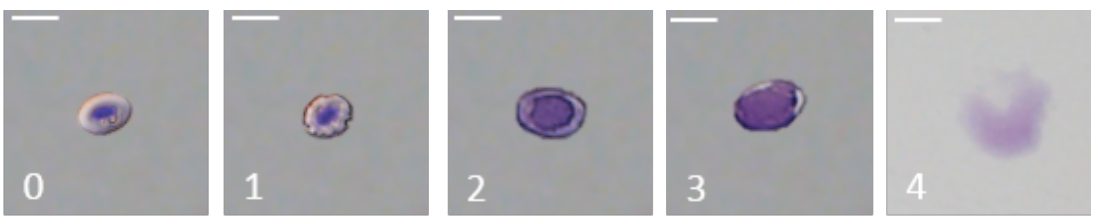


Figure 5.1. Examples of the different stages of haemolysis. Scale bar represents 15 µm.

5.1.4. Statistical Analysis

Outliers were defined using the median absolute deviation (MAD) method detailed in (Leys et al., 2013) and outliers within three deviations of the median were removed. Shapiro-Wilk normality tests were conducted on all data sets; since data were normally distributed, one-way ANOVAs were used to test for significant differences between the number of cells at each stage of haemolysis in the control and venom-exposed blood. A two-way ANOVA was used to test for significant differences between the different timepoints from the start of the experiment. All statistical analyses were conducted in IBM SPSS Statistics (25.0.0.2).

5.2. Results

The haemolytic effects of *C. capillata* venom on the RBCs of *S. salar* were investigated using light microscopy by scoring RBCs based on their stain morphology (Figure 5.2, Table 5.1). The results have been expressed as percentages of RBCs (Table 5.2). Throughout the experiment, the control had mostly healthy RBCs (38%) or RBCs with a crenulated cell membrane (57%) (stages 0 to 1). Some RBCs showed signs of damage and were scored at stages 2 (1.7%), 3 (1.1%) and 4 (1.9%). The low abundance of RBCs scored at stages 2 – 4 of haemolysis suggests that the damage might be an artefact of drying in suboptimal blood smears as opposed to haemolysis. Crenulation may also be an artefact of drying. On average, RBCs were healthy at 0 minutes but crenulated (i.e. stage 1) at every timepoint thereafter (Figure 5.3).

Damage was immediately observed in the blood exposed to venom. At 0 minutes, most RBCs (59 ± 38%) were at stage 2 of haemolysis where the RBC was swollen; only 6 ± 9% of RBCs were healthy. For the duration of the experiment, the stages of haemolysis ranged from 2 – 4 with a peak in RBCs at stage 4 of haemolysis, where they were nucleated ghosts, at 3 minutes (41 ± 39%). On average, RBCs were at stage 2 or 3 of haemolysis, where the RBC and nuclei were swollen, respectively (Figure 5.3).

Overall, significantly more RBCs were healthy in the control than in the venom-exposed blood at 0 minutes ($P < 0.001$), 2 minutes ($P < 0.05$), 3 minutes ($P < 0.001$), 8 minutes ($P < 0.01$), 10 minutes ($P < 0.001$) and 20 minutes ($P < 0.001$). Conversely, there was a significant number of RBCs at stage 4 of haemolysis, i.e. the final stage, in the venom-exposed blood at 0 minutes ($P < 0.001$), 2 minutes ($P < 0.001$), 3 minutes ($P < 0.001$), 8 minutes ($P < 0.01$) and 20 minutes ($P < 0.05$). There were no significant differences with time (Two-way ANOVA, $P > 0.05$).

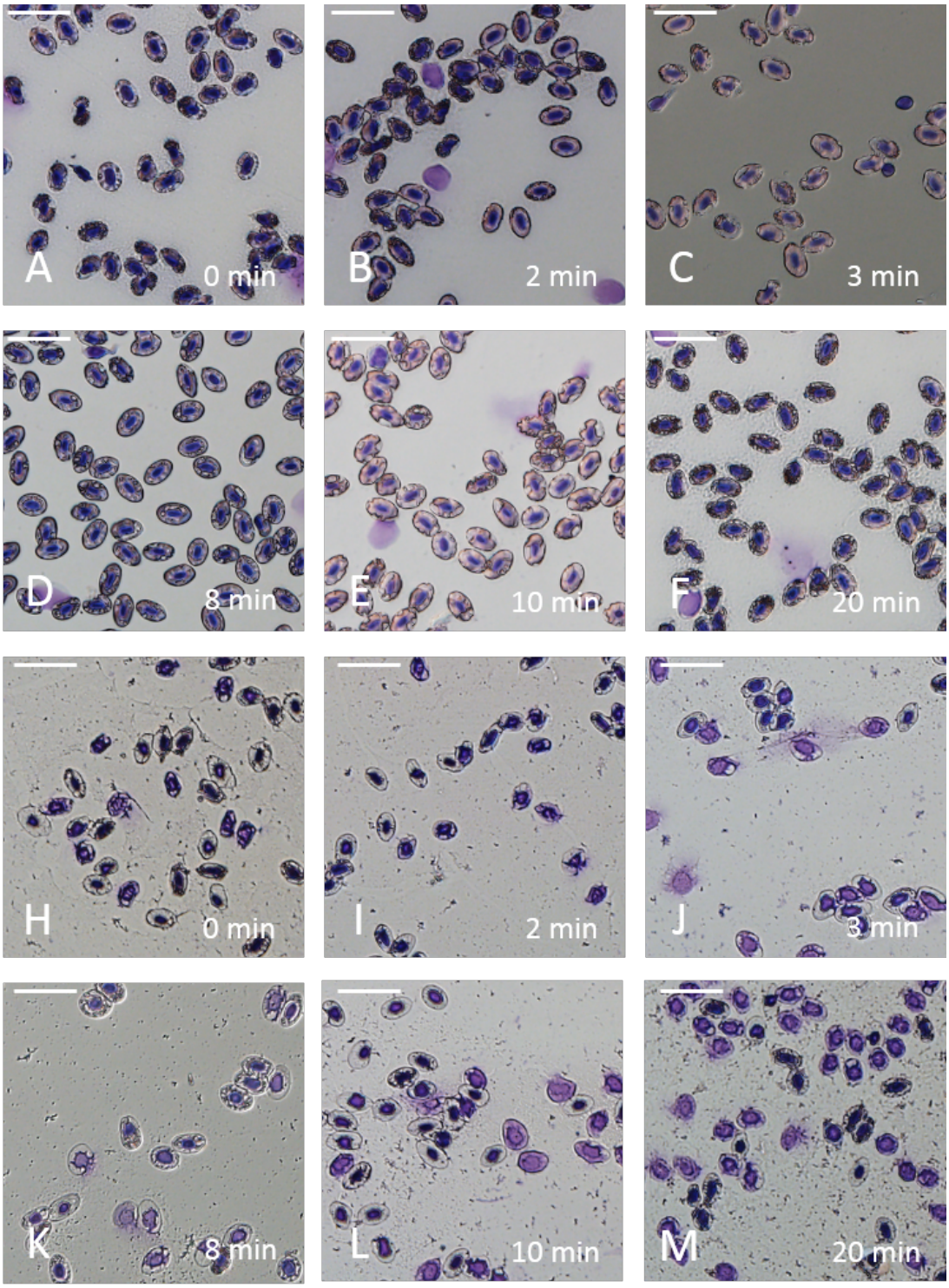


Figure 5.2. Representative sample of blood smears of the control (A – F) and blood exposed to venom (H – M). Scale bar represents 30 µm.

Table 5.2. Frequency of RBCs (%; mean ± SD) at each stage of haemolysis in the control and venom-exposed blood with time from the start of the experiment. Each

blood-smear field was divided into eight sub-fields and the total number of cells at each stage of haemolysis were counted and averaged across the sub-fields.

Time (min)	Stage of haemolysis	Control (% cells)	Venom (% cells)	*P* Value
0	0	65±45	6±8	< .001
	1	35±45	8±13	< .001
	2	0	59±39	< .001
	3	0	4±5	< .05
	4	0	23±26	< .001
2	0	15±13	0	< .05
	1	50±42	10±17	< .001
	2	11±20	52±45	< .001
	3	15±20	25±34	< .001
	4	9±15	13±17	< .05
3	0	53±39	0	< .001
	1	47±39	0	< .001
	2	0	27±26	< .05
	3	0	32±24	< .001
	4	0	41±39	< .001
8	0	22±27	0	< .01
	1	53±36	0	< .05
	2	0	52±45	< .001
	3	17±24	23±17	= .212
	4	8±8	25±29	< .01
10	0	49±40	0	< .001
	1	47±40	0	< .001
	2	1±1	54±37	< .01
	3	0	14±13	< .001
	4	3±4	32±37	= .328
20	0	42±31	0	< .001
	1	51±30	1±4	< .001

	2	4±3	31±31	< .001
	3	0	50±33	< .001
	4	2±2	18±30	< .05

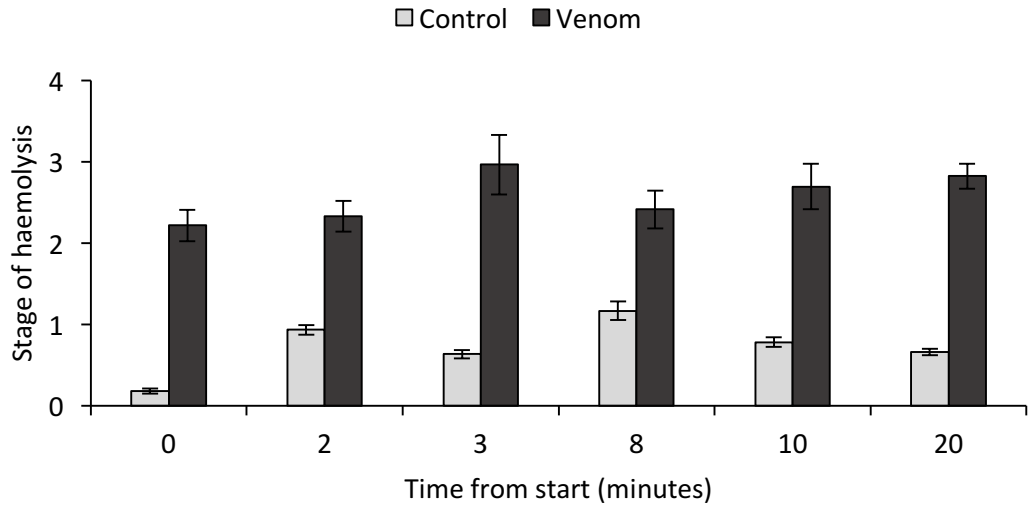


Figure 5.3. Mean stage of haemolysis of control and venom-exposed blood with time from the start of the experiment (mean ± SE). Each blood-smear field was divided into eight sub-fields and the total number of cells at each stage of haemolysis were counted and averaged across the sub-fields.

5.3. Discussion

In this study, we demonstrated a simple *ex vivo* approach to assess the effects of jellyfish on marine-farmed fish at a cellular level. In our experiment, RBCs from *S. salar* showed distinct morphological changes with the onset of haemolysis immediately after exposure to *C. capillata* venom. These results imply that even short-term exposure to jellyfish could result in immediate and significant damage in marine-farmed fish. Immediate damage was also demonstrated in gilthead seabream (*Sparus aurata*) exposed to *Pelagia noctiluca* (Bosch-belmar et al., 2016) where significant gill damage was observed only 3 hours after exposure to macerated *Pe. noctiluca* which continued to increase over time with a peak after 48 hours (Bosch-belmar et al., 2016). In our experiment, because we examined the damage on a cellular level, damage became apparent seconds after exposure, however, Bosch-

belmar et al. (2016) examined the gross pathology of the gills so it took some time (3 hours) before the damage became apparent (Bosch-belmar et al., 2016). Clearly a component of the venom acts fast to inflict damage to the gills. Gills are particularly susceptible to damage because they are made up of thin, delicate filaments of tissue (Laurent, Hoar, & Randall, 1984). Each filament contains capillaries for gas and ion exchange, i.e. water is pumped through the gills to carry away waste products, making them a prime target for venom cytolytic porins. Haemolysis and cytolysis have immediate effects on ion homeostasis, due to the release of intracellular contents, and disruption of gas exchange. Both induce an osmoregulatory response, such as increased opercular movements or hyperventilation, to compensate for the ion imbalance and build-up of CO_2 (Wendelaar Bonga & Lock, 1992), i.e. more water is pumped through the gills to carry away the surplus waste products. If fish do not die directly from the immediate effects of venom toxins, they might die within a few hours from respiratory failure or later from secondary bacterial infections (Ferguson et al., 2010).

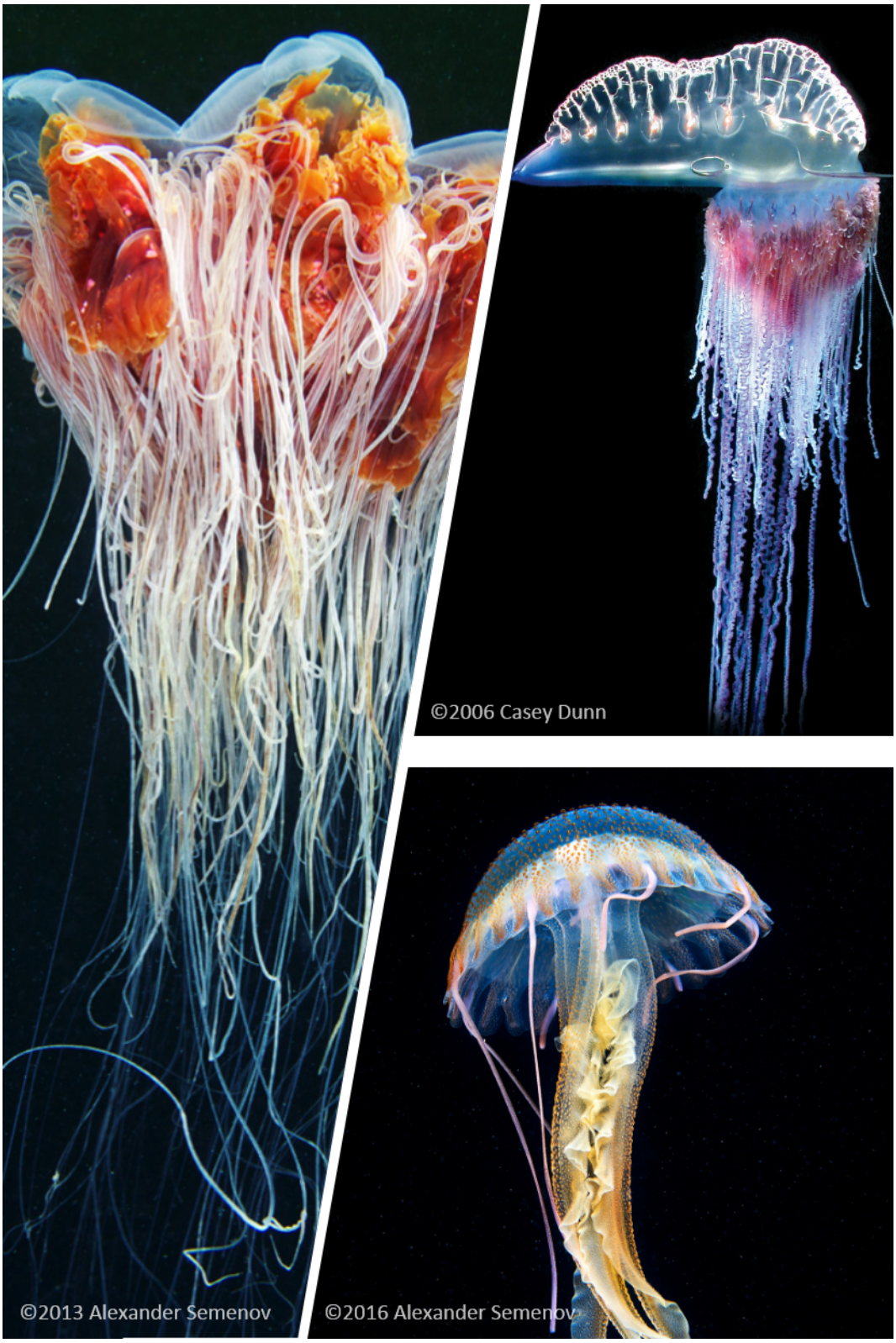
The observed immediate haemolysis is consistent with fast-acting porins, as opposed to venom enzymes such as proteases and lipases which can also lead to haemolysis and cytolysis but act more slowly. In addition, haemolysis by *C. capillata* has been shown to cause a dose-dependent increase in blood potassium in *S. salar*, reflective of haemolysis induced by porins (Powell et al., 2018; Yanagihara & Shohet, 2012). Therefore, this evidence suggests that haemolysis in *C. capillata* is caused by a porin class of venom protein. Furthermore, all jellyfish venoms analysed to date contain porins, so clearly they play a key role in envenomation (Yanagihara, Wilcox, Smith, et al., 2016). Venom porins and their associated effects are potentially lethal but have been shown to be inhibited by zinc gluconate (Yanagihara & Shohet, 2012). Zinc gluconate is a stable compound that is non-toxic at low doses. Importantly, it has been shown to prolong survival time in mice following venom injection (Yanagihara & Shohet, 2012). However, zinc gluconate was found to activate zinc dependent metalloproteinases (MMPs). Another divalent cation, copper gluconate, inhibits porins at lower concentrations and does not activate MMPs (patent #US10172883B2) and is now used in a first-aid treatment for humans in jellyfish

envenomations. Taken together, these findings suggest that prophylactic administration of copper gluconate may have utility in preventing fish mortalities in the event of a jellyfish bloom. The conserved nature of porins in all jellyfish species also means that a treatment which inhibits porins could be widely applicable to more species than just *C. capillata.* However, further research is required to determine whether copper gluconate has any adverse effects on the health of salmon and indeed if its ingestion is enough to reduce venom activity following envenomation by a bloom of jellyfish.

Several species of jellyfish, including *C. capillata*, have been implicated in both large scale fish kill events and the more chronic problem of gill damage in marine-farmed fish. While some species of jellyfish are relatively innocuous, several species clearly represent a serious threat to the aquaculture industry. With aquaculture predicted to increase worldwide to meet the global demand for fish protein (the production of farmed salmon is expected to increase by 40% by the year 2030 (Food and Agriculture Organization of the United Nations, 2018)) and evidence suggesting that jellyfish blooms are increasing in some areas (Brotz et al., 2012), the threat of jellyfish to aquaculture and the associated significant losses could be expected to increase in the future (Baxter et al., 2011b). Every year, in Ireland, jellyfish kill an average of 12% of farmed salmon (Rodger et al., 2011). While Ireland is a minor player in the global salmon production (12,000 tonnes) (BIM, 2019), what happens in Ireland is a microcosm of what happens globally. For example, large fish kill events have been recorded in Canada, Norway, Scotland and Chile (Palma et al., 2007; Rodger et al., 2011), the four largest producers of farmed salmon. Furthermore, such jellyfish induced mortalities are not limited to salmon. Sea bass and sea bream which are extensively farmed in the Mediterranean are also known to suffer fish kill events due to jellyfish (Bosch-Belmar et al., 2014). Our data therefore have a global relevance.

While this study has provided some initial insights, it warrants further investigation. For example, the experiment should be repeated to include more replicate blood smears to reduce the chance that damaged RBCs occurred as a result of drying of suboptimal blood smears. However, there were significant differences

between the control and venom-exposed blood which would suggest that the venom damaged the RBCs and that they were not artefacts of drying. Additionally, more than one field of each blood smear should be examined to determine that the RBCs were uniform across the entire blood smear. In addition, the experiment should be repeated with different doses of venom as it may not be the case that the dose of venom used in this study was equivalent to a lethal envenomation. Indeed, lethal venom doses are often administered to properly elucidate the specific pathophysiological changes that occur in an envenomation (e.g. Yanagihara and Shohet, 2012).



Chapter 6 – General Discussion

6. General Discussion

Reports of jellyfish blooms and their consequences on human activities such as tourism, fishing, aquaculture and power generation (Purcell, Uye, & Lo, 2007) have increased globally during the last half-century. As a result, speculation that jellyfish have been increasing as a result of anthropogenic impacts such as climate change, eutrophication, overfishing, translocation and habitat modification (Purcell et al., 2007; Richardson, Bakun, Hays, & Gibbons, 2009) is abundant. As a result of this concern, there has been a substantial increase in jellyfish research (Brotz et al., 2012; Condon et al., 2013; Dong et al., 2010). We now know that there is no evidence of a global increase in jellyfish populations but that they are more numerous and widespread in certain areas (Brotz et al., 2012). Indeed, most global reviews of jellyfish populations show evidence of numerous localized increases as opposed to global increases (Mills, 2001; Purcell et al., 2007). In addition, we now know that jellyfish undergo larger, global oscillations every 20 years and that a rising phase during recent decades may have contributed to the perception of a global increase even though there is no robust evidence to suggest that this is the case (Condon et al., 2013). Overall, the discipline has come a long way from global concerns of "a more gelatinous future" (Richardson et al., 2009) and is now maturing. However, despite this growing body of work, there are still critical knowledge gaps associated with the abundance of jellyfish and mitigation measures to reduce the impact of localized increases. Generally, there is limited information on how the defining characteristic of jellyfishes – their stinging, impacts on coastal industries such as recreation, tourism and aquaculture. For example, in many coastal areas, jellyfishes pose a threat to public safety. Because of a lack of information on sting management, many ineffective and potentially dangerous measures are still widely recommended. In addition, there is a lack of data on jellyfish abundance and their broad-scale distributions (Pauly, Graham, Libralato, Morissette, & Palomares, 2008; Purcell, 2009). This lack of data has hindered the development of effective mitigation measures. For example, without information on jellyfish abundance and their broad-scale distributions, it is difficult to integrate jellyfishes into forecasting models. Forecasting models might be used to forecast blooms on a local scale or to determine

if localized increases are expected to become a regular occurrence under future climate scenarios (Prieto et al., 2015). However, due to the lack of abundance data, forecasting models of jellyfish abundance and distribution are limited although several attempts have been made (Canepa et al., 2015; Decker et al., 2007). This deficit has become increasingly obvious in recent decades which saw an increase in the number of forecasting models of harmful algal blooms (HABs) (Allen, Smyth, Siddorn, & Holt, 2008; Hamilton, McVinish, & Mengersen, 2009; Walsh, Penta, Dieterle, & Bissett, 2001). Like jellyfishes, localized high biomass blooms of algae have become more numerous and widespread as a result of climate change (e.g. Edwards, Johns, Leterme, Svendsen, & Richardson, 2006). Although forecasting models are desired, they will only mitigate the impacts of localized increases if there are additional effective mitigation measures in place. For example, if jellyfishes are forecasted to reach a coastline, to protect public safety, beach closures can be enforced, and effective sting management can be administered. However, in the case of aquaculture, effective mitigation measures are limited, and more research is required in the area of mitigation. More specifically, there is a requirement for research to be undertaken into effective jellyfish envenomation therapeutics. However, a greater understanding of the pathophysiology of fish envenomation is required before these can be developed.

Within Ireland, many of these broader limitations also apply and in fact Ireland can be considered a microcosm of this global issue. For example, even though Ireland is a minor player in the global salmon aquaculture industry (12,000 tonnes produced in Ireland (BIM, 2019) vs. 1.2 million tonnes produced in Norway (Iversen, Asche, Hermansen, & Nystøyl, 2020)), Ireland has experienced one of the largest fish kill events on record. In 2007, a massive bloom of mauve stinger (*Pelagia noctiluca*) killed 250,000, or approximately 1,000 tonnes of harvest-sized (~4 kg), Atlantic salmon (*Salmo salar*) (Doyle et al., 2008). By our estimates, this cost the Irish salmon aquaculture industry approximately €10 million. In Ireland, gill disorders are one of the most significant causes of mortality in the salmon aquaculture industry, resulting in an annual average mortality of 12% (Rodger et al., 2011) or a cost of approximately €13 million. These losses may be exacerbated in the future as global aquaculture

output is predicted to increase by 40% in the coming years (Food and Agriculture Organization of the United Nations, 2018). As a result, there will likely be an increase in negative interactions between aquaculture and jellyfish (Haberlin, 2018).

In Ireland, open water swimming has become an increasingly popular sport with approximately 3,000 swimmers regularly taking part in open water swimming events. Most of these events take place along coastlines such as the 40 foot in Sandycove, County Dublin, which is also a popular bathing location. During a previous survey of this area, 51% of swimmers (*N* = 77) said that they had been stung by a jellyfish and three said that they required medical attention (Doyle et al., 2017). There is no database in Ireland which reports the number of jellyfish stings, but it is approximately 10 to 100 person per year (Doyle et al., 2017). This is a small fraction of the global number of jellyfish stings per year, which is approximately 150 million (Boulware, 2006). While few envenomations occur in Ireland, a number of beach closures have been enforced during peak stranding events of *C. capillata* (Doyle et al. 2017) and, most recently, *Ph. physalis* (pers. comm. T. K. Doyle). If open water swimming continues to increase in popularity, we might expect to see more beach closures. In areas of the Pacific, such as Waikiki in Honolulu, Hawaii, where up to 800 envenomations have occurred in one day (Thomas et al., 2001), jellyfish are not only a public health issue but also a threat to tourism (Gershwin & Dabinett, 2009). To address the impacts of jellyfish on aquaculture and public safety in Ireland, this thesis aimed to demonstrate directed research required to underpin mitigation measures such as improved sting management, forecasting models and tentative steps towards the development of effective jellyfish envenomation therapeutics for the treatment of injured fish (Figures 6.1, 6.2).

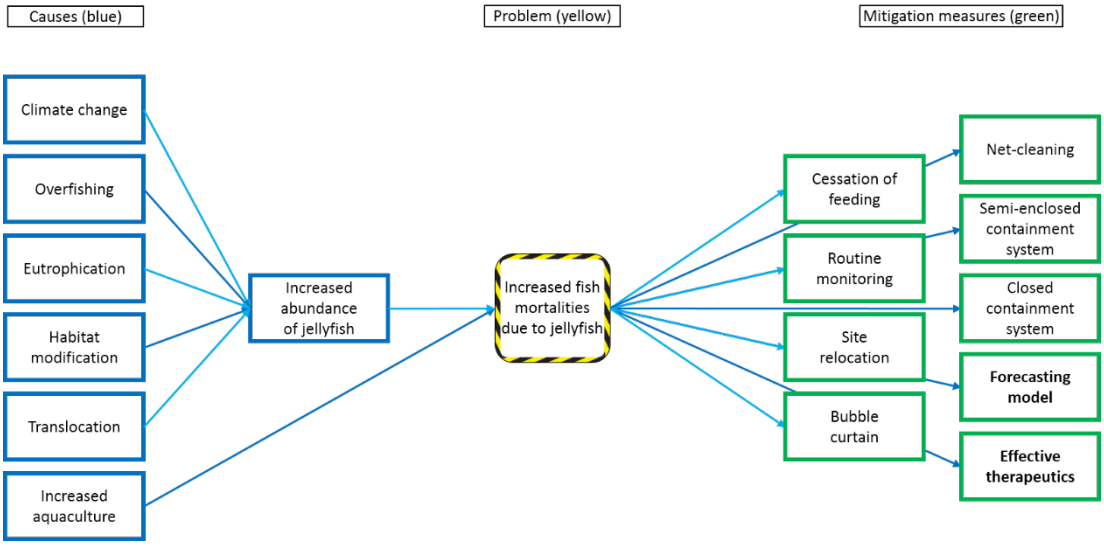


Figure 6.1. Bow-tie diagram showing the cause-effect relationship between anthropogenic impacts and increased aquaculture (blue) on aquaculture (yellow hazard box). Mitigation measures (green) can reduce or prevent these impacts. The mitigation measures alluded to in this thesis are highlighted in bold.

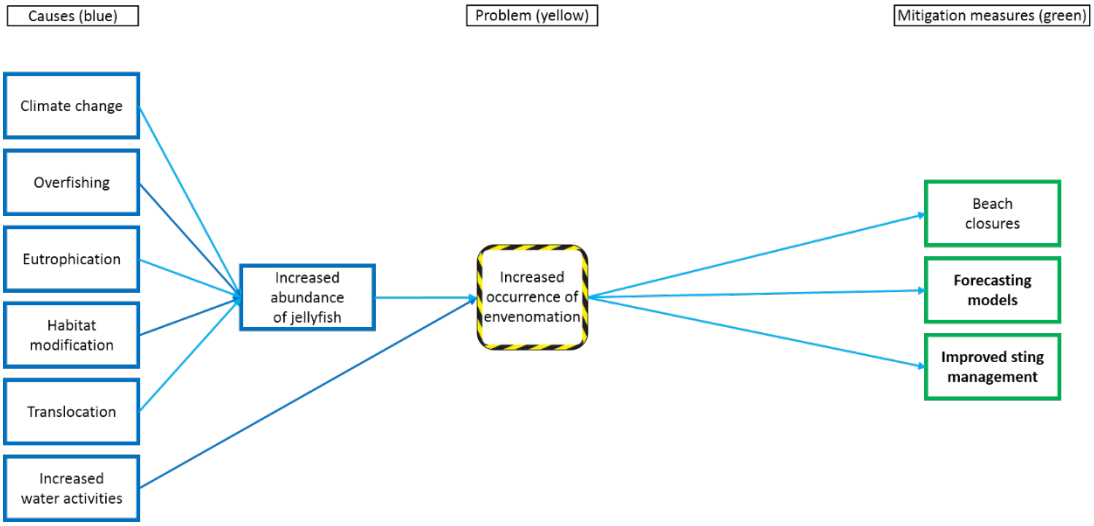


Figure 6.2. Bow-tie diagram showing the cause-effect relationship between anthropogenic impacts and increased open water swimming (blue) on envenomation (yellow hazard box). Mitigation measures (green) can reduce or prevent these impacts. The mitigation measures alluded to in this thesis are highlighted in bold.

One area where there has been considerable debate has been the issue of sting management. There has been a large amount of research on the treatment of jellyfish stings, but confusion still exists as to what the most effective first aid

management is. Most current recommendations are founded on relatively weak evidence (Cegolon et al., 2013) lacking rigorous scientific support (Doyle et al., 2017). For example, current recommendations from the Australian Resuscitation Council (ARC) are based on a literature review (Australian Resuscitation Council, 2010) and some of the literature is almost 40 years old (Hartwick, Callanan, & Williamson, 1980). As a result, the ARC currently recommends ice for tropical species (Australian Resuscitation Council, 2010) even though there are ample amounts of evidence supporting the use of hot water (Wilcox et al., 2017; Wilcox & Yanagihara, 2016b, 2016a; Yanagihara & Wilcox, 2017). In Ireland, current recommendations are based on guidelines drawn up by the Jellyfish Action Group of Ireland and Wales which includes members of the Beaumont Poison Centre, Pre-hospital Emergency Care experts, hospital A&E consultants, local GPs, and water safety officers under the INTERREG EcoJel Project. The guidelines for sting management were developed in 2008 and were based on a literature review (carried out and presented by Dr Tom Doyle) and a committee decision. Therefore, one of the key components of this thesis was to rigorously test the different recommended treatments using recently developed, readily available and reproducible envenomation assays (Chapter 3; Doyle et al., 2017; Wilcox et al., 2017). Several rinse solutions including seawater, vinegar and StingNoMore® Spray, to inhibit cnidae discharge, as well as hot packs and ice packs, to reduce venom activity, were tested for their efficacy in treating *C. capillata*, *Pe. noctiluca* and *Ph. physalis* stings. These are the three most venomous species in Ireland. Results from several assays indicated that the most effective management for these species was a two-step protocol of: 1) rinsing with vinegar or Sting No More ® Spray for 30 seconds and, 2) 40 minutes or longer treatment with hot packs or hot water immersion (45^{o}C) (Chapter 3; Doyle et al., 2017; Wilcox et al., 2017). These recommendations contradict current recommendations of rinsing with seawater and cold packs (Health Service Executive, 2018). In some assays, seawater and cold packs worsened stings (Chapter 3; Doyle et al., 2017; Wilcox et al., 2017). By testing the different recommended treatments, this study has simplified the development of first aid management for jellyfish stings in Ireland. It is hoped that these recommendations will be adopted by local authorities. However, randomized clinical trials may be required to ensure that the treatments have no adverse effects.

While investigating the various treatments it became very apparent that several treatments (i.e. urine and isopropanol) caused all nematocyst types to discharge whereas other treatments (i.e. vinegar) only caused one or two types of nematocyst (i.e. a-isorhiza and eurytele) to discharge (Chapter 3; Doyle et al., 2017). Some nematocyst types (e.g. large A-isorhiza and O-isorhiza) are potentially more toxic than others and several treatments (i.e. urine and isopropanol) caused these nematocysts to discharge. Therefore, a greater understanding of the abundance of these types (i.e. large A-isorhiza and O-isorhiza) was essential to further elucidate the inefficacy of certain treatments (i.e. urine and isopropanol). Even though it was revealed that large A-isorhiza and O-isorhiza were low in abundance in *C. capillata* (12% and 7% of nematocysts counted), there were still 45 million of them in total (Chapter 4). Therefore, the discharge induced by ineffective treatments could be potentially detrimental. Conversely, vinegar caused a-isorhiza and eurytele nematocysts to discharge (Chapter 3; Doyle et al., 2017). Of all the nematocysts in *C. capillata*, a-isorhiza nematocysts were the smallest in size (Chapter 4) and studies suggest that they have short tubules (Colin & Costello, 2007) so they may not contribute to sting severity (Kitatani et al., 2015). Even though eurytele nematocysts have longer tubules (Colin & Costello, 2007), they were low in abundance (7% of total nematocysts counted, Chapter 4). Therefore, the discharge of these types might be negligible and might not contribute to sting severity. In addition, as seen when vinegar was applied to adherent cnidae in our simple gelatine sting model (Chapter 3), of the cnidae that discharged in response to vinegar application, all the tubules discharged non-productively upwards into the vinegar droplet and did not impale the gelatine. Therefore, discharge caused by the application of vinegar might not result in functional venom delivery (Chapter 3; Doyle et al., 2017). Therefore, both Chapter 3 and Chapter 4 demonstrate that any discharge caused by the addition of vinegar may not contribute significantly to sting severity. In addition to quantifying the abundance of the different nematocyst types, this thesis also provided insights into the total amount of nematocysts possessed by *Pe. noctiluca* and *C. capillata* and the venom dose that an injured person would receive during an encounter with *C. capillata* (Chapter 4). Overall, *C. capillata* possessed three orders of magnitude more nematocysts than *Pe. noctiluca*. Furthermore, an encounter with *C. capillata* could

result in the injection of approximately 9.4 mg of venom which is 100 million times the volume of venom injected by a honeybee (Chapter 4). Clearly, the amount of venom injected has some bearing on sting severity and may explain many of the serious *C. capillata* envenomations in Ireland. Indeed, *C. capillata* stings are associated with more severe systemic symptoms, including a severe Irukandji-like syndrome (Doyle et al., 2017), whereas *Pe. noctiluca* stings are associated with more local dermal reactions (Kokelj & Burnett, 1990). While these differences in sting severity may be due to the potency of *C. capillata* venom, the significant differences in the numbers of nematocysts and amounts of venom suggest that these differences in sting severity are most likely due to the size of *C. capillata* themselves.

During this thesis, four unprecedented strandings of *Ph. physalis* were documented. In the Mediterranean, it has been shown that wind patterns and ocean currents are the main mechanisms controlling their drift (Prieto et al., 2015). Therefore, it was decided to investigate whether a Lagrangian particle-tracking model, forced by wind and ocean currents, could be used to forecast *Ph. physalis* blooms (Chapter 2). The model incorporated coastal and offshore observations of *Ph. physalis* collected during the largest mass strandings event documented in Ireland in the past 150 years. Hindcasting stranded *Ph. physalis* from the Irish coastline suggested that the 2016 mass stranding event originated from an extensive source area located over the European basin including the Porcupine Seabight. However, stranded individuals ultimately originated from the North Atlantic Current (NAC). Forecasting particles from the Porcupine Seabight, where *Ph. physalis* were observed prior to stranding, resulted in particles stranding in a pattern that was 82% similar to actual strandings. Due to the fact that 1) *Ph. physalis* were observed in the Porcupine Seabight weeks before the 2016 stranding event and, 2) both the hindcast and forecast model highlighted the Porcupine Seabight as an important area of *Ph. physalis* strandings in Ireland, the model clearly has great potential as a forecasting model to forecast jellyfish blooms and strandings based on offshore observations to highlight areas most likely to be impacted. With some minor adjustments the model might be applied to other jellyfish species. The forecasting model could be used to provide advanced notice to local authorities or fish farms to enforce beach closures

or implement mitigation measures to protect the health of fish stock. In addition, the model might be used to determine if unprecedented strandings are expected to become a regular occurrence under future climate scenarios. Indeed, a GAM analysis used in this study revealed that wind speeds above 6 ms^{-1} had an increasingly positive effect on the drift speeds of *Ph. physalis*. Therefore, if wind speeds are expected to increase under future climate scenarios, greater numbers of *Ph. physalis* may continue to disperse into areas outside of their normal range. Furthermore, Prieto et al., (2015) suggests that the North Atlantic Oscillation (NAO) has a strong influence on the intensity of winds in the North Atlantic and that negative NAO indices are strongly correlated with conditions sufficient to cause increased *Ph. physalis* strandings. As a result, if projected NAO patterns in future climate scenarios are increasingly negative, *Ph. physalis* strandings may occur more frequently (Prieto et al., 2015).

Another area of jellyfish research that required further investigation was the issue of jellyfish blooms and their detrimental effects on fish. Indeed, a greater understanding of the links between harmful jellyfish and Atlantic salmon was urgently needed before a mitigative jellyfish envenomation therapeutic could be developed. Therefore, by exploring the pathogenic effects of *C. capillata* in Atlantic salmon, this thesis took tentative steps towards the development of such a treatment (Chapter 5). Importantly, this pilot study revealed that *C. capillata* venom immediately caused significant haemolysis which suggests that even short-term exposure to jellyfish venom can result in immediate and significant damage to exposed tissues (gill, skin and eyes) in farmed fish. The rapidity with which *C. capillata* venom took effect suggests that the venom might contain fast-acting porins. Venom enzymes such as lipases and proteases also cause haemolysis but act more slowly. Venom porins are inhibited by copper gluconate, a treatment for humans in jellyfish envenomations. Therefore, the prophylactic administration of copper gluconate may have potential as a mitigation measure to prevent fish mortalities.

This thesis aimed to fill gaps in the knowledge of harmful jellyfish in Ireland and provide some suggestions as to how their negative impacts might be mitigated in the future either through forecasting models, sting management or an effective

jellyfish envenomation therapeutic for farmed salmon. In the future, we may see an increased abundance of jellyfish as a result of climate change. These changes will have important consequences for coastal activities in Ireland such as recreation, tourism and aquaculture. Ireland is considered a microcosm of the global issue. Therefore, the changes that we see in Ireland might be exacerbated globally. Managing these changes remains a substantial challenge and mitigation measures require fundamental and targeted research such as was presented in this thesis. Therefore, it is hoped that the research presented here has added to the global knowledge base with which some of these mitigation measures can be developed.

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Appendices

Appendix 1 – Supplementary Material for Chapter 2

Table A.2.1. List of coastal observations of Portuguese man of war *Physalia physalis*. Total number of *Ph. physalis* sightings along the Irish coastline. N/A: Not available. Pers. comm.: Personal communication.

Year	Month	Day	Locality	Abundance of *Ph. physalis*	Reference
1834	03	13	Ardmore, Co. Waterford	1	(Thompson, 1835)
1881	08	N/A	Castlerock, Co. Derry	1	(Johnson, 1921)
1907	05	N/A	Cooscroum, Dingle Bay, Co. Kerry	1	(Delap, 1924)
1907	08	24	At sea, 50°16'N 11°27'W	1	(Stelfox, 1936)
1921	02	27	Bantry Bay, Co. Cork	N/A	(Delap, 1921)
1934	N/A	N/A	Hook Tower Lighthouse, Co. Wexford	1	(Stelfox, 1936)
1934	11	23	Tragumina, Skibbereen, Co. Cork	1	(Stelfox, 1936)
1935	10	02	Hook Tower Lighthouse, Co. Wexford	1	(Stelfox, 1936)
1945	09	19 – 24	Tramore, Co. Waterford	N/A	(Walker, 1946)
1945	10	N/A	Ballyteige Burrow, Co. Wexford	N/A	(Wilson, 1947)
1958	09	07	Brittas Bay, Co. Wicklow	1	(Friel & Roche, 1959)
1958	10	N/A	Outer Galway Bay, Co. Galway	N/A	(Boyd, Céidigh, & Wilkinson, 1973)
1958	10	N/A	Kilkieran Bay, Co. Galway	N/A	(Boyd et al., 1973)

1958	11	23	Fountainstown Strand, Co. Cork	2	(Atkins, 1959)
1965	09	26	Cape Clear Island, Co. Cork	3	(Sharrock, 1967)
1965	09	26	Cape Clear Island, Co. Cork	1	(Sharrock, 1967)
1965	09	30	Cape Clear Island, Co. Cork	2	(Sharrock, 1967)
1965	10	02	Cape Clear Island, Co. Cork	1	(Sharrock, 1967)
1967	10	15	Ineermore, Cape Clear Island, Co. Cork	96	(Sharrock, 1968b)
1967	10	15	Ineerbeg, Cape Clear Island, Co. Cork	84	(Sharrock, 1968a)
1967	10	16	Foiladda, Cape Clear Island, Co. Cork	20	(Sharrock, 1968b)
1968	10	10	Cape Clear Island, Co. Cork	16	(Sharrock, 1969)
1968	10	11	Cape Clear Island, Co. Cork	40	(Sharrock, 1969)
1968	10	17	Cape Clear Island, Co. Cork	1	(Sharrock, 1969)
1968	10	18	Cape Clear Island, Co. Cork	153	(Sharrock, 1969)
1968	10	19	Cape Clear Island, Co. Cork	234	(Sharrock, 1969)
1968	10	31	Cape Clear Island, Co. Cork	2	(Sharrock, 1969)
1968	10	23	Blasket Islands, Co. Kerry	1	(O'Riordan, 1969)
1968	10	18	Dingle Harbour, Co. Kerry	1	(O'Riordan, 1969)
1968	10	27	Ventry, Co. Kerry	2	(O'Riordan, 1969)
1989	11	N/A	Ballyteige Burrow, Co. Wexford	1	Pers. comm.

1995	10	24	Tacumshin Lake, Co. Wexford	1	Pers. comm.
1995	10	31	Tacumshin Lake, Co. Wexford	1	Pers. comm.
1995	11	22	Rostonstown, Co. Wexford	1	Pers. comm.
1995	12	03	Tacumshin Lake, Co. Wexford	1	Pers. comm.
1996	11	07	Inch Beach, Dingle Bay, Co. Kerry	4	Pers. comm.
1996	11	10	Long Strand, Galley Head, Co. Cork	1	Pers. comm.
1996	11	10	Ballydonegan Beach, Co. Cork	46	Pers. comm.
1996	11	10	Allihies, Co. Cork	3	Pers. comm.
1996	11	11	Long Strand, Ballinskelligs, Co. Kerry	1	Pers. comm.
1996	11	11	Glenbeigh Beach, Dingle Bay, Co. Kerry	3	Pers. comm.
2008	02	05	Lohar, Waterville, Co. Kerry	2	Pers. comm.
2008	09	06	Blasket Islands, Co. Kerry	1	Pers. comm.
2012	08	29	Tramore Beach, Co. Waterford	3	Facebook
2015	08	05	Barley Cove Beach, Co. Cork	1	Facebook
2016	08	05	N/A	1	Facebook
2016	08	21	White Strand, Co. Clare	2	Facebook
2016	09	07	Inchydoney Beach, Co. Cork	1	Facebook
2016	09	08	The Warren, Co. Cork	1	Facebook

2016	09	08	Inchydoney Beach, Co. Cork	1	Facebook
2016	09	08	Long Strand, Co. Cork	6	Facebook
2016	09	09	Clonea Beach, Co. Waterford	2	Facebook
2016	09	09	N/A	100	Facebook
2016	09	10	Dunworley Bay, Co. Cork	2	Facebook
2016	09	12	Fennell's Bay, Co. Cork	5	Facebook
2016	09	12	Cape Clear Island, Co. Cork	1	Facebook
2016	09	12	Long Strand, Co. Cork	51	Facebook
2016	09	13	Tra na Lan Beach, Co. Cork	2	Facebook
2016	09	14	Beacon Beach, Co. Cork	1	Facebook
2016	09	14	Cape Clear Island, Co. Cork	5	Facebook
2016	09	15	Inchydoney Beach, Co. Cork	2	Facebook
2016	09	16	Cape Clear Island, Co. Cork	1	BirdWatch Ireland
2016	09	17	Fenit Beach, Co. Kerry	1	Facebook
2016	09	18	Ventry Beach, Co. Kerry	8	Facebook
2016	09	20	Kilkee, Co. Clare	1	Facebook
2016	09	20	Ballyheigue Beach, Co. Kerry	1	Facebook
2016	09	22	Finnavara, Co. Clare	0	Beach survey

2016	09	22	Spanish Point, Co. Clare	0	Beach survey
2016	09	22	Quilty, Co. Clare	0	Beach survey
2016	09	22	Kilkee, Co. Clare	0	Beach survey
2016	09	23	The Warren, Co. Cork	1	Facebook
2016	09	23	Keel Beach, Co. Mayo	N/A	Facebook
2016	09	24	Cape Clear Island. Co. Cork	68	BirdWatch Ireland
2016	09	25	Castletownsend, Co. Cork	1	Facebook
2016	09	25	St. Finian's Bay, Co. Kerry	3	Facebook
2016	09	25	St. Finian's Bay, Co. Kerry	52	Facebook
2016	09	25	Farranamanagh Beach, Co. Cork	3	Facebook
2016	09	25	Derrynane, Co. Kerry	2	Facebook
2016	09	25	Dinish Island, Co. Cork	2	Facebook
2016	09	26	Cape Clear Island, Co. Cork	60	Facebook
2016	09	26	Roche's Point, Co. Cork	8	Facebook
2016	09	26	Keel Beach, Co. Mayo	3	Facebook
2016	09	26	Dooega, Co. Mayo	3	Facebook
2016	09	26	Kincasslagh Harbour, Co. Donegal	1	Facebook
2016	09	26	Clifden, Co. Galway	0	Beach survey

2016	09	26	Salthill, Co. Galway	0	Beach survey
2016	09	26	Dog's Bay Beach, Co. Galway	0	Beach survey
2016	09	26	Derrynane, Co. Kerry	8	Beach survey
2016	09	27	Cape Clear Island, Co. Cork	98	BirdWatch Ireland
2016	09	27	Long Strand, Co. Cork	2	Facebook
2016	09	27	Inch Strand, Co. Cork	2	Facebook
2016	09	27	Ardmore, Co. Galway	4	Facebook
2016	09	29	Chimney Cove, Co. Cork	22	Facebook
2016	09	30	Garrarus, Co. Waterford	1	Facebook
2016	09	30	Bunowen Bay, Co. Galway	3	Facebook
2016	09	30	Kilfarassy/Kilmurrin/Benvoy Strand, Co. Waterford	11	Facebook
2016	09	30	Fanore, Co. Clare	14	Facebook
2016	09	30	Tra Bhui, Co. Sligo	3	Facebook
2016	09	30	Long Strand, Co. Cork	163	Facebook
2016	09	30	Keel Beach, Co. Mayo	40	Facebook
2016	09	30	Tramore Beach, Co. Waterford	2	Facebook
2016	09	30	Rossaveal, Co. Galway	1	Facebook
2016	09	30	N/A	20	Facebook

2016	10	01	Omey Island, Co. Galway	7	Facebook
2016	10	01	Seafield, Co. Clare	20	Facebook
2016	10	01	Slade, Co. Wexford	1	Facebook
2016	10	01	Rostonestown, Co. Wexford	N/A	Facebook
2016	10	02	Gurteen Strand, Co. Galway	1	Facebook
2016	10	02	Glen Strand, Co. Kerry	56	Facebook
2016	10	02	Garretstown Beach, Co. Cork	10	Facebook
2016	10	02	Ballinskelligs, Co. Kerry	2	Facebook
2016	10	03	Schull, Co. Cork	15	Facebook
2016	10	03	Church Bay, Co. Cork	20	Facebook
2016	10	03	Galley Cove, Co. Cork	27	Facebook
2016	10	03	Dooneen Strand, Co. Cork	10	Facebook
2016	10	03	Inchydoney Beach, Co. Cork	2	Facebook
2016	10	04	Cartfotine Strand, Co. Cork	20	Facebook
2016	10	04	Myrtleville Beach, Co. Cork	1	Facebook
2016	10	04	Bunmahon, Co. Waterford	1	Facebook
2016	10	04	Ardmore, Co. Waterford	10	Facebook
2016	10	04	Ballytrent Beach, Co. Wexford	7	Facebook

2016	10	05	Galley Cove, Co. Cork	102	Facebook
2016	10	05	Cape Clear Island, Co. Cork	121	BirdWatch Ireland
2016	10	05	Rosslare Strand, Co. Wexford	10	Facebook
2016	10	05	Rosslare Strand, Co. Wexford	20	Facebook
2016	10	06	Cobh, Co. Cork	1	Facebook
2016	10	07	Graball Beach, Co. Cork	1	Facebook
2016	10	07	Youghall, Co. Cork	7	Beach survey
2016	10	07	Ardmore, Co. Waterford	27	Beach survey
2016	10	08	Fennell's Bay, Co. Cork	10	Facebook
2016	10	08	Cape Clear Island, Co. Cork	28	BirdWatch Ireland
2016	10	08	Ballybrannigan Strand, Co. Cork	1	Facebook
2016	10	08	Inny Beach, Co. Kerry	1	Facebook
2016	10	08	Curracloe Beach, Co. Wexford	10	Facebook
2016	10	08	Barrys Cove, Co. Cork	1	Facebook
2016	10	08	Galley Cove, Co. Cork	20	Facebook
2016	10	09	Cape Clear Island, Co. Cork	147	BirdWatch Ireland
2016	10	09	Inchydoney Beach, Co Cork	1	Facebook
2016	10	09	Morriscastle Beach, Co. Wexford	1	Facebook

2016	10	09	Curracloe Beach, Co. Wexford	1	Facebook
2016	10	09	Curracloe Beach, Co. Wexford	2	Facebook
2016	10	09	Cape Clear Island, Co. Cork	20	Facebook
2016	10	09	Horseshoe Harbour, Co. Cork	30	Facebook
2016	10	10	Castletownbere, Co. Cork	10	Facebook
2016	10	10	Lawlor's Strand, Co. Waterford	1	Facebook
2016	10	10	Baltimore Harbour, Co. Cork	20	Facebook
2016	10	11	Cape Clear Island, Co. Cork	20	BirdWatch Ireland
2016	10	12	Youghal, Co. Cork	N/A	Facebook
2016	10	12	Cape Clear Island, Co. Cork	30	BirdWatch Ireland
2016	10	13	Cape Clear Island, Co. Cork	14	BirdWatch Ireland
2016	10	13	Courtmacsherry Beach, Co. Cork	1	Facebook
2016	10	14	Cape Clear Island, Co. Cork	150	BirdWatch Ireland
2016	10	15	Myrtleville Beach, Co. Cork	1	Facebook
2016	10	15	Cape Clear Island, Co. Cork	3	BirdWatch Ireland
2016	10	15	Baltimore Harbour, Co. Cork	2	Facebook
2016	10	16	Cape Clear Island, Co. Cork	30	BirdWatch Ireland
2016	10	16	Roches Point, Co. Cork	1	Facebook

2016	10	18	Cape Clear Island, Co. Cork	18	BirdWatch Ireland
2016	10	26	Youghal, Co. Cork	0	Beach survey
2016	10	26	Ardmore, Co. Waterford	0	Beach survey

Table A.2.2. List of weather conditions and visual counts of *Ph. physalis* from the *Oceanic Endeavour* traversing the Celtic Sea during 04/08/2016 – 21/09/2016. Where there are multiple lines for one transect, each line represents one consecutive sample period. The count provided represents one whole transect. Location (longitude and latitude), depth, speed (knots), wind direction, wind force (Beaufort scale), sea state, swell, visibility and glare were recorded every hour. Sea state: G = glassy (like a mirror), S = slight (no or few white caps), C = choppy (many white caps), R = rough (large waves, foam crests, spray); Swell: O = low (< 2 m), M = medium (2 – 4 m), L = large (> 4 m); Visibility: P = poor (< 1 km), M = moderate (1 – 5 km), G = good (> 5 km).

Transect #	Date	Start Longitude (W)	Start Latitude (N)	Depth (m)	End Longitude (W)	End Latitude (N)	Depth (m)	Speed (knots)	Wind direction	Wind force (Beaufort)	Sea state	Swell	Visibility	Abundance of *Ph. physalis*
1	04/08/2016	12.68	49.99	2397	12.52	50.02	2274	4.3	S	2	S	O	G	11
2	08/08/2016	12.78	49.96	2451	12.71	49.97	2462	4.7	S	3	S	O	G	6
3	13/08/2016	13.26	49.72	2026	13.38	49.69	2171	4.6	S	2	S	O	G	5
4	17/08/2016	12.75	49.94	2450	12.64	49.95	2392	4.6	SE	4	S	O	G	7
5	14/09/2016	13.34	49.74	2203	13.24	49.77	2263	4.8	S	4	C	O	G	36

		13.24	49.77	2263	13.12	49.79	2469	4.8	SE	4	C	O	G	
		13.12	49.79	2469	12.98	49.81	2393	4.6	SE	5	C	O	G	
		12.98	49.81	2393	12.89	49.82	2438	4.4	SE	4	C	O	G	
		12.89	49.82	2438	12.75	49.85	2445	4.5	SE	5	C	O	G	
		12.75	49.85	2445	12.70	49.87	2428	2.8	SE	5	C	O	G	
6	15/09/2016	12.25	49.81	1774	12.37	49.79	1879	4.5	SE	5	C	O	G	8
7	16/09/2016	12.15	49.69	1349	12.04	49.71	1290	4.5	SE	5	C	M	G	6
8	17/09/2016	13.44	49.46	2979	13.34	49.51	1772	4.6	NE	5	S	O	G	13
		13.34	49.51	1772	13.22	49.54	1646	4.6	NE	5	S	O	G	
		13.22	49.54	1646	13.16	49.55	1653	4.6	NE	4	S	O	G	
9	17/09/2016	12.77	49.62	1810	12.67	49.64	1879	4.6	NE	5	C	O	G	26
		12.67	49.64	1879	12.57	49.66	1858	4.4	NE	5	C	O	G	
		12.57	49.66	1858	12.45	49.68	1717	4.2	NE	5	C	O	G	
		12.45	49.68	1714	12.36	49.69	1589	4.3	NE	5	C	O	G	
10	18/09/2016	12.02	49.83	1435	12.12	49.82	1504	4.3	S	6	C	O	M	4
11	18/09/2016	12.58	49.74	2067	12.69	49.72	2148	4.3	S	6	C	O	G	8
		12.69	49.72	2148	12.80	49.70	2189	4.6	S	6	C	O	G	
		12.80	49.70	2189	12.91	49.67	2057	4.8	S	6	C	O	G	
12	19/09/2016	12.43	49.76	1814	12.55	49.74	2006	4.5	SE	3	S	O	G	2
13	19/09/2016	12.67	49.72	2127	12.77	49.69	2157	4.7	SE	3	S	O	G	3
14	20/09/2016	11.90	49.80	1251	11.99	49.84	1423	4.2	NE	5	C	M	G	0

15	21/09/2016	12.46	49.65	1628	12.37	49.67	1527	4.7	SE	4	C	M	G	1
		12.37	49.67	1527	12.22	49.69	1420	4.8	SE	4	C	M	G	
16	21/09/2016	12.12	49.71	1340	12.07	49.72	1287	4.8	SE	4	C	O	G	3
		12.07	49.72	1287	12.01	49.72	1223	4.8	SE	4	S	O	G	

Appendix 2 – Paper 1

Evaluation of *Cyanea capillata* Sting Management Protocols Using *Ex Vivo* and *In Vitro* Envenomation Models

Doyle, T. K., Headlam, J. L., Wilcox, C. L., MacLoughlin, E., & Yanagihara, A. A.

Published in *Toxins,* 2017, 9(7).

Appendix 2 – Paper 2

Assessing the Efficacy of First-Aid Measures in *Physalia* sp. Envenomation, Using Solution- and Blood Agarose-Based Models

Wilcox, C. L., Headlam, J. L., Doyle, T. K., & Yanagihara, A. A.

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